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REVIEW

Immunotropic Effects and Proposed Mechanism of Action for 3-Hydroxy-3-methylglutaryl-coenzyme A Reductase Inhibitors (Statins)

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Abstract—Inhibitors of HMG-CoA reductase (statins) are the major group of lipid-lowering drugs. Along with hypocholesterolemic activity, statins exhibit anti-inflammatory and immunomodulatory properties that expand their clinical use, particularly, in the treatment of chronic inflammatory and autoimmune disorders. In this review, we critically analyze the data of statin effects on immune cells (e.g., monocytes and T cells) involved in the development of atherosclerosis and other chronic inflammatory diseases. We (i) discuss the properties of statins and routes of cell entry, as well as their major intracellular targets; (ii) evaluate the data on the effects of statins on the subset composition of circulatory monocytes, ability of monocytes to migrate to the site of inflammation (cell motility and expression of adhesion molecules and chemokine receptors), production of cytokines, matrix metalloproteinases, and reactive oxygen species by monocytes/macrophages, and antigen-presenting activity in peripheral blood monocyte-derived dendritic cells; and (iii) summarize the data on the regulation of proliferation and differentiation of various $CD4^+$ T cell subsets (type 1/2/17 helper T cells and regulatory T cells) by statins.

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Keywords: HMG-CoA reductase inhibitors, statins, monocytes, macrophages, T cells

STATINS: PROPERTIES AND MECHANISM OF ACTION

Statins are the major group of lipid-lowering drugs widely used in clinical practice. Statins act as competitive inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, a key enzyme of cholesterol synthesis that catalyzes conversion of HMG-CoA to mevalonate. Statins lower the content of atherogenic lipoproteins in the blood thereby decreasing the risk of development of cardiovascular diseases and subsequent complications.

In addition to their hypolipidemic effects, statins stimulate NO production in endothelial cells and exhibit anti-inflammatory, anticoagulant, antioxidant, and antiarrhythmic activities [1]. However, the use of statins is

Abbreviations: CD, cluster of differentiation; CRP, C-reactive protein; DCs, dendritic cells; ERK1/2, extracellular signal-regulated kinase-1/2; FPP, farnesyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate; GM-CSF, granulocyte macrophage colony stimulating factor; GTPase, guanosine triphosphate hydrolase; HLA-DR, human leukocyte antigen – antigen D-related; HMG-CoA reductase, 3-hydroxy-3-methylglutaryl-coenzyme A reductase; ICAM-1, intracellular adhesion molecule 1; IL, interleukin; INF-γ, interferon-γ; LFA-1, lymphocyte function-associated antigen 1, integrin family of adhesion molecules; LPS, lipopolysaccharide; Mac-1, macrophage-1 antigen, integrin family of adhesion molecules; MAP kinase, mitogen-activated protein kinase; MCP-1, monocyte chemoattractant protein-1; MIP, macrophage inflammatory protein, cytokine; MMP, matrix metalloproteinase; mTOR, mammalian target of rapamycin, protein kinase; PHA, phytohemagglutinin; RANTES, regulated on activation, normal T-cell expressed and secreted (chemokine); TGFβ, transforming growth factor-β; Th1/2/17, type 1/2/17 helper T cells; TIMP, tissue inhibitor of metalloproteinase; TLR4, Toll-like receptor 4; TNF, tumor necrosis factor; Treg, regulatory T lymphocytes; VLA-4, very late antigen-4, integrin family of adhesion molecules.

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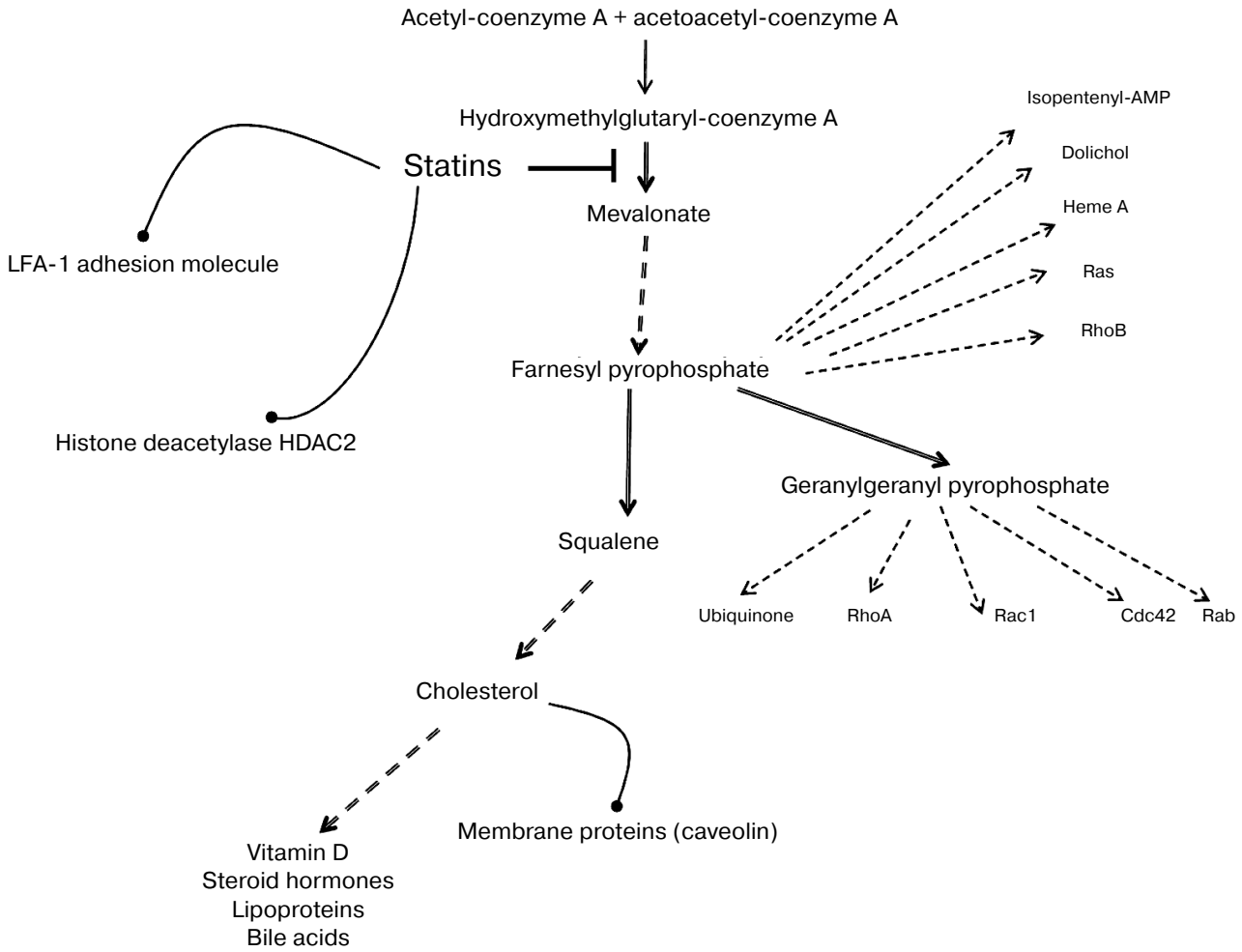
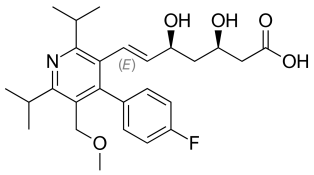
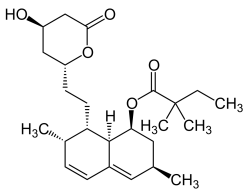
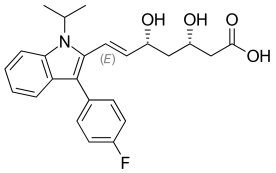
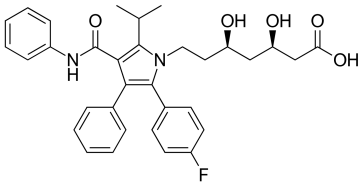
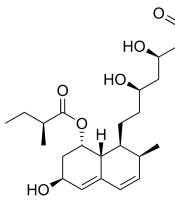
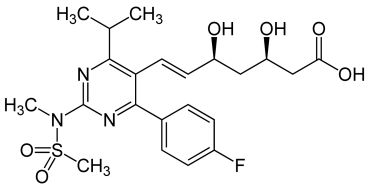
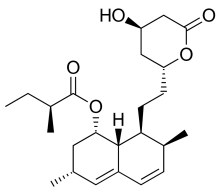
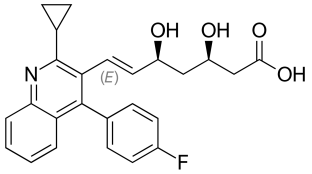
also associated with some negative consequences the most common of which are myopathy, hepato- and nephrotoxicity, and increased risk of developing diabetes mellitus and neurological disorders [2]. The pleiotropic effects of statins are mostly related to the downregulation of production of mevalonate and its derivatives, including farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP) (figure). FPP and GGPP are involved in post-translational protein modification (prenylation), when FPP and GGPP are bound to cysteine residues in the *C*-terminal CaaX motif by the enzymes farnesyltransferase and geranylgeranyl transferase, respectively. GGPP also modifies proteins with the CC or CXC motifs in a reaction catalyzed by geranylgeranyl transferase II. Prenylation is required for membrane anchoring and functioning of a broad spectrum of biological molecules [3]. Among them are small GTPases Ras and RhoB (modified by farnesylation) and Rho, Rac, Cdc42, and Rab (modified by geranylgeranylation). Collectively, these small GTPases are involved in all key signaling pathways controlling proliferation, adhesion, migration, apoptosis, cytokine production, and other cell processes. In addition, prenylation is also used to modify heme A and ubiquinone of the mitochondrial respiratory chain, nuclear lamina proteins, dolichol involved in *N*-glycosylation of membrane-bound proteins, and other molecules (figure), whose deficiency might result in cell death. It is believed that function of prenylation is directly linked to the toxic complications (myopathy, liver cell damage) observed in a long-term use of statins [2]. Lowering cholesterol concentration in the cell membrane by statins might promote redistribution of membrane proteins and alter activity of membrane receptors, as it was demonstrated for caveolin 3 and β-adrenoreceptors [4]. Finally, cholesterol is necessary for the synthesis of vitamin D3 and steroid hormones. It is assumed [2] that by suppressing production of these compounds, high doses of statins contribute to the increased risk of developing autoimmune diseases and sexual disorders.

Statins directly bind to and modulate activity of some proteins. Histone deacetylase 2 (HDAC2) is of special interest among the so-called mevalonate-independ-



Major intracellular targets for statins (see explanation in the text)

Table 1. Statin structure and properties

Statin	Chemical formula	Method of preparation	Properties, predicted log*P** value	Bioavailability, %
Cerivastatin		synthetic	lipophilic, 1.5-1.75	60
Simvastatin		semisynthetic	lipophilic, 1.5-1.75	5
Fluvastatin		synthetic	lipophilic, 1.0-1.25	24
Atorvastatin		synthetic	lipophilic, 1.0-1.25	14
Pravastatin		semisynthetic	hydrophilic, –0.75-(–1.0)	17
Rosuvastatin		synthetic	hydrophilic, –0.25-(–0.5)	20
Lovastatin		natural (from *Aspergillus terreus*)	lipophilic, 3.9-4.1	5
Pitavastatin		synthetic	lipophilic, 2.9-3.8	51

* *P* is *N*-octanol/water partition coefficient.

Table 2. Protein transporters of statins

Transporter	Tissue expression	Statins
OATP1A2	brain, kidney, liver, intestine	rosuvastatin, pitavastatin, lovastatin
OATP1B1	liver	rosuvastatin, pitavastatin, cerivastatin, fluvastatin, pravastatin, simvastatin, atorvastatin, lovastatin
OATP1B3	liver	rosuvastatin, pitavastatin, atorvastatin, fluvastatin, pravastatin
OATP2B1	liver, placenta, heart, skin, muscles	rosuvastatin, pravastatin, atorvastatin, pitavastatin
NTCP	liver	rosuvastatin, pitavastatin, fluvastatin

ent statin targets (figure). In cultured cancer cells, lovastatin inhibits HDAC2 activity, presumably by directly binding to its catalytic site, and causes accumulation of acetylated H3 histone and upregulation of expression of cyclin-dependent protein kinase inhibitor p21. Therefore, statins may be directly involved in the regulation of gene expression [5].

In addition, statins target the lymphocyte function-associated antigen 1 (LFA-1, αLβ2, CD11a/CD18; figure) adhesion molecule of the integrin family involved in lymphocyte adhesion and migration and transduction of the costimulation signal to T cells during antigen presentation. Activation of integrins, including LFA-1, is accompanied by conformational changes resulting in the increased affinity of these molecules to their substrates. Binding to statins (lovastatin, simvastatin, but not pravastatin) prevents such conformational changes in the adhesion molecules [6], which, according to the author's opinion, might explain the anti-inflammatory activity documented for some statins.

The range of the pleiotropic activity of statins depends on their chemical properties and pharmacokinetic parameters, as well as cell sensitivity to these compounds.

Based on physical and chemical properties, statins can be divided into lipophilic and hydrophilic [7] (Table 1). Hydrophilic statins dissolve better in an aqueous phase, while lipophilic statins dissolve better in a lipid phase.

Statins are taken orally; they are easily absorbed in the intestine and captured in the liver. Statins mainly target hepatocytes, where they elicit their hypolipidemic effect. In the liver, statins are partially degraded and excreted in the bile. On average, the bioavailability of statins, i.e., the percentage of active compound found in the blood stream, does not exceed 25% (Table 1) [8, 9]. The ability of statins to enter various cell types depends on their chemical properties and expression of membrane transporters in the cells. Proteins of the OATP (organic-anion-transporting polypeptide) and NTCP (Na^+-taurocholate co-transporting polypeptide families (Table 2) play a major role in the active transport of statins into the cells. Hepatocytes express all types of transporters from these protein families; certain members of the OATP family were also found in the membranes of enterocytes, endothelial cells, and skeletal muscle cells [10-19]. Some studies demonstrated [20] that the transporter-mediated hepatic uptake of statins (e.g., atorvastatin) is suppressed in the presence of blood serum proteins.

Along with active transporter-mediated transfer, statins may penetrate into the cells by passive diffusion across the plasma membrane. Obviously, this route is preferentially used by lipophilic statins. Using immobilized artificial membranes (IAMs), it was demonstrated that the comparative affinity of various statins to the lipid bilayer decreases in the following order: pravastatin < atorvastatin < simvastatin [21]. Hydrophilic statins can also diffuse across the cell membranes. It was shown that at high doses, rosuvastatin and pravastatin are transferred into cultured enterocytes and hepatocytes via both active and passive transport [20, 22]. Therefore, when investigating the mechanism of statin action in cell cultures, the ability of these drug to enter various types of cells, as well as their pharmacokinetics properties, should be taken into consideration.

Some of the most important manifestations of the pleiotropic action of statins are their anti-inflammatory and immunomodulatory activities. Here, we critically analyze the data on the effects of statins on immune cells (monocytes and T cells) that play a key role in developing atherosclerosis and other chronic inflammatory diseases.

STATINS AND MONOCYTES/MACROPHAGES

Monocytes/macrophages are essential for triggering inflammatory processes. When responding to chemotactic stimuli released by the cells at the site of inflammation, monocytes migrate from the blood to tissues to differentiate into macrophages. Macrophages phagocytize

foreign and modified molecules, participate in antigen presentation to T cells, and produce cytokines, growth factors, proteolytic enzymes, reactive oxygen species, and other molecules.

There are three subsets of circulating monocytes [23] that differ in the expression levels of CD14 (a component of receptor complex recognizing lipopolysaccharide (LPS)) and CD16 (low-affinity receptor for the Fc-fragment of IgG, FcγRIII glycoprotein). Among them, classical monocytes dominate (over 80% of total monocyte population); they are characterized by a high level of CD14 expression but lack the CD16 expression (i.e., display $CD14^{++}CD16^{-}$ phenotype). Intermediate monocytes have a high level of CD14 expression and moderate level of CD16 expression ($CD14^{++}CD16^{+}$ phenotype); non-classical monocytes exhibit moderate expression levels for both markers ($CD14^{+}CD16^{+}$ phenotype). Some researchers believe that $CD16^{+}$ monocytes are more mature cells [24]; however, there are also data that, on the contrary, $CD16^{+}$ exhibit the most pronounced inflammatory potential [25]. Moreover, it was suggested [26] that the function of classical monocytes is phagocytosis, while the role of non-classical monocytes is antigen presentation and production of inflammatory cytokines, particularly IL-1β and tumor necrosis factor (TNF). Intermediate monocytes are in a transition state and display the properties of both classical and non-classical monocytes. It was found that the relative content of intermediate and non-classical monocytes in the blood increases in autoimmune and inflammatory disorders (e.g., atherosclerosis) [25]. Kashiwagi et al. [27] demonstrated that elevated amount of $CD14^{+}CD16^{+}$ monocytes in the blood is associated with atherosclerotic plaque instability.

The data on the effects of various HMG-CoA reductase inhibitors on the absolute or relative content of monocyte subsets in the blood are scarce and controversial. Earlier studies showed that treatment of hypercholesterolemic patients with fluvastatin increases the population of $CD16^{+}$ monocytes [24]. However, Jaipersad et al. [28] found that simvastatin and atorvastatin failed to cause any significant changes in the absolute numbers of monocyte subsets in the blood. Temporal cessation of statin therapy for two weeks had no effect on the amount of $CD14^{++}CD16^{-}$, $CD14^{++}CD16^{+}$, and $CD14^{+}CD16^{++}$ monocytes in the blood, as well as on the expression of the CCR2 receptor for the chemoattractant protein 1 (MCP-1) in patients with stable coronary artery disease. However, expression of the vascular endothelial growth factor receptor type 2 (VEGFR2), angiopoietin receptor Tie2, and Toll-like receptor TLR4 was upregulated. In authors' opinion, this indicates that statins regulate the angiogenic and inflammatory potentials of monocytes. Previously, we demonstrated [29] that administration of high doses of atorvastatin (80 mg/day) for 7 days caused no significant changes in relative and absolute amounts of classical, non-classical, and intermediate monocytes in the peripheral blood.

Imanishi et al. [30] found that statins (rosuvastatin and atorvastatin) prevent increase in the amount of circulating $CD14^{+}CD16^{+}$ monocytes in patients with unstable angina pectoris who received statin treatment compared to patients who did not. Coen et al. [31] followed the changes in the amount of $CD14^{+}CD16^{+}$ monocytes in rosuvastatin-treated hypercholesterolemic patients and found that the population of these cells was the lowest in patients that participated in the exercise training program, thereby suggesting the synergistic anti-inflammatory effect of exercise in combination with statin therapy. In HIV patients, rosuvastatin administration for 24 weeks promoted the decrease in the blood level of tissue factor-expressing $CD14^{+}CD16^{+}$ monocytes and reduced the concentration of the monocyte activation marker sCD14 [32]. The authors believe that administration of statins in addition to the basal therapy might contribute to prevention of cardiovascular diseases in HIV patients. Several lines of evidence suggest that the shift in the ratio between monocyte subsets toward $CD14^{+}CD16^{+}$ cells might be induced by percutaneous coronary intervention (PCI), which subsequently leads to peri-procedural myocardial injury. Therefore, pre-procedural statin administration might prevent expansion of inflammatory monocytes and have a cardioprotective effect [33].

Fildes et al. [34] suggested that the modality of statin-mediated effects on the relative contents of the monocyte subsets depends on physical and chemical properties of statins. Statin administration after heart transplantation decreased the total population of circulating monocytes. However, patients receiving atorvastatin had fewer classical $CD14^{++}$ monocytes; patients receiving pravastatin had fewer non-classical and intermediate $CD16^{+}$ monocytes.

Therefore, the data on the statin-induced effects on the circulating monocyte phenotype remain quite controversial. Perhaps, this is due to the use of non-matched groups of patients and volunteers in the studies, inclusion of patients with various nosologies (acute coronary syndrome, stable angina), as well as multiple dose regimens and courses of statin administration.

The impact of statins on the monocyte/macrophage effector functions, such as adhesion, migration, antigen presentation, and production of cytokines, metalloproteases, and reactive oxygen species have been investigated in clinical and experimental studies in cell cultures.

It was found that treatment of human promonocytic THP-1 cell line with simvastatin (16-20,000 nM) for 16 h resulted in the suppression of the cell ability to migrate in response to the major monocyte chemoattractant MCP-1 [35]. This effect of simvastatin was fully reversed by mevalonate and its derivatives (FPP, GGPP), but not by ubiquinone. The authors did not discuss the observed phenomenon; however, we suggest that it can be

explained by statin-mediated downregulation of the MCP-1 receptor expression. Thus, Veillard et al. [36] showed that simvastatin inhibits INF-γ-induced expression of the chemokine receptors CCR1, CCR2, CCR4, and CCR5 (as assessed by changes in the levels of the corresponding mRNAs) in human primary macrophages and that the effects of simvastatin were related to the reduced intracellular GGPP level.

Statin-induced expression of chemokine receptors in monocytes was also observed in clinical studies. For instance, in patients with acute coronary syndrome, simvastatin downregulated expression of the fractalkine receptor CX3CR1 in a dose- and time-dependent manner [37]. Simvastatin contributed to the reduced expression of the CCR2 receptor of MCP-1 in circulating monocytes in healthy volunteers [38]. We found [29] that monocytes and lymphocytes of the ischemic heart disease patients treated with high doses of atorvastatin (80 mg/day) for 7 days had a decreased density of the CCR5 receptor for the chemokines RANTES (regulated on activation, normal T-cell expressed and secreted), MIP (macrophage inflammatory protein)-1α, and MIP-1β. Altogether, these data show that cells from statin-treated patients have a limited ability to migrate to the inflammation site.

This reduced cell ability to migrate into body tissues might be due to the downregulated expression of adhesion molecules. Weber et al. [39] demonstrated that lovastatin (1-10 μM) promotes a decrease in the CD11b (αM-subunit of β2-integrin Mac-1) content at the surface on human cultured blood-derived monocytes; similar changes were observed in hypercholesterolemic patients treated with lovastatin or simvastatin for 6 weeks. The decrease in the integrin density was accompanied with suppression of the monocyte adhesion to the endothelial cell monolayer. Stulc et al. [40] assessed expression of L-selectin and integrins Mac-1, LFA-1, and VLA-4 (α4β1, CD49d/CD29) in leukocytes from hypercholesterolemic patients before and after atorvastatin therapy (20 mg/day, 12 weeks) and found that atorvastatin decreased the levels of LFA-1 and VLA-4, but not of the other adhesion molecules. Treatment with atorvastatin of normocholesterolemic patients with coronary heart disease resulted in a decreased expression of integrins Mac-1 and LFA-1, as well as the immunoglobulin superfamily adhesion molecule ICAM-1 [41]. On the other hand, Cerda et al. [42] showed that simvastatin administered to hypercholesterolemic patients caused no changes in the amounts of selectin and integrin mRNAs in mononuclear cells. Atorvastatin and simvastatin downregulated expression of L-selectin, selectin ligand PSGL-1, and VLA-4 in the THP-1 monocytic cells only when used at a high non-physiological dose (10 μM). No impact of the assayed statins on the β2-integrin expression was observed. Hence, currently there are no data unambiguously proving that statins suppress expression of monocyte adhesion molecules.

There are very few studies on the effect of statins on monocyte motility. Montecucco et al. [43] observed that pretreatment of peripheral blood monocytes with atorvastatin or simvastatin (1 μM, 2 h) suppressed their C-reactive protein (CRP)-induced migration. Inhibitory analysis and estimation of the levels of phosphorylation of signaling molecules demonstrated that this suppression was mediated by inhibition of the mitogen-activated protein (MAP) kinase ERK 1/2. Treatment with L-mevalonate or FPP, but not GGPP, reversed the statin-induced effect on the CRP-mediated functions. Finally, no data on the impact of statins on the monocyte chemotaxis in response to other stimuli (e.g., chemokines) are yet available.

Therefore, the impact of statins on the monocyte/macrophage migratory activity is determined by suppressing the ability of monocytes/macrophages to sense chemotactic signals (e.g., via suppression of chemokine receptor expression) and, probably, by reducing the activity of adhesion molecules rather than by directly affecting the cell motility.

Statins regulate production of cytokines including chemokines in monocytic cells. In cultured human INFγ-stimulated macrophages, simvastatin reduced the amounts of secreted MCP-1 and decreased the levels of mRNAs for MCP-1 and other chemokines such as MIP-1α and MIP-1β [36]. Fluvastatin and lovastatin inhibited in a dose-dependent manner the LPS-induced secretion of IL-6 in THP-1 monocyte cells [44]. Similarly, atorvastatin downregulated production of TNF, IL-6, IL-1β and expression of the LPS receptor TLR4 [45]. Montecucco et al. [43] showed that atorvastatin and simvastatin decreased the CRP-induced secretion of MCP-1, MIP-1α, and MIP-1β by peripheral blood monocytes. It was mentioned above that statin-induced suppression of the CRP-dependent effects including the impact on cytokine production and cell motility was due to L-mevalonate/FPP-mediated inhibition of ERK1/2. Clinical studies demonstrated that treatment of patients with isolated hypercholesterolemia with simvastatin for 3 months resulted in the suppression of the LPS-induced secretion of pro-inflammatory cytokines TNF, IL-1β, and IL-6 by the peripheral blood monocytes [46, 47]. Moreover, patients with moderate hypercholesterolemia treated for 8 weeks with pravastatin and simvastatin tended to have lower amounts of the LPS-stimulated MCP-1 chemokine in the blood (monocytes being the major source of MCP-1 in this case) as compared to placebo-treated patients, but the difference was statistically insignificant [47].

Therefore, some studies show that statins suppress production of pro-inflammatory cytokines (IL-1β, TNF, IL-6) and chemokines. On the contrary, Kuijk et al. [48, 49] observed that simvastatin upregulated IL-1β at the posttranslational level in the LPS-stimulated THP-1 promonocytic cells. The authors explain this phenomenon by activation of the Rac1/PI3K/Akt pathway under

conditions of isoprenoid deficiency, resulting in the emergence of active caspase-1 that processes of the key pro-inflammatory cytokine IL-1β from an inactive precursor to an active molecule. Keiner et al. [50] found that lipophilic simvastatin, atorvastatin, and lovastatin, but not hydrophilic pravastatin, stimulated secretion of IL-1β, TNF, MCP-1, and IL-8 by human monocytes. Lindholm and Nillson [51] demonstrated that in the absence of endotoxin stimulation, simvastatin treatment significantly increased the secretion of IL-1β and IL-8 from macrophages, whilst inhibiting the secretion of TNF and having no significant effect on IL-6 secretion. These data support the idea that statins display a complex effect and that the cell response to the drug depends on statin type and cell microenvironment. However, it should be noted that the above-described effects were observed when statins were used at high doses (10 μM) that significantly exceed their predicted levels in the blood serum [52]. It may be assumed that this mechanism of statin action might take place in the liver (for example, Kupffer cells), i.e., in body tissues with the highest concentration of statins.

The impact of statins on cytokine production might depend on type of affected cells. Lovastatin enhanced LPS-induced TNF production via the mevalonate-dependent pathway in murine RAW264.7 macrophages. In particular, it reduced the amount of geranylgeranylated Rho proteins (RhoA, Cdc42, Rac1) bound to the plasma membrane, but did not alter the content of farnesylated G-proteins of the Ras family. Endotoxin-stimulated cells treated with lovastatin displayed a more pronounced ERK1/2 activation, while ERK1/2 inhibitors cancelled the statin-triggered effects [53]. Similar effect of lovastatin was observed in murine dendritic cells (DCs) derived from the bone marrow myeloid cells, where it stimulated LPS-induced production of IL-6, TNF, and IL-12 via the mevalonate-dependent pathway. Moreover, lovastatin treatment also caused simultaneous increase in the mRNA levels for the granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-2, IL-6, MCP-1, and TNF [54]. Lee et al. showed that murine RAW264.7 cells and THP-1 human monocytic cells respond differently to simvastatin treatment: the drug inhibited INF-γ-induced cyclooxygenase-2 expression in RAW264.7 cells but not in THP-1 cells [55]. According to [56, 57], human leukocytes are more sensitive to statins than rodent leukocytes due to species-specific features of the HMG-CoA reductase activity regulation.

As seen from above, the data on the statin-induced *in vitro* effects on cytokine expression in monocytes/macrophages are highly controversial due, probably, to the discrepancies between the used cell models and differences in the experimental settings, concentrations of statins used, and duration of exposure to statins.

Inflammatory cytokines elevate the ratio between matrix metalloproteinases (MMPs) and endogenous tissue inhibitors of metalloproteinases (TIMPs) at the site of inflammation. Increasing the activity of MMPs in an atherosclerotic plaque might result in the rupture of its fibrous cap and following thrombosis [58, 59]. These data demonstrate the importance of studying statin-induced effects on the MMP and TIMP expression in monocytes/macrophages and MMP activity in cell culture *in vitro*.

Fluvastatin and cerivastatin suppress MMP-9 activity in human macrophages derived from the peripheral blood monocytes [60, 61]. In the same cell model, rosuvastatin suppresses MMP-7 activity [62]. Using inhibiting of protein synthesis with actinomycin D, it was shown that statins act post-translationally via blocking the MMP secretion. Similar data were obtained in rabbit foam macrophages, where cerivastatin at a concentration close to the physiological one inhibited secretion of MMP-1, -3, -9, but did not affect the levels of the corresponding mRNAs [63]. In all these cases, isoprenoids GGPP and FPP reversed the inhibitory action of statins. A single study [64] uncovered the effects of statins on MMP and TIMP expression in the THP-1 cell line: atorvastatin inhibited the CRP-induced expression of MMP-1, -2, -9 via the mevalonate-dependent pathway without significantly affecting the level of TIMP-1 mRNA. However, it should be noted that such effect was observed only when atorvastatin was applied at a sufficiently high concentration (>10 μM).

There are very few studies on the effect of statins on the production of reactive oxygen species in macrophages. Delbosc et al. [65] demonstrated that simvastatin (10-50 μM) suppresses phorbol-12-myristate-13-acetate-induced production of superoxide anion by NADPH oxidases in THP-1 cells. This effect is mediated by the FPP-dependent inhibition of prenylation of the NADPH oxidase p21 rac subunit resulting in the impaired assembly of the active enzyme at the plasma membrane inner surface. However, in a culture of human blood-derived monocytes, lipophilic statins (atorvastatin, lovastatin, simvastatin, 10 μM) exhibited a mild but significant stimulatory effect on the production of reactive oxygen species via the mevalonate-dependent pathway [50].

It should be emphasized that in the majority of the above-mentioned studies, statins were used at the concentrations substantially exceeding their maximum amounts in the blood serum (<100 nmol/liter), thus allowing some researchers [57] to question the relevance of the applied models and reliability of the *in vitro* data for interpreting pleiotropic statin-related effects *in vivo*. Moreover, some studies merely describe but do not investigate the molecular mechanism of statin action. Obviously, an impact of physiological concentrations of statins on the effector functions of monocyte/macrophage including production of reactive oxygen species, cytokines, MMPs, and other inflammatory mediators requires further investigation.

Monocytes bridge innate and adaptive immunity via phagocytizing foreign molecules and presenting antigens to T cells. A number of studies have examined the effect of statins on antigen presentation in peripheral blood monocyte-derived DCs. Treatment of DCs with statins suppressed maturation of these cells induced by various factors (LPS, oxidized low density lipoprotein, TNF, IL-1β, and prostaglandin E2) and inhibited expression of the major histocompatibility complex class II receptor HLA-DR and molecules involved in costimulation of T cells, such as CD80/CD86 (B7-1/2), CD40, and CD83 [66-69]. Pre-incubation of the LPS-stimulated DCs with simvastatin and atorvastatin (0.1-1 μM) suppressed the ability of these cells to activate T helper cells resulting in reduced proliferative response, lowered INF-γ and IL-2 secretion, and suppressed differentiation towards the type 1 T helpers [67].

Leuenberger et al. [69] explained the impact of statins on the antigen presentation in DCs by changes in the cytoskeleton. It was found that in maturing DCs, atorvastatin (1-5 μM) markedly reduced the amount of intracellular F-actin presumably due to altered Rho GTPase activity. Cytoskeleton rebuilding results in the impaired intracellular exosome transport required for the surface expression of various molecules involved in antigen presentation in DCs. This impaired exocytosis after exposure to statins might be due to a decreased activity of small Rab GTPases that bind to the exosomal membranes via GGPP and regulate intracellular membrane trafficking [70].

When treated with statins, DCs derived from peripheral blood monocytes were not only able to efficiently present antigens to T cells, but also promoted expansion of T cells with the suppressor phenotype [69]. So far, there is no evidence that such statin-related effects on the differentiation on antigen-presenting cells exist *in vivo*. Nonetheless, the data obtained may be used to develop new approaches to cell therapy of autoimmune and allergic diseases.

STATINS AND T CELLS

Along with the impact on T cells via affecting the DC phenotype and activity, statins can influence lymphocytes directly. Here, we summarize the data on the effects of statins on the proliferation and migration of T cells and differentiation of the major subsets of T helper ($CD4^+$) cells.

Earlier studies demonstrated that HMG-CoA reductase inhibitors, such as lovastatin and statin precursor compactin, inhibit phytohemagglutinin (PHA)-induced T cell proliferation via the mevalonate-dependent pathway [71, 72]. Kurakata et al. [73] compared the anti-proliferative effects of hydrophobic simvastatin and hydrophilic pravastatin at different modes of lymphocyte activation (PHA, IL-2, mixed lymphocyte culture). In all the cases, simvastatin was more efficient than pravastatin (IC_{50} ~ 0.013 vs. 5.6 μM, respectively), which is presumably related to its higher potential to penetrate through the lymphocyte plasma membrane in the absence of specific transporter proteins. These observations were confirmed by Overton et al. [74] who showed that atorvastatin and, to a lesser extent, rosuvastatin, but not pravastatin, strongly suppressed superantigen (staphylococcal enterotoxin B)-stimulated proliferation of T helper $CD4^+$ and cytotoxic $CD8^+$ cells from HIV patients. Mevalonate partially reversed the inhibitory effects of statins. Unfortunately, the authors did not specify the concentration of statins used in the study.

The impact of statins on the number of circulating T cells expressing activation markers such as HLA-DR and CD38 was investigated in the clinical studies in HIV patients (the number of $HLA\text{-}DR^+CD38^+$ cells has a prognostic value and is estimated to assess the activity of the HIV infection). Treatment with rosuvastatin and atorvastatin caused a reduction in the relative content of peripheral blood $CD38^+HLA\text{-}DR^+$ T cells. However, it should be noted that the effect of rosuvastatin was observed only after 48 weeks of treatment, whereas the effect of atorvastatin was found already after 8 weeks of treatment [32, 75] suggesting that the latter has a greater immunomodulatory potential. Unfortunately, the authors have not investigated the mechanism of statin action on T cells.

Statins can affect lymphocyte migratory activity via direct impact on LFA-1 and mevalonate-dependent inhibition of small G-proteins. As mentioned above, statin (lovastatin, etc.) binding to the integrin dimer α-subunit hinders LFA-1 activation. The anti-inflammatory effect of the lovastatin derivative LFA878 is mediated by the LFA-1 inhibition, as it was shown in the carrageenan-induced rat paw edema assay [76]. Waiczies et al. [77] found that pre-incubation with atorvastatin suppresses SDF-1-induced chemotaxis of human peripheral blood mononuclear cells by about 50%. The effect of atorvastatin on the lymphocyte migration was due to the impaired geranylgeranylation of RhoA that prevented its anchoring to the membrane and resulted in cytosolic localization of this GTPase. As a result, activation of Rho-stimulated kinase ROCK1 was inhibited resulting in altered actin filament organization and impaired cell motility.

The majority of statin studies are aimed at investigating the influence of statins on the effector and regulatory T cell subsets. Type 1 helper T cells (Th1), type 2 helper T cells (Th2) and T helpers 17 (Th17) represent the major effector populations among Th cells. Th1 cells are characterized by expression of the transcription factors T-bet and STAT4. By producing INF-γ, IL-2, TNF, GM-CSF, and other cytokines, Th1 cells activate macrophages and NK cells and stimulate proliferation of cytotoxic T cells.

Moreover, Th1 cells trigger cell-mediated adaptive immunity and enhance delayed-type hypersensitivity reactions via activating macrophages [78]. Th2 cells express transcription factors GATA3 and STAT6 and produce IL-4, IL-5, and IL-13 that induce B cell proliferation and differentiation and stimulate antibody production. Th2 cells are involved in allergic reactions by playing a crucial role in the regulation of IgE synthesis [78]. The balance between T cell subsets *in vivo* is maintained via specific cytokines: Th1 cells suppress Th2 cell activity and *vice versa* [79]. Multiple studies demonstrated that Th1 cells exhibit elevated activity in atherosclerosis and other Th1-dependent chronic inflammatory disorders [80-85]. Among minor T cell subsets in the peripheral blood, Th17 cells are characterized by production of neutrophil-recruiting cytokines IL-17A and IL-17F, as well as IL-22 and IL-26 [86-88]. T cell differentiation towards Th17 phenotype is controlled by the transcription factors RORγt and RORC. It was shown that Th17 cells play an important role in pathogenesis of various autoimmune diseases, including multiple sclerosis, systemic lupus erythematosus, and rheumatoid arthritis. The severity of tissue damage in these diseases correlates with the amount of Th17 cells [89, 90].

Regulatory T cells (Tregs), type 3 T helper cells, and type 1 regulatory T cells are $CD4^+$ T cells involved in maintaining immune homeostasis. Among them, Tregs display the highest immunosuppressive activity. Natural Tregs undergo maturation in the thymus, whereas induced Tregs are developed at the periphery during immune response. Expression of the transcription factor Foxp3 is necessary for Treg maturation and functioning. Tregs produce the anti-inflammatory cytokines IL-10, transforming growth factor-β (TGF-β), and IL-35, ensure the tolerance to autoantigens, and control exuberant immune response by preventing development of inflammatory and autoimmune reactions [79, 91].

A number of experimental studies modelling various autoimmune diseases have demonstrated that statins promote skewing from the Th1- to Th2-type immune response, thereby having a beneficial effect on the disease progression [92]. It was shown that mice with experimental autoimmune encephalomyelitis (model of human multiple sclerosis) treated with atorvastatin had an improved motor activity along with the elevated production of Th2 cell cytokines (IL-4, IL-5, IL-10) and decreased secretion of Th1 cell cytokines (IL-2, IL-12, INF-γ, TNF). The atorvastatin-related mechanism of immunomodulation included mevalonate-dependent upregulation of the transcription factor STAT6 involved in the IL-4-dependent differentiation of naïve (Th0) T cells towards the Th2 phenotype, which was paralleled with the transcription downregulation of STAT4 participating in the IL-12-dependent Th1 cell differentiation. However, exact intracellular pathways involved in the modulation of the activity of these transcription factors in response to atorvastatin (mevalonate deficiency) were not clarified [93]. Atorvastatin administered to mice with experimental autoimmune encephalomyelitis and rats with experimental autoimmune myocarditis converted T cells from the Th1 to Th2 phenotype, as demonstrated by measuring signature cytokines in the blood serum [94, 95]. Lovastatin produced similar effect on the Th1/Th2 cell ratio in mice with the delayed-type hypersensitivity: the levels of mRNAs for INF-γ, IL-12, IL-1, and TNF at the site of inflammation decreased, while the levels for the IL-4, IL-10, and TGFβ mRNAs were upregulated [72]. Nath et al. [96] observed that in naïve T cells of mice with experimental autoimmune encephalomyelitis, lovastatin stimulated expression of the GATA3 transcription factor, increased phosphorylation of STAT6, and downregulated phosphorylation of Janus kinase 2 and STAT4, thereby creating the conditions for preferential differentiation of Th0 cells into Th2 cells upon stimulation with myelin fragments both *in vivo* and *in vitro*. Moreover, lovastatin suppressed the activity of nuclear transcription factors T-bet and NF-κB in activated T lymphocytes, inhibited the synthesis of INF-γ in Th1 cells, and stabilized production of IL-4 in Th2 cells. Dunn et al. [97] found that in mice with experimental autoimmune encephalomyelitis, atorvastatin shifts T cell differentiation towards the Th2 phenotype by altering RhoA geranylgeranylation and Ras farnesylation, which impairs signal transduction from the T cell receptor to the ERK1/2 and p38 kinases and c-fos proto-oncogene and eventually alters proliferation of T cells *in vitro* and their differentiation potential.

Statins modulate Th1/Th2 differentiation not only by directly targeting T cells, but also via regulating DC functions. In particular, Arora et al. [98] demonstrated that the phenotype and the properties of murine DCs were modified in response to simvastatin, so that the contact of these cells with $CD4^+$ lymphocytes triggered expression of GATA3 and downregulated expression of T-bet in the latter. As a result, primed T cells produced IL-4, IL-5, and IL-13, but not INF-γ. The statin-induced effects in DCs were reversed in the presence of mevalonate.

According to other studies, modulation of the T helper cell differentiation, in particular, the shift in Th1/Th2 balance towards the Th2 phenotype, is not the only mechanism underlying the beneficial action of statins in *in vivo* models of autoimmune disorders. Thus, orally administered atorvastatin did not induce Th2 cells in the spleen of experimental animals. Moreover, atorvastatin positively affected the course of experimental autoimmune encephalomyelitis in $STAT6^{-/-}$ mice unable exhibit the Th2 cell-type immune response [99]. The authors suggested that the anti-inflammatory effect of statins was mediated by suppression of the Th1 cell activation and proliferation. The absence of selective Th2 cell-targeted activity of statins has been documented in

other studies as well. For instance, in rats with experimental autoimmune myocarditis, fluvastatin inhibited expression of both Th1- and Th2-produced cytokines [100]. Simvastatin suppressed expansion of Th1, but not Th2 cells in mice with collagen-induced arthritis [101]. Lovastatin administered to rodents with experimental autoimmune uveoretinitis suppressed the T cell proliferation and inhibited INF-γ production but did not affect cytokine expression by Th2 cells [102, 103]. Finally, in mice with experimental autoimmune uveitis, atorvastatin did not affect the production of both Th1 and Th2 cytokines [104]. The authors explained the lack of cell response to atorvastatin by the low penetration of the drug into the ocular tissues, as well as by possible natural resistance of the studied mouse strain to immunomodulatory stimuli. Altogether, these data prove that the action of statins on the subsets of effector T cell is non-selective.

A number of studies provided evidence that the main targets for the immunosuppressive action of statins are Th1 and Th17 cells, i.e., subpopulations with the highest pro-inflammatory potential. It was shown that simvastatin orally administered to mice with experimental autoimmune encephalomyelitis improved the clinical course of the disease by suppressing infiltration of mononuclear leukocytes (including Th1 and Th17 cells) in the central nervous system. Moreover, the animals demonstrated decreased T cell proliferative activity in the spleen and upregulated cerebral expression of the anti-inflammatory TGF-β together with reduced expression of the pro-inflammatory cytokines and chemokines, such as IL-6, IL-12p40, IL-12p70, RANTES, and MIP-1β [105]. Treatment of mice with experimental autoimmune myocarditis with pitavastatin for 3 weeks resulted in lowered phosphorylation of STAT3 and STAT4 in the heart and suppressed production of INF-γ and IL-17 by cardiac $CD4^+$ T cells [106]. Moreover, cultured T cells treated with pitavastatin failed to differentiate towards Th1 and Th17 phenotypes due to the inhibition of signature transcription factors T-bet and RORγt, respectively. Atorvastatin therapy in mice with experimental autoimmune glomerulonephritis promoted a decrease in the amounts of Th1 and Th17 cells in the renal tissues and regional lymph nodes draining the kidneys [107].

It should be noted that in animal studies, statins are commonly used at a concentration of 1-100 mg/kg body weight, whereas the therapeutic dose for humans does not exceed 1 mg/kg body weight. The use of high doses of statins in rodents is justified by their pharmacodynamics resistance and a need to reach high drug concentrations in order to inhibit the mevalonate pathway [108-110]. Nonetheless, the differences in the activity of statins in animals and humans should be taken into consideration to properly interpret experimental data.

Despite multiple studies conducted in animals, very few publications are available that investigate the mechanism of statin action on the subsets of effector T cells in humans. For instance, it was shown that simvastatin (10 mg/day for 12 weeks) lowered the Th1/Th2 cell ratio in the peripheral blood of rheumatoid arthritis patients. The immunomodulatory effect of statins was accompanied with improved clinical state of the patients, decreased the erythrocyte sedimentation rate, and reduced the contents of CRP and rheumatoid factor in the blood serum [111]. Simvastatin downregulated via the mevalonate pathway expression of the Th17 cell-associated transcription factor RORC and suppressed secretion of IL-17 and IL-21 involved in IL-17 autocrine regulation by $CD4^+$ T cells isolated from patients with multiple sclerosis and healthy donors [112]. These data confirm that statins suppress Th1 and Th17 cell differentiation and activation in humans; however, this topic requires further investigation.

Treg cells are another important target of statins, which has been proven in several experimental and clinical studies. Thus, simvastatin *in vitro* induced expression of the transcription factor Foxp3 in spleen $CD4^+$ cells purified from $RAG^{-/-}$ transgenic mice with impaired lymphocyte maturation. Apart from directly upregulating Foxp3 expression, simvastatin enhanced TGF-β-mediated effect on the induction of peripheral Treg cells by inhibiting methylation of the *Foxp3* gene promoter and suppressing activation of the Smad6 and Smad7 proteins. The effects of simvastatin were found to be related to a blockade of protein geranylgeranylation [113]. Similar results were obtained in a culture of murine spleen $CD4^+$ T cells, where simvastatin promoted maturation of $Foxp3^+CD4^+$ T cells along with blockade of Th17 cell differentiation. The observed effect was reproduced by adding geranylgeranyltransferase inhibitor GGTI-289, but not farnesyltransferase inhibitor FTI-227, suggesting that intracellular protein geranylgeranylation may be involved in skewing T cell differentiation towards Th17 rather than Treg cells [114]. Rosuvastatin administered intravenously to rats with myocardial infarction (ischemia-reperfusion model) upregulated expression of the transcription factor Foxp3 in both the myocardium and the spleen and increased the content of myocardial Treg cells. Other effects of rosuvastatin administration were lower levels of cardiac troponin I in the blood serum and reduced size of infarcted area after ischemia-reperfusion. The effects of rosuvastatin were fully reversed by mevalonate [115]. Simvastatin administered intragastrically to $ApoE^{-/-}$ mice significantly increased the content of Tregs and upregulated Foxp3, TGF-β, and IL-10 in aortic atherosclerotic lesions [116]. Atorvastatin administered for 7 days to sensitized mice with atopic asthma increased IL-10 concentration in the lung tissue and caused accumulation of Tregs in mediastinal lymph nodes accompanied with a significant decrease in the peribronchial inflammation [117].

Mausner-Fainberg et al. [118] assessed the effects of statins on the number of $Foxp3^+$ Treg cell in the samples

of peripheral blood mononuclear cells from donors. It was found that atorvastatin (but not mevastatin or pravastatin) promoted accumulation of activated $CD4^+Foxp3^+$ Tregs. Similarly, atorvastatin increased the content of Tregs in the primary culture of lymphocytes from rheumatoid arthritis patients [119]. Inhibitory analysis demonstrated that the effect of atorvastatin might be mediated by the blockade of the intracellular PI3K/Akt/mTOR signaling pathway and ERK1/2 kinase. ERK1/2 kinase is a target for small GTPases Ras and Rho, and its activity may be affected by statins via inhibition of GGPP/FPP synthesis. Meng et al. [116] observed that addition of simvastatin to cultured mononuclear cells purified from the peripheral blood of patients with acute coronary syndrome resulted in accumulation of Tregs and increase in their immunosuppressive potential. However, it should be noted that such *in vitro* effects were observed when statins were used at a concentration of $\geqslant$5-10 μM. It cannot be ruled out that the observed effects were, at least, partially due to the depletion (apoptosis) of primarily $Foxp3^-$ cells in the presence of high statin concentrations rather than to differentiation of $Foxp3^+$ Tregs from $Foxp3^-$ T cells.

A number of clinical studies prove that statins display immunomodulatory activity related to Treg cell expansion. In particular, simvastatin or pravastatin administered for 8 weeks increased 2 to 3 times the relative content of Tregs in the peripheral blood of hypercholesterolemic patients [118]. Atorvastatin therapy (12 weeks) in patients with rheumatoid arthritis resulted in improved clinical parameters (decreased erythrocyte sedimentation rate and CRP concentration) and elevated number of active Tregs cells, as well as in improved overall health state of the patients [119]. Atorvastatin and lovastatin administered at standard doses to healthy volunteers upregulated expression of Foxp3, TGF-β, and IL-10 by Tregs and increased Treg count in the peripheral blood [120].

Changes in the parameters of cell-mediated immunity in response to statins may be observed even after a short-term therapy or upon the increase in the drug dose. Treatment of patients with acute coronary syndrome with atorvastatin for 2 weeks was accompanied with an elevated content of circulating Tregs and increased inhibitory effect of these cells on proliferation of effector T cells. Moreover, statin-treated patients had lower levels of INF-γ and increased levels of TGF-β and IL-10 in the blood serum. The Treg cell count directly correlated with the TGF-β concentration and inversely correlated with the INF-γ level, which allowed the authors to suggest the anti-inflammatory effect of atorvastatin [121]. It was found that patients with ST-segment elevation myocardial infarction who received atorvastatin (80 mg/day) before primary percutaneous coronary intervention had higher Treg cell count in the peripheral blood, higher levels of mRNAs for Foxp3 and TGF-β, but lower INF-γ in peripheral mononuclear cells, as compared to patients treated with a standard dose of statin (20 mg/day) [122]. We have demonstrated [29] that the relative Treg count was higher in patients that received a long-term atorvastatin (20 mg/day) therapy than in patients who did not. Moreover, increasing atorvastatin dose from 20 to 80 mg/day for 7 days resulted in further elevation of the Treg cell count in the peripheral blood.

Thus, the use of statins may be associated with certain changes in the cell-mediated immune response, in particular, reduced amount of effector T cells (mainly Th1 and Th17) along with the increased relative and absolute counts of active Treg cells. It seems that skewing the immune balance towards its regulatory arm is one of the main mechanisms underlying the anti-inflammatory effects of statins.

Statins as HMG-CoA reductase inhibitors are among the most commonly prescribed pharmaceuticals. Along with the major lipid-lowering effect, they exhibit a broad range of pleiotropic activities, mostly mediated via suppression of mevalonate synthesis and, therefore, formation of its derivatives (cholesterol, GGPP, and FPP) involved in post-translational modification of intracellular proteins, including small GTPases and mitochondrial proteins. Statin-related mevalonate-independent effects are associated with unique properties of particular molecules and, apparently, are not class-specific.

Cell response to statins mainly depends on the physical and chemical properties of statin molecules, as well as the drug sensitivity of the affected cells. In was shown that leukocytes (particularly monocytes and lymphocytes) lack proteins transferring statins into the cells; therefore, the action of statins in these cells is determined by the ability to passively penetrate across the plasma membrane. Classification of statins into lipophilic and hydrophilic according to their ability/inability to diffuse in the cell membrane should be used with care, as hydrophilic statins can enter the cells in the absence of specific protein transporters, although it requires higher drug concentrations and longer cell exposure to the drug compared to lipophilic statins. Moreover, the extent of lipophilicity/hydrophilicity differs in different statins (Table 1). For instance, based on its water/lipid solubility, atorvastatin is closer to hydrophilic rosuvastatin than to lipophilic lovastatin or pitavastatin, which probably explains its less pronounced pleiotropic effects as compared to other lipophilic statins. Finally, pharmacokinetic properties of statins should be taken into consideration when studying statin-related pleiotropic effects in cell cultures, so that the *in vitro* concentrations of the tested drugs are comparable with their amounts in the human blood serum.

Despite numerous experimental and clinical studies of the immunomodulatory activity of statins, the obtained data remain controversial, particularly, those on the effect

of statins on the leukocyte subset composition and monocyte properties. Based on the clinical and experimental studies that used statins at physiological concentration, it was suggested that statins modulate migration of monocytes to the site of inflammation by downregulating expression of chemokine receptors and, to a lesser extent, adhesion molecules. Effects of statins on other parameters of monocytes/macrophages, e.g., cytokine production, require further investigation. Statins also affect T cell-mediated immune response. For example, animals treated with statins have a decreased amount of effector T cell subsets, mainly Th1 and Th17, as well as an increased content of active Treg cells. Statin-mediated skewing of immune balance towards pro-inflammatory T cells was confirmed in several clinical studies including our pilot studies. However, there is lack of data comparing effects of different statins on immunological parameters in humans. We believe that comparative analysis of immunotropic activity of lipophilic and hydrophilic statins, as well as the dose-effect relationship, is of high priority.

The immunomodulatory and anti-inflammatory effects of statins targeting differentiation and activity of immune cells may be beneficial in patients with chronic inflammatory and autoimmune diseases. On the other hand, in immunocompromised patients and individuals at a high risk of oncological diseases, such additional immunosuppression would be undesired. Therefore, it is extremely important to continue examining the mechanism of statin action in order to come closer to the development of patient-oriented personalized medicine.

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