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Statin therapy in autoimmunity: From protein prenylation to immunomodulation

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Abstract

Statins have been prescribed extensively for their cholesterol-lowering properties and efficacy in cardiovascular disease. Compelling evidence now exists, however, that statins also possess extensive immunomodulatory properties that operate independently of lipid lowering. Consequently, much attention has been directed towards their potential as therapeutic agents in the treatment of autoimmune disease. Modulation of post-translational protein prenylation seems to be a key mechanism by which statins alter immune function. In this article, the effect of statin therapy on immune function, and how this impacts on the pathogenesis of autoimmune disease, will be reviewed alongside current opinion of the key biological targets.

The 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, generically referred to as statins, have emerged as the leading therapeutic regimen for treating HYPERCHOLESTEROLEMIA and reducing cardiovascular morbidity and mortality. The clinical use of statins has become so prevalent that they are now prescribed to more than 25 million people worldwide, with the number estimated to rise rapidly. These compounds exert their biological effect by inhibiting HMG-CoA reductase, which is an upstream rate-limiting enzyme in the cholesterol synthesis pathway (FIGURE 1). The consequent reduction in circulating LOW-DENSITY LIPOPROTEIN (LDL) CHOLESTEROL, which provided the original rationale for treating cardiovascular disease, was until recently believed to be the major therapeutic effect¹. It has become increasingly apparent, however, that the beneficial effects of statins in cardiovascular medicine cannot be ascribed solely to their lipid-lowering properties^{2,3}. So, just as it emerged that coronary disease comprises a major inflammatory component^{4,5}, so it became evident that statins modulate the immune response⁶. This revelation resulted in extensive clinical and laboratory studies that generated compelling evidence that statins possess comprehensive immune-modulating properties that affect many facets of the inflammatory response. Because of the diverse effects of statins on the immune system, considerable interest has arisen in their therapeutic potential for treating autoimmune disease⁷⁻¹⁰. Statins are particularly attractive as their safety profile is generally good and, as they are administered orally, provide an additional advantage over parenterally administered agents. As is discussed below, the pleiotropic actions of statins and their ability to attenuate experimental inflammatory disease is impressive but the relative importance of each modified pathway in bestowing improved clinical outcome remains poorly understood. Here, we highlight, in the context of autoimmune disease, the different facets of the immune response that are modulated by statins and review current opinion regarding their mechanism of action, with particular reference to the inhibition of protein prenylation.

Statin pharmacology

The statin family of drugs comprises naturally occurring (lovastatin, mevastatin, pravastatin and simvastatin) and synthetic members (fluvastatin, atorvastatin, cerivastatin and rosuvastatin) which differ in their LIPOPHILICITY, half-life, and potency. All statins, independent of their structural differences, bind to HMGCoA reductase at nanomolar concentrations leading to competitive displacement of the natural substrate HMG-CoA. This competitive inhibition results in a failure to catalyze the conversion of HMG-CoA to L-mevalonic acid which in turn prevents the downstream biosynthesis of cholesterol (FIGURE 1). Not only is cholesterol synthesis reduced, but low intracellular concentrations activate sterol responsive element binding proteins that lead to increased transcription of the LDL receptor gene and subsequent cell surface expression. As LDL receptor uptake of cholesterol by the liver is a key factor in determining circulating levels, this reduces further blood LDL cholesterol. A reduction in circulating cholesterol is a primary clinical objective in treating hypercholesterolemia as it removes one of the principle risk factors for atherosclerosis. Despite an unambiguous association between lipid-lowering and improved clinical outcome in patients with coronary atherosclerosis, not all the benefit can be attributed to cholesterol reduction. In particular, the inflammatory component of atherosclerosis appears to respond to statin-mediated effects that are independent of reduced cholesterol. In recent years, numerous reports have highlighted the important role played by intermediate metabolites of the cholesterol biosynthetic pathway in the pathogenesis of coronary disease¹¹, and consequently their potential as immunomodulators in other immune-mediated diseases. Foremost amongst the alternative downstream pathways affected by statins are those that depend on the supply of intermediate products of the cholesterol biosynthetic pathway (FIGURE 1). In particular, the 15 carbon farnesyl pyrophosphate (FPP) and the 20 carbon geranylgeranyl pyrophosphate (GGPP). These isoprenoid pyrophosphates have attracted considerable attention as they serve as adjuncts in post-translational (ISO)PRENYLATION of a variety of important cell signaling proteins¹². Prenylation occurs on proteins containing a C-terminal CaaX motif, and it has been estimated that there are in excess of 100 known and hypothetical prenylated CaaX proteins encoded in the human genome. Amongst these proteins are approximately 40 members of the small GTPase family of molecular switch proteins that include Rho, Rac and Cdc42. These small proteins cycle between an inactive GDP-bound state and an active GTP-bound state and play a crucial role in controlling multiple signaling pathways, many of which are involved in reorganization of the cytoskeleton¹³. Prenylation, however, is not confined to proteins containing the CaaX sequence, but also occurs on members of the Rab family of Ras-related G proteins, of which there are in excess of 60 proteins, most of which contain a CC or CxC C-terminal sequence in place of the CaaX motif. The Rab family of small GTP-binding proteins participate as essential signaling elements in controlling intracellular membrane trafficking¹³. For all prenylated proteins identified to date, and they constitute up to 2% of total cellular protein, the lipophilic prenyl group enables these proteins to anchor to cell membranes, which in most cases is an essential requirement for their biological function. In addition to promoting membrane interactions, prenylation also appears to play a major role in crucial protein-protein interactions. By inhibiting HMGCoA reductase, statins reduce the availability of these intermediate metabolites and hence the activity of key cell signaling molecules. It is not surprising, therefore, that given the central role these small GTP-binding proteins play in determining cell function that the immune response will be modified independently of their lipid lowering effect (TABLE 1).

Modulation of immune function by statins

While it has been appreciated for the past 15 years that statins have antiproliferative effects on lymphocytes and other cell types^{14,15}, more recent studies indicate that statins also have

immunomodulatory properties that alter the function of both T cells and antigen presenting cells (APC) (FIGURE 2). In 1997, Pahan *et al.*¹⁶ observed that *in vitro* treatment of macrophages and resident central nervous system (CNS) APCs (such as microglia and astrocytes) with lovastatin prevented expression of tumour-necrosis factor (TNF) and interleukin-1 (IL-1). Inhibition of these pro-inflammatory mediators raised the possibility that statins may provide benefit in neuroinflammatory disease and, by extrapolation, other autoimmune disorders. This concept was first tested by Singh and colleagues, who showed that lovastatin reduced mononuclear cell infiltration into the brain and attenuated the clinical signs of experimental autoimmune encephalomyelitis (EAE), the animal model of multiple sclerosis^{17,18}. Since then, further *in vitro* studies have shown that statins suppress a number of key functions of the immune system which influence the development of autoimmune disease. The testing of statin therapy in animal models of inflammatory disease and, more recently in clinical trials, has provided tantalizing evidence that this class of drug may be of benefit to patients with diseases such as multiple sclerosis, rheumatoid arthritis and systemic lupus erythematosus (SLE).

MHC class II expression

Of particular relevance to immune modulation in T-cell-mediated inflammatory disease, is the observation that statins inhibit interferon- γ (IFN- γ)-inducible expression of MHC class II molecules by APCs (human monocyte-macrophages and saphenous vein endothelial cells) and prevent antigen presentation to CD4⁺ T cells¹⁹ (FIGURE 2). This finding was of special importance as earlier studies raised the possibility that autoimmune disease may be treated with antibodies specific for MHC class II molecules²⁰⁻²⁴. Indeed, Feldmann had hypothesized that a critical step in the pathophysiology of autoimmune disease was the aberrant expression of MHC class II molecules in tissues where it is not constitutively expressed²⁵. Increased MHC class II expression has been demonstrated in several autoimmune diseases, including multiple sclerosis, rheumatoid arthritis, myocarditis and SLE. Therapeutic targeting of MHC class II molecules, therefore, is an attractive strategy for modifying autoimmune disease, and the recent seminal finding by Kwak and colleagues¹⁹ reinforces this view. Statins inhibit IFN- γ -inducible expression of the MHC class II transactivator (CIITA), the master regulator for MHC class II expression¹⁹, with atorvastatin being more potent than either lovastatin or pravastatin. Although these observations suggested that statins inhibit MHC class II upregulation through selective inhibition at one IFN- γ -inducible CIITA promoter, pIV, more recent data indicate that statins also inhibit IFN- γ -inducible CIITA expression at pI, indicating that statins are not selective for pIV, but inhibit IFN- γ -inducible CIITA expression in general²⁶. Interestingly, inhibition of MHC class II by simvastatin can be reversed in human microvascular endothelial cells by both mevalonate and GGPP, but not squalene, suggesting the involvement of small GTPases in the inhibitory event²⁷. Although statins clearly inhibit induced MHC class II expression, it appears that constitutive expression by mature professional APCs is largely unaffected. These data provided a compelling rationale for the use of statins in treating T-cell-mediated disease and has resulted in various studies exposing further the pleiotropic effects of such drugs in an autoimmune setting.

Co-stimulatory molecule expression

For an effective T-cell response to antigen presentation, not only do T cells require antigen presented in the context of MHC class II molecules but they also require recognition of other co-stimulatory molecules. In professional APCs, both simvastatin and atorvastatin prevent cytokine-induced maturation, resulting in a failure to express mature levels of CD83, CD40, CD86, HLA-DR and CC-chemokine receptor 7 (CCR7)²⁸. Unsurprisingly, the ability of these statin-treated dendritic cells (DCs) to induce T-cell proliferation was greatly reduced and, consistent with this being mediated by an effect on protein prenylation, could be

reversed by either mevalonate or GGPP. In the CNS, microglial cells are purported to act as tissue-resident APCs and expression of co-stimulatory molecules are also modulated by statins. *In vitro* treatment of microglial cells with atorvastatin results in inhibition of IFN- γ -inducible expression of CD40, CD80 and CD86 molecules²⁶. Moreover, in vascular endothelial cells, statins reduce cytokine-induced CD40 mRNA and protein expression, which may²⁹ or may not³⁰ be reversed by mevalonate. Overall, these data suggest that statins inhibit cytokine-mediated activation of co-stimulatory gene expression (FIGURE 2). Of particular relevance to SLE, which is a progressive autoimmune disease characterized by the production of high levels of autoantibodies, atorvastatin also reduces the expression of MHC class II molecules and the co-stimulatory molecules CD80 and CD86 by B cells. Concomitant with this was impaired antigen presentation and T-cell response³¹ and a reduction of disease intensity in an experimental animal model.

Ag presentation and T cell proliferation

A prerequisite for antigen presentation by APCs is endocytosis of antigen, its internal processing and subsequent presentation by MHC class II molecules on the cell surface. It is clear that this process is governed in part by remodeling of the cytoskeleton and as such requires the input of small GTPases. In DCs, the endocytic pathway requires activation of Rac and Cdc42³², and accordingly the process may be subject to modification by statins. In agreement with this hypothesis, constitutively active Cdc42 and Rho enhance the ability of DCs to present antigen to T cells. Any reduction in small GTPase activity will also have a significant impact on other functions that require modification of the actin cytoskeleton. For example, both Rac and Cdc42 are involved in the formation of the T-cell receptor complex as it binds to the MHC class II complex on APCs to form the immune synapse, whereas Ras, Rho, Rac1 and Cdc42 are all involved in cell-cycle progression and proliferation. The observation that statin inhibition of antigen-induced T-cell proliferation is linked to negative regulation of cell-cycle progression, is consistent with inhibition of GTPase-mediated cell proliferation³³. Given the multiple targets in T-cell activation and proliferation where statins may exert an effect (FIGURE 2), it is not surprising that one of the most common features of statin treatment in experimental autoimmune disease is loss of T-cell proliferation (TABLE 2). Moreover, lovastatin, simvastatin and mevastatin have all been shown to inhibit, in a dose-dependent manner, the proliferation of peripheral-blood mononuclear cells (PBMCs) harvested from untreated or IFN- γ treated patients with multiple sclerosis³⁴.

It is possible that MHC class II expression may also be affected by statins in a cholesterol-dependent manner as it has been postulated that reduced cholesterol might affect the integrity of cell membrane lipid rafts. These structures, which are believed to be important microdomains for the assembly of signaling complexes, have been reported to be disrupted by simvastatin leading to a loss of MHC class II lipid raft association³⁵. On the contrary, it has been shown that in Jurkat cells lipid rafts were only disrupted when statins were applied in the absence of serum³⁶ and furthermore, *in vivo* data indicates that treatment of mice with atorvastatin (10 mg/kg) has no effect on T cell cholesterol content³⁷. The effect of statins on lipid raft formation and consequential signaling, therefore, remains uncertain.

T-cell phenotype

In most cases reported to date, statin administration *in vivo* has been found to prevent induction of experimental T helper 1 (T_H1)-mediated autoimmune diseases (TABLE 2). More importantly, however, statins can modulate disease progression after induction of disease. In a chronic-relapsing model of multiple sclerosis, experimental autoimmune encephalomyelitis (EAE), statin treatment commenced after onset of the acute phase was found to reverse disease²⁶, and when initiated during disease remission prevented relapse^{26,33,38}. A major effector mechanism responsible, at least in part, for this statin-

mediated disease amelioration appeared to be through a change in the profile of T-cell phenotype (FIGURE 2). Following atorvastatin treatment of EAE, which resulted in a decrease in CNS inflammation and T-cell proliferation, Youssef *et al.*²⁶ showed that there was a concomitant shift in the pattern of T-cell cytokine secretion. More specifically, there was a significant induction in the secretion of anti-inflammatory T_H2 cytokines (IL-4, IL-5 and IL-10) and phosphorylation (activation) of STAT6 (signal transducer and activator of transcription 6), which is involved in IL-4-dependent T_H2-cell differentiation. Conversely, phosphorylation of STAT4, which is required for IL-12-dependent T_H1-cell differentiation, was inhibited and secretion of IL-2, IL-12, IFN- γ and TNF was reduced. Furthermore, by means of an adoptive transfer model it was shown that these Th2-differentiated cells were able to protect recipient mice from developing disease. This data was corroborated by Stanislaus *et al.*³⁹, who showed that in comparison to untreated EAE rats, lovastatin-treated animals expressed decreased levels of IFN- γ , whereas IL-10 was markedly increased. The fact that this was a key effector mechanism in alleviation of T_H1-mediated autoimmune disease was further elaborated by Nath *et al.*⁴⁰, who reported that statin treatment might also promote T-cell expression of GATA3 (GATA-binding protein 3), a transcription factor involved in T_H2-cell differentiation, and downregulate activation of nuclear factor- κ B (NF- κ B) and T-bet, a T-box transcription factor associated with T_H1-cell differentiation⁴¹⁻⁴³. This same group also showed a concomitant increase in the levels of T_H2 cytokine transcripts⁴⁴. In addition, Aktas and co-workers³³ found that atorvastatin, at the same time as inhibiting the development of chronic EAE and reducing autoreactive T-cell proliferation, caused a strong attenuation of the T_H1 immune response, with evidence for increased secretion of T_H2 cytokines. The capacity for statins to cause a shift in T-cell phenotype, from T_H1 to T_H2 predominance, is not however restricted to EAE but has also been observed in a T_H1-driven model of experimental autoimmune myocarditis (EAM)⁴⁵.

Notwithstanding these intriguing findings, not all studies have observed a statin-induced bias towards a T_H2 cytokine profile. In experimental autoimmune uveitis (EAU), lovastatin⁴⁶, but not atorvastatin⁴⁷, was found to attenuate disease and inhibit T-cell IFN- γ production with little effect on T_H2 cytokines. Similarly, high-dose simvastatin treatment of collagen-induced arthritis resulted in a significant suppression of collagen-specific T_H1 humoral and cellular immune responses⁴⁸ without any evidence of a corresponding upregulation of T_H2 cytokines. In an identical arthritis model, however, atorvastatin and rosuvastatin had no effect, whereas the same regimen of simvastatin resulted in attenuation of disease but was associated with serious side effects⁴⁹. Nevertheless, more acceptable doses of atorvastatin were efficacious in a rat model of adjuvant-induced arthritis⁵⁰, although cytokine profiles in this study were not reported. Additionally, fluvastatin treatment of EAM was found to decrease T_H1 cytokines⁵¹, but this also coincided with a significant reduction in the T_H2 cytokines IL-4 and IL-10. Conversely, *in vitro* studies with human PBMCs from patients from multiple sclerosis revealed that simvastatin, lovastatin and mevastatin all failed to induce either a T_H1 or T_H2 bias but enhanced both IFN- γ and IL-12³⁴. Furthermore, when cytokine profiles were determined in anti-CD3-activated T cells from simvastatin-treated multiple sclerosis patients where clinical improvement was observed, no cytokine alteration was observed⁵². Despite such variations, the most consistent and robust finding throughout the animal studies are a beneficial suppression of the T_H1 response. Any reported deviations are likely to be explained by differences in the model system and statin used. In light of this, it is interesting to note that in Brown Norway rats, which are less susceptible to EAE due to an inherent T_H2 bias⁵³, atorvastatin fails to alleviate disease⁵⁴.

How the effect of statins on the T_H1/T_H2 balance is mediated remains to be fully elucidated. What is clear is that atorvastatin-mediated T_H2 differentiation can be reversed by mevalonate, suggesting that either mevalonate or its metabolites promote T_H1

differentiation²⁶. In addition, recent studies have shown that specific isoprenoid intermediates modulate this effect via differential farnesylation and prenylation of Ras and Rho molecules³⁷. The role of GTPase function in statin-mediated alleviation of disease is further endorsed by the finding that prenyl transferase inhibitors also attenuate EAE in a similar manner to statins⁵⁵. It is of interest to note that in mevalonate kinase deficiency, characterized by periodic fever, neuralgic pain in the joints and hyperproduction of IgD, abnormal production of isoprenoids mediates many of the autoimmune abnormalities^{56,57}.

Leukocyte adhesion molecules

Leukocyte adhesion to the vascular wall and subsequent migration into the tissue is central to the pathogenesis of autoimmune disease (FIGURE 3). This multi-step process is comprised of adhesive and signalling events that begin with transient leukocyte tethering to the endothelial cell surface and culminate in leukocyte infiltration into the sub-endothelial space and beyond. Both leukocytes and endothelial cells participate pro-actively in this complex event and each requires specific activation signals before successful leukocyte extravasation can occur. Of paramount importance to this process is the expression, in the correct configuration and state of activation, of cell adhesion molecules as these play a central and co-operative role in dictating the pattern and extent of leukocyte recruitment.

The expression of the integrin lymphocyte function-associated antigen 1 (LFA-1; CD11a/CD18) on lymphocytes, which binds to the immunoglobulin superfamily molecule intercellular adhesion molecule 1 (ICAM-1), is one of the key adhesion molecules involved in both adhesion/migration and costimulation. Surprisingly, certain statins are capable of altering the binding capacity of LFA-1 to ICAM-1 independently of either cholesterol lowering or inhibition of mevalonate and its metabolites. This is achieved through direct binding to the so-called lovastatin binding site (L-site) on the extracellular domain of LFA-1, which blocks binding to its cognate binding partner and inhibits both lymphocyte adhesion and costimulation⁵⁸. However, the significance of the L-site is controversial, as several studies indicate that statin-mediated inhibition of APC-induced pro-inflammatory cytokines, inhibition of upregulation of costimulatory molecules and T_H2-cell differentiation can be reversed by mevalonate^{26,33}. This indicates that, although statins may interact directly with LFA-1 and possibly other proteins involved in T cell regulation, many critical components in immune regulation are influenced by the mevalonate pathway.

One of the most consistent outcomes of statin therapy in autoimmune disease is a reduction in leukocyte infiltration to the target organ (TABLE 2). This may be mediated, at least in part, by a direct effect on adhesion molecule expression resulting in an impaired ability of leukocytes to adhere to and migrate across the vascular barrier. In support of this, several reports have now revealed that statins are able to modulate the expression of both leukocyte and endothelial-cell adhesion molecules. In monocytes, the integrin Mac-1 (CD11b/CD18) is involved in adhesion to endothelial cells through its interaction with ICAM-1. Treatment of monocytes with lovastatin *in vitro* has been shown to reduce the surface expression of CD11b and consequently monocyte adhesion. This was not due to steric hindrance, as co-incubation with mevalonate reversed the effects⁵⁹. In a similar study, treatment of monocytic U937 cells with cerivastatin significantly decreases their ability to adhere to activated human umbilical vein endothelial cell (HUVEC) under conditions of flow without affecting rolling. This decrease in adhesive properties corresponded with downregulation of surface expression of the integrin chains CD11a and CD49d (a subunit of a very late antigen 4; VLA-4) by U937 monocytic cells and an apparent alteration of RhoA function⁶⁰. Moreover, simvastatin, atorvastatin, and cerivastatin were found to downregulate PBMC expression of CD18, and CD11a at both the mRNA and protein level and decrease binding to TNF- α stimulated HUVECs⁶¹.

Endothelial cell adhesion molecules

Statins can also modulate endothelial-cell adhesion molecule expression, with the weight of evidence tending towards the effect being inhibitory. This is particularly true for both P- and E-selectin, where downregulation of the latter is reported to be mediated by inhibition of Rho and subsequent gene expression⁶². Numerous studies have also shown that statins reduce both constitutive and induced expression of ICAM-1, which is directly involved in sustaining both leukocyte adhesion and diapedesis. Contrary to this, Sadeghi *et al.*⁶³ reported that simvastatin augmented cytokine-induced ICAM-1 expression, and that this could be reversed by co-administration of GGPP or mevalonate. In the case of VCAM-1 expression, it has recently been reported that lovastatin inhibition of VCAM-1 on brain endothelial cells, mediated through inhibition of the PI3 kinase/Akt/NF κ B pathway, results in a significant reduction in monocyte transendothelial cell migration⁶⁴. The ability of statins to inhibit NF κ B activation is in agreement with other studies, but at odds with reports where Akt is activated (see below). Nevertheless, inhibition of NF κ B appears to be a robust outcome and raises the prospect of modulating other pro-inflammatory endothelial-cell-derived molecules also under control of this transcription factor. This assertion is supported by the finding that in human endothelial cells statins decrease CXCL8 (also known as IL-8) and CCL2 (also known as MCP-1) production⁶⁵⁻⁶⁷, two chemokines that contribute to the process of leukocyte recruitment.

Leukocyte motility and migration

The Rho family of GTPases, of which Rho, Rac and Cdc42 are the best characterized, regulate actin and microtubule dynamics, and consequently are central to virtually all processes that involve motility (TABLE 1). It is hardly surprising that statins, which affect Rho GTPase function, also influence leukocyte motility and migration. Stabilisation of directional movement by cells (chemotaxis) requires external cues such as chemokines⁶⁸ and this process is controlled by Cdc42. Thus, in chemotaxis of macrophages along a chemotactic gradient, inhibition of Cdc42 results in random movement and loss of directionality (chemokinesis), whereas inhibition of Rac blocks all cell movement⁶⁹. Also impacting on this process is the observation that statins downregulate expression of chemokine receptors in B cells, T cells and macrophages^{34,67}, which will result in a loss of OUTSIDE-IN SIGNALING. Consistent with small GTPases being involved in cytokine induced chemokine receptor expression, Veillard *et al.* demonstrated that similar effects could be achieved with geranylgeranyl transferase inhibitors⁶⁷. Lymphocytes also use the small GTPase Rap-1, which is activated within seconds following treatment with the chemokines CXCL12 (also known as SDF1) and CCL21 (also known as SLC). This results in enhanced integrin-mediated adhesion to endothelial cell ICAM-1 and VCAM-1, as well as increasing leukocyte motility and transendothelial migration⁷⁰.

Leukocyte migration, especially once the cell breaches the vascular barrier, is also reliant on matrix metalloproteinase (MMP) expression, to ease the passage through the basal lamina and extracellular matrix beyond. As with many other pro-inflammatory molecules, statins also impair MMP secretion by leukocytes. This is especially true for immune cells of the monocyte/macrophage lineage, where MMP-9 secretion is inhibited in a dose-dependent manner, an effect which could be reversed by the simultaneous addition of exogenous mevalonate⁷¹. It was postulated that as Rab GTPases are essential for regulating membrane traffic and protein secretion, statin modulation of prenylation may underlie the mechanism through which inhibition is achieved. By contrast, secretion of MMP-9 from human saphenous vein smooth-muscle cells can be inhibitory by blocking the RhoA/ROCK pathway⁷², and secretion of MMP-9 from the monocytic cell line THP-1 can be achieved with a geranylgeranyl transferase inhibitor and C3 exoenzyme implicating Rho in the inhibitory process⁷³. In addition, cerivastatin-mediated inhibition of monocyte MMP-9

secretion was found to be accompanied by attenuation of NF- κ B translocation into the nucleus, and could be reversed by FPP, thus implicating Ras⁷⁴. Together, these data show that multiple small GTPase signaling pathways are involved in MMP-9 production and as such may represent the mechanism by which statins exert their effect. An alternative mechanism, however, has also been proposed whereby atorvastatin-mediated inhibition of MMP-9 (as well as CCL2 and TNF) in monocytes may be mediated through activation of the nuclear receptor transcription factor peroxisome-proliferator-activated receptor- γ (PPAR- γ)⁷⁵. Interestingly, PPAR- γ expression is decreased by pro-inflammatory cytokines but is induced by the T_H2 cytokine IL-4. Contrary to an inhibitory effect on secretion, exposure of MMPs to statins may actually increase their proteolytic capacity, especially that of MMP-2⁷⁶.

Endothelial cell adhesion molecule receptor signaling

Endothelial-cell adhesion molecules serve not only as docking structures but also initiate signaling cascades that are requisite for successful leukocyte penetration through the vascular barrier. In this regard, ICAM-1 fulfils an important role in controlling transvascular migration through the induction of signalling cascades that mediate endothelial-cell reorganisation of the actin cytoskeleton. Thus, on engagement of ICAM-1 to its cognate binding partners (LFA-1 on lymphocytes and Mac-1 on monocytes), there is propagation of divergent signalling pathways, the functional consequences of which are subject to mounting investigation. To date, it has been shown that ICAM-1-mediated signalling results in tyrosine phosphorylation of cytoskeletal-associated proteins, activation of MAP kinases (p38, ERK and JNK) and the transcription factors NF- κ B and c-fos, upregulation of ICAM-1 and VCAM-1 gene expression and reorganisation of the actin cytoskeleton to facilitate lymphocyte migration^{77,78}. In brain endothelium, Rho GTPases appear to be a central upstream signalling component in the ICAM-1-mediated pathway responsible for sustaining lymphocyte migration⁷⁹⁻⁸¹, and as such represent an intriguing target for statins. Indeed, statins have been shown to inhibit Rho prenylation in endothelial cells, which results in inhibition of the ICAM-1-mediated pathway and transvascular lymphocyte migration³⁸. The fact that this effect could be reversed by endothelial cells expressing Rho with a myristoylation site, which renders the cell insensitive to loss of isoprenylation³⁸, lends considerable weight to the assertion that endothelial ICAM-1-mediated signalling via Rho is of fundamental importance to lymphocyte extravasation in the CNS.

VCAM-1 also transduces signals to the endothelial cell and initiates signalling cascades that are essential to leukocyte, especially monocyte, migration. Again, the activation of small GTPases is key and therefore susceptible to statin inhibition. Engagement of VCAM-1 results in calcium-mediated Rac1 activation⁸², an increase in NADPH oxidase activity that in turn results in reactive oxygen species (ROS) production, actin reorganisation, loss of vascular endothelial-cadherin (VE-cadherin) cell-cell contact and reduction in leukocyte transmigration^{83,84}. This endothelial-cell pathway can be blocked by statins, which inhibit the prenylation of Rac1 and in so doing diminish the production of ROS⁸⁵, which are powerful activators of the NF- κ B/REL family of transcription factors. As the NF- κ B family of transcription factors are pivotal in controlling inflammatory and immune responses, including the expression of endothelial-cell adhesion molecules⁸⁶, downregulation of this pathway will have an impact on disease progression.

The VCAM-1/Rac1-mediated disruption of the endothelial-cell junction provides an important insight into the potential role of small GTPases in controlling vascular permeability and potentially leukocyte migration. Statins may exert an important effect on cell-junction integrity through their indirect inhibitory effect on small GTP-binding proteins. It is now well established that both Rho and Rac modulate the actin cytoskeleton, which in endothelial cells performs an important scaffold function at the cortical region stabilising

cell-cell junctions. Induction of rho-mediated actomyosin contraction can result in cell-junction separation that may be an essential element in transendothelial-cell leukocyte migration. Rac activation in endothelial cells, in contrast to epithelial cells, leads to the promotion of junctional disassembly⁸⁷ and, as described above, loss of VE-cadherin-mediated cell-cell contact. Thus, a reduction in Rho and Rac prenylation may result in an inability of the endothelial cell to regulate the cell junction and facilitate leukocyte diapedesis. Interestingly, expression of the chemokine receptor CCR2 by brain endothelial cells, which is a receptor for CCL2, initiates junctional opening in a Rho-dependent manner⁸⁸. This raises the possibility that statins, which not only reduce CCL2 expression, will also inhibit this Rho/Rho kinase-mediated barrier opening.

eNOS and NO production

Many studies have attributed the beneficial effect of statins in atherosclerosis to their role in upregulating endothelial nitric oxide synthase (eNOS), which is decreased in dysfunctional endothelium. Additionally, statins also reduce oxidised LDL which has been shown to inhibit eNOS transcription and protein synthesis. In autoimmune disease, however, the role of eNOS and its product nitric oxide (NO) in either the pathogenesis or resolution of disease is less obvious. Nevertheless, there is evidence that maintenance of vascular NO production is beneficial, as it prevents leukocyte chemotaxis⁸⁹ and downregulates leukocyte adhesion and migration at the vascular wall⁹⁰⁻⁹². These effects of NO may be mediated by inhibition of NF- κ B activity, thereby downregulating cytokine-induced adhesion molecule expression⁹³. Statins can increase eNOS expression and both basal and stimulated NO production⁹⁴, either by inhibiting the Rho/Rho kinase pathway that negatively regulates eNOS expression or by stimulating Akt- and AMP-activated protein kinase, which activate eNOS^{95,96}. It has been proposed that inhibition of Rho prenylation by statins is a key factor in upregulating eNOS gene expression and stability⁹⁷⁻⁹⁹, as the effects can be reversed by GGPP. Interestingly, inhibition of protein prenylation with a geranylgeranyltransferase I inhibitor results in an increase in eNOS activity and NO production, with a concomitant decrease in NADPH oxidase activity and ROS production¹⁰⁰. It is important to note, however, that the production of NO is not always beneficial. In the brain, pro-inflammatory cytokines induce iNOS expression in astrocytes, which, it is believed, contributes to oligodendrocyte degeneration in multiple sclerosis. Unlike in endothelial cells, in which statins enhance the expression of eNOS, lovastatin has been shown to inhibit cytokine-mediated astrocytic expression of iNOS and NO production *in vitro*, which was reversible by mevalonate and FPP¹⁶, as well as in EAE *in vivo*¹⁷.

Efficacy of statins in human autoimmune disease

It is currently too early to predict with any confidence whether the promising data derived from statin therapy in experimental animal models of autoimmune disease can be translated successfully into the clinic. Certainly most experimental data directs us to believe that alone, or more probably in combination with other therapeutic approaches, statins will improve clinical outcome. The relative safety of these agents and their ease of delivery also provides a compelling case for their evaluation in the clinical setting. In multiple sclerosis, the benefit of statin treatment in patients is only just beginning to be properly evaluated, although the outcome from two small open-label trials have already been reported. In a purely observational study of seven patients with relapsing–remitting multiple sclerosis, Sena *et al.*¹⁰¹ reported that lovastatin treatment over a 12 month period resulted in a reduction in the mean number of GADOLINIUM-ENHANCING LESIONS but with no accompanying decrease in the EXPANDED DISABILITY STATUS SCALE (EDSS) SCORE. In a separate open-label study involving 28 patients over a 6 month period, treatment with simvastatin reduced significantly the number and volume of gadolinium-enhancing lesions by 44% and 41% respectively⁵². A concern in interpreting the results

from this trial, however, is that the observed reduction in enhancing lesions may reflect a statistical regression to the mean. It is generally agreed that placebo-controlled trials are necessary to establish whether statins will be efficacious in multiple sclerosis. In this regard, a larger phase II placebo-controlled trial (funded by the Immune Tolerance Network of the NIH) is testing whether Lipitor (atorvastatin) can reduce the risk of developing multiple sclerosis activity in patients who have experienced their first clinical CNS demyelinating event, a “clinically isolated syndrome”. Other human autoimmune diseases are also receiving attention. McCarey and colleagues¹⁰² conducted a 6 month randomised double-blind placebo-controlled trial with atorvastatin in patients with rheumatoid arthritis. Although the outcome was modest, atorvastatin had a significant effect on disease activity. The authors quite correctly state that further studies are needed to establish what benefit may be derived from long-term treatment. Finally, in a very preliminary study, short-term simvastatin treatment of three patients with SLE resulted in significant reduction in proteinuria, providing a tentative indication that statins may offer some clinical benefit¹⁰³.

Together these clinical trials provide some encouragement although it remains to be seen whether any such benefit is mediated by the same mechanisms that have been proposed in animal studies. In particular, the effect of statins on patient T cell phenotype and cytokine production remains unclear and is awaited with anticipation. Whatever the mechanism, a number of issues relating to clinical application remain outstanding and need to be addressed. Firstly, differences exist between statins with respect to their pharmacokinetics and potency¹⁰⁴ and it is still unclear which statin will provide the best outcome with respect to immune modulation in autoimmune disease. Secondly, the doses applied experimentally to elicit a beneficial effect are considerably greater than those currently recommended for the treatment of cardiovascular disease. Some comfort, however, can be gained from the observation that circulating levels of active statin in mice were found to be much lower than could be predicted from the dose⁴⁶. Nonetheless, the circulating concentration still exceeded that which would be acceptable in patients over an extended period and could lead to serious side effects such as RHABDOMYOLYSIS. The case for combining statins in a reduced dosage with other disease modifying agents, so that efficacy may be retained whilst toxicity diminished, is therefore compelling. In fact, data in the EAE model indicate that Lipitor can enhance the efficacy of glatiramer acetate (Copaxone)¹⁰⁵. Finally, many of the therapeutic effects of statin therapy appear to be mediated through modulation of isoprenoid biosynthesis and consequently small GTPase activity but not all of these effects are necessarily beneficial. Thus, GTPases may also act as negative regulators of inflammation, and inhibition of these pathways by statins will result in promotion of a pro-inflammatory state. This may explain those reports where a pro-inflammatory response to statins is observed. It is likely that cell type, the statin used, the dose and duration will all determine which outcome predominates.

Concluding remarks

Most data now indicate that the greatest therapeutic attribute of statins is their ability to modulate a broad range of pro-inflammatory immune mechanisms through inhibition of small GTPases and other prenylated proteins. Given the enormous repertoire of cell functions mediated by prenylated proteins, it is not surprising that modulation by statins will have a diverse effect on immune function. However, the ability to induce downregulation without provoking complete inhibition of these critical signaling proteins is fundamental to their efficacy as complete blockade of these molecular switches would, in most cases, be lethal. What statins are able to accomplish is partial inhibition of an upstream common denominator of multiple regulatory signaling networks controlling the immune system. Through subsequent attenuation of protein prenylation, many pro-inflammatory pathways are modulated without adversely affecting other critical pathways necessary for cell

survival. The pleiotropic nature of statins is impressive and the weight of data demonstrating efficacy in animal models of autoimmune disease provides us with a compelling rationale to translate such work into the clinical setting. Although caution must be applied, the high degree of patient tolerance to statins and their simplicity of delivery make them a highly attractive addition to currently available immunosuppressive drugs.

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Glossary

HYPERCHOLESTEROLEMIA

A clinical condition in which there is abnormally high circulating levels of blood cholesterol that can be a significant contributing factor towards cardiovascular disease.

LOW-DENSITY LIPOPROTEIN (LDL) CHOLESTEROL

Cholesterol is carried through the bloodstream by proteins in the form lipoproteins. There are five different lipoproteins with cardiovascular risk being associated with high circulating low-density lipoprotein cholesterol.

LIPOPHILICITY

The measure of a molecules ability to dissolve in lipid (oil) as apposed to water. Lipophilic or 'lipid-loving' molecules show a preference for dissolving in lipids.

(ISO)PRENYLATION

Prenylation (or isoprenylation) is the post-translational modification of a protein through the addition of an isoprenoid lipid; namely the 15-carbon farnasyl or 20-carbon geranylgeranyl lipid moiety derived from the cholesterol synthesis pathway.

OUTSIDE-IN SIGNALING

The initiation of an intracellular signaling pathway through extracellular ligand engagement of a cell surface receptor.

GADOLINIUM-ENHANCING LESIONS

Damaged areas (lesions) detected by magnetic resonance imaging (MRI) that have been enhanced by the intravenous administration of a contrast agent (gadolinium) to increase the sensitivity of the MRI scan.

EXPANDED DISABILITY STATUS SCALE (EDSS) SCORE

A widely used neurological and functional scoring system for judging the clinical status of people with multiple sclerosis.

RHABDOMYOLYSIS

Severe muscle toxicity resulting in the breakdown of muscle fibres. A potential side effect of statins, either in monotherapy or in combination therapy.

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Figure 1.

The cholesterol synthesis pathway and protein prenylation. Statins inhibit the conversion of HMGCoA to L-mevalonate through competitive inhibition of the rate limiting enzyme HMGCoA reductase. This inhibition results in a decrease in the downstream biosynthesis of cholesterol and other intermediate metabolites including the isoprenoids farnesyl pyrophosphate and geranylgeranyl pyrophosphate. These isoprenoid pyrophosphates serve as essential adjuncts in the posttranslational modification of numerous key proteins that act as molecular switches including the small GTPases Ras, Rac and Rho. The posttranslational modification enables these signaling proteins to associate with membranes which are requisite for most of their biological function.

Figure 2.

The effect of statins on T-cell/antigen presenting cell function. Statins inhibit cytokine-inducible expression of MHC class II molecules and co-stimulatory molecules on antigen-presenting cells (APCs) and prevent antigen presentation to CD4⁺ T cells. T-cell proliferation is abrogated through modulation of GTPase-linked regulation of cell cycle progression and proliferation. In addition, the effect of statins on cytoskeletal organization will interfere with formation of the immune synapse. Statins also alter the T-cell profile by inhibiting the secretion of pro-inflammatory cytokines through phosphorylation of STAT4 (signal transducer and activator of transcription 4) and induction of the transcription factor T-bet which are required for T_H1 differentiation. Conversely, statins may also enhance the secretion of anti-inflammatory T_H2 cytokines via the activation of both STAT6 and GATA3 (GATA-binding protein 3), which are involved in T_H2-cell differentiation.

Figure 3.

The effect of statins on leukocyte adhesion and migration and endothelial cell immune function. Cell-adhesion molecule expression on leukocytes and endothelia are attenuated by statins, resulting in reduced adhesion and transvascular migration. In addition, statins inhibit chemokine and matrix metalloproteinase (MMP) secretion, which will further interfere with leukocyte migration. In the endothelium, adhesion molecule signaling necessary for leukocyte migration is blocked through the modulation of Rho and other small GTPases. This may also result in stabilization of the endothelial cell-cell junction. The effect of statins on the cytoskeleton alters leukocyte motility and directional migration in response to chemotactic gradients.

Table 1

Summary of the role of prenylated proteins in mediating immune function

Immune function	Prenylated protein (activated state)	Required for:
Leukocyte motility	Rac1, Cdc42	Forward movement through formation of membrane protrusions (lamellipodia and filopodia)
	Rac1, Cdc42, RhoA	Formation of focal complexes and podosomes
	Cdc42	Directed migration, macrophage and lymphocyte chemotaxis
	RhoA	Leukocyte tail retraction during transmigration
	Rap1	Increased leukocyte substrate motility and migration
	Rac1	LFA-1 induced Rac1 activation required for T cell motility
Antigen uptake, processing and presentation	Cdc42, Rac	Remodelling of the cytoskeleton during Ag endocytosis; Remodelling of the actin cytoskeleton for the formation of the immunological synapse between T cell and APC
	Rab (eg Rab4, 5 and 7)	Ag processing and presentation
	Rho, Cdc42	Ag presentation
	Rac1, Rac2	Mature dendritic cell dendrite formation, polarized short-range migration to T cells and T cell priming
	Rap1	Regulation of LFA-1 avidity for ICAM-1
Leukocyte activation, proliferation and function	Rap1	T cell receptor clustering and enhanced LFA-1 avidity for ICAM-1 following activation by T cell receptor engagement; Increased integrin-mediated (eg LFA-1 and VLA-4) adhesion following chemokine activation
	Ras	Regulation in mature lymphocytes of transcription factors that regulate cytokine genes. Occurs following rapid activation of Ras upon T cell receptor engagement and some cytokines
	Rac1	Determines T cell fate in the thymus; Modulates actin cytoskeletal dynamics required for receptor clustering, coalescence of adhesion molecules and signalling receptors and formation of immunological synapse. Rac1 is activated following T cell activation; CD28 activation of T cells; IL-2 production and T cell proliferative response following TCR engagement.
	Rac2	CD4+ T cell differentiation into Th1 subtype through activation of the IFN- γ gene
	RhoA	Regulates B cell receptor signalling and cell proliferation
	Ras, Rho, Rac1, Cdc42	Promotes cell-cycle progression and proliferation
	Rho, Rac	PKC dependent AP-1 and NF κ B activation and subsequent proliferation; JNK activation which is important for Th cell differentiation; Cytoskeletal reorganisation, receptor clustering and IL-2 secretion following CD3 stimulation; Upstream activator of NF κ B/REL family of transcription factors; NF κ B activation in monocytes
	Rho, Rac, Cdc42	Inhibits cytotoxic T lymphocyte- and Fas-induced apoptosis
	Rab27a	Exocytosis of lytic granules in cytotoxic T lymphocytes
Phagocytosis	Rac1, Cdc42	Formation of the phagocytic cup
	Rac	Induction of NADPH oxidase production of superoxide mediated phagosome function
	Rho	Superoxide formation during phagocytosis
	Rho, Rap1	Complement-mediated phagocytosis
Leukocyte transvascular migration	Rap1	Chemokine receptor mediated lymphocyte integrin activation and transvascular migration on ICAM-1 and VCAM-1; PECAM-1-mediated leukocyte signalling to enhance integrin adhesion to ICAM-1 and VCAM-1
	Ras, Rho	Monocyte MMP9 expression required for leukocyte migration
	RhoA	Induction of LFA-1 high affinity state
	Rap1 & 2	SDF-1 induced B cell migration

Immune function	Prenylated protein (activated state)	Required for:
	Rap	B cell LFA-1 and VLA-4 mediated adhesion
	Cdc42, Rac1	Chemokine-induced lymphocyte polarization and directional migration
	Rac2	Neutrophil migration
Endothelial cell immune function	Rho	Generation and maintenance of the endothelial cell docking structure; ICAM-1-mediated endothelial cell signalling required for lymphocyte transvascular migration; Endothelial cell actin cytoskeletal reorganisation necessary for monocyte migration; Opening of the endothelial cell junction, possibly triggered through endothelial cell CCR2 activation; Negative regulation of eNOS and NO production; Adhesion receptor clustering on endothelial cells for monocyte adhesion and spreading; LPS induced ICAM-1 and TNF- induced E-selectin expression in endothelial cells
	Rho, Rac1	Induction of ICAM-1, VCAM-1 and E-selectin expression through activation of NF B family of transcription factors
	Rac1	VCAM-1-mediated endothelial cell signalling required for NADPH oxidase activation, ROS production, junctional opening and monocyte transvascular migration
	Rap1	Enhanced endothelial cell junction assembly reducing leukocyte transmigration

Table 2

Summary of published data reporting the outcome of statin treatment in animal models of autoimmune disease

Disease model	Statin	Animal Model	Effect	Reference
Experimental Autoimmune Encephalomyelitis (EAE)	Atorvastatin, 1 and 10 mg/kg/day oral	Mouse SJL/J (Ag: PLP) C57BL/6 (Ag: MOG) B10.PL (Ag: MBP)	Attenuation of disease. Shift from Th1 to Th2 cytokine profile. Inhibition of T cell proliferation. Decrease in leukocyte infiltration. Inhibition of co-stimulatory molecules.	14
	Atorvastatin 10 mg/kg/day oral or s.c.	Mouse SJL/J (Ag: PLP)	Attenuation of disease. Decrease in leukocyte infiltration and blockade of Th1 immune response with increase in Th2 cytokines. Inhibition of T cell proliferation.	21
	Atorvastatin 10 mg/kg/day oral	Mouse C57BL/6 (Ag: MOG)	Attenuation of disease. Decrease in leukocyte infiltration. Blockade of Th1 immune response.	31
	Lovastatin 10 mg/kg/day i.p.	Mouse Biozzi ABH (spinal cord homogenate)	Attenuation of disease. Inhibition of leukocyte migration across the blood-brain barrier.	23
	Lovastatin 2 and 5 mg/kg/day i.p.	Mouse SJL/J (Ag: PLP)	Attenuation of disease. Inhibits proinflammatory cytokine biosynthesis. Shift towards a Th2 dominant T cell response.	25
	Lovastatin 2 mg/kg/day i.p.	Rat Lewis (Ag: MBP)	Attenuation of disease. Inhibits expression of iNOS and proinflammatory cytokines.	5
	Lovastatin 2 mg/kg/day i.p.	Rat Lewis (Ag: MBP)	Attenuation of disease. Shift from Th1 to Th2 cytokine profile.	24
	Lovastatin 2 mg/kg/day i.p.	Rat Lewis (Ag: MBP)	Attenuation of disease. Reduction in mononuclear cell infiltration and inflammatory cytokines. Downregulation of LFA-1 expression	6
	Lovastatin 2 mg/kg/day i.p.	Rat Lewis (Ag: MBP)	Attenuation of disease. Decrease in leukocyte infiltration. Increase in Th2 cytokine transcripts.	29
	Simvastatin 20 mg/kg/day oral	Rat Brown Norway (Ag: MOG)	No effect on disease.	39
Experimental arthritis	Simvastatin 10-40 mg/kg/day i.p.	Mouse DBA/1 (Ag: collagen)	Attenuation of disease. Suppression of Th1 response.	33
	Atorvastatin, 1 or 100 mg/kg/day oral. Rosuvastatin, 0.2 or 2 mg/kg/day s.c. Simvastatin, 40 mg/kg/day i.p. or oral	Mouse DBA/1 (Ag: collagen)	No effect on disease with atorvastatin or rosuvastatin. Simvastatin attenuated disease with side effects.	34
	Atorvastatin 1-10 mg/kg/day oral	Rat Holtzman (adjuvant)	Attenuation of disease.	35
Experimental Autoimmune Uveoretinitis (EAU)	Lovastatin 20 mg/kg/day i.p. Atorvastatin 10 mg/kg/day oral	Mouse B10RIII (Ag: IRBP)	Attenuation of disease. Decrease in leukocyte infiltration. Modulation of Th1 immune response. Oral atorvastatin only resulted in mild attenuation of disease.	31
	Atorvastatin 1 or 10 mg/kg/day oral	Mouse B10RIII (Ag: IRBP)	No effect on disease.	32
Experimental Autoimmune Myocarditis (EAM)	Atorvastatin 1 or 10 mg/kg/day oral	Rat Lewis (Ag: porcine cardiac myosin)	Improved cardiac function with decrease in cardiac inflammation. Decrease in Th1 and increase in Th2 cytokines.	30
	Fluvastatin 3.75 or 7.5 mg/kg/day oral	Rat Lewis (Ag: porcine cardiac myosin)	Improved cardiac function with decrease in CD4 ⁺ T cell infiltration. Decrease in Th1 cytokines and inhibition of NF- κ B.	36
Experimental systemic lupus erythematosus (SLE)	Atorvastatin 30 mg/kg/day i.p.	Mouse Female NZB/W F ₁ (spontaneous)	Improved clinical outcome with reduced glomerular injury. Decreased MHC class II and costimulatory molecule expression and T cell proliferation	19