Bioactive lipids as key regulators in atherosclerosis

Bot, M.

7 FTY720, a Synthetic Sphingosine 1-Phosphate Analogue, Inhibits Development of Atherosclerosis in Low-Density Lipoprotein Receptor Deficient Mice

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Abstract

Background: Numerous *in vitro* studies suggest that sphingosine 1-phosphate (S1P), a bioactive lysosphingolipid associated with high-density lipoproteins, accounts at least partly for the potent anti-inflammatory properties of high-density lipoprotein and, thereby, contributes to the anti-atherogenic potential attributed to high-density lipoproteins. The present study was undertaken to investigate whether modulation of S1P signaling will affect atherosclerosis in a murine model of disease.

Methods and Results: Low-density lipoprotein receptor deficient mice on a cholesterol-rich diet were given FTY720, a synthetic S1P analogue, at low (0.04 mg/kg per day) or high (0.4 mg/kg per day) doses for 16 weeks. FTY720 dose–dependently reduced atherosclerotic lesion formation, both in the aortic root and brachiocephalic artery, and almost completely blunted necrotic core formation. Plasma lipids remained unchanged during the course of FTY720 treatment. However, FTY720 lowered blood lymphocyte count (at a high dose) and significantly interfered with lymphocyte function, as evidenced by reduced splenocyte proliferation and interferon-γ levels in plasma. Plasma concentrations of pro-inflammatory cytokines such as tumor necrosis factor-α, interleukin (IL)-6, IL-12 and regulated on activation normal T cell expressed and secreted were reduced by FTY720 administration. Moreover, lipopolysaccharide-elicited generation of nitrite/nitrate and IL-6, two markers of classical (M1) macrophage activation, was inhibited, whereas IL-4-induced production of IL-1 receptor antagonist, a marker of alternative (M2) macrophage activation, was augmented in peritoneal macrophages from FTY720-treated low-density lipoprotein receptor deficient mice.

Conclusions: The present results demonstrate that an S1P analogue inhibits atherosclerosis by modulating lymphocyte and macrophage function, and these results are consistent with the notion that S1P contributes to the anti-atherogenic potential of high-density lipoprotein.

Introduction

The biologically active lysosphingolipid sphingosine 1-phosphate (S1P) is an important lipid mediator generated from phospholipids on cell activation and is present in plasma and extracellular fluid in high nanomolar concentrations[1,2]. A large body of evidence has documented the pleiotropic effects of S1P in various cell types and organs. S1P was demonstrated to interfere with proliferation, migration and cytokine secretion by lymphocytes and to prevent their recirculation from lymphoid compartments to peripheral sites of antigen presentation[3,4]. These immunomodulatory activities likely account for the beneficial effects exerted by S1P and its analogues in animal models of inflammatory diseases such as ulcerating colitis, viral myocarditis, endotoxin-induced lung injury, or autoimmune encephalomyelitis[5-8]. In the vasculature, S1P was found to affect survival, proliferation, migration, and activation of endothelial and smooth muscle cells and thus regulate vascular development and vessel contraction[1,9]. S1P evokes these diverse cellular responses by binding to members of a family of homologous G-protein-coupled receptors designated as S1P receptors. $S1P_1$ and $S1P_4$ were found to preponderate in lymphocytes, whereas other cells present in vascular wall mainly express $S1P_1$, $S1P_2$ and $S1P_3$ receptors[9,10].
Numerous epidemiological and clinical studies have documented an inverse relationship between high-density lipoprotein (HDL) and the progression of atherosclerosis, but the mechanisms underlying the anti-atherogenic effects of HDL are not entirely clear. Recent investigations have revealed that HDL serves as a carrier of biologically active lysosphingolipids, including S1P[11,12]. Moreover, S1P *in vitro* was found to emulate several anti-atherogenic effects attributed to HDL, including inhibition of endothelial apoptosis and stimulation of cell movement, inhibition of the expression of adhesion molecules, and stimulation of nitric oxide generation[13-15]. Although these data may point to an atheroprotective activity of S1P, the effect of HDL-associated lysosphingolipids on atherosclerosis has not yet been addressed in an *in vivo* setting. In the present study, we evaluated the effect of 2-amino-2-[2-(4-octylph enyl)ethyl]propane-1,3-diol hydrochloride (FTY720), a high-affinity agonist of $S1P_1$, $S1P_3$, $S1P_4$ and $S1P_5$ receptors[16], on the development of diet-induced atherosclerosis in low-density lipoprotein (LDL) receptor deficient ($LDLr^{-/-}$) mice. FTY720 was originally derived as an immunomodulatory compound exerting beneficial effects in several animal models of chronic inflammation[17,18]. In addition, FTY720 was reported to interact with endothelial cells and to exert several potentially anti-atherogenic effects, such as enhancing adherens junction assembly and endothelial barrier function or promoting nitric oxide generation and vasorelaxation[18,19]. Here, we show that FTY720 retards the development of atherosclerosis independent of changes in total plasma or HDL cholesterol.

Methods

FTY720 determination, application and distribution
FTY720 was determined in full blood and fractionated plasma lipoproteins using our previously reported high-performance liquid chromatography-atmospheric pressure chemical ionization-tandem mass spectrometry method for human whole blood, ex-

actly as described previously[20]. In order to minimize any potential biological matrix differences, weighed-in standard and control material was prepared from mouse blood, plasma and lipoprotein fractions. In brief, the method requires samples to be subjected to liquid-liquid extraction, in the presence of an internal standard (Y-32919, an analogue of FTY720) and detected by selected reaction monitoring (FTY720: *m/z* 308.3→255.3, Y-32919: *m/z* 324.3→159.3). FTY720 shows few toxic effects in either animal or human studies[21,22]. Accordingly, no adverse effects or significant changes in body weight were observed in the present investigation in treated animals during FTY720-application period. Because of rapid initial absorption (time of occurrence for maximum drug concentration ≈2 to 24 hours), extensive volume of distribution, and exceptionally long elimination half-life (≈7 days), FTY720 blood concentrations remain stable after administration, with little fluctuation during the dosing interval[21-23]. Consistent with these pharmacokinetic properties, twice- or thrice-weekly administration of FTY720 is sufficient to suppress inflammatory processes in various mouse models[24,25]. In the present study, we found that repeated intraperitoneal injection of the compound (20 µg/LDLr$^{-/-}$ mouse, thrice-weekly) produces FTY720 plasma concentration of 67.5 ± 8.7 ng/mL, which is within the known therapeutic concentration range[23]. Examination of FTY720 distribution among lipoprotein fractions isolated from LDLr$^{-/-}$ mouse plasma spiked with 50 ng/mL of the compound revealed that 32%, 24% and 44% were present in very-low-density lipoprotein, LDL, and HDL, respectively. Minor amounts of FTY720 were found in plasma fractions containing no lipoproteins.

Animals

Female LDLr$^{-/-}$ mice on a C57BL/6J background were purchased from The Jackson Laboratory, Bar Harbor, ME, and housed under pathogen-free conditions at the Center for Animal Studies of the University Clinic of Münster. Mice (8 weeks old, 20 to 25 g, female) were put on an atherogenic high-fat diet (1.25% cholesterol, 7% cocoa butter; Harlan Winkelmann, Borchen, Germany) for 16 weeks. Animals were separated randomly into three groups (n=8 mice/group). The first and the second group received 3 weekly intraperitoneal injections of 2.0 µg FTY720 (approximately 0.04 mg/kg per day; low dose) and 20 µg FTY720 per mouse (approximately 0.4 mg/kg per day; high dose), and the third control group was injected with the same volume (0.1 mL) of saline. Blood samples for blood cell count, lipid, and lipoprotein profiling were collected retroorbitally at the days 0, 7, 30, 60 and 90 of the study. At the end of the 16-week treatment period, mice were bled by retroorbital vein puncture under complete anesthesia with ketamine. Peritoneal macrophages were isolated, and tissues were collected for further analysis. The experimental protocol was approved by the local animal research committee.

Histology and lesion analysis

Exsanguinated animals were subjected to *in situ* perfusion fixation with formaldehyde (4% weight/volume) through the left cardiac ventricle. For analysis of spontaneous atherosclerosis, the aortic root and the brachiocephalic artery were removed and embedded in Tissue-Tek. Cryosections of the brachiocephalic artery (5 µm thick) were prepared and stained with hematoxylin (Sigma Diagnostics, Zwijndrecht, the Netherlands) and eosin (Merck Diagnostica, Darmstadt, Germany). For the aor-

tic root, 10 µm cryosections were prepared and stained with Oil Red O (Sigma, St. Louis, Mo). Cross-sections with maximal stenosis were used for morphometric analysis on a DM-RE microscope with Leica Qwin image-analysis software (Leica Microsystems B.V., Rijswijk, the Netherlands), as described previously[26,27].
Corresponding sections were stained immunohistochemically with antibodies directed against mouse macrophages (monoclonal mouse IgG_{2a}, clone monocyte + macrophage antibody-2 [MOMA-2], dilution 1:50; Serotec, Kidlington, Oxford, UK), vascular smooth muscle cells (monoclonal mouse IgG_{2a}, clone 1A4, dilution 1:50; Sigma) and lymphocytes (purified antimouse CD3 molecular complex, 17A2, dilution 1:50; BD Biosciences Pharmingen, San Diego, Calif). Sections were stained for collagen using Picrosirius Red (Sigma). Macrophage-, vascular smooth muscle cell- and collagen-positive areas were determined by computer-assisted color-gated measurement and were related to the total intimal surface area. For lymphocytes, the number of CD3-positive cells was assessed in 5 consecutive sections and averages were used for analysis.

Lipid analysis and lipoprotein fractionation
Plasma total cholesterol, HDL cholesterol and triglycerides were determined enzymatically using commercially available kits (Roche, Mannheim, Germany). Plasma lipoproteins were fractionated using Smart™ chromatographic system (Pharmacia, Uppsala, Sweden) equipped with a Superose 6 column (3.2 mm × 30 mm). Plasma was fractionated at a constant flow rate of 50 µL/min, using a buffer containing 150 mmol/L NaCl and 1 mmol/L EDTA, pH 8.0. In total, 36 fractions with a volume of 50 µL each were collected. Total cholesterol content of effluent was determined using enzymatic colorimetric assay (Roche Diagnostics, Mannheim, Germany).
For spiking experiments, two $LDLr^{-/-}$ mice on cholesterol-rich diet were exsanguinated and plasma was obtained by spinning down at 4000 rpm for 10 minutes. Plasmas from two animals were pooled and 125 µl aliquots were spiked with FTY720 to the final concentration of 50 ng/mL. Samples were than incubated for 30 minutes at 37°C and lipoprotein fractions were obtained using Smart™ chromatographic system as described above. FTY720 concentration in each lipoprotein fraction was determined by HPLC-tandem mass spectrometry.

Cytokine determination
Plasma tumor necrosis factor (TNF)-α, interleukin (IL)-6, and interferon (IFN)-γ levels were quantified by commercially available ELISAs (R&D Systems, Wiesbaden, Germany). Semiquantitative determination of pro-inflammatory cytokines was performed using a Cytokine Array I from Raybiotech Inc (Norcross, GA).

Leukocyte differential count and lymphocyte subtyping
Differential leukocyte count was performed manually (Pappenheim staining) in a routine hospital laboratory. Lymphocyte subtyping was performed by flow cytometry.
For lymphocyte subtyping, whole blood was anti-coagulated with citrate, incubated for 30 minutes with fluorescein isothiocynate (FITC)- or phycoerythrin (PE)-conjugated antibodies against CD3, CD4, CD8 or CD19 (5.0 µg/mL, Nanotools, Tenningen, Germany), and fixed for 30 minutes with 0.4% formaldehyde in phosphate buffered saline (PBS). Thereafter, cells were centrifuged for 10 minutes at 1700 rpm and

erythrocytes were lysed in a buffer containing 0.15 mol/L NH_4Cl, 10 mmol/L $NaHCO_3$, 0.1 mmol/L EDTA (pH 7.4). The remaining cells were washed twice in PBS and analyzed on a Beckton-Dickinson flow cytometer (BD Science, San Jose, CA) equipped with a 488 nm argon laser.

Splenocyte proliferation
The [^{3}H]thymidin incorporation rate was used to estimate proliferation of splenocytes stimulated with phorbol myristate acetate or concanavalin A. Spleens were removed and passed through a 70 µm mesh filter. Harvested cells were suspended in RPMI1640 containing 10% (v/v) fetal calf serum (FCS), 2 mmol/L L-glutamine, and 50 µmol/L β-mercaptoethanol, washed, and erythrocytes were lysed as described above. Splenocytes were washed twice, counted and seeded in 96-well plates (5 x 10^5 cells/well). Cells were stimulated for 40 hours with phorbol myristate acetate (10 µmol/L) or concanavalin A (2 µg/mL; both Sigma, Deisenhoffen, Germany). After 24 hours [^{3}H]thymidin (5.0 µCi/well; GE Healthcare, Braunschweig, Germany) was added and cells were incubated for the final 16 hours. Thereafter, cells were centrifuged (5 min, 5000 rpm), washed 3 x with PBS, lysed with 0.1 mol/L NaOH, and cell-associated radioactivity was determined by scintillation spectrometry.

Functional characterization of peritoneal macrophages
Peritoneal macrophages were harvested as previously described[28]. Cells were suspended in DMEM containing FCS (10% v/v) and 2 mmol/L L-glutamine, seeded in a 24-well plate at a density of 0.5 x 10^6 cells/mL. After 4 hours non-adherent cells were removed and remaining macrophages were incubated for 24 hours in the absence or presence of lipopolysaccharide (LPS; 50 ng/mL; Sigma, Deisenhoffen, Germany) or IL-4 (10 ng/mL; Peprotech, London, UK). Nitrite concentration in the cell medium was determined colorometrically using Griess Reagent System (Promega, Madison, WI). IL-6 and IL-1 receptor antagonist (IL-1RA) were determined by commercially available ELISA according to the instructions of manufacturer (R&D Systems, Wiesbaden, Germany).

Statistical analysis
Values are expressed as mean ± SD unless indicated otherwise. Comparisons between control and treated mice were made by 1- or 2-way ANOVA for independent samples or repeated measures as appropriate. Non-Gaussian distributed data were analyzed by Kruskall-Wallis test. Pair-wise comparisons of sample means were performed with Tukey HSD test. Trend significance analysis was performed with linear regression using actual doses as independent variables with ANOVA for the significance of the obtained correlation coefficient. A level of $p<0.05$ was considered significant.

Results

FTY720 retards atherosclerotic lesion development in LDLr$^{-/-}$ mice
Aortic root and brachiocephalic plaque size was determined as a measure of the effect of FTY720 on atherogenesis in high-fat diet-fed LDLr$^{-/-}$ mice. Morphometric

quantification of Oil Red O-stained aortic root lesions revealed decreased atherosclerosis after FTY720 treatment, with significant trend towards reduction of lesion area with increasing doses of the compound (r=-0.508; P=0.011; Figure 1A).

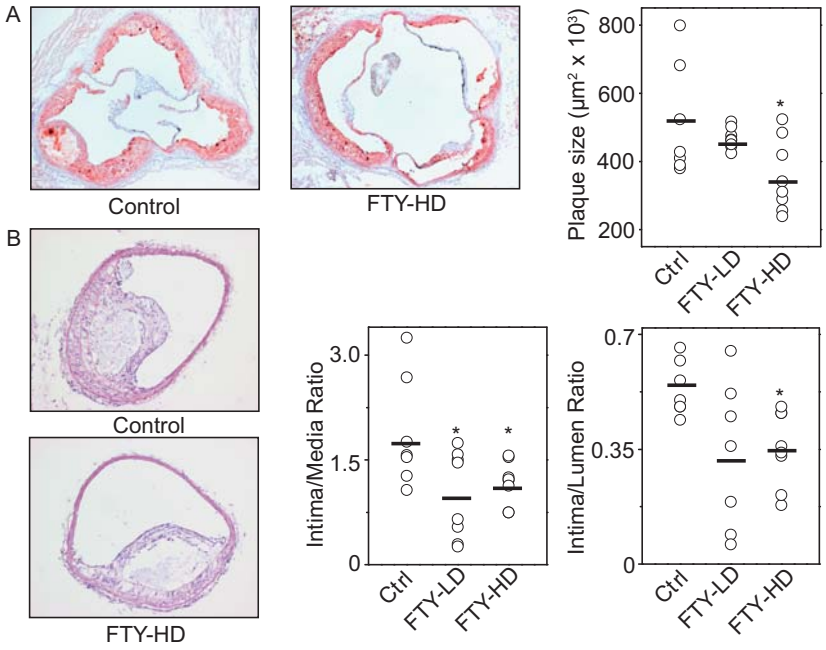

Figure 1. Effect of FTY720 on lesion development in LDLr$^{-/-}$ mice. LDLr$^{-/-}$ mice were placed on a cholesterol-rich diet, and saline (n=7; Ctrl), low doses (2.0 μg per mouse; n=8; FTY-LD) or high doses of FTY720 (20 μg per mouse; n=8; FTY-HD) were administered 3 times a week for 16 weeks. The mice were euthanized and bled, and the aortic root and brachiocephalic artery were fixed, sectioned, and used for morphometric analysis. (A) Aortic root lesions. Left panel, representative Oil Red O staining of aortic root lesions from control and FTY-HD mice. Right panel, distribution of atherosclerotic lesion area. (B). Brachiocephalic artery lesions. Left panel, representative hematoxylin and eosin staining of brachiocephalic artery lesions from control and FTY-HD mice. Right panel, distribution of intima/media and intima/lumen ratio. *P<0.05 (FTY-LD or FTY-HD vs. control).

The group receiving low doses of FTY720 (approximately 0.04 mg/kg per day) showed a slight decrease in lesion area that did not reach statistical significance. However, high doses of FTY720 (approximately 0.4 mg/kg per day) led to significant reductions in lesion area compared with control mice. A similar pattern was observed for the brachiocephalic artery plaques (Figure 1B). Because the variance in brachiocephalic vessel area was rather high, the intima/media ratio (reflecting the relative vessel wall thickening) and the intima/lumen ratio (reflecting degree of stenosis) were considered to be more reliable parameters. Both ratios were found to be significantly reduced after FTY720 treatment.

FTY720 affects atherosclerotic plaque morphology

Although FTY720 treatment attenuated lesion formation in the aortic root as well

as the brachiocephalic artery, it did not significantly affect the plaque collagen or anti-smooth muscle antibody content (not shown). Plaques from mice treated with FTY720 at a high dose (FTY-HD mice) tended to be richer in MOMA-positive macrophages, but this tendency could be completely ascribed to a lower progression stage of these plaques (Figure 2A). Indeed, FTY treatment dose-dependently impaired necrotic core formation ($p<0.01$ in high dose-treated compared with untreated mice, Figure 2B). Further analysis showed that high-dose FTY720 treatment substantially reduced intraplaque content of CD3-positive T cells (Figure 2C). Taken together, our data suggest that FTY720 treatment slows down necrotic core formation and dampens intraplaque inflammation, 2 major determinants of reduced plaque stability.

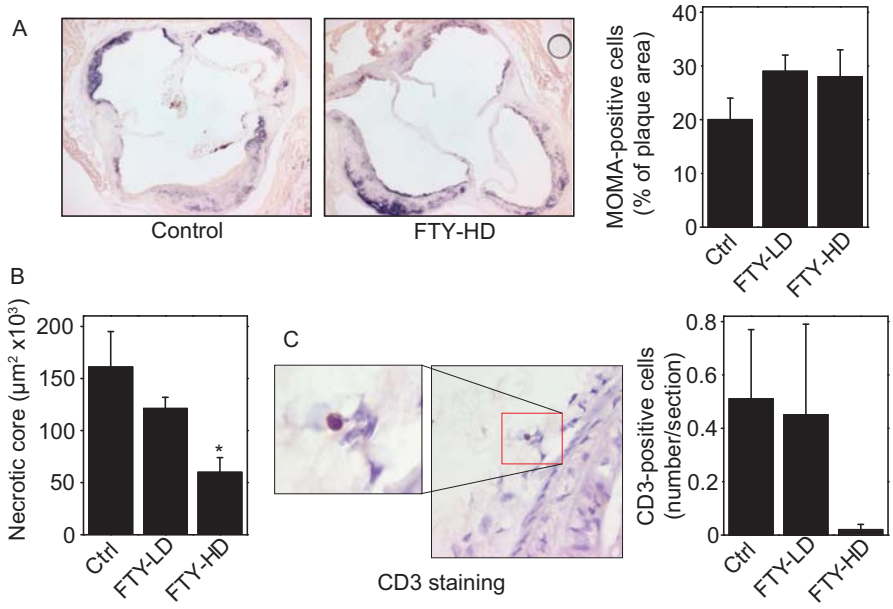

Figure 2. Effect of FTY720 on the morphology of atherosclerotic plaques in LDLr$^{-/-}$ mice. Aortic root and brachiocephalic artery lesions of controls (n=7 mice; Ctrl) or animals treated with low doses of FTY720 (n=8 mice; FTY-LD) or high doses of FTY720 (n=8 mice; FTY-HD) were stained for macrophages (MOMA-2) or T cells (CD3). (A) Left panel, representative MOMA-2 staining (x200) from control and FTY-HD-treated mice. Right panel, bar graph showing the content of macrophages in the atherosclerotic plaque that was calculated as percentage of staining area relative to the total plaque size. (B) Bar graph showing the size of necrotic core of aortic lesion in control and FTY720-treated animals. *P<0.01 (FTY-HD versus control). (C) Left panel, demonstration of CD-3 staining in atherosclerotic plaque. Right panel, bar graph showing the number of CD3-positive cells per section.

FTY720 does not affect plasma lipid profile

To assess potentially anti-atherogenic effects of FTY720 on lipid metabolism, we monitored plasma lipid profiles in LDLr$^{-/-}$ animals during the treatment period. As shown in Figure 3, total cholesterol, triglycerides, and HDL cholesterol concentrations in LDLr$^{-/-}$ mice on chow diet were approximately 300 mg/dL, 150 mg/dL, and 120 mg/dL, respectively. When fed an atherogenic diet, total cholesterol, triglycer-

ides, and HDL cholesterol levels were increased to approximately 1400 mg/dL, 450 mg/dL, and 400 mg/dL, respectively. No significant changes (by repeated-measures ANOVA) in the plasma lipid profile were observed between treatment groups (Figure 3D).

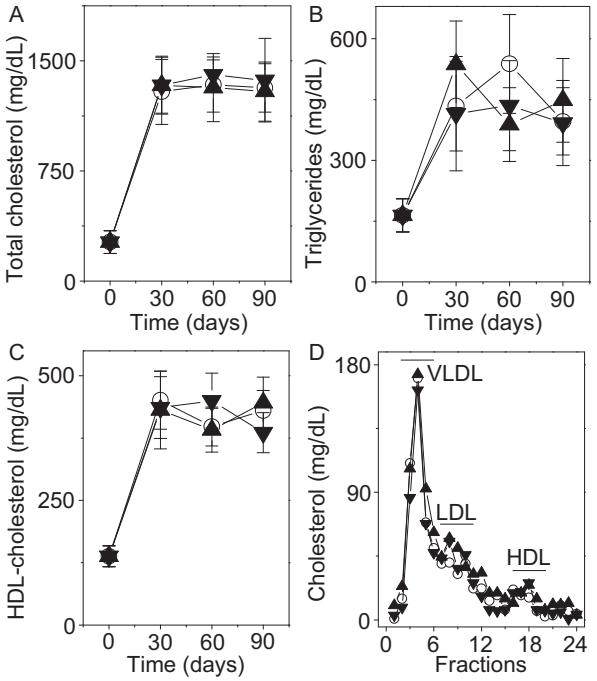

Figure 3. Effect of FTY720 on plasma lipid levels. Plasma was obtained from LDLr$^{-/-}$ mice after 0, 30, 60 and 90 days of controls (n=7 mice; ○), low-dose (n=8 mice; ▼) or high-dose (n=8 mice; ▲) FTY720 treatment, and total cholesterol (A), triglyceride (B), and HDL-cholesterol (C) levels were determined using routine laboratory procedures. (D) Demonstration of chromatographic lipoprotein profile in plasma obtained from animals treated with high (▲) or low (▼) doses of FTY720 or in untreated animals (○). Repeated-measures ANOVA revealed no significant main effects of FTY720 doses on plasma total cholesterol, HDL-cholesterol, or triglyceride levels.

FTY720 affects lymphocyte count and profile

To investigate whether FTY720 affects lesion formation by modulating peripheral immunity, we monitored leukocyte abundance and analyzed lymphocyte subpopulations by flow cytometry. As shown in Figure 4, the relative amount of lymphocytes was markedly decreased, whereas that of neutrophils was relatively increased in LDLr$^{-/-}$ mice treated with high doses of FTY720 ($P<0.001$ by repeated-measures ANOVA). In addition, a rise in B-cell number ($CD19^+$) and a dramatic drop in T-helper to cytotoxic T-cell ratio ($CD4^+/CD8^+$) were registered in FTY720-HD-treated animals ($P<0.001$ by repeated-measures ANOVA). In contrast, low doses of FTY720 did not alter the leukocyte profile or the B-cell counts, but they significantly decreased the $CD4^+/CD8^+$ ratio ($P<0.001$ by repeated-measures ANOVA) (Figure 4).

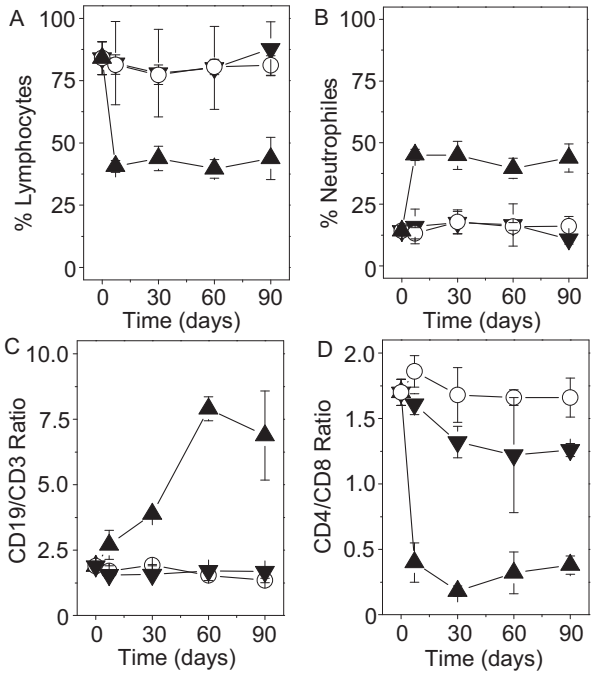

Figure 4. Effect of FTY720 on the blood leukocyte profile. LDLr$^{-/-}$ mice on cholesterol-rich diet were given saline (n=7; ○), or FTY720 at low (n=8; ▼) or high doses (n=8; ▲). Blood was collected retroorbitally from 3 randomly selected animals in each group at day 0, 7, 30, 60 and 90 of the treatment. Relative lymphocyte (A) and neutrophil (B) counts in peripheral blood were determined in blood smears using routine laboratory procedures. CD19^{+}/CD3^{+} (C) and CD4^{+}/CD8^{+} (D) ratios were determined using flow cytometry as described in the Methods section. Values represent mean ± SD. Repeated-measures ANOVA revealed significant main effects of HD-FTY720 lymphocyte and neutrophil counts and CD19^{+}/CD3^{+} and CD4^{+}/CD8^{+} ratios (P<0.001 for each dependent variable) and a significant main effect of LD-FTY720 on CD4^{+}/CD8^{+} ratio (P<0.001).

FTY720 suppresses lymphocyte proliferation

In addition to lymphocyte counts and subset pattern, we also addressed the influence of FTY720 on lymphocyte function. To this end, murine splenocytes from control and FTY720-treated animals were stimulated with phorbol myristate acetate (10.0 µmol/L) or concanavalin A (2.0 µg/mL), and the proliferation rate was determined in a [^{3}H]thymidin-incorporation assay. As shown in Figure 5, both agonists potently stimulated proliferation of splenic cells from control animals. By contrast, the mitotic response of splenocytes obtained from LDLr$^{-/-}$ mice treated with either high or low FTY720 doses to phorbol myristate acetate or concanavalin A was almost completely abrogated.

FTY720 modulates plasma cytokine profile

Because lymphocyte activation may be associated with an altered secretion of several inflammatory mediators, we next examined the effects of FTY720 treatment on the plasma cytokine profile. At both doses, FTY720 treatment of LDLr$^{-/-}$ mice led to a marked reduction of the plasma levels of IL-12 and regulated on activation normal T

cell expressed and secreted, 2 key mediators of lymphocyte function (Figure 6A). In addition, plasma levels of soluble TNF-receptor (TNF-R), a member of the inflammatory cytokine network, were reduced by FTY720 treatment. Plasma concentrations of IFN-γ, a cytokine released from lymphocyte upon activation, were seen to be dramatically decreased in low- and high-dose FTY720-treated versus control animals (Figure 6B). Finally, FTY720 substantially reduced plasma concentrations of IL-6 and TNF-α, two markers of macrophage-dependent inflammatory processes (Figure 6B).

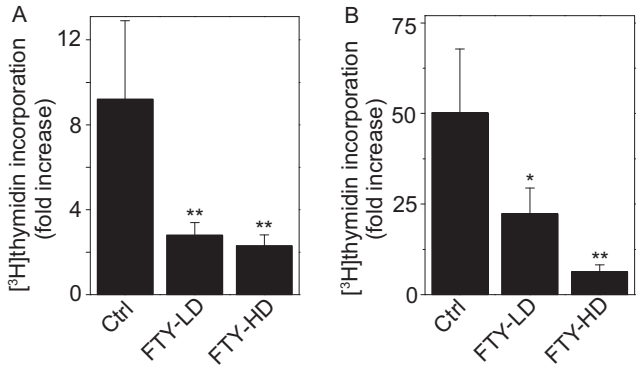

Figure 5. Effect of FTY720 on the mitotic response of splenocytes to phorbol myristate acetate or concanavalin A. Splenocytes were isolated from controls (n=7 mice; Ctrl) or animals treated with FTY720 at low doses (n=8 mice; FTY-LD) or high doses (n=8 mice; FTY-HD). Cells were seeded in 96-well plates and stimulated for 40 hours with phorbol myristate acetate (10 µmol/L; A) or concanavalin A (2 µg/mL; B) (as described in the Methods section). Cell proliferation was determined as [^{3}H]thymidin incorporation in the final 16 hours of incubation. Values are shown as mean ± SD. *P< 0.05, **P< 0.01 (FTY-LD or FTY-HD vs. control).

FTY720 modulates macrophage activation

Because FTY720 reduced the plasma levels of macrophage-derived pro-inflammatory cytokines, its effect on macrophage activation by pro-inflammatory stimuli was investigated as well. To this purpose, peritoneal macrophages obtained from control and FTY720-treated animals were stimulated for 24 hours, with LPS inducing a classical (M1) type of macrophage activation characterized by high nitric oxide and IL-6 production, or with IL-4 promoting an alternative (M2) type of macrophage activation, with high secretion levels of the anti-inflammatory cytokine IL-1RA[29]. As demonstrated in Figure 7, stimulation of macrophages with LPS induced the secretion of the M1 mediators IL-6 and nitric oxide, whereas the release of IL-1RA, an M2-type activation marker, was markedly elevated in IL-4-elicited macrophages. Both basal and LPS-stimulated production of IL-6 and nitric oxide were attenuated in macrophages obtained from FTY720 treated LDLr$^{-/-}$ mice. Conversely, treatment with FTY720 significantly amplified both basal and IL-4-induced release of IL-1RA from macrophages. Collectively, these results indicate that long-term treatment of LDLr$^{-/-}$ mice with FTY720 favors an M2-type macrophage-activation profile.

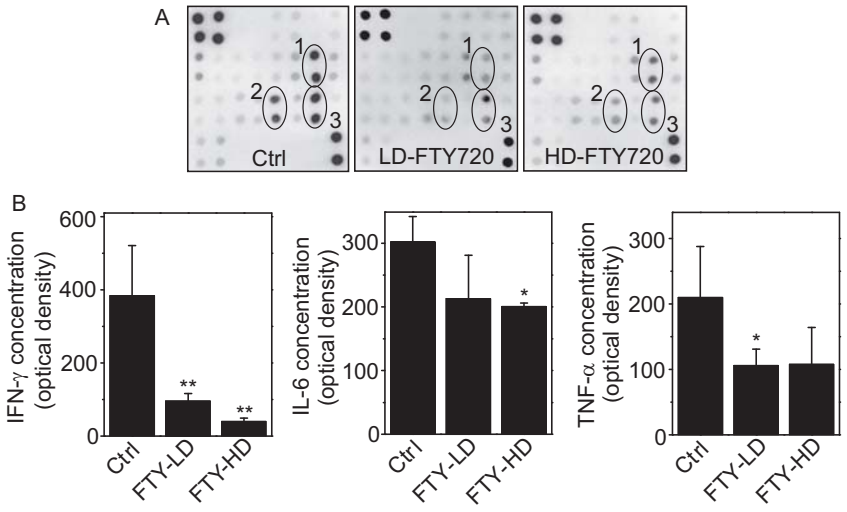

Figure 6. Effect of FTY720 on pro-inflammatory cytokine response *in vivo*. (A) Cytokine profiles of pooled plasma from 4 mice of control (n=7 mice; Ctrl), low-dose FTY720 (n=8 mice; FTY-LD) or high-dose FTY720 (n=8 mice; FTY-HD) treatment groups were determined semi-quantitatively by a RayBiotech cytokine array. Shown are arrays representative of one out of two determinations, in which IL-12 (1), regulated on activation normal T cell expressed and secreted (2) and TNF-R (3) were determined. (B) Plasma levels of pro-inflammatory cytokines IL-6, TNF-α and IFN-γ were determined in each animal by commercially available ELISAs as described in the Methods section. Values are shown as mean ± SD. *P< 0.05, **P< 0.01 (FTY-LD or FTY-HD vs. control).

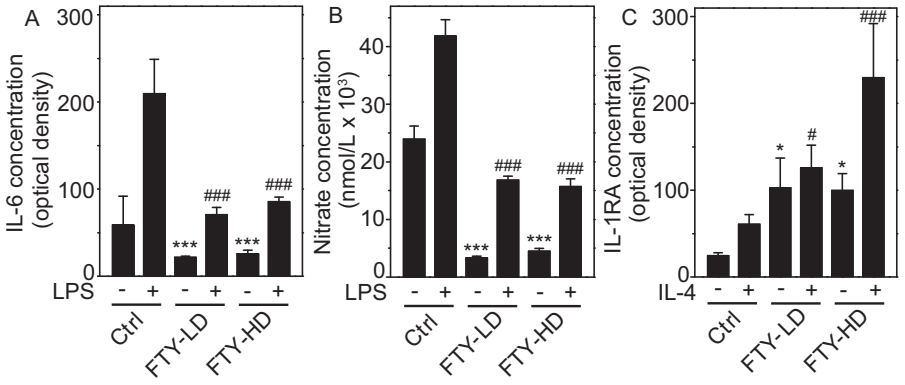

Figure 7. Effect of FTY720 on cytokine secretion by peritoneal macrophages. Peritoneal macrophages were collected for controls (n=7 mice; Ctrl) or animals treated with FTY720 at low doses (n=8 mice; FTY-LD) or high doses (n=8 mice; FTY-HD). Cells were established in culture as described in the Methods section, and incubated for 24 hours in the absence or presence of (A,B) LPS (50 ng/mL) or (C) IL-4 (10 ng/mL). Nitrite concentration in the cell medium was determined colorometrically. IL-6 and IL-1RA were determined using commercially available ELISAs. Values are shown as mean ± SD. *P<0.05; ***P<0.001 (FTY-LD or FTY-HD vs. control; unstimulated cells). #P<0.05; ###P<0.001 (FTY-LD or FTY-HD vs. control; cells stimulated with LPS or IL-4).

Discussion

The recent identification of S1P as an integral component of HDL has generated widespread interest in the potential anti-atherogenic effects of this compound[13]. However, the effects of S1P on the development of atherosclerotic lesions in animal models of atherosclerosis have not been addressed to date. Here, we demonstrate that FTY720, a synthetic analogue of S1P and a potent agonist of 4 out of 5 S1P receptors ($S1P_1$, $S1P_3$, $S1P_4$, and $S1P_5$)[16], preferentially distributes into HDL and inhibits the development of atherosclerotic lesions in $LDLr^{-/-}$ mice on a cholesterol-rich diet. These observations provide direct evidence for an atheroprotective effect of S1P-receptor agonists and are consistent with the notion that S1P contributes to the anti-atherogenic potential attributed to HDL.

Because FTY720 did not change HDL and total cholesterol levels or the lipoprotein profile, its capacity to protect against atherosclerosis seems to be independent from alterations in lipid metabolism. It might be related to the ability of this compound to interfere with atherosclerosis-relevant functions of inflammatory cells. FTY720 was previously shown to produce lymphopenia by sequestering lymphocytes from blood into lymph nodes, thereby preventing their recruitment into sites of inflammation[18,30]. In the present study, we were able to recapitulate FTY720-induced lymphopenia in $LDLr^{-/-}$ mice and, in addition, to demonstrate markedly reduced $CD3^+$-cell infiltration into atherosclerotic lesions. Lymphocytes are crucially involved in the propagation of inflammatory processes within the arterial wall, and T-cell deficiency has been repeatedly reported to attenuate atherogenesis[31-33]. Thus, it is conceivable that the decreased lymphocyte availability partly accounts for the reduction of atherosclerosis seen in animals treated with a high dose of FTY720. In addition, long-term FTY720 administration favorably affected the lymphocyte subset profile by disproportionally reducing pro-atherogenic $CD4^+$ T cells[34,35].

Although FTY720 most effectively reduced atherosclerosis when administered at high doses, the atheroprotective effects of this compound were still evident after low doses of FTY720 that only slightly affected blood lymphocyte counts and T-cell subset pattern. Apparently, altered T-cell trafficking and sequestration cannot be held fully accountable for the reduced atherosclerosis in FTY720-treated animals. $S1P_1$- and $S1P_4$-receptor-mediated signaling was previously demonstrated *in vitro* to suppress the response of lymphocytes to mitogenic stimuli and the secretion of T cell-specific cytokines, including IL-2 and IFN-γ[36-38]. In the present study, splenocyte proliferation and IFN-γ levels in plasma were sharply reduced after FTY720 treatment, indicating some interference with lymphocyte function under *in vivo* conditions. The suppression of IFN-γ production after long-term FTY720 administration may be instrumental in the anti-atherogenic effects exerted by this compound. IFN-γ is abundantly present in atherosclerotic lesions, and substantially reduced plaque formation was observed in IFN-γ- or IFN-γ-R-deficient $ApoE^{-/-}$ mice[39-42]. IFN-γ is critically engaged in the development of Type 1 T-cell polarization. The present data demonstrate that other important mediators of lymphocyte type 1 polarization, such as IL-12 and regulated on activation normal T cell expressed and secreted, are decreased in FTY720-treated animals, suggesting that signaling via S1P receptors may attenuate Th1 immune responses. In this regard, our data agree with previous findings showing decreased levels of Th1 cytokines and Th1 immunoglobulin isotypes in animals treated with

FTY720 or overexpressing $S1P_1$ receptor[43,44]. In addition, both S1P and FTY720 were shown to suppress the production of Th1 cytokines by isolated T cells while enhancing the production of Th2 cytokines such as IL-10[29,45]. Because Th1 cells are considered to represent a particularly pro-atherogenic subset within the $CD4^+$ T-cell population[46], FTY720 may be expected to impair atherogenesis by attenuating Th1 response and by skewing the immune response toward Th2 response.

In contrast to lymphocytes, accumulation of macrophages within the atherosclerotic plaque was not significantly changed in FTY720-treated animals, although the necrotic core formation in FTY720-treated mice was dose-dependently blunted, and the plaque phenotype in these mice was less progressed. Analogous to lymphocytes, however, FTY720 did modulate macrophage function *in vivo*, even at low doses. Plasma levels of pro-inflammatory cytokines such as TNF-α, TNF-R, and IL-6, which are abundantly secreted by activated macrophages, were markedly reduced in $LDLr^{-/-}$ mice treated with low or high doses of FTY720. In addition, peritoneal macrophages from FTY720-treated animals displayed a decreased response to LPS, an established inducer of classical (M1)-type macrophage activation. Conversely, long-term administration of FTY720 enhanced the IL-4-elicited production of IL-1RA, a marker of alternative (M2)-type macrophage activation. Collectively, these observations suggest that the FTY720-induced polarization of lymphocytes towards a Th2 response is paralleled by a complementary M1→M2 switch in macrophages. Because M2 macrophages constitute a rich source of anti-inflammatory factors[29], these cells may dampen inflammatory responses within the vascular wall and, thereby, counteract the formation of atherosclerotic lesions.

S1P signals through five cognate S1P receptors, 4 of which ($S1P_{1-4}$) were seen to be expressed in the vasculature and may be potentially involved in the pathogenesis of atherosclerosis. A contribution of $S1P_2$ can be excluded as FTY720 is only a very poor agonist of this receptor. The preponderance of $S1P_1$ and $S1P_4$ in cells of hematopoietic origin such as lymphocytes and macrophages, both major targets of FTY720, suggests that these 2 receptors may play a prominent role in the atheroprotective activity of FTY720. This notion is further strengthened by the observation that $S1P_1$ is a preferential FTY720-binding partner and signal-transducing receptor[16]. Recent studies have suggested that $S1P_1$ is mainly responsible for S1P-elicited anti-inflammatory responses such as inhibition of leukocyte adhesion to endothelium, vascular permeability or T-cell trafficking[47-49]. In addition, $S1P_1$ mediates protective effects of FTY720 on ischemia-reperfusion injury[43,50]. In this context, it is of interest that HDL effectively prevents ischemia-reperfusion injury in the kidney and heart and that a substantial portion of these effects is not attributable to proteins present in HDL because it is not fully mimicked by apolipoprotein A-I-containing liposomes[51,52]. Whereas anti-atherogenic effects of HDL, S1P, and FTY720 might be primarily mediated by $S1P_1$, the involvement of other S1P receptors cannot be entirely dismissed. For instance, S1P- and FTY720-induced signaling via $S1P_3$ receptors has been previously demonstrated to stimulate NO generation and to inhibit pro-inflammatory NADPH-oxidase activity in endothelial and smooth muscle cells, respectively[12,19] (Nofer *et al.* unpublished observations, 2005). In addition, $S1P_3$ has been shown to serve as a functional HDL receptor, because its endothelial nitric oxide synthase-stimulating and NADPH-oxidase-inhibiting effects were markedly attenuated in $S1P_3$-deficient mice. Recently, $S1P_3$ was also suggested to mediate pro-

tective effects of HDL on myocardial injury in mice[53]. Our present study emphasizes the necessity of further research to delineate the exact contribution of the individual S1P-signaling pathways to the atheroprotective effects of HDL, S1P, and FTY720, and warrants further evaluation of more selective S1P-receptor agonists as potential HDL surrogates and modulators of human cardiovascular disease.
In conclusion, the present data show that signaling via S1P receptors attenuates the development of atherosclerosis in an animal model of disease, and this effect can be attributed to modulation of the function of T cells and macrophages. Because S1P is an integral component of HDL, our results strengthen the notion that this lysosphingolipid importantly contributes to anti-inflammatory and anti-atherogenic effects exhibited by HDL.

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Clinical perspective

High-density lipoprotein (HDL) is an atheroprotective agent and is thus an interesting therapeutic target in atherosclerotic vascular disease. However, because HDL quantity in plasma does not necessarily reflect its functional capacity, direct application of the protective constituents of HDL may be of greater therapeutic benefit than elevating plasma concentrations of HDL holoparticles. Sphingosine 1-phosphate (S1P) is a natural component of HDL and exhibits strong anti-inflammatory properties. Several potentially anti-atherogenic effects of HDL *in vitro* were shown to be fully or partially emulated by S1P. However, the anti-atherogenic effects of S1P had not previously been examined *in vitro*. The present study documents that FTY720, a synthetic analogue of S1P and a potent agonist of 4 out of 5 S1P receptors, inhibits development of atherosclerosis in low-density lipoprotein receptor deficient mice. The anti-atherogenic effects of FTY720 were not related to changes in the plasma levels of atherogenic lipoproteins but, rather, reflected the modulatory effects of this compound on the functional polarization of lymphocytes and macrophages. These results constitute the first demonstration that signaling via S1P receptors attenuates the development of atherosclerosis, warranting further evaluation of S1P-receptor agonists as potential HDL surrogates and modulators of human atherosclerotic cardiovascular disease.

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