Quantitative and Qualitative Differences in Proatherogenic NKT Cells in Apolipoprotein E–Deficient Mice

Amy S. Major, Michael T. Wilson, Jennifer L. McCaleb, Yan Ru Su, Aleksandar K. Stanic, Sebastian Joyce, Luc Van Kaer, Sergio Fazio, MacRae F. Linton

**Background**—Atherosclerosis is a disease marked by lipid accumulation and inflammation. Recently, atherosclerosis has gained recognition as an autoimmune-type syndrome characterized by increased activation of the innate and acquired immune systems. Natural killer T (NKT) cells have characteristics of both conventional T cells and NK cells and recognize glycolipid antigens presented in association with CD1d molecules on antigen-presenting cells. The capacity of NKT cells to respond to lipid antigens and modulate innate and acquired immunity suggests that they may play a role in atherogenesis.

**Methods and Results**—We examined the role of NKT cells in atherogenesis and how the atherosclerotic environment affects the NKT cell population itself. The data show that CD1d-deficiency in male apolipoprotein E–deficient (apoE0) mice results in reduction in atherosclerosis, and treatment of apoE0 mice with α-galactosylceramide, a potent and specific NKT cell activator, results in a 2-fold increase in atherosclerosis. Interestingly, we demonstrate that α-galactosylceramide–induced interferon-γ responses and numbers of NKT cells in apoE0 mice show age-dependent qualitative and quantitative differences as compared with age-matched wild-type mice.

**Conclusions**—Collectively, these findings reveal that hyperlipidemia and atherosclerosis have significant effects on NKT cell responses and that these cells are proatherogenic. (Arterioscler Thromb Vasc Biol. 2004;24:2351-2357.)

**Key Words:** inflammation ■ atherosclerosis ■ NKT lymphocytes ■ cytokines ■ autoimmunity

Recent studies of innate immunity in chronic inflammatory diseases have focused on natural killer T (NKT) cells. NKT cells are a subset of T lymphocytes that share common surface receptors with both conventional T cells and NK cells. Most are CD4+ or CD4-CD8+ and express varying levels of CD1d (NK1.1 in mice). NKT cells are present in both humans and mice, and are found in lymphoid organs and tissues, have a restricted T cell receptor (TCR) expression (Vα14-Jα18/Vβ8 in mice and Vα24-Jα18/Vβ11 in humans), and recognize glycolipid antigens presented in the context of the major histocompatibility complex (MHC) class I-like molecule CD1d. Although a physiological ligand for NKT cells is still not known, these cells respond strongly to the marine sponge–derived glycolipid α-galactosylceramide (α-GalCer), which specifically binds to CD1d and selectively activates invariant NKT cells. Once activated, NKT cells rapidly produce large amounts of cytokines, including interleukin (IL)-4 and IL-10, which are associated with an antiinflammatory T helper 2 (Th2) response, and interferon (IFN)-γ and tumor necrosis factor-α, which are associated with a proinflammatory Th1 response. Recent studies have shown that activation of NKT cells by in vivo administration of α-GalCer, or its synthetic homolog KRN7000, has anti-metastatic activities and suppresses inflammation in chronic autoimmune diseases such as type 1 diabetes and experimental autoimmune encephalomyelitis in mice. Because atherosclerosis is a lipid-associated disease and has many characteristics in common with other autoimmune disorders, we hypothesized that NKT cells regulate immunity and progression of lesion growth in the artery wall. This hypothesis is consistent with the recent findings that CD1d is expressed in human atherosclerotic lesions and that numbers of CD4+ NK1.1+ cells producing IL-4 are enhanced in the artery plaques of apolipoprotein E–deficient (apoE0) mice treated with lipopolysaccharide. We have investigated the role of NKT cells in atherosclerosis using the apoE0 mouse model of spontaneous hyperlipidemia and atherosclerosis. Our results indicate that the dyslipidemic/atherosclerotic environment has significant effects on the NKT cell population and that NKT cells are proatherogenic.

**Methods**

**Animals**

All mice used in these studies were male. NKT cell–deficient CD1d0 and Jα180 mice on the C57BL/6 (B6) background have been previously described. ApoE0 mice on the B6 background were...
purchased from The Jackson Laboratory (Bar Harbor, Me). CD1d<sup>+</sup>, apoE<sup>+</sup> double mutant (CD1d<sup>-</sup>apoE<sup>-</sup>) mice were generated by crossing apoE<sup>-</sup> mice with CD1d<sup>+</sup> mice. All mice were maintained in microisoler cages according to the guidelines of the Vanderbilt University Institutional Animal Care and Use Committee (Nashville, Tenn).

**Flow Cytometry**

Single cell suspensions were stained using anti-CD4, -B220, -NK1.1, and -TCR<sub>B</sub> (all from BD Pharmingen, San Diego, Calif) and CD1d<sup>-</sup>/α-GalCer tetramers<sup>6</sup> and analyzed using a FACSCalibur flow cytometer and CELLQuest software (Becton Dickinson). B220<sup>+</sup> cells were excluded from the analysis by electronic gating.

**Atherosclerosis Studies**

Animals were age- and cholesterol-matched at baseline. Atherosclerosis in apoE<sup>-</sup> and CD1d<sup>-</sup>apoE<sup>-</sup> mice was assessed at 16 weeks of age in animals that were maintained on normal chow diet. The effect of NKT cell activation by α-GalCer was examined starting at 4 weeks of age; mice were treated twice weekly for 10 weeks with an IP injection of 4 μg per mouse per injection (200 μL total volume) of α-GalCer or vehicle (0.05% polysorbate-20 in PBS). Two or 10 weeks after the last treatment (at 16 or 24 weeks of age, respectively) mice were euthanized and atherosclerosis assessed. Atherosclerosis was measured by oil red-O staining in the proximal aorta and by en face analysis in distal aortas.<sup>10</sup>

**In Vivo and In Vitro Stimulation of NKT Cells With α-GalCer**

ApoE<sup>-</sup> mice and control CD1d<sup>+</sup> and B6 mice were injected IP with a single injection of 4 μg α-GalCer (KRN7000 obtained from the Kirin Brewery Co Ltd, Gunma, Japan) or vehicle. At 2 and 8 hours after injection, mice were bled and serum levels of IFN-γ and IL-4 were measured by ELISA. Spleen cells (2×10<sup>5</sup> per well) were incubated with 25 mg/ml α-GalCer in RPMI-1640 medium (supplemented with 10% FCS, 50 μmol/L 2-mercaptoethanol, 2 μmol/L glutamine, antibiotics, and 10 mmol/L HEPES) for 48 or 72 hours as previously described.<sup>11</sup>

**ELISA**

For details, please see the online Methods, available at http://atvb.ahajournals.org.

**Polymerase Chain Reaction and Real-Time RT-PCR**

DNA isolated from splenocytes was subjected to semiquantitative polymerase chain reaction (PCR) amplifying the V<sub>α</sub>14J<sub>ε</sub>18 TCR gene rearrangement. Relative amounts of V<sub>α</sub>14J<sub>ε</sub>18 were normalized to the 18S genomic DNA. Total RNA was isolated from the aortas (arch to iliac bifurcation) of vehicle- and α-GalCer–treated mice at 16 weeks time point. For real-time RT-PCR methods, please see the online Methods.

**Histology and Immunohistochemistry**

For details, please see the online Methods.

**Statistical Analyses**

Statistical analyses were performed using a Student <i>t</i>-test; <i>P</i>≤0.05 was considered statistically significant. Normal distribution of atherosclerosis data were determined using a Kolmogorov–Smirnov test.

**Results**

**Absence of CD1d-Restricted NKT Cells Results in Reduced Atherosclerosis in ApoE<sup>-</sup> Mice**

CD1d<sup>+</sup> mice lack the MHC class I–associated molecule CD1d, which is necessary for normal development of invariant NKT cells.<sup>7</sup> We examined whether NKT cell deficiency would modulate the development of atherosclerosis. Flow cytometric analyses demonstrated the absence of NKT cells in the liver of CD1d<sup>-</sup>apoE<sup>-</sup> mice (Figure 1A). Absence of NKT cells had no effect on serum cholesterol or triglyceride levels (data not shown). However, at 16 weeks of age we observed a significant 68% decrease in atherosclerosis in CD1d<sup>-</sup>apoE<sup>-</sup> compared with apoE<sup>-</sup> mice (Figure 1B).

**In Vivo Activation of NKT Cells With α-GalCer Exacerbates Atherosclerosis in ApoE<sup>-</sup> Mice**

We determined whether specific activation of NKT cells, using α-GalCer treatment in vivo, would enhance atherogenesis. Starting at 4 weeks of age, apoE<sup>-</sup> mice were given either vehicle (0.05% polysorbate in PBS) or α-GalCer (4 μg per mouse per injection) twice weekly for a period of 10 weeks. Mice were allowed to rest 2 or 10 weeks and were euthanized at 16 and 24 weeks of age. No significant differences in...
serum cholesterol or triglycerides were observed (data not shown). As reported in numerous studies (reviewed by Wilson et al1), repeated injection of α-GalCer led to dramatic decreases in NKT cell numbers and responses at 16 weeks of age (Figure 1C and 1D). These decreases were present at 24 weeks of age as demonstrated by reduced NKT cell responses (Figure 1D, right). Assessment of atherosclerosis in the aortic sinus at 16 weeks of age demonstrated ∼2-fold increase in atherosclerotic lesion area in the α-GalCer–treated mice compared with vehicle-treated mice (Figure 1E, left). Macrophages appeared to make up the majority of the lesion in both groups, as measured by MOMA-2 staining (data not shown). An increase in atherosclerosis was also present at 24 weeks of age in the proximal and distal aortas (Figure 1E, right, and Figure 1F).

In Vivo Activation of NKT Cells With α-GalCer Results in Local Cytokine Production in the Aorta

Recently, several studies have shown that NKT cells are present in atherosclerotic lesions.4,12,13 In fact, Tupin et al showed that a single injection of α-GalCer results in rapid increases in NKT cell–associated cytokine production in aortic tissue. However, because these latter studies were conducted in 5-week-old mice, it was not established whether activation of NKT cells can effect the cytokine production in aortas where early lesions are present. To evaluate this, the 16-week-old vehicle-treated animals were divided into 2 groups. Sixteen hours before being euthanized, 5 mice received an additional injection with vehicle and 5 mice received a single IP injection of 4 μg per mouse α-GalCer. Real-time RT-PCR was performed to detect mRNA for IFN-γ, IL-4, and IL-10. Because these mice had early atherosclerosis but were naïve to α-GalCer, this experiment was designed to examine the effects of increased activation of aortic NKT cells after 1 injection of α-GalCer. We observed ∼80-fold and 60-fold increase in IFN-γ and IL-10, respectively, 16 hours after a single injection of α-GalCer relative to aortas from vehicle-injected mice (Figure 1A, available online at http://atvb.ahajournals.org). α-GalCer injection induced an even more dramatic yet substantial increase in IL-4 with mRNA levels increasing ∼5- to 6-fold relative to vehicle control. We did not detect any changes in cytokine mRNA in the aortas of NKT cell–deficient CD1d−/− mice (data not shown), demonstrating that increases in cytokine mRNA are NKT cell–mediated. These are the first data demonstrating that in vivo activation of NKT cells by a single injection of α-GalCer has dramatic effects on the local cytokine environment of aortas with established atherosclerotic lesions.

Repeated administration of α-GalCer to mice of the B6 background has been shown to skew the immune response toward a Th2-type phenotype.1 In addition, previous studies of NKT cells and atherosclerosis looked at the effects of chronic stimulation with α-GalCer immediately after the last injection.13 This is a time point that would be greatly influenced by the acute effects of α-GalCer and may not represent the long-term changes in the aortic environment as a result of chronic NKT cell activation. Therefore, we analyzed cytokine mRNA in the aortas of 16-week-old mice 2 weeks after the final α-GalCer injection. Th2 polarization was observed in the aortas of apoE0 mice receiving repeated injection with α-GalCer. Using real-time RT-PCR we observed a 6-fold increase in IL-4 and IL-10 message levels after 20 injections of α-GalCer compared with the vehicle controls (Figure 1B). In contrast, there was only a slight (1.5- to 2-fold) increase in IFN-γ message levels in α-GalCer–treated animals. Consistent with these results, we observed no differences in the percentage of cells expressing the MHC class II molecule I-Aq (Figure 1C).

To determine whether the changes in the aortas of α-GalCer–treated apoE0 mice reflected a general change in the overall resting cytokine profile, we analyzed message levels for IFN-γ, IL-4, and IL-10 in spleens of vehicle and α-GalCer–treated mice at 16 weeks of age. We observed that α-GalCer–treated animals had similar levels of IFN-γ, IL-4, and IL-10 mRNA in spleens (Figure 1D). Likewise, we did not see any significant difference in the total or oxidized low-density lipoprotein–specific IgG1 or IgG2a between the vehicle and α-GalCer–treated groups (data not shown).

Age-Dependent Changes in the NKT Cell Population of ApoE0 Mice

Many studies have shown that mice and humans with autoimmune diseases have reduced NKT cell numbers and functions.3,14–16 Although recent studies,12,13 including this one, have emerged showing that activation of NKT cells with α-GalCer results in increased atherosclerosis, the experiments in CD1d-deficient mice demonstrate that NKT cells, without enhanced stimulation, are proatherogenic. Therefore, we assessed the homeostatic state of NKT cells in apoE0 mice as compared with wild-type C57Bl/6 controls. This is inherently important given that NKT cells recognize glycolipids, and several species of glycolipids have been shown to be increased during hyperlipidemia/atherosclerosis in both humans and animals.17,18 For this purpose, we compared NKT cell numbers in B6 and apoE0 mice at 4, 8, 16, and 24 weeks of age. These ages represent times of prelesion, early, intermediate, and complex atherosclerotic lesions, respectively.19 In the spleen, NKT cell numbers were normal at 4 weeks in apoE0 mice but started to decline slightly at 8 weeks compared with B6 controls (Figure 2A). We found similar results in the liver, except there was no decline in NKT cells at 8 weeks (data not shown). At 16 weeks of age, apoE0 mice had a 50% decrease in NKT cells in spleen and liver, a trend that continued until 24 weeks of age (Figure 2A and data not shown).

PCR experiments amplifying the Vα14Jα18 gene rearrangement in spleens showed that young naïve apoE0 mice (8 weeks) had similar amounts of NKT cell–associated DNA compared with C57Bl/6 mice (Figure 2B). However, as apoE0 mice age and hyperlipidemia/atherosclerosis progresses, much less of the Vα14Jα18 gene rearrangement was present in spleens of the apoE0 mice (Figure 2B). Calculations of the relative amount of Vα14Jα18 rearrangement to the 18S genomic DNA demonstrated that NKT cell numbers were reduced by 2-fold (Figure 2C), data that correlate well with the flow cytometry analyses. These data indicate that the reduction of NKT cells in apoE0 mice is not due to decreased TCR expression, as is the case for acute NKT cell activa-
tion, but instead is the result of physical reductions in NKT cells. Decreases in NKT cell numbers are likely caused by changes in circulating lipoproteins during atherosclerosis and are not specific to the apoE0 mouse because, similar to Nakai et al, we observed changes in NKT cell numbers and functions in C57BL/6 mice fed an atherosclerotic diet compared with control animals fed a chow diet (data not shown).

ApoE0 NKT Cells Exhibit Functional and Phenotypic Differences Compared With Wild-Type Mice

Because NKT cell numbers decreased in older apoE0 mice, we determined whether changes in cytokine production in response to α-GalCer are affected. As expected, cell proliferation and cytokine production after in vitro α-GalCer stimulation of splenocytes was suppressed in old apoE0 mice compared with B6 age-matched controls (Figure 3A). Interestingly, splenocytes of young apoE0 mice produced significantly more IFN-γ in response to α-GalCer as compared with B6 animals (Figure 3B, upper left). There was no significant difference in IL-4 production between the 2 groups. Similar to in vitro cytokine responses, immediate cytokine production after in vivo administration of α-GalCer resulted in increased IFN-γ production in young apoE0 mice compared with controls and age-dependent decreases in IFN-γ and IL-4 production (data not shown).

It has been shown that after chronic in vivo stimulation with α-GalCer, percentages of CD4+ and NK1.1+ NKT cells decrease dramatically. To determine whether this occurred in aging apoE0 mice, we compared the percentage of CD4+ and NK1.1+ NKT cells in the spleens and livers of 4-week-old and 16-week-old apoE0 mice to age-matched B6 controls (Figure 4A and 4B). We observed that percentages of CD4+ NKT cells were similar in young apoE0 and B6 mice (Figure 4A). However, older apoE0 mice had a significant reduction in CD4+ NKT cells in spleens compared with age-matched B6 mice (65±0.6 versus 76±0.3, respectively). This change in CD4+ NKT cells was also apparent in livers of older apoE0 mice compared with B6 mice (70±0.4 versus 76±0.6, respectively). These data suggest that NKT cells of apoE0 mice may be chronically stimulated during the atherosclerotic process. Analyses of NK1.1 (Figure 4B) revealed that at 4 weeks of age, apoE0 mice, as compared with B6 mice, had...
NKT cells can modulate specific adaptive immunity by producing both Th2-associated cytokines, such as IL-4 and IL-10, and Th1-type cytokines, such as IFN-γ. This panel of cytokines includes factors with proatherogenic (IL-4 and IFN-γ) and antiatherogenic (IL-10) properties. Acute activation of NKT cells after a single injection of α-GalCer demonstrated increases in IFN-γ (Th-1), IL-4, and IL-10 (Th2) message levels in the aortas of 16-week-old apoE0 mice with established atherosclerosis. The lack of such a dramatic increase in IL-4 mRNA may reflect the time point analyzed; IL-4 message likely peaks much earlier than 16 hours after α-GalCer treatment, which is the case for IL-4 protein in serum. Finding an abundance of α-GalCer–induced mRNA in the aortas of apoE0 mice for both IFN-γ and IL-10 somewhat complicates the interpretation of the results. It is possible that induction of IL-10 in the aorta after α-GalCer treatment is age-dependent. This would be consistent with reports in systemic lupus erythematosus–susceptible mice where induction of Th1 or Th2 responses is dependent on the age at which α-GalCer treatment is initiated. Therefore, one might hypothesize that initiation of α-GalCer treatment in older apoE0 mice may have a protective effect against atherosclerosis. Further experiments will be necessary to determine whether this phenomenon also applies to atherosclerosis.

Interestingly, real-time RT-PCR analysis in 16-week-old apoE0 mice also showed that repeated injection of α-GalCer resulted in greater increases in IL-4 and IL-10 in the aorta 2 weeks after the last treatment. Because the 2-week “rest” period represents a time when the immediate effects of α-GalCer would no longer be present, these data suggest that α-GalCer resulted in overt changes in the aortic cytokine environment. That the spleen cytokine mRNA profile in these mice did not differ between the vehicle and α-GalCer group supports the hypothesis that the differences in the aorta are site-specific. Because IL-4 has been associated with upregulation of CD36 and 12-lipoxygenase, the Th2 bias in the aorta after repeated α-GalCer administration does not entirely deviate from a proatherogenic role for chronic NKT cell stimulation. This is further supported by data reported by Nakai et al.12 that demonstrate that an α-GalCer analog, OCH, which preferentially stimulates Th2 responses in NKT cells, is as atherogenic as α-GalCer. One may argue, however, that the mRNA data are confounded by the presence of greater lesion area in the α-GalCer–treated animals. In fact, we do see more atherosclerosis in the distal aortas of 24-week-old α-GalCer–treated mice compared with vehicle controls. However, normalizing the cytokine message levels to the lesion area would not change the qualitative differences between the α-GalCer and vehicle animals, with the α-GalCer animals containing more Th2–associated transcripts compared with the vehicle controls. Additionally, similar I-Aβ sclerosis is not relevant to the human condition. However, we initiated these studies expecting that α-GalCer treatment would lead to decreased disease. Therefore, α-GalCer represented a possible therapeutic for the treatment of atherosclerosis. In addition, α-GalCer is being suggested as a therapeutic for cancer and autoimmunity, thus the understanding of any adverse effects of exposure to this compound is imperative.

***Discussion***

In most studies of autoimmunity, NKT cells have been associated with protection against disease. Interestingly, our current study and recent studies of others12,13 demonstrate a proatherogenic role for CD1d and CD1d-restricted NKT cells in apoE0 mice using 2 different experimental approaches. We observed a dramatic 68% reduction in atherosclerosis, in the absence of changes in serum lipoproteins, in NKT cell–deficient CD1d–apoE0 mice compared with apoE0 controls. This observation was confirmed by demonstrating that in vivo activation of NKT cells, through administration of α-GalCer, exacerbates disease to a similar extent in 16-week-old apoE0 mice and to a lesser extent in 24-week-old apoE0 mice. The decreased effect in 24-week-old mice may be because of a 10-week “rest” period between the last injection and the time of euthanization. Alternatively, it may indicate that NKT cells are more important in early atherosclerosis, a finding similar to studies in Rag2 mice.13 A proatherogenic role for NKT cells is consistent with recent reports that CD1d is expressed in human atherosclerotic lesions4 and that IL-4–producing NK1.1+ cells are present in the atherosclerotic lesions of lipopolysaccharide-treated apoE0 mice. As α-GalCer is not a physiological ligand for NKT cells in mammals, one may argue that studying its effects on athero-
staining (an indirect measurement of IFN-γ production) between the groups is consistent with no differences in IFN-γ message.

Perhaps most interesting in this study is the finding that the NKT cells of apoE0 mice exhibit quantitative and qualitative differences compared with wild-type animals. Ours is the first study to characterize changes in NKT cell numbers and functions during spontaneous hyperlipidemia as seen in apoE0 mice. We observed age-dependent decreases in the numbers of NKT cells in the liver and spleen of apoE0 mice compared with B6 controls (Figure 2). The reduction in number of NKT cells in aging apoE0 mice is consistent with studies demonstrating that T lymphocyte deficiency affects early, but not advanced, lesion development in mice.21,26 These data are also similar to studies of NKT cells in mice with genetic susceptibility to other inflammatory diseases, such as type 1 diabetes and experimental autoimmune encephalomyelitis.3 Unlike these latter studies, however, NKT cell deficiencies were observed at all ages, suggesting a genetic mechanism, whereas in apoE0 mice numbers of NKT cells at an early age (before the onset of atherosclerosis) were normal but declined as disease progressed. Therefore, one may hypothesize that environmental factors are involved in changes in NKT cell numbers and functions in apoE0 mice. Similar findings have been made in humans with a variety of autoimmune diseases, such as type 1 diabetes and experimental autoimmune encephalomyelitis.3

In conclusion, our study describes novel discoveries regarding immune-mediated mechanisms of atherosclerosis as well as potential effects of the atherogenic environment on immunity. We show that NKT cells have proatherogenic potential and that increased serum lipoproteins or atherosclerosis is associated with changes in the dynamics of the NKT cell population. NKT cells recognize glycolipid antigens and can greatly influence both innate and adaptive immune responses.2,3 Therefore, our studies reveal a critical link between atherosclerosis-associated dyslipidemia/disease, inflammation, and antigen-specific immunity.

Acknowledgments

This work was supported by American Heart Association Grant 0330412N (to A.S.M), the Vanderbilt Discovery Grants Program (to A.S.M and L.V.K.), National Institutes of Health (NIH) Grants AI50953, NS44044, HL68774 (to L.V.K.), AI42284 (to S.J.), HL57986, HL65709 (to S.F.), HL65405, and HL53989 (to M.F.L.), and by the Lipid, Lipoprotein, and Atherosclerosis Core of the Vanderbilt Mouse Metabolic Phenotyping Centers (NIH DK59637-01).

References


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Arterioscler Thromb Vasc Biol. 2004;24:2351-2357; originally published online October 7, 2004;
doi: 10.1161/01.ATV.0000147112.84168.87

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

**ELISA.** IFN-\(\gamma\) and IL-4 were measured in culture supernatants using purified and biotinylated antibodies and compared to purified IFN-\(\gamma\) and IL-4 as standards (all from BD Pharmingen). Optical density was determined at 405 nm. Oxidized LDL (oxLDL)-specific IgG\(_1\) and IgG\(_{2a}\) isotypes in mouse serum were measured as previously described\(^1\).

**Real-time RT-PCR.** In the aortas of 16-week-old mice, the vehicle-treated mice were divided into two groups. One group received an additional injection of vehicle and the other received a single i.p. injection of \(\alpha\)-GalCer. Sixteen hours later, mice were sacrificed, aortas dissected and cleaned and total RNA was isolated. Message levels for IFN-\(\gamma\), IL-4, IL-10 and 18S ribosomal mRNA were measured using the Assay on Demand primer and probe sets (Applied Biosciences). Normalization was made to the 18S ribosomal RNA. Results are expressed as the relative increase in message level in the \(\alpha\)-GalCer treated animals using the vehicle treated group as the calibrator. The increases were calculated using the \(\Delta\Delta CT\) method and are expressed as \(2^{-\Delta\Delta CT}\).

**Histology and immunohistochemistry.** Immunohistochemistry with the macrophage-specific marker MOMA-2 was performed as previously described\(^1\). Detection of MHC class II (I-A\(^b\)) was performed on acetone fixed frozen sections of the aortic sinus using biotin-conjugated mouse anti-mouse I-A\(^b\) (Pharmingen). Positive cells were visualized using the ABC Elite system followed by DAB (both from Vector Laboratories). Data are expressed as \%I-A\(^b\) positive cells per total cells in atherosclerotic lesions. Four sections/mouse were analyzed.
Reference

Figure legend

**Fig. 1.** Effects of short-term and long-term NKT cell activation on cytokine profiles in aortas of apoE^0^ mice. *(A)* 16 week old vehicle-treated mice were given either an additional injection of vehicle or a single i.p. injection of α-GalCer. mRNA levels for IFN-γ, IL-4 and IL-10 were analyzed by real-time RT-PCR. Results are the means and standard error of 5 mice per group. *(B)* Comparison of mRNA levels for IFN-γ, IL-4 and IL-10 in aortas of apoE^0^ mice following long-term (20 injections) α-GalCer treatment as analyzed by real-time RT-PCR. Results are the means and standard error of 5 mice per group. *(C)* Immunohistochemistry analysis for the presence of I-A^b^ class II molecules in the atherosclerotic lesions. Bars are the mean and standard error of 6-8 mice per group. *(D)* Comparison of mRNA levels for IFN-γ, IL-4 and IL-10 in spleens of 16 week old apoE^0^ mice following long-term (20 injections) α-GalCer treatment as analyzed by real-time RT-PCR. Results are the means and standard error of 3-4 mice per group.