Lipophilic statins suppress cytotoxicity by freshly isolated natural killer cells through modulation of granule exocytosis

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Abstract

NK cells, a component of the innate immune system, provide a first line of defense against viral infections and malignancies, interact with the adaptive immune system and have a role in rejection of allogeneic bone marrow transplants and solid allo- and xenotransplants. Immunoregulatory activity by the anti-hypercholesterolemia agents, 3-hydroxy-3-methyl-glutaryl Coenzyme A (HMG-CoA) reductase inhibitors, known as statins, has recently been reported. We analyzed the effects of three statins on human NK cell cytotoxicity. Two lipophilic statins (simvastatin and fluvastatin) suppressed the cytotoxic activity of fresh and IL-2-stimulated NK cells, while pravastatin, a hydrophilic statin, did not. Suppression was not associated with changes in intracellular perforin, granzyme A or granzyme B levels, or with changes in expression of leukocyte function-associated antigen-1, an integrin known to regulate NK activity and reported to be altered by statin treatment. Decreased cytoloytic activity was associated with decreased CD107a surface expression, indicating that the exocytosis pathway was compromised by simvastatin and fluvastatin but not by pravastatin. Mevalonate, the immediate downstream product of HMG-CoA reductase, partially reversed the effect of lipophilic statins on cytotoxicity and CD107a expression. Lipophilic statins also suppressed the release of the granule component, granzyme B, by IL-2-activated NK cells following stimulation with K562. That lipophilic statins suppress NK cell activity through inhibition of the exocytosis pathway suggest an additional potential role for statins in inhibition of transplantation responses.

Introduction

NK cells are lymphocytes of the innate immune system that are involved in early defense against virus infection and malignant transformation (1). NK cells also have important roles in regulating the adaptive immune system through direct action on T cells (2) and B cells (3), and through their production of cytokines and interactions with dendritic cells and T cells (4, 5). Recently, roles have been described for NK cells in rejection of allogeneic bone marrow (6), organ allografts (6, 7) and many types of xenografts (8–12). It is in the context of these latter roles that down-regulation of NK cell activity could be beneficial, and we have been interested in the potential for negative regulation of NK activity in such systems (13–15).

Inhibitors of 3-hydroxy-3-methyl-glutaryl Coenzyme A (HMG-CoA) reductase, known as statins, are used clinically for reducing blood cholesterol levels, and much evidence has accumulated prompting recognition of their pleiotropic effects such as effects on endothelial function, inflammation, coagulation and plaque vulnerability (16–19). For example, evidence suggests that statins may reduce the incidence of cardiac rejection, coronary vasculopathy, and increase survival in recipients of heart allografts (20–22), and have a beneficial effect on renal allograft survival (23). Anti-cancer effects have also been suggested (17, 24–26), though the literature is conflicting (27). Recently, the immunomodulatory activity of statins has received increasing attention. Several
studies have suggested that statins act on the immune system through a variety of pathways. For example, lipophilic statins have been reported to block leukocyte function antigen-1 (LFA-1)-mediated adhesion and co-stimulation of lymphocytes in a manner unrelated to inhibition of HMG-CoA reductase, but via binding to a novel allosteric site within LFA-1 (28, 29).

In the present paper, we have examined the effect of lipophilic statins, simvastatin and fluvastatin, and the hydrophilic statin, pravastatin (30), on NK cell cytotoxic activity. We report that simvastatin and fluvastatin but not pravastatin suppress the cytotoxic activity of NK cells and IL-2-activated NK cells at concentrations that do not affect cell viability or proliferation. Mevalonate, the immediate downstream product of HMG-CoA reductase, partially restored the cytotoxic activity of NK cells supporting the role of statin-mediated suppression of HMG-CoA reductase activity in suppressing NK cell activity. Suppression of cytotoxic activity of NK cells was associated not with expression of LFA-1, perforin or granzyme A or B, but was associated with down-regulation of processes involved in granule exocytosis.

**Methods**

**Reagents**

Human recombinant IL-2 (rIL-2) was provided by Chiron (Emeryville, CA, USA). Simvastatin, pravastatin and fluvastatin were purchased from Calbiochem (San Diego, CA, USA) as sodium salts, and sodium mevalonic acid lactone was from Sigma (St Louis, MO, USA). Lipophilic statins were dissolved in dimethyl sulfoxide (DMSO) which was then diluted 1:1000 in medium. The hydrophilic statin was dissolved in an aqueous solution, but 10 μl of DMSO was added to 10 ml of the final solution. mAbs to CD2–FITC (Leu-5B), CD11a–PE (clone S5.2), CD18–PE (clone 6.7), CD16–PE (clone 3G8), CD56–allophycocyanin (APC) (clone NCAM16.2) and CD107a–FITC (clone HA43) were purchased from BD Biosciences (San Jose, CA, USA), as were FITC-conjugated anti-human granzymes A and B mAbs (clones CB9 and Gb11, respectively). FITC-conjugated anti-human perforin mAb was purchased from Ancell (clone delta G9; Bayport, MN, USA). RPMI 1640 and buffers were purchased from Cambrex Bioscience (formerly BioWhittaker, Walkersville, MD, USA). Standard RPMI 1640 medium was prepared with 10% FCS (HyClone Laboratories, Logan, UT, USA), 50 μg ml⁻¹ gentamicin sulfate and 2 mM glutamine.

**Cell cultures**

The NK-sensitive target cell line, K562, was purchased from the American Type Culture Collection (Manassas, VA, USA), and maintained in standard RPMI 1640 medium. Porcine aortic endothelial cells (PAECs) and human aortic endothelial cells (HAECs) were obtained from Cambrex and cultured in EBM2, a medium formulated for endothelial cells (ECs; Clonetics, Walkersville, MD, USA). Both were used between passages 5 and 13.

**Purification of human NK cells and cell-surface analyses**

Fresh human NK cells were isolated from buffy coats obtained from de-identified healthy donors, under an exemption from the Food and Drug Administration Institutional Review Board, as by-products of blood collected for patient care (Blood Bank, National Institutes of Health, Bethesda, MD, USA) using RosetteSep NK cell cocktail (Stem Cell Technologies, Vancouver, Canada) according to the manufacturer’s instructions. To ascertain NK cell purity, cells were stained with anti-CD56–PE and CD16–FITC and results read on a FACS Caliber (Beckton-Dickinson, Mountain View, CA, USA). Purity of cells expressing CD16 and/or CD56 was consistently in the range of 85–95%. Typically, CD3⁺, CD14⁺ and CD20⁺ cells represented 0.38 ± 0.12%, 1.24 ± 0.60% and 10.07 ± 3.50% of the populations, respectively (n = 11). Purified NK cells were cultured with standard RPMI 1640 medium with or without IL-2 (100 U ml⁻¹, ∼2 nM) and with statins as described in Results. In some experiments, we attempted to reverse the effects of statins by including mevalonate at a final concentration of 1 mM during the last 16 h of culture. In some experiments, NK cells were stimulated by culture with K562 cells (1:1 ratio) in standard RPMI 1640 medium for 1 h followed by analysis of cell-surface CD107a expression by flow cytometry. The conditions for each independent experiment were set up using cells from a single healthy blood donor, and all were treated at the same time and analyzed by flow cytometry in the same session. The forward scatter–side scatter gating strategy consisted of gating on the live lymphocyte population (specifically NK lymphocytes in this case because the NK cells were 85–95% pure before culture). The much larger K562 cells were excluded from the lymphocyte gate using a K562 alone sample. Doublets with K562 and larger cells in general were also not included in this analysis. Leukocyte integrin chains, CD11a and CD18 (LFA-1 subunits), were identified by staining with fluorescence-tagged mAb using standard methodology. CD107a, the membrane expression of which reflects granule exocytosis (31), was assessed on NK cells by staining with anti-CD107a–FITC and gating on NK cells stained with CD16–PE and CD56–APC. After pre-culture with or without IL-2 and each statin, NK cells were co-cultured with K562 to stimulate cytotoxicity at a ratio of 1:1 for 1 h, and then stained. Flow cytometric data were analyzed using FlowJo software (TreeStar, Ashland, OR, USA).

**Intracellular staining for perforin and granzymes**

After culture with or without IL-2 and with or without statins, NK cells were fixed with 2% buffered formaldehyde and permeabilized with a buffer containing 0.3% saponin. After pre-incubation with human IgG at 25 μg ml⁻¹, the cells were stained with FITC-conjugated mAb against human perforin, granzyme A or granzyme B to detect the cytosolic proteins. Staining was visualized on a Becton–Dickinson FACSCaliber. Cells were analyzed using FlowJo software.

**Granzyme B ELISA**

Purified NK cells were cultured as above for 3 days with 100 U ml⁻¹ IL-2 in the absence or presence of statins. Cells were collected and stimulated with K562 at 1:1 ratio for 1 h. Supernatants of the stimulation cultures were collected and granzyme B was assayed using Granzyme B kit (Diaclone, Stamford, CT, USA) according to the manufacturer’s instructions.
Cell viability and proliferation assay

Cell viability was analyzed using trypan blue dye exclusion. Proliferation was assessed by $[^{3}H]$thymidine ($[^{3}H]$Tdr) incorporation. Triplicate aliquots of 100 μl were taken from 3-day stimulation cultures and added to 96-well flat-bottomed plates cultured for an additional 6–16 h in the presence of 1.0 μCi of $[^{3}H]$Tdr (6.7 Ci mol$^{-1}$; PerkinElmer, Boston, MA, USA). Cells were harvested on to glass filters (Skatron, Sterling, VA, USA), placed in scintillant and counted in a scintillation counter (Wallac, Turku, Finland). Data are expressed as counts per minute (c.p.m.).

Cytotoxicity assay

Cytotoxicity by NK cells after culturing with or without IL-2 and each statin was assessed in standard $^{51}$Cr-release assay using target cells labeled with $^{51}$Cr and each statin was assessed in standard $^{51}$Cr-release assay. Effector cells were incubated with 5000 target cells at effector-to-target (E:T) ratios ranging from 20 to 0.3:1 in quadrupling dilutions and in a final volume of 200 μl per well. Supernatants were harvested using a Skatron system, and counted in an automated gamma counter. Maximum release of $^{51}$Cr was obtained by lysis of the cells in 1 M HCl. Spontaneous $^{51}$Cr release was determined in wells containing labeled target cells incubated with medium alone. Results are shown as percent release: (c.p.m. in experimental sample – c.p.m. in spontaneous release)/(c.p.m. in maximum release – c.p.m. in spontaneous release) at all E:T ratios tested.

Reverse transcription–PCR for Fas ligand (CD95L) mRNA

Fas ligand (CD95L) (FasL) mRNA expression was assessed using reverse transcription (RT)–PCR. Total RNA was isolated from cells using RNeasy Micro Kit (Qiagen) according to the manufacturer’s instructions. The sequences of the primer pairs in this experiment were as follows: human glyceraldehyde-3-phosphate dehydrogenase (G3PDH; used as an internal control), 5′-GCT CAG ACA CCA TGG GGA AGG T-3′ and 5′-GTG GTG GTG GAG GCA TTG CTG A-3′; and human FasL, 5′-GTG CCC AGA AGG CCT GGT CAA AGG-3′ and 5′-TTG CAA GAT TGA CCC CGG AAG TAT-3′. Amplifications were performed in a thermocycler (Applied Biosystems) as follows: 94°C, 3 min, followed by 25 (G3PDH) or 35 (FasL) cycles (94°C, 1 min; 57°C, 1.5 min and 72°C, 2 min). Aliquots of each amplification were analyzed by electrophoresis in 2% acrylamide–Tris-borate gels. The lanes were normalized to G3PDH mRNA levels as described (32).

Results

Lipophilic statins suppress the cytotoxic activity of NK cells cultured with and without rIL-2

Purified NK cells were cultured in the presence or absence of human rIL-2 (100 U ml$^{-1}$), and with or without pravastatin (8 or 40 μM), simvastatin (2 or 10 μM) or fluvastatin (2 or 10 μM) for 3 days, and the cytotoxic activity was assessed against K562 target cells. This time point was selected because it gave clear and consistent results. As shown in Fig. 1, simvastatin (Fig. 1A) and fluvastatin (Fig. 1B) but not pravastatin (Fig. 1C) suppressed the cytotoxic activity in a dose-dependent manner. The same pattern was observed when NK cells were cultured in the presence of these statins without IL-2 (Fig. 1D). These concentrations of statins affected neither viability of NK cells cultured with or without IL-2 (Fig. 2A) nor NK cell proliferation (Fig. 2B). These results show that the lipophilic statins, simvastatin and fluvastatin, but not the hydrophilic pravastatin, can suppress the cytotoxic activity of NK cells cultured with or without IL-2. The data suggest that the lipophilic but not hydrophilic statins negatively regulate NK cell activity. That lipophilic statins down-regulate NK cytolytic activity whether or not cells are cultured with IL-2 supports the conclusion that the effect is, indeed, on the cells themselves.

Since ECs are physiological target cells during the processes of graft rejection as well as atherosclerosis, we examined the effect of statins on NK cytotoxicity against both HAECs and PAECs, representing possible targets in allografts/atherosclerosis and xenografts, respectively. The results shown in Fig. 3 demonstrate that the lipophilic statin, simvastatin, strongly suppressed IL-2-activated NK activity against both target cells; pravastatin had no effect on activity against PAEC targets, and only marginally reduced activity against HAEC. Consistent results were obtained with cells cultured without IL-2 (data not shown).

Mevalonate partially restores the cytotoxicity of NK cells treated with lipophilic statins

To determine whether the inhibition of cytotoxic activity by lipophilic statins is related to their effect on HMG-CoA reductase, we assessed whether treatment with mevalonate, the immediate downstream enzymatic product, reverses their effect on IL-2-activated NK cells (Fig. 4). Purified NK cells were cultured with or without IL-2 and each statin for 3 days. For the final 16 h (a typical mevalonate treatment interval from the literature), each statin treatment group was divided into two subgroups, and mevalonate was added to one of them at a final concentration of 1 mM. Mevalonate reversed, at least in part, the simvastatin and fluvastatin inhibition of cytotoxic activity of IL-2-activated NK cells against K562 (Fig. 4D and E). It had no effect on the statin-unaltered cytotoxicity mediated by pravastatin-treated cells (Fig. 4C). These results support that the lipophilic statins’ effect for suppressing cytotoxicity of NK cells depends on the inhibition of HMG-CoA reductase.

The effect of lipophilic statins on mediators of NK cytotoxicity

We proceeded to investigate the mechanism by which lipophilic statins inhibit activity of NK cells, concentrating on IL-2-activated cells. We first asked whether lipophilic statins inhibited cytotoxicity through a direct effect on the cytotoxic mechanisms. The most well-described mechanisms of NK cell lysis involve directed exocytosis of granules containing perforin and granzymes or induction of apoptosis in target cells through FasL, especially on IL-2-activated NK cells (33, 34). FasL mRNA expression was assessed after culture with IL-2 by RT–PCR. The data in Fig. 5 show that culture with statins (lipophilic, fluvastatin or hydrophilic, pravastatin) had no effect on FasL mRNA expression when included during the 3-day culture of NK cells with IL-2. We also attempted to examine FasL surface expression but could not identify an
We then analyzed expression of perforin and granzymes A and B by intracellular staining and flow cytometry using fluorescence-conjugated mAbs. While perforin levels increased; 2-fold as indicated by fluorescence intensity [from median fluorescent intensity (MFI) of 94 to MFI of 210] after stimulation with IL-2, statin treatment had no further effect on perforin levels (Fig. 6A). Furthermore, though the increase in granzyme A (Fig. 6B) and granzyme B (Fig. 6C) were marginally increased after IL-2 stimulation, consistent with the lack of effect on perforin expression, statins did not alter the expression of either granzyme.

Effect of the lipophilic statins on the expression of a cell-surface adhesion molecule, LFA-1 does not explain inhibition of cytotoxicity

Leukocyte function-associated antigen (LFA-1), which comprises CD11a and the common β2 integrin chain, CD18, is involved in early signaling for cytotoxic activity of NK cells (35, 36) and is important during IL-2 activation of NK cells (37, 38). Furthermore, it has been reported that PBMCs of hypercholesterolemic patients who were treated with simvastatin expressed lower amounts of CD18 and CD11a mRNAs compared with pre-treatment values and that surface expression of CD18/CD11a on CD14+ monocytes decreased significantly.

Fig. 1. Cytotoxic activity by purified NK cells cultured with or without IL-2 against K562 cells is reduced by lipophilic but not by hydrophilic statins. Purified NK cells were cultured with or without 100 U ml\(^{-1}\) of IL-2 in the absence or presence of statins at the indicated concentrations for 3 days as described in Methods. The cytotoxic activity of NK cells up-regulated with IL-2 (same control is shown in A–C), and was suppressed by simvastatin (A) or fluvastatin (B) in a dose-dependent manner, but not by pravastatin (C). Simvastatin and fluvastatin but not pravastatin suppressed cytotoxicity when cells were cultured without IL-2 in the presence of these statins (D). Data are from one experiment but are representative of at least six independent experiments which were conducted independently using NK cells isolated from different donors. Though magnitudes of differences varied among donors, all experiments consistently showed inhibition by simvastatin and fluvastatin but not by pravastatin. Error bars are standard errors of replicate samples.

Fig. 2. Statins have no effect on viability or proliferation of IL-2-stimulated NK cells. (A) Viability of NK cells after culturing without IL-2 or with IL-2 in the absence or presence of statins for 3 days was assessed by trypan blue dye exclusion, and no significant differences were observed. Data are shown as percent viable cells and are average results from three separate experiments. Error bars represent standard errors. (B) Likewise, none of the statins (at concentrations of 10 µM for fluvastatin and simvastatin and 40 µM for pravastatin) affected the proliferative response of NK cells to IL-2. Data are shown as averages from three separate experiments, and error bars are standard errors.
We therefore analyzed CD11a and CD18 on NK cells by flow cytometry after culturing with or without IL-2 and each statin for 3 days. As shown in Fig. 7(A), IL-2 stimulation increased CD18 surface expression slightly, and fluvastatin suppressed its expression, compatible with decreasing cytotoxic activity. However, simvastatin, which is very effective in suppressing IL-2-activated NK activity, had only a marginal effect on CD18 expression. CD11a expression was scarcely affected by IL-2 stimulation and the level following IL-2 stimulation was not affected by statin treatment (Fig. 7B). Thus, two lipophilic statins, both of which suppressed IL-2-activated NK activity, exhibited differing magnitudes of effects on CD18 expression but none on CD11a. Therefore, LFA-1 (CD18/CD11a) expression does not appear to explain the consistent marked inhibition of cytotoxic activity by simvastatin and fluvastatin and hence probably does not completely explain their common effect on NK cell cytotoxic activity.

Effect of statins on exocytosis pathway

Since our results suggest involvement of lipids in the inhibition of NK cytotoxicity by lipophilic statins, but did not affect cytosolic expression of lytic mediators, we next assessed the effect of statins on the release mechanism, or exocytosis of lytic granules in response to stimulation with target cells. The lytic granules are surrounded by a lipid bilayer containing lysosomal-associated membrane glycoproteins (LAMPs), including CD107a (LAMP-1) (40). These proteins are not normally found on the cell surface (41), but analyzing CD107a expression on the NK cell surface after stimulation with target cells to induce exocytosis provides an indirect measurement of release of cytolytic granules. We first assessed

*Fig. 3.* Cytotoxic activity by IL-2-activated purified NK cells against ECs is reduced by a lipophilic statin. NK cells were treated as in Fig. 1, and tested for cytotoxicity against HAEC and PAEC. Results are representative of four independent experiments. Error bars are standard errors of replicates within the one representative experiment; some error bars are too small to be visualized.

*Fig. 4.* Mevalonate reverses the statin-mediated inhibition of cytotoxic activity by IL-2-activated NK cells. Mevalonate (1 mM) was added to the culture medium during the last 16 h of the 3-day culture without (A) or with 100 U/ml IL-2 (B), and 40 µM pravastatin (C), 10 µM simvastatin (D) or 10 µM fluvastatin (E). Mevalonate at least partially restored the cytotoxicity of IL-2-stimulated NK cells that had been down-regulated by simvastatin or fluvastatin. The data are representative of three separate experiments using NK cells from three different donors.
CD107a expression on IL-2-stimulated NK cells, treated or not treated with statins, and then stimulated, or not, by coculture with K562. This experiment was repeated four times, and in three additional experiments, we also tested whether 1 mM mevalonate, added at the beginning of the third day of culture, could reverse the effect of statins on CD107a expression. We used flow cytometric analysis, gating on CD16+/CD56+ NK cells, and measured CD107a expression by three-color staining (Fig. 8). The results revealed that pretreatment with simvastatin or fluvastatin but not pravastatin at low (Fig. 8A) or high (Fig. 8B) concentrations significantly suppressed CD107a expression on NK cells. Interestingly, in contrast to the data shown in Fig. 1, even the lower concentrations of lipophilic statins markedly decreased CD107a expression. This difference is probably attributable in part to differences between donors and in part to the difference in assays (i.e. cytotoxicity versus flow cytometry) which results in differing dose responses. The expression of CD107a, as well as its suppression by statins, was of greater magnitude after effector cells had been co-cultured with K562. Addition of 1 mM mevalonate rescued CD107a expression, and the reversal of reduced CD107a expression by mevalonate is consistent with its rescue of NK-mediated cytotoxicity from the reduction induced by lipophilic statins. It is interesting that only in IL-2-treated effector cells not cultured with K562, regardless of statin concentration, the addition of mevalonate seemed to reduce baseline CD107a expression (from 11 to 6%); however, the levels of CD107a in statin-cultured cells were nevertheless all equivalent to the reduced baseline, suggesting rescue even under these conditions. These findings support the notion that the lipophilic statins reduce lysis by NK cells by a mechanism involving release of cytoxic granules.

To confirm that granule exocytosis is inhibited by lipophilic statins, we measured secretion of the granule component, granzyme B, in response to stimulation with K562 target cells. Granzyme B was measured in supernatants of K562-stimulated NK cells that had been cultured with IL-2 with and without statins. Although statins did not significantly alter the intracellular expression of IL-2-stimulated NK cells (Fig. 6), the lipophilic statins, simvastatin and fluvastatin, but not the hydrophilic pravastatin, markedly decreased the levels of granzyme B secreted in response to stimulation with target cells (Table 1). These results provide additional support for the conclusion that lipophilic but not hydrophilic statins suppress human NK cytotoxicity through a mechanism that reduces exocytosis of cytoxic granules.

**Discussion**

Statins are HMG-CoA reductase inhibitors that are widely used as therapeutic agents for hypercholesterolemia. Recently, evidence of their pleiotropic effects, meaning outside of the ability to reduce cholesterol synthesis, has emerged, such as anti-proliferative effects on smooth muscle and up-regulation of endothelial nitric oxide synthase (16–18, 42). Notably, reports that show statins act as immunomodulators are accumulating (43, 44), with both pro- (45–47) and anti-inflammatory effects (28, 29, 45, 48–50) reported. Therefore, we were interested in the possibility that statins could suppress the cytotoxic activity of NK cells, which play an important role, for example, in rejection of allogeneic bone marrow and xenotransplants (6), and emerging evidence has supported a role for NK cells in rejecting non-bone marrow transplants (6, 7).

We now report that two lipophilic statins (simvastatin and fluvastatin), but not a hydrophilic statin (pravastatin), strongly suppressed NK cytotoxic activity in a dose-dependent manner. The suppression of cytotoxicity is associated with suppression of granule exocytosis in response to K562 target cell stimulation as demonstrated by reduced CD107a surface expression and suppression of granzyme B release. The effect of lipophilic statins on exocytosis is consistent with that of Yamakuchi et al. (51), who reported that simvastatin suppresses exocytosis of Weibel–Palade bodies by ECs. One obvious possible explanation for the different effects we observed between the lipophilic and hydrophilic statins is the difference in diffusion into cells. While both lipophilic and hydrophilic statins are used clinically (30) and exhibit similar pharmacodynamics (52), the lipophilic statins are generally taken up much more widely by passive diffusion into a broad range of tissues and cells compared with hydrophilic statins (53). Intuitively, one would think that their differences in lipid solubility may influence the ability of statins to exert their cholesterol-lowering and pleiotropic effects. However, the low density lipoproteins-lowering potency per milligram of drug has been reported as fluvastatin (lipophilic) > lovastatin = pravastatin (hydrophilic) > simvastatin (lipophilic) > atorvastatin > rosvastatin, arguing against the lipid solubility as being crucial for clinical outcome. In addition, to examine whether alteration of lipid membrane components may explain the effect observed on exocytosis, we used choler toxin B staining (55) to analyze surface expression of GM1.
ganglioside, a membrane glycolipid, on fresh NK cells treated with statins. While we detected a slight decrease in the frequency of cholera toxin-positive cells among those treated with lipophilic but not hydrophilic statins, the results did not reach significance (data not shown), suggesting that difference in GM1 ganglioside composition does not explain the differences between the statins.

It has been reported that PBMCs from hypercholesterolemic patients treated with simvastatin expressed lower amounts of mRNAs encoding CD18 (β2 leukocyte integrin common chain) and CD11a (LFA-1 unique chain) compared with pre-treatment values and that surface expression of CD18/CD11a on CD14+ monocytes decreased (39). Additionally, LFA-1 transduces an early activating signal to NK cells (35, 36) and is activated during IL-2 stimulation of NK cells (37, 38). Therefore, we tested whether lipophilic statins affect LFA-1 expression in association with their suppression of NK cell cytotoxic activity. However, we found that while fluvastatin decreased CD18 expression on human NK cells, simvastatin did so only marginally, but both lipophilic statins markedly suppressed NK cell activity. Thus, the effect on CD18 expression did not correlate with or explain all the suppression of NK activity, and suggest that the somewhat differing effect of simvastatin on CD18 expression on monocytes and NK cells may be referable to cell type. Also, in contrast to the findings of Rezaie-Majd et al. (39), in human monocytes, but consistent with those of Wan et al. (29), who tested a statin-like compound on murine neutrophils, neither
Moreover, it has also been reported that the means by which statins inhibit LFA-1 activity is independent of suppressing cholesterol synthesis (28, 29). It should be noted that our findings do not exclude the possibility that statins affect expression of the other β2 integrin α chains that dimerize with CD18, namely CD11b and CD11c, which also may affect NK cell-mediated cytotoxicity.

Our findings show that both the lipophilic statin-mediated inhibition of NK cytotoxicity measured as 51Cr release from target cells and the statin-mediated inhibition of exocytosis measured as CD107a expression on CD16+CD56+ NK cells is reversed by mevalonate, the specific HMG-CoA reductase product (26). These results support the conclusion that inhibition of HMG-CoA reductase is necessary and sufficient for the effect of lipophilic statins on NK cell activity. Thus, the suppression of cytotoxicity and exocytosis is mediated, at least in part, through the same upstream pathway as inhibition of cholesterol synthesis, namely inhibition of HMG-CoA reductase. However, it is quite possible that the effect on cytotoxicity branches off from the HMG-CoA reductase cholesterol-synthesizing pathway downstream of mevalonic acid, and may not be directly related to cholesterol synthesis.

It is interesting that while our data clearly demonstrate an inhibitory effect of lipophilic statins on NK cells, which as components of the innate immune system are believed to provide a first line of defense against tumorogenesis, statins have also been proposed to have anti-cancer activity (17, 24–26). Though conflicting conclusions have been reached concerning the anti-cancer effects of statins (27), it is possible that anti-cancer activity operates through an effect on ECs, and thereby angiogenesis through effects on endothelial nitric oxide synthase (56, 57). Another possible mechanism for the anti-tumor effects is enhancement of up-regulated IFN-γ-induced MHC class I expression (58), which could stimulate increased CTL activity while potentially inhibiting NK activity. These potentially opposing effects of statins on cancer need to be explored more fully.

That statins inhibit NK cytotoxicity is consistent with a possible future role for these drugs in suppression rejection of allografts and xenografts. It would therefore also be of interest to determine whether lipophilic statins suppress lytic activity mediated by other important mediators of graft rejection, such as cytotoxic T cells. Though in our study we used pharmacological levels of statins [10–40 μM, equivalent to ~4–20 mg l⁻¹, or one to two orders of magnitude greater than Cmax of 40–180 μg l⁻¹, depending on the statin (52)], these concentrations were similar to those reported by other investigators performing studies ex vivo. Moreover, Hillyard et al. (59) identified similar alterations of function in lymphocytes obtained from patients treated with therapeutic doses.
of simvastatin, thus supporting the feasibility of clinical intervention for inhibiting NK cell activity.

Acknowledgements

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Abbreviations

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<tr>
<th>APC</th>
<th>allophycocyanin</th>
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<tr>
<td>c.p.m.</td>
<td>counts per minute</td>
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<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
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<tr>
<td>EC</td>
<td>endothelial cell</td>
</tr>
<tr>
<td>E:T</td>
<td>effector-to-target</td>
</tr>
<tr>
<td>FasL</td>
<td>Fas ligand (CD95L)</td>
</tr>
<tr>
<td>G3PDH</td>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>HAEC</td>
<td>human aortic endothelial cell</td>
</tr>
<tr>
<td>HMG-CoA</td>
<td>3-hydroxy-3-methyl-glutaryl Coenzyme A</td>
</tr>
<tr>
<td>[3H]Tdr</td>
<td>[3H]thymidine</td>
</tr>
<tr>
<td>LAMP</td>
<td>lysosomal-associated membrane glycoprotein</td>
</tr>
<tr>
<td>LFA-1</td>
<td>leukocyte function-associated antigen-1</td>
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<tr>
<td>MFI</td>
<td>median fluorescent intensity</td>
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<tr>
<td>PAEC</td>
<td>porcine aortic endothelial cell</td>
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<tr>
<td>rIL-2</td>
<td>recombinant IL-2</td>
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<td>RT</td>
<td>reverse transcription</td>
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Table 1. Granzyme B release by NK cells activated with IL-2 (3 days) and stimulated with K562 (1 h, 1:1 ratio) is reduced by including simvastatin or fluvastatin (lipophilic) but not by pravastatin (hydrophilic) during the 3-day culture

<table>
<thead>
<tr>
<th>Statin treatment</th>
<th>Supernatant granzyme B concentration (pg ml⁻¹)</th>
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<tbody>
<tr>
<td></td>
<td>Experiment 1</td>
</tr>
<tr>
<td>None</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>Simvastatin (10 µM)</td>
<td>206 ± 16</td>
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<tr>
<td>Fluvastatin (10 µM)</td>
<td>239 ± 75</td>
</tr>
<tr>
<td>Pravastatin (40 µM)</td>
<td>&gt;1000</td>
</tr>
</tbody>
</table>

Values shown are averages ± SDs of replicate samples.

Fig. 8. CD107a expression on NK cells is altered by lipophilic (simvastatin and fluvastatin) but not by hydrophilic (pravastatin) statin. (A) NK cells were cultured for 3 days with or without 100 U ml⁻¹ IL-2 plus no statin treatment (black bar), 8 µM pravastatin (gray bar), 2 µM simvastatin (diagonal striped bar) or 2 µM fluvastatin (vertical striped bar). For mevalonate rescue, NK cells from the same donor were treated with the same conditions except that 1 mM mevalonate was added to the culture media for the last 16 h of the 3-day culture. The cells were harvested and cultured with or without K562 cells at a 1:1 ratio for an additional 1 h. Cells were stained with CD107a-FITC mAb, fixed and analyzed by flow cytometry for cell-surface CD107a expression on the CD16⁺CD56⁺ double-positive NK cell population. (B) NK cells from the same donor were cultured exactly as in (A) except statin concentrations were increased to 40 µM pravastatin (gray bar), 10 µM simvastatin (diagonal striped bar) or 10 µM fluvastatin (vertical striped bar). These data are representative of three independent experiments. The effect of statins on CD107a expression was also analyzed in four additional experiments, without testing for mevalonate reversal, with consistent results to those shown here. The conditions for each independent experiment were set up using cells from a single healthy blood donor, and all were treated at the same time and analyzed in the same session by flow cytometry.

References

in hypercholesterolemic patients after treatment for 12 weeks.


