Sphingosine-1-Phosphate Analogue FTY720 Causes Lymphocyte Redistribution and Hypercholesterolemia in ApoE-Deficient Mice


Objective—Resident immune cells are a hallmark of atherosclerotic lesions. The sphingolipid analogue drug FTY720 mediates retrafficking of immune cells and inhibits their homing to inflammatory sites. We have evaluated the effect of FTY720 on atherogenesis and lipid metabolism.

Methods and Results—ApoE−/− mice on a normal laboratory diet received oral FTY720 for 12 weeks, which led to a 2.4-fold increase in serum cholesterol (largely VLDL fraction) and a 1.8-fold increase in hepatic HMGCoA reductase mRNA. FTY720 increased plasma sphingosine-1-phosphate and induced marked peripheral blood lymphopenia. A discordant modulation of B, T and monocyte cell numbers was found in peripheral lymphoid organs. Overall depletion of T cells was accompanied by a relative (2-fold) increase in regulatory T cell content paralleled by a similar increase in effector memory T cells (CD4+CD45RACD62Llo) as absolute numbers of both subpopulations remained essentially unchanged. Lymphocyte function was unaltered as indicated by anti-OxLDL antibodies and T cell proliferation. There were no changes in atherosclerotic lesions in early and established atherosclerosis.

Conclusions—FTY720 mediated peripheral lymphocyte depletion and retrafficking without altering function and overall balance of pro- and antiatherogenic lymphocyte populations. A net decrease in lymphocyte numbers occurred concomitantly with a more proatherogenic hypercholesterolemia resulting in unaltered atherogenesis. (Arterioscler Thromb Vasc Biol. 2007;27:2392-2399.)

Key Words: atherosclerosis ■ immune system ■ immunosuppressive therapy ■ leukocytes ■ vascular biology

Atherosclerosis is a chronic inflammatory disease elicited by lipid retention and modification in the arterial intima. Several inflammatory cell types such as macrophages and T-lymphocytes are believed to be intrinsically involved in the initiation and progression of arterial wall lesions.1–3 Accumulating evidence suggests an important role for endogenous lysosphingolipids such as sphingosine-1-phosphate (S1P) in modulating immune cell trafficking, initiating angiogenesis, preserving vascular integrity, and enhancing eNOS-induced vasodilatation. All these effects are thought to be mediated via G protein–coupled receptors expressed on a variety of cell types.4–6 The immunomodulator FTY720 acts as a sphingosine-1-phosphate mimic and was shown effective in experimental models of transplantation and autoimmunity7–9 with promising results in clinical trials for prevention of kidney graft rejection10 and multiple sclerosis.11 In vivo, FTY720 undergoes rapid phosphorylation to form the active compound FTY720-P, a sphingosine-1-phosphate (S1P) analogue. FTY720-P is an agonist of 4 of the 5 currently known G protein–coupled sphingolipid receptors (S1P1, S1P3, S1P4, S1P5).12 FTY720 mediates redistribution of lymphocytes and other immune cells preventing their egress to inflamed tissues, while concomitantly preserving the functionality of lymphocytes.13,14 Two recent reports have shown a protective effect on atherosclerosis for FTY720 in the LDLr−/−mice and ApoE−/− mouse model under conditions of a high-fat diet, attracting the effect to a decreased recruitment of inflammatory cells into the lesion. However, as perturbations occur on administration of a high-fat diet encompassing a pronounced monocytosis with a massive increase of macrophages17 and T cells in lesions,18,19 it appeared of great interest to analyze the protective potential of FTY720 in a more physiological mouse model of spontaneously developing atherosclerosis with less extreme lipid levels (ApoE−/− mice fed normal laboratory diet). This is the first study to show that hypercholesterolemia is induced by FTY720 treatment, which possibly counteracts its antiatherogenic effect on immune cell distribution.
Materials and Methods
For detailed Materials and Methods, please see the supplemental materials (available online at http://atvb.ahajournals.org).

Animals
Male ApoE−/− mice were obtained from Jackson Laboratories (Bar Harbor, Me). Ethical permission was obtained from the University of Heidelberg. Mice were fed a normal laboratory diet (ssniff Spezialdiäten GmbH). FTY720 (Novartis) was continuously administered for 12 weeks to the animals by drinking water calculated at a daily dose of 3 mg/kg/d. 8-week-old mice (n = 7/group) and 26-week-old mice (n = 10/group) were treated for 12 weeks with FTY720 to evaluate lesions in the aortic root and innominate artery.

Tissue Processing
For RNA isolation the aortic arch, a predilection site for lesion development in ApoE−/− mice, was dissected and snap-frozen. Lesion area and fractional area of the lesion were quantified and the results were expressed as the average of 8 sections per mouse.

Immunohistochemistry
Light microscopy was performed on 10-µm cryosections adjacent to the ORO-stained sections. Primary antibodies (CD4, CD8, CD19, CD68, vascular cell adhesion molecule [VCAM]-1, I-Ab; all rat anti-mouse by BD Pharmingen) were titrated to optimum performance and applied to acetone-fixed cryosections followed by detection with the ABC alkaline phosphatase kit (Vector Laboratories). A thresholding technique using computerized ImagePro analysis on the aortic root sections was implemented.

Flow Cytometry
Flow cytometry was performed on a CyAn (Dako) after staining with the appropriate Ab; data were analyzed using Summit v4.3 software. Primary labeled antibodies used were CD19, CD3, CD4, CD8, F4/80, CD25, CD62L, CD44 from Pharmingen, FoxP3-PE was from eBioscience. F4/80, CD25, CD62L, CD44 from Pharmingen, FoxP3-PE was from BioLegend.

Functional Immune Assays
Splenocytes were harvested and cultured in duplicate in a 96-well plate at 5×10^4 cells per well after red blood cells lysis. Cells were incubated for 72 hours in the absence or presence of anti-CD3 antibody (1 µg/mL) followed by incorporation of 3H-thymidine during the last 18 hours. ELISA methods were used to quantitate serum Ig isotypes to Ox-LDL.

Real-Time Polymerase Chain Reaction
RNA was isolated from the aortic arch using the RNeasy kit (Qiagen). Reverse transcription was performed using the Boehringer reagent class B type I (SR-BI), and LDL receptor-related protein (LRP1). Figure 1C and 1D illustrates that HMGCoA reductase transcript levels normalized to β-actin were up-regulated (1.8-fold) in liver tissue of FTY720-treated animals. When the distribution of Treg and memory effector T cells within the CD4 population was examined we found a significant (2-fold) relative increase among total CD4+ T cells for both, regulatory T cells, and memory effector T cells in the

Statistical Analysis
Values are expressed as mean±SEM unless otherwise indicated. Nonparametric Mann–Whitney U test was used to compare individual groups of animals. A level of P<0.05 was considered significant.

Results
Early Atherogenesis
Biochemical Markers and Drug Levels
FTY720 was administered continuously via the drinking water to 8-week-old ApoE−/− mice for a period of 12 weeks. A dose of 3 mg/kg/d yielded plasma drug levels of 3.08±1.99 ng/mL (mean±SD). FTY720 administration caused a 70% reduction in peripheral blood lymphocyte counts but did not influence other blood cells such as monocytes (supplemental Table I). Body weight did not differ between groups when fed a normal laboratory diet. However, there was a 2.4-fold increase in total serum cholesterol levels (supplemental Table I) with a marked elevation of the VLDL fraction (Figure 1A). Triglyceride levels were unaltered. To elucidate the mechanism leading to isolated hypercholesterolemia the effect of FTY720 on lipid metabolism was evaluated. Administration of FTY720 was associated with a significant increase in plasma levels of the natural analogue of FTY720-P—sphingosine-1-phosphate (S1P)—in the treated group (1318±39.0 ng/mL versus 1158±54.9 ng/mL, P=0.0476; Figure 1B). As sphingosine had previously been shown to induce HMGCoa reductase we analyzed mRNA from liver and intestinal tissue—2 key organs in lipid metabolism. Both hepatocytes and intestinal epithelial cells were recently shown to express S1P receptors Some genes involved in cholesterol/VLDL synthesis and uptake are sterol regulatory element-binding proteins (SREBPs), HMGCoa reductase, microsomal triglyceride transfer protein (MTP), scavenger receptor class B type I (SR-BI), and LDL receptor-related protein (LRP1). Figure 1C and 1D illustrates that HMGCoa reductase transcript levels normalized to β-actin were up-regulated (1.8-fold) in liver tissue of FTY720-treated animals (1.20±0.15 versus 0.68±0.05; P=0.033). These data indicate that FTY720 interfered with hepatic cholesterol metabolism.

Cellular Composition in Immune Organs
Two distinct lymph node sites (axillary and inguinal), spleen, and peripheral blood were analyzed for cellular composition. FTY720 mediated a redistribution of B cells (CD19+) from peripheral blood into spleen and lymph nodes whereas T cells (CD4+ and CD8+) were depleted in all 3 lymphoid tissues (Figure 2A and 2B, spleen data not shown). The percentage of monocytes was unchanged in blood and spleen, but increased in lymph nodes. Analysis of regulatory T cells (CD4+FoxP3+) and memory effector T cells (CD4+CD44hiCD62Llo) showed no difference between groups when expressed as percentage of total parenchymal cells. However, when the distribution of Treg and memory effector T cells within the CD4+ T population was examined we found a significant (2-fold) relative increase among total CD4+ T cells for both, regulatory T cells, and memory effector T cells in the
FTY720 treated animals (Figure 2C through 2F). This effect was attributable to the decline in total CD4+ T cell numbers on FTY720 administration whereas absolute cell numbers for Treg and memory effector cells remained essentially unchanged (supplemental Figure II). Data on Treg and memory effector T cells in blood are not shown as total CD4+ cell content was diminished to less than 1% of total cells in the FTY720 group obviating accurate interpretation and reasonable statistical analysis because of minimal cell numbers. As both subpopulations showed the same relative increase (2-fold), the overall balance of regulatory T cells and memory effector T cells was maintained in the FTY720 treated group. These data show a discoordinate modulation of lymphocyte populations with a preserved overall balance of pro-and antiatherogenic T cells.

**Serum Antibody and Cytokines Levels**

Isotype analysis of serum antibodies against OxLDL, implicated in the pathogenesis of atherosclerosis, showed no significant differences between the 2 groups except for the subclass IgG1, which was lower in the treated group (0.28±0.03 versus 0.17±0.02, p = 0.004; Figure 3A). Serum cytokine levels for interleukin (IL)-5, IL-10, and IFN-γ were similar in treated and control animals (data not shown). Anti-CD3 induced splenic T cell proliferation was not influenced by FTY720 administration as illustrated in Figure 3B.
Thus, functional properties of B and T cells remained essentially unaltered by FTY720 treatment.

**Lesion Size, Cellular Composition, and Cytokine Pattern**

Lesion size was measured to determine the effect of FTY720 on de novo atherogenesis. Morphometric analysis in the aortic root did not show any effect of FTY720 on lesion size ($10.2 \times 10^4 \pm 1.4 \times 10^4 \, \mu m^2$ in controls versus $12.5 \times 10^4 \pm 2.5 \times 10^4 \, \mu m^2$ in treated group; $P = 0.66$) or fractional area of the lesion in ($10.9 \pm 1.6\%$ versus $14.3 \pm 2.2\%; P = 0.34$) (Figure 4A and 4B and supplemental Figures III and IV). Immunohistochemical analysis of lesion composition yielded no significant differences in T, B cells or macrophage content (supplemental Table II). Expression of cytokine-induced genes (I-A$^\beta$, VCAM-1) was not different. RT-PCR analysis of the aorta showed no significant differences in cytokine/mediator pattern (supplemental Figure V).

**Advanced Atherosclerosis**

A second group of ApoE$^{-/-}$ mice on normal laboratory diet with established atherosclerotic lesions (26 weeks old) was treated orally with FTY720 at 3 mg/kg/d for 12 weeks. Similar results with respect to significant lymphopenia and changes in the lipid profile were found as in the early atherosclerosis experiment. All other parameters displayed no difference, comparable with the results in early atherosclerosis (supplemental Table III).

**Lesion Size, Cellular Composition, and Cytokine Pattern**

Quantitative analysis of the aortic root showed no significant differences between the 2 groups. Lesion size was similar comparing controls with treated animals ($43.6 \times 10^4 \pm 4.0 \times 10^4 \, \mu m^2$ versus $38.5 \times 10^4 \pm 2.7 \times 10^4 \, \mu m^2, P = 0.15$) and also the fractional area of the lesion ($24.7 \pm 2.1\%$ versus $23.6 \pm 1.2\%, P = 0.26$). Immunohistochemical analysis of the advanced lesions detected no differences in T, B cell or macrophage content comparing treated animals with controls (data not shown). To evaluate whether FTY720 had any effect on plaque stability in advanced atherosclerosis we examined the innominate artery. Lesion size, fractional area of the lesion, plaque thickness, thickness of the fibrous cap, amount of calcification, and intraplaque hemorrhage were not

![Figure 3](http://atvb.ahajournals.org/)

**Figure 3.** Controls (black bars), FTY720 (open bars). A, Serum anti-OxLDL antibodies. OD values. B, Splenocyte proliferation assay.

![Figure 4](http://atvb.ahajournals.org/)

**Figure 4.** Controls (black circles), FTY720 (open circles). A, Lesion size in aortic root (ORO stain). B, Fractional area of the lesion.
different between the 2 groups (data not shown). RT-PCR analysis of the aorta showed no significant differences in cytokine/mediator pattern (supplemental Figure VI).

Discussion
The concept of atherosclerosis as an inflammatory disease is supported by an increasing amount of data suggesting immunomodulation may provide an effective tool to interfere with the development and progression of atherosclerosis.1,2 In this study, the immunomodulatory sphingolipid analogue FTY720 mediated a pronounced peripheral lymphopenia, however without altering the immunologic balance toward a more protective profile leaving atherogenesis unchanged. This appears attributable to the novel finding of a 2.4-fold increase in cholesterol associated with proatherogenic fractions, primarily VLDL, on administration of the drug.

Three aspects appear noteworthy as to why administration of FTY720 has not been associated with hypercholesterolemia up to present. First, with respect to method only very few groups have performed a detailed plasma lipoprotein analysis as in our study. All currently analyzed clinical trials have not reported cholesterol levels and thus may have missed such an effect.10,11 Second, the choice of animal model may influence the detected effects. In this respect, the ApoE−/− mouse presents a unique model—by means of its disturbed lipid clearance resulting in a massive elevation of plasma cholesterol levels primarily attributable to an increase in cholesterol-rich VLDL—and chylomicron remnant particles. ApoE−/− (and also LDLr−/−) mice are sensitive to a high-fat diet, which leads to a marked increase in non-HDL cholesterol levels.35 It is therefore likely that the effect of FTY720 on plasma cholesterol levels as observed in our study is masked on administration of a high-fat diet. This appears attributable to the novel finding of a 2.4-fold increase in cholesterol associated with proatherogenic fractions, primarily VLDL, on administration of the drug.

Despite drug-induced hypercholesterolemia, lesions were not larger in the treated ApoE−/− mice. This suggests a separate attenuating effect on atherosclerosis of FTY720 mediated by immunomodulation. Corroborating a previous study in C57BL/6J mice, we found a diminished peripheral lymphocyte pool. This suggests a separate attenuating effect on atherosclerosis of FTY720 mediated by immunomodulation. Corroborating a previous study in C57BL/6J mice, we found a diminished peripheral lymphocyte pool, which the authors from that study35 attributed predominantly to a decreased release of naïve lymphocytes from the thymus on long-term treatment with FTY720. Future work needs to delineate how long-term treatment differs from short-term treatment to explain this generalized peripheral lymphopenia. Two options are conceivable—either preserved inhibition of thymic lymphocyte egress38 attributable to differential chemokine requirements in distinct lymphoid compartments37 with long-term administration of FTY720, or peripheral depletion via ie, apoptosis.39 Interestingly, we found maintained numbers of Treg and effector memory T cells in lymphoid tissues which argues against a “conventional” lymphodepletion and indicates a functional expansion of effector cells within an overall diminished peripheral lymphocyte pool. Thus, the ability to mount a systemic immune response was not disabled as evidenced by unaltered atherosclerosis-related OxLDL antibody profiles and splenic T cell proliferation. As to the source of Treg and effector memory cells, 2 options are conceivable. Either a thymic-derived natural Treg pool may be constantly self-regenerating in the periphery, or both CD4+ T cell subtypes are continuously regenerated in the periphery after antigen-induced activation. Our finding that effector memory T cell numbers were preserved indicates a peripheral source of at least the memory T cells as they are not derived from the thymus but rather are the result of peripheral antigen activation and subsequent continuous self-renewal. Recent data indicate that peripheral Treg may originate from memory T cells.39 This may help explain our finding of preserved Treg counts and effector memory T cells as Treg would be regenerated from the peripheral memory T cell pool. In support of our findings, previous data show that FTY720 mediated sequestration of effector memory T cells into lymph nodes8 and promoted accumulation of natural regulatory T cells,40 the latter exerting protective effects on atherosclerosis.41 However, lymphocyte retrafficking did not translate into a protective effect on atherosclerosis in our study which is in contrast to 2 very recent studies.15,16 Three reasons may account for the discrepancy between these studies and the present one. First, in both studies animals were fed a high-fat diet which in itself causes a pronounced monocytesis and accumulation of macrophages and T cells in lesions.17–19 In addition, hypercholesterolemia strongly promotes lymphocyte and macrophage activation.42 Thus, the suppressive effect of FTY720 on inflammatory cell trafficking5,12 as well as lymphocyte activation15 may be facilitated under such conditions. Of note, inhibitory effects of FTY720 on splenic T cell proliferation were only observed in LDLr−/− mice exposed to high-fat diet but not when fed a normal laboratory diet (Nofer et al, unpublished results). We found a further increase in S1P plasma levels on FTY720 administration to high-fat fed mice when compared with control animals and FTY720-treated mice on normal diet (data not shown). This might translate into an enhanced effect of S1P on atherosclerosis-related effects (ie, eNOS-induced vasorelaxation4 and reduced lymphocyte activation) in high-fat fed mice. Our data are supported by the recent finding that FTY720 inhibits sphingosine-1-phosphate lyase, the enzyme responsible for S1P degradation.43 Normal lipopidemic C57BL/6J mice had significantly lower sphingolipid levels compared with hypercholesterolemic ApoE−/− mice.44 Second, the treatment period in both studies was extended (16 and 20 weeks, respectively) enabling detection of even small protective effects on atherosclerosis. Third, the drug level was nearly 25-fold higher compared with our study (68 ng/mL versus 3 ng/mL) in 1 group which showed a decrease in lesion size in the aorta in LDLr−/− mice. Interestingly, in that study a second treatment group with a drug level more in line with levels obtained in our study, no protective effect on atherosclerosis could be detected in the aortic root.15 In the other study drug levels were not measured.16

Our study illustrates a link between sphingolipid and cholesterol metabolism and extends previous data.30,43–45 We
show that the sphingosine-1-phosphate (S1P) analogue FTY720 mediates an increase in S1P levels which is associated with increased hepatic HMG-CoA reductase gene expression leading to increased serum cholesterol levels. Further evidence for an interaction between sphingolipid and cholesterol metabolism comes from a recent study which showed that statins—HMG-CoA reductase inhibitors—induce endothelial S1P receptors and mediate vasorelaxation by enhanced eNOS production.\(^{36}\) To establish the effect of sphingosine-1-phosphate agonists such as FTY720 on atherogenesis, it seems preferable to avoid extreme conditions such as the administration of high-fat diets to genetically hyperlipidemic animals. This will help extrapolate data derived from ongoing clinical trials. From the currently available data it appears safe to assume that FTY720 neither attenuates nor increases atherosclerosis in ApoE\(^{-/-}\) mice.

Acknowledgments

We are grateful for the excellent technical assistance by Nadine Wambsganss, Inger Bodin and Ingrid Törnberg.

Sources of Funding

Grants supporting this work were from Novartis (Germany) and Deutsche Forschungsgemeinschaft (KL1398/2-1) to R.K. and T.J.D. (DE591/5-5/5-6), ADUMED Medical Research Foundation to J.R.N., Swedish Research Council, the Grönberg, the Novo Nordisk (DE591/5-5-5-6), ADUMED Medical Research Foundation to Grönberg, Inger Bodin and Ingrid Törnberg.

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We are grateful for the excellent technical assistance by Nadine Wambsganss, Inger Bodin and Ingrid Törnberg.

Disclosures

None.

References


References


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Arterioscler Thromb Vasc Biol. 2007;27:2392-2399; originally published online August 30, 2007;
doi: 10.1161/ATVBAHA.107.149476

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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Materials and Methods:

Drug
FTY720 (2-amino-2-(2-[4-octylphenyl]ethyl)-1,3-propanediol) was synthesized by chemical derivatization of myriocin (ISP-1), a metabolite of the ascomycete *Isaria sinclairii*. Unlike myriocin, FTY720 does not inhibit serine-palmitoyl-transferase, the first enzyme in sphingolipid biosynthesis. FTY720 is rapidly phosphorylated in vivo and the FTY720-phosphate metabolite (FTY720-P) is the biologically active principle. FTY720-P triggers signalling by a novel class of G protein coupled receptors 12.

Animals
Male ApoE−/− mice were obtained from Jackson Laboratories (Bar Harbor, Maine, USA) (strain name B6.129P2-Apoetm1Unc/J). All animal work was approved by the regulatory authorities of the University of Heidelberg. Mice were fed a normal laboratory diet by ssniff Spezialdiäten GmbH, Soest, Germany (www.sniff.de), catalogue number V1185-340. FTY720 (kindly provided by V. Brinkmann, Novartis, Basel, Switzerland) was dissolved in water and continously administered for 12 weeks to the animals by drinking water calculated according to weekly drinking consumption for each mouse at a daily dose of 3 mg/kg/day, a dose shown to be effective in preventing cardiac allograft rejection in a murine model 7. Drinking water was exchanged once a week. In a first experiment, 8 week old mice (n = 7/group) were used to analyze the effect on de novo atherosclerosis development in the aortic root (early atherogenesis) 20. In a second experiment, 26 week old mice (n = 10/group) were treated for 12 weeks with FTY720 in the same manner as the younger animals to evaluate the effect on established atherosclerotic lesions in the aortic root and innominate artery 21. After 12 weeks of treatment mice were anesthesized, (Avertin, Aldrich, Milwaukee, WI, USA) and
blood was collected from the inferior vena cava, and animals were sacrificed by exsanguination. The animals were perfused with 10 mL ice cold PBS at physiological pressure via the left ventricle.

Tissue processing

For analysis of atherosclerotic lesion development, the heart and aorta extending to the abdomen were dissected, embedded in Tissue Tek® O.C.T. medium (Sakura Finetek Europe BV, Zoeterwoude, Netherlands) and snap-frozen in liquid nitrogen and stored at – 80°C until further use. For RNA isolation the aortic arch extending to the diaphragm was dissected and snap-frozen in liquid nitrogen including the lesser curvature of the aortic arch previously described as a predilection site for lesion development in ApoE +/- mice. The root of the aorta was serially cryosectioned (10 µm). From the area in which three aortic valve cusps are detected, alternate sections were collected for quantification of lesion size or immunohistochemical staining, respectively. A total of 8 sections at 100 µm intervals were Oil Red-O (ORO) stained and counterstained with hematoxylin. Quantitation of the lesions was performed by one blinded observer using ImagePro software (Media Cybernetics, Silver Spring, MD, USA) with an intra-observer variability of < 5%. Lesion area and fractional area of the lesion (ratio of lesion surface area per vessel surface area) were quantified and the results were expressed as the average of 8 sections per mouse. To evaluate plaque stability in advanced atherosclerosis, additional analysis of the innominate/brachiocephalic artery was performed in the older (38 weeks) animals using Movat and von Kossa stains. Evaluation of lesions and histological analysis to assess intraplaque hemorrhage and calcification was performed as previously described.
**Immunohistochemistry**

Light microscopy was performed on 10 µm cryosections adjacent to the ORO-stained sections. Primary antibodies (CD4, CD8, CD19, CD68, VCAM-1, I-A^b (=murine MHC II); all rat anti-mouse by BD Pharmingen, Heidelberg, Germany) were titrated to optimum performance and applied to acetone-fixed cryosections. After a washing step, the secondary antibody (a biotinylated rabbit anti-rat immunoglobulin antibody) and ABC alkaline phosphatase kit (Vector Laboratories, Burlingame, CA) were used. All sections were counterstained with hematoxylin. Species-matched isotype controls replacing the first antibody by a non-binding IgG were used as negative controls. The area stained for specific cell type per atherosclerotic lesion was determined for each animal at the level of the greatest lesion size. A thresholding technique using computerized ImagePro analysis on the aortic root sections was implemented.

**Flow cytometry**

Cells from three lymphoid tissues were analyzed (spleen, inguinal lymph node and blood). Flow cytometry was performed on a CyAn™ (Dako, Glostrup, Denmark) after staining with the appropriate Ab; data were analyzed using Summit v4.3 software. Box plots of 10000 stained cells were evaluated by grid analysis for individual cell populations after appropriate compensation. Primary labeled antibodies used (CD19, CD3, CD4, CD8, F4/80, CD25, CD62L, CD44) were from Pharmingen (Heidelberg, Germany). FoxP3-PE was from eBioscience (San Diego, CA, U.S.A.). Cell clusters of interest were plotted against CD3.
Functional immune assays

For proliferation assays splenocytes from individual mice were harvested and cultured in duplicate in a 96 well plate at $5 \times 10^5$ cells/well after osmotic lysis of red blood cells. These cells were incubated for 72 hours in the absence or presence of anti-CD3 antibody (1µg/mL). The incubation included incorporation of $^3$H-thymidine during the last 18 hours for evaluation of cell proliferation. ELISA methods were used to quantitate serum Ig isotypes to Ox-LDL. For analysis of anti-OxLDL Ig isotypes, polystyrene microtiter plates were coated overnight at 4°C with 5 µg/ml of OxLDL (copper oxidized) in a coating buffer with 100 mM NaHCO$_3$ and 1 mg/ml Na$_2$EDTA. Plates were then washed thoroughly with PBS with 0.05% Tween-20 and incubated with PBS with 5% BSA for 30 min at room temperature to prevent nonspecific binding. Mouse sera were incubated for 1 h at 37°C in duplicate wells, using a serum dilution of 1/100 in PBS-Tween. After rinsing, conjugated antibodies to IgM, IgG, IgG2a (all alkaline phosphatase-conjugated), or IgG1 (peroxidase-conjugated) were added to the wells for a 1-h incubation at 37°C, plates were again washed, and enzyme activities were developed using $p$-nitrophenyl phosphate (Sigma-Aldrich, Stockholm, Sweden) or o-phenylenediamine dihydrochloride (Sigma-Aldrich, Stockholm, Sweden) to visualize alkaline phosphatase and horseradish peroxidase, and read at 405 and 450 nm, respectively.

Real-Time Polymerase Chain Reaction

Total cellular RNA was isolated from the aortic arch extending distally from the aortic root to the abdomen from each animal by using the RNeasy kit (Qiagen, Hilden, Germany). Reverse transcription of 2 µg total RNA was performed using the Boehringer cDNA kit (Roche Diagnostics, Mannheim, Germany) according to manufacturer's instructions. cDNA was diluted 1:10 and 2 µL used for PCR. The Roche real-time PCR kit with SYBR Green (Roche Diagnostics, Mannheim, Germany) was used for quantitative PCR (LightCycler). PCR
conditions were: 55 cycles total, each with 95°C for 5 seconds, 57°C for 10 seconds and 72°C for 12 seconds. Primer sequences were previously published [24-28]. Data were analyzed on the basis of the relative expression method with the formula $2^{-\Delta\Delta C_T}$, where $\Delta\Delta C_T = \Delta C_T (\text{sample}) - \Delta C_T (\text{calibrator} = \text{average } C_T \text{ values of all samples})$, and $\Delta C_T$ is the $C_T$ of the target gene subtracted from the $C_T$ of the housekeeping gene ($\beta$-actin).

**Hematological and biochemical parameters**

Whole blood (EDTA) was analyzed for cellular content by Cell Dyn 3500 hemocounter (Abbott, Delkenheim, Germany). Lipid assays. Serum total cholesterol and triglycerides were determined in 3 µL serum aliquots from the individual animals using a Monarch Automated Analyzer (ILS Laboratories Scandinavia AB, Stockholm, Sweden). Size-fractionation of serum lipoproteins by Fast Performance Liquid Chromatography (FPLC) was performed using a micro-FPLC column (30 x 0.32 cm Superose 6B from Amersham Pharmacia, Uppsala, Sweden) coupled to a system for on-line separation and subsequent detection of cholesterol [29]. In brief, 10 µL of serum from each individual were injected into the system at a flow rate of 40 µL/min. Specific reagents for cholesterol were purchased from Roche Diagnostics GmbH, Mannheim, Germany. FTY720 serum levels were determined at Novartis (Basel, Switzerland) by HPLC. S1P plasma levels were determined as described [6]. BD™ Cytometric Bead Array technique (Becton Dickinson and Company, Franklin Lakes, NJ, U.S.A.) was used to measure cytokine levels in serum.

**Statistical analysis**

Values are expressed as mean ± standard error of the mean (SEM) unless otherwise indicated. Non-parametric Mann-Whitney U test was used to compare individual groups of animals. A level of $p < 0.05$ was considered significant.
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<th>Control</th>
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<td><strong>Total cholesterol level (mg/dl)</strong></td>
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<td>614 ± 171</td>
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<td><strong>Triglyceride level (mg/dl)</strong></td>
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<td>4.23 ± 0.72</td>
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<td><strong>Absolute lymph. count</strong> (10^3/µl)</td>
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<tr>
<td><strong>Erythrocytes</strong> (10^6/µl)</td>
<td>9.63 ± 0.57</td>
<td>10.27 ± 0.55</td>
<td>n.s.</td>
</tr>
<tr>
<td><strong>Thrombocytes</strong> (10^3/µl)</td>
<td>1239 ± 239</td>
<td>1450 ± 185</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

All values are shown as mean ± standard deviation. * = significant, n.s. = not significant.
Table II:

**Cellular composition and inflammatory markers in aortic lesions at 20 weeks**

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<tr>
<th></th>
<th>Control</th>
<th>FTY720</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CD4 (cells/mm²)</strong></td>
<td>101 ± 13</td>
<td>88 ± 14</td>
<td>n.s.</td>
</tr>
<tr>
<td><strong>CD8 (cells/mm²)</strong></td>
<td>47 ± 12</td>
<td>42 ± 14</td>
<td>n.s.</td>
</tr>
<tr>
<td><strong>CD19 (cells/mm²)</strong></td>
<td>&lt; 10</td>
<td>&lt; 10</td>
<td>n.s.</td>
</tr>
<tr>
<td><strong>I-Aᵇ (cells/mm²)</strong></td>
<td>342 ± 83</td>
<td>575 ± 97</td>
<td>n.s.</td>
</tr>
<tr>
<td><strong>CD68 (% of lesion)</strong></td>
<td>26.4 ± 2.2</td>
<td>25.3 ± 3.0</td>
<td>n.s.</td>
</tr>
<tr>
<td><strong>VCAM-1 (% of lesion)</strong></td>
<td>38.7 ± 3.8</td>
<td>37.0 ± 5.8</td>
<td>n.s.</td>
</tr>
</tbody>
</table>
### Table III:

**Advanced atherosclerosis: Body weight, lipid profile and lymphocytes at 38 weeks**

<table>
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<th>FTY720</th>
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</tr>
</thead>
<tbody>
<tr>
<td><strong>Body weight</strong> (g)</td>
<td>35.3 ± 1.17</td>
<td>33.6 ± 0.70</td>
<td>n.s.</td>
</tr>
<tr>
<td><strong>Total cholesterol level</strong> (mg/dl)</td>
<td>423.0 ± 48.7</td>
<td>790 ± 57.3</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td><strong>Triglyceride level</strong> (mg/dl)</td>
<td>102.6 ± 15.3</td>
<td>124.5 ± 18</td>
<td>n.s.</td>
</tr>
<tr>
<td><strong>Absolute lymph. count</strong> (10³/µl)</td>
<td>2.60 ± 0.76</td>
<td>0.8 ± 0.32</td>
<td>&lt; 0.01</td>
</tr>
</tbody>
</table>

All values are shown as mean ± standard deviation. * = significant, n.s. = not significant.

Cholesterol was not determined by FPLC in this study but by conventional lipid assay.
Figure I
Figure II

Flow cytometric analysis of cellular distribution in peripheral lymphoid tissues. Illustrated from left to right: total cell gate (inguinal lymph node); double staining for CD4+FoxP3+ T cells gated on R1; as an excerpt of the middle graph: CD4+FoxP3+ T cells shown only among CD4+ T cells. I) Isotype controls. Upper panel: IgG-FITC, lower panel: IgG-PE. II) Inguinal lymph node sample from the control group (upper panel) and the FTY720 group (lower panel). In the FTY720 group a decrease in total CD4+ T cell number is noted compared with controls (middle graph, R2+R14). However, the absolute number of Treg remains constant (middle graph, R2).
**Figure III and IV:**

Early atherosclerosis. Morphometric analysis in aortic root. **III)** Representative oil-red O stain from control group. **IV)** Representative oil-red O stain from FTY720 group.
Figure V:

Early atherosclerosis. Cytokine and inflammatory mediator mRNA in aorta. Controls (black bars), FTY720 (open bars). Normalized expression of target gene is expressed relative to normalized expression of calibrator sample (formula $2^{-\Delta\Delta C_T}$).
Figure VI:

Advanced atherosclerosis. Cytokine and inflammatory mediator mRNA in aorta. Controls (black bars), FTY720 (open bars). Normalized expression of target gene is expressed relative to normalized expression of calibrator sample (formula $2^{-\Delta\Delta C_T}$).
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Please insert brief explanatory information describing each relationship in each category in the chart below. Note these definitions of the categories: "Research Grant" includes principal investigator, collaborator or consultant and pending grants as well as grants already received. "Other Research Support" includes receipt of drugs, supplies, equipment or other in-kind support. "Honoraria" includes speaking fees for symposia and other meetings or occasions. "Expert Witness" includes serving as an expert witness, consultant or otherwise providing a deposition, testimony, or other information, analysis or document for a lawsuit, government agency proceeding, grand jury, or other legal proceeding, even if the case did not go to trial. "Ownership Interest" includes stocks, stock options, partnership, membership, or other equity position in an entity regardless of the form of the entity, any option or right to acquire such position, or any rights in any patent or other intellectual property.

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B. Each author is required to classify each disclosed relationship as either "Significant" or "Modest". A relationship is considered to be "Significant" if (a) the person receives $10,000 or more during any 12 month period, or 5% or more of the person’s gross income; or (b) the person owns 5% or more of the voting stock or share of the entity, or owns $10,000 or more of the fair market value of the entity. A relationship is considered to be "Modest" if it is less than "Significant" under the preceding definition.

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If additional space is necessary, please provide additional detail on an attached sheet.
2. **Institutional Conflict of Interest**

Your institution or employer: __________________________________________________________

Institutional conflict of interest is defined as any financial interest held by your employer or academic institution in any business or entity having an interest in the topic addressed in the manuscript.

Are you aware that your academic institution or employer has any financial or ownership interest directly related to the topic of this manuscript?

[ ] No
[ ] Yes. Please describe in the space below or on an attached sheet

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Author’s signature: [Signature]
Date: 21 May 2007