

Theme Issue Article

Effect of natural killer T cell activation on initiation of atherosclerosis

Gijs H. M. van Puijvelde; Eva J. A. van Wanrooij; Arnaud D. Hauer; Paula de Vos; Theo J. C. van Berkel; Johan Kuiper

Division of Biopharmaceutics, Leiden University, Leiden, the Netherlands

Summary

It has been shown that natural killer T (NKT) cell activation accelerates atherosclerosis in apoE^{-/-} mice. ApoE is, however, an important mediator in the presentation of lipids which may complicate conclusions on the role of NKT cells in atherosclerosis. Treatment of LDLr^{-/-} mice with α -GalCer during Western-type diet feeding is therefore of interest. Atherosclerosis was induced by Western-type diet feeding and collar placement around the carotid arteries in both LDLr^{-/-} and apoE^{-/-} mice. Subsequently, the mice were treated twice a week with α -GalCer. This resulted in an 84% reduction in plaque size in LDLr^{-/-} mice ($P < 0.05$), while no effect was observed in apoE^{-/-} mice. In-vitro incubation of splenocytes with α -GalCer showed that LDLr^{-/-} splenocytes proliferated stronger than apoE^{-/-} splenocytes. This

is reflected in a larger increase in production of cytokines and especially IL-10 after in-vitro stimulation with α -GalCer in LDLr^{-/-} mice compared with apoE^{-/-} splenocytes. Additionally, feeding a Western-type diet for 1.5 weeks induced a strong increase in the number of NKT cells in LDLr^{-/-} mice and this increase was slower and less prominent in apoE^{-/-} mice. Administration of α -GalCer to LDLr^{-/-} mice in combination with Western-type diet feeding reduced plaque formation, but this effect was not seen in apoE^{-/-} mice. This may be explained by the decreased presentation of lipids on CD1d molecules due to the lack of apoE. In this study we proved for the first time that NKT cells may also act in an atheroprotective manner.

Keywords

Atherosclerosis, cytokines, immune response, NKT cells

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Introduction

Atherosclerosis is a chronic inflammatory disease of the vasculature in which both the innate and the adaptive immune system are involved. T cells, B cells, monocytes and dendritic cells (DCs) are present in atherosclerotic plaques of mice and humans (1, 2, 36). The inflammatory response in atherosclerosis is mainly driven by Th1 cells, producing pro-atherogenic cytokines such as interleukin (IL)-12 and interferon (IFN)- γ (3–6). Th2 cells may counteract the Th1 cells by producing anti-atherogenic cytokines such as IL-5, IL-10 and IL-13. The Th2 cytokine IL-4, however, has pro-atherogenic properties in initial stages of atherosclerosis (6–9). In addition regulatory T cells may also regulate the process of atherosclerosis. More recently, natural killer T (NKT) cells have been reported to play a role in the inflammatory process of atherosclerosis. NKT cells represent a subset of T cells expressing receptors such as NK1.1 (CD161) found on NK cells. The majority of NKT cells express a semi-in-

variant T cell receptor (TCR) composed of a V α 14-J α 18 α -chain paired with a V β 8 or V β 2 β -chain. This TCR is unique because of its specificity for (glyco)lipid antigens. (Glyco)lipid antigens are presented by the MHC class I like molecule CD1d, which is expressed on most antigen presenting cells (APCs). Upon stimulation, NKT cells are able to produce large amounts of both Th1 cytokines (IFN- γ and IL-12) and Th2 cytokines (IL-4, IL-5, IL-10 and IL-13). This makes NKT cells a unique T cell population with potentially both pro- and anti-inflammatory properties (10).

Chemokines play an important role in attracting different cell types into the atherosclerotic lesion (11). Chemokines involved in atherosclerosis such as CXCL12 may also be responsible for the recruitment of NKT cells to the site of injury (12). Consequently, NKT cells are found in atherosclerotic plaques of both humans (13, 14) and atherosclerosis-prone (LDLr^{-/-} and apoE^{-/-}) mice (15–18). In human lesions, NKT cells co-localise with CD1d-expressing DCs in the shoulder regions, which may con-

Correspondence to:

Prof. Dr. J. Kuiper
LACDR, Div. of Biopharmaceutics, Gorlaeus Laboratories
Einsteinweg 55, PO Box 9502, 2300 RA Leiden, The Netherlands
Tel.: +31 71 5274378, Fax: +31 71 5276032
E-mail: j.kuiper@chem.leidenuniv.nl

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tribute to plaque destabilisation. The NKT cells represent 2% of the total lymphocyte population within the lesion (13, 14). Initial studies show that NKT deficiency combined with apoE^{-/-} or LDLr^{-/-} deficiency results in an attenuation of atherosclerosis (15–17, 19) while the adoptive transfer of NKT cells into RAG1^{-/-}LDLr^{-/-} mice accelerates atherosclerosis (20). These studies indicate that the endogenous activation of NKT cells during atherosclerosis is a pro-atherogenic process. Activation of NKT cells using a synthetic ligand, α -galactosylceramide (α -GalCer), significantly increases atherosclerosis (15–17). The administration of α -GalCer causes an increase in pro-atherogenic (IFN- γ , IL-4) cytokines (16, 17), which is in contrast to studies on a number of other Th1-mediated autoimmune diseases (autoimmune diabetes [21], experimental autoimmune encephalomyelitis [22] and colitis [23]) in which repeated injections with α -GalCer polarises the adaptive immune response towards a protective Th2-like response (24, 25).

Interestingly it has been recently shown that apoE is important in lipid antigen presentation via CD1 molecules, which may complicate conclusions on the effect of α -GalCer activation of NKT cells on atherosclerosis in apoE^{-/-} mice (26). Therefore it is of major interest to determine the effect of α -GalCer activation of NKT cells combined with their endogenous activation via hyperlipidemia on atherosclerosis in LDLr^{-/-} mice.

In this study, we show that α -GalCer reduced atherosclerosis in LDLr^{-/-} mice but was not effective in apoE^{-/-} mice. When compared with LDLr^{-/-} splenocytes, apoE^{-/-} splenocytes showed a lower proliferative response and a dampened cytokine production towards α -GalCer. In addition, we show that a cholesterol-rich Western-type diet increased NKT cell numbers in liver and spleen of LDLr^{-/-} mice. Our findings suggest that in combination with endogenous activation of NKT cells, treatment with α -GalCer can be protective against atherosclerosis.

Materials and methods

Animals

All animal work was approved by the regulatory authority of Leiden University and carried out in compliance with the Dutch government guidelines. Male LDLr^{-/-} and apoE^{-/-} mice were obtained from the Jacksons Laboratory (Bar Harbor, ME, USA). All mice were kept under standard laboratory conditions and were fed a normal chow diet or a 'Western-type' diet containing 0.25% cholesterol and 15% cocoa butter (Special Diet Services, Witham, Essex, UK). All mice used were 10–12 weeks of age, and diet and water were administered *ad libitum*.

Glycolipids

α -Galactosylceramide (α -GalCer; KRN7000) and the control analogue β -galactosylceramide (β -GalCer) were developed and manufactured by the Pharmaceutical Research Laboratory of Kirin Brewery Co. (Gunma, Japan). Both α -GalCer and β -GalCer were dissolved in water. For intraperitoneal and intravenous injections, both glycolipids were diluted in 0.9% NaCl.

Effect of α -GalCer on lesion formation

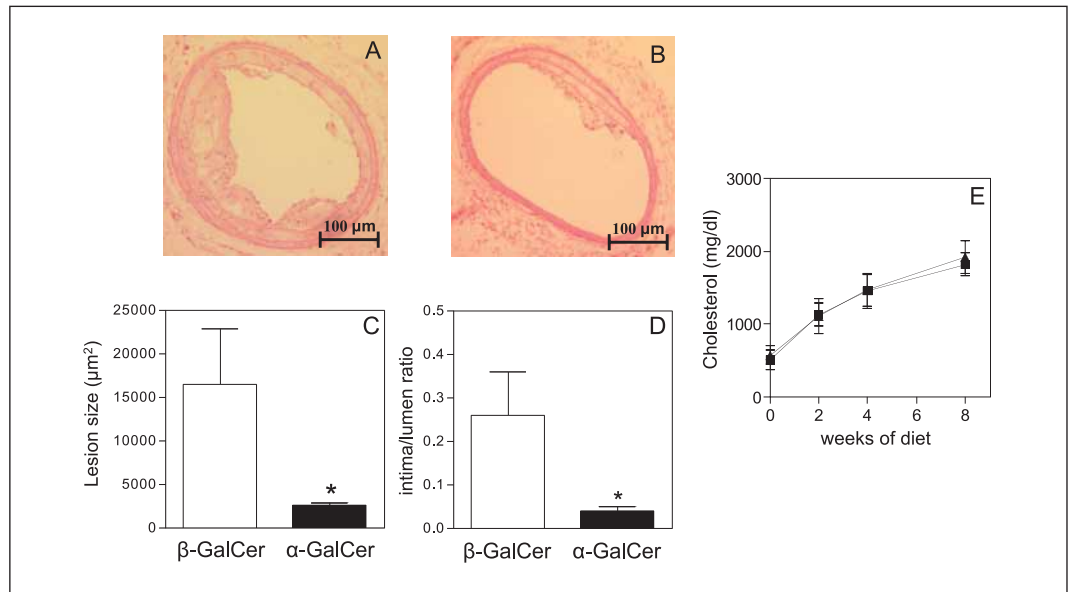
To determine the effect of α -GalCer on the initiation of atherosclerosis, atherosclerosis was induced in LDLr^{-/-} and apoE^{-/-}

mice. The mice were fed a Western-type diet for two weeks and after two weeks, atherosclerosis was induced by placement of perivascular collars prepared from elastic tubing (0.3 mm inside diameter; Dow Corning, Midland, MI, USA), around both carotid arteries (method described by von der Thüsen et al. [27]). For this surgery the mice were anaesthetised by a subcutaneous injection of ketamine (60 mg/kg; Eurovet Animal Health), fentanyl citrate (1.26 mg/kg; Janssen Animal Health) and fluanisone (2 mg/kg; Janssen Animal Health). The diet response was followed by measuring the cholesterol levels in serum of these mice. Total cholesterol levels were quantified spectrophotometrically using an enzymatic procedure (Roche Diagnostics, Mannheim, Germany). Immediately after collar placement, the mice were injected twice a week with 2 μ g of β -GalCer or α -GalCer. All injections were performed half intravenously (i.v.)/half intraperitoneally (i.p.) and continued for seven weeks. Subsequently, the mice were anaesthetised by a subcutaneous injection with ketamine-hypnorm and exsanguinated by femoral artery transection. The mice were perfused and fixated through the left cardiac ventricle with phosphate-buffered saline (PBS) and FormalFixx. Common carotid arteries and both carotid bifurcations were removed for analysis as described by von der Thüsen et al. (27). The arteries were embedded in OCT compound (TissueTek; Sakura Finetek, Zoeterwoude, The Netherlands) and 5- μ m sections were made on a Leica CM 3050S Cryostat (Leica Instruments, Milton Keynes, UK) proximally of the place of collar occlusion. These cryosections were stained with hematoxylin (Sigma Diagnostics, St. Louis, MO, USA) and eosin (Merck Diagnostica, Munich, Germany). Plaque areas were measured using a Leica DM-RE microscope and LeicaQwin software (Leica Imaging Systems, Cambridge, UK). Plaque composition (macrophages, T cells, smooth muscle cells and collagen) was determined using (immuno) histochemistry as described before (8, 34).

Spleen cell proliferation assay

To test the responsiveness of splenocytes to α -GalCer, spleens from LDLr^{-/-} and apoE^{-/-} mice were dissected and single cell suspensions were obtained by squeezing the spleen through a 70 μ m cell strainer (Falcon, The Netherlands). The erythrocytes were eliminated by incubating the cells with erythrocyte lysis buffer (0.15 M NH₄Cl, 10 mM NaHCO₃, 0.1 mM EDTA, pH 7.3). Subsequently, the splenocytes were cultured in triplicate at 2·10⁵ cells per well of a 96-well round-bottom plate in the presence or absence of different concentrations of α -GalCer. RPMI 1640 (with L-Glutamine) supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, 100 μ g/ml streptomycin (all from BioWhittaker Europe) and 50 μ M 2-mercaptoethanol was used as culture medium. The splenocytes were incubated for 48 hours (h) in a humidified atmosphere (37°C; 5% CO₂). Cultures were pulsed for the final 16 h with [6-³H]-thymidine (1 μ Ci/well, sp. act. 24 Ci/mmol; Amersham Biosciences, The Netherlands). The amount of [6-³H]-thymidine incorporation was measured using a liquid scintillation analyser (Tri-Carb 2900R). The magnitude of the proliferative response is expressed as stimulation index (SI) defined as the ratio of the mean counts per minute (min) of triplicate cultures with α -GalCer to the mean counts/min in culture medium without α -GalCer.

Figure 1: Multiple injections with α -GalCer reduce plaque formation in LDLr^{-/-} mice. LDLr^{-/-} mice were treated twice a week with 2 μ g of α -GalCer (n=11) or β -GalCer (n=11) after collars were placed around the carotid arteries. During the experiment, the mice were fed a Western-type diet. Representative cryosections of plaques from β -GalCer (A) and α -GalCer (B) treated mice were stained with hematoxylin and eosin. Using computer assisted morphometric analysis plaque size (C) and intima/lumen ratio (D) were determined. During the experiment total serum cholesterol levels were monitored (E). * p<0.05



Cytokine assays

To determine the cytokine production by splenocytes, splenocytes were isolated from LDLr^{-/-} and apoE^{-/-} mice and incubated with 100 ng/ml α -GalCer in medium. After 0, 24 and 48 h culture supernatants were collected for ELISA assays to measure the IFN- γ , IL-10 and IL-4 production conform the manufacturers protocols (eBioscience, Halle-Zoersel, Belgium). To determine the in-vivo response to α -GalCer, LDLr^{-/-} and apoE^{-/-} mice were fed a Western-type diet and α -GalCer was injected i.p. and i.v. (1:1) twice a week during seven weeks. At 72 h after the last injection the mice were sacrificed and the spleen and the mediastinal lymph nodes were dissected. Mononuclear cells were isolated using Lympholyte (Cedarlane, ON, Canada) conform the manufacturer's protocol. The cells were stimulated o/n with anti-CD3 and anti-CD28 coated onto a 96-well plate. Cells were subsequently stained with APC-conjugated anti-CD3 (0.125 μ g/sample) for 30 min and subsequently fixated and permeabilised for 20 min. Then, the cells were stained with PE-conjugated anti-IL-4 mAb, PE-conjugated anti-IL-10 mAb and PE-conjugated anti-IFN- γ mAb (eBioscience) for 30 min. Cells were washed two times and analysed immediately by flow cytometry on a FACSCalibur. All data were analysed with CELLQuest software (BD Biosciences, Breda, The Netherlands). IL-10 was determined in serum using a commercial ELISA (eBioscience).

Effect of Western-type diet on NKT cells

To investigate the effect of Western-type diet on the amount of NKT cells, LDLr^{-/-} and apoE^{-/-} mice were fed a Western-type diet for 0, 1.5 and 4.5 weeks. After diet feeding, the mice were sacrificed and the liver and spleen were dissected. Mononuclear cells were isolated from these organs using Lympholyte (Cedarlane, ON, Canada) conform the manufacturer's protocol. Subsequently, the cells were stained with PerCP-conjugated CD3 and FITC-conjugated NK1.1 antibodies (eBioscience) for 30 min. Cells were washed two times and analysed immediately by flow cytometry on a FACSCalibur. All data were analysed with

CELLQuest software (BD Biosciences).

Statistical analysis

All data are expressed as mean \pm standard error of the mean (SEM). The two-tailed student's t-test was used to compare all data. P-values less than 0.05 are considered to be statistically significant.

Results

α -GalCer protects against atherosclerosis in LDLr^{-/-} mice, but not in apoE^{-/-} mice

Two mouse models for atherosclerosis were used to investigate the effect of multiple injections of α -GalCer on plaque formation. Male LDLr^{-/-} and apoE^{-/-} mice, fed a Western-type diet for two weeks, were equipped with perivascular collars around both carotid arteries to induce atherosclerosis. After surgery, diet was continued and mice were treated (twice a week for seven weeks) by combined i.p. and i.v. injections (50%/50%) of 2 μ g α -GalCer or β -GalCer (control). Representative examples of plaques of control and α -GalCer-treated LDLr^{-/-} mice are shown in Figure 1A and B, respectively. Treatment with α -GalCer led to a 84.1% reduction in plaque size in LDLr^{-/-} mice, when compared with β -GalCer treatment (Fig. 1C; 2,621 \pm 263 μm^2 vs. 16,488 \pm 6,286 μm^2 , respectively; p<0.05). α -GalCer treatment also resulted in an 85.7% reduction in intima/lumen ratio (Fig. 1D). On the other hand α -GalCer treatment of apoE^{-/-} mice did not significantly affect plaque size (Fig. 2A-C). In addition, the intima/lumen ratio (Fig. 2D) was not significantly affected in apoE^{-/-} mice. In both studies no effect of α -GalCer on total plasma cholesterol levels (Figs. 1E and 2E) and body weight (data not shown) was observed. Both in the lesions in the carotid arteries of LDLr^{-/-} mice and apoE^{-/-} mice no significant changes were observed in plaque morphology. Macrophages, smooth muscle cells, T cells and collagen were present to the same extent.

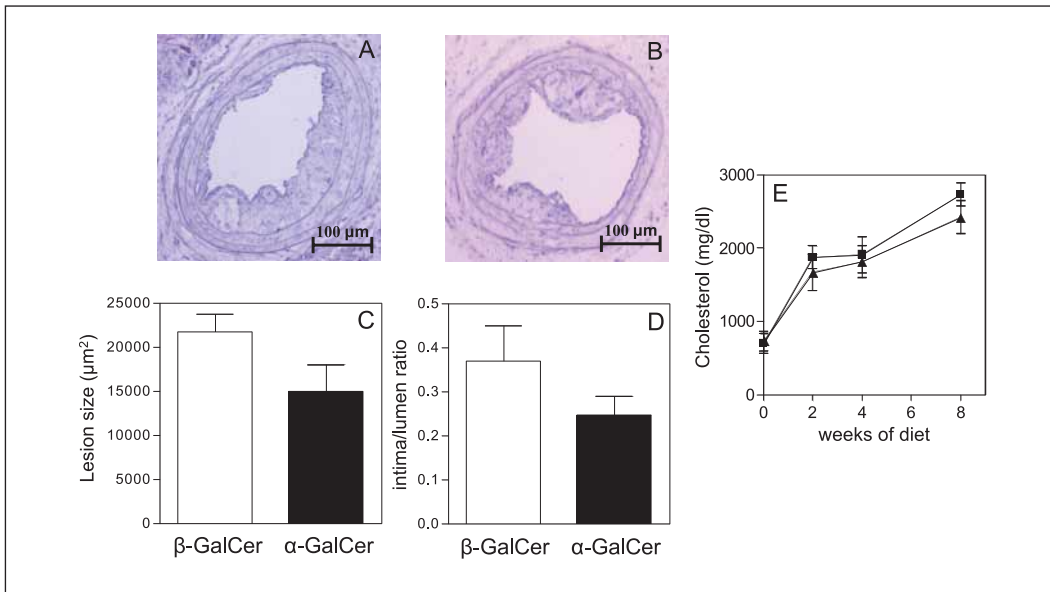


Figure 2: Multiple injections with α -GalCer have no significant effect on atherosclerosis in ApoE^{-/-} mice. ApoE^{-/-} mice were treated with 2 μg of α -GalCer (n=9) or β -GalCer (n=9) just like the LDLr^{-/-} mice in Figure 1. Representative cryosections of plaques from β -GalCer (A) and α -GalCer (B) treated apoE^{-/-} mice were stained with hematoxylin and eosin. Plaque size (C) and intima/lumen ratio (D) were determined. During the experiment total serum cholesterol levels were monitored (E).

In-vitro effects of α -GalCer

To investigate the effect of α -GalCer on proliferation of spleen cells, splenocytes were isolated from LDLr^{-/-} and apoE^{-/-} mice. Splenocytes from LDLr^{-/-} mice respond to α -GalCer with a significant proliferative response. Incubation with 100 and 500 ng/ml of α -GalCer induced a 15- to 22-fold increase in proliferation (Fig. 3, left graph, $p < 0.01$). In addition, the response of splenocytes of apoE^{-/-} mice was present, but was much lower. Incubation with 100 ng/ml of α -GalCer had no significant effect, while 500 ng/ml of α -GalCer caused a 5.2-fold increase in proliferation (Fig. 3, middle graph, $p < 0.05$).

In addition, the cytokine production of splenocytes from LDLr^{-/-}

and apoE^{-/-} mice in response to α -GalCer was determined. Stimulation of LDLr^{-/-} splenocytes with 100 ng/ml of α -GalCer for 48 h resulted in an increase in the production of IL-10 (101 ± 13 pg/ml vs. $1,559 \pm 133$ pg/ml), IL-4 (36 ± 9 pg/ml vs. 585 ± 100 pg/ml) and IFN- γ (39 ± 13 pg/ml vs. 381 ± 80 pg/ml) when compared with cells cultured without α -GalCer (Fig. 4, upper panel). Incubation of splenocytes of apoE^{-/-} mice with 100 ng/ml of α -GalCer also led to an increase in production of IL-10 (5 ± 1 vs. 16 ± 1 pg/ml), IL-4 (10 ± 4 vs. 92 ± 22 pg/ml) and IFN- γ (1.0 ± 0.5 vs. 9 ± 1 pg/ml) (Fig. 4, lower panel). When compared with each other, the relative increase in IL-10 production by LDLr^{-/-} splenocytes is rather high compared with the splenocytes from apoE^{-/-} splenocytes (15.6-fold vs. 3.2-fold) while the relative increase in production of IL-4 and IFN- γ is approximately the same.

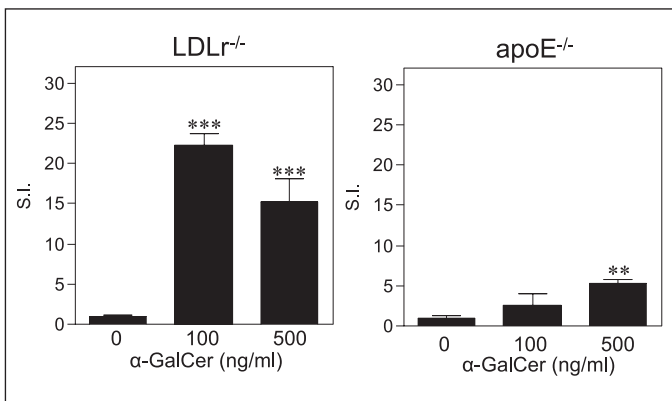


Figure 3: Proliferative response of splenocytes to α -GalCer.

Splenocytes were isolated from LDLr^{-/-} and apoE^{-/-} mice (10–12 weeks of age) and were incubated for 48 h with 100 or 500 ng/ml of α -GalCer. As a control, non-stimulated cells were used. Cells were pulsed with [³H]-thymidine for the final 16 h, and the amount of proliferation was measured. Data are shown as the stimulation index (S.I.) \pm SEM. The S.I. is defined as the ratio of the mean counts per minute of triplicate cultures with α -GalCer to the mean counts per minute in culture medium without α -GalCer. ** $p < 0.01$, *** $p < 0.001$

In-vivo effects of α -GalCer on cytokine production

To determine whether injections with α -GalCer affected the cytokine profile in LDLr^{-/-} and apoE^{-/-} mice, the cytokine production by CD3⁺T cells in spleen and mediastinal lymph nodes was determined after multiple injections (50% i.p./50% i.v.) with α -GalCer and β -GalCer (control). In control treated LDLr^{-/-} mice, $1.20 \pm 0.13\%$ and $0.76 \pm 0.13\%$ of the CD3⁺T cells in the spleen produced IFN- γ and IL-10, respectively. There was no effect on CD3⁺IFN- γ ⁺ cells ($1.66 \pm 0.28\%$) and CD3⁺IL-4⁺ cells (data not shown) after multiple injections of α -GalCer in LDLr^{-/-} mice, but a significant increase in CD3⁺IL-10⁺ cells ($2.12 \pm 0.32\%$; $p < 0.01$) was observed (Fig. 5A, upper panel). In the mediastinal lymph nodes, the percentage of CD3⁺IL-10⁺ lymphocytes increased after multiple α -GalCer injections ($0.46 \pm 0.12\%$ vs. $1.42 \pm 0.11\%$, respectively; $p < 0.05$). No effect on CD3⁺IFN- γ ⁺ lymphocytes ($0.52 \pm 0.06\%$ vs. $0.56 \pm 0.12\%$) and CD3⁺IFN- γ ⁺ cells (data not shown) was observed (Fig. 5A, lower panel). In the spleen and mediastinal lymph nodes of β -GalCer-treated apoE^{-/-} mice no significant effects were observed after multiple injections with α -GalCer (Fig. 5B). In addition to the in

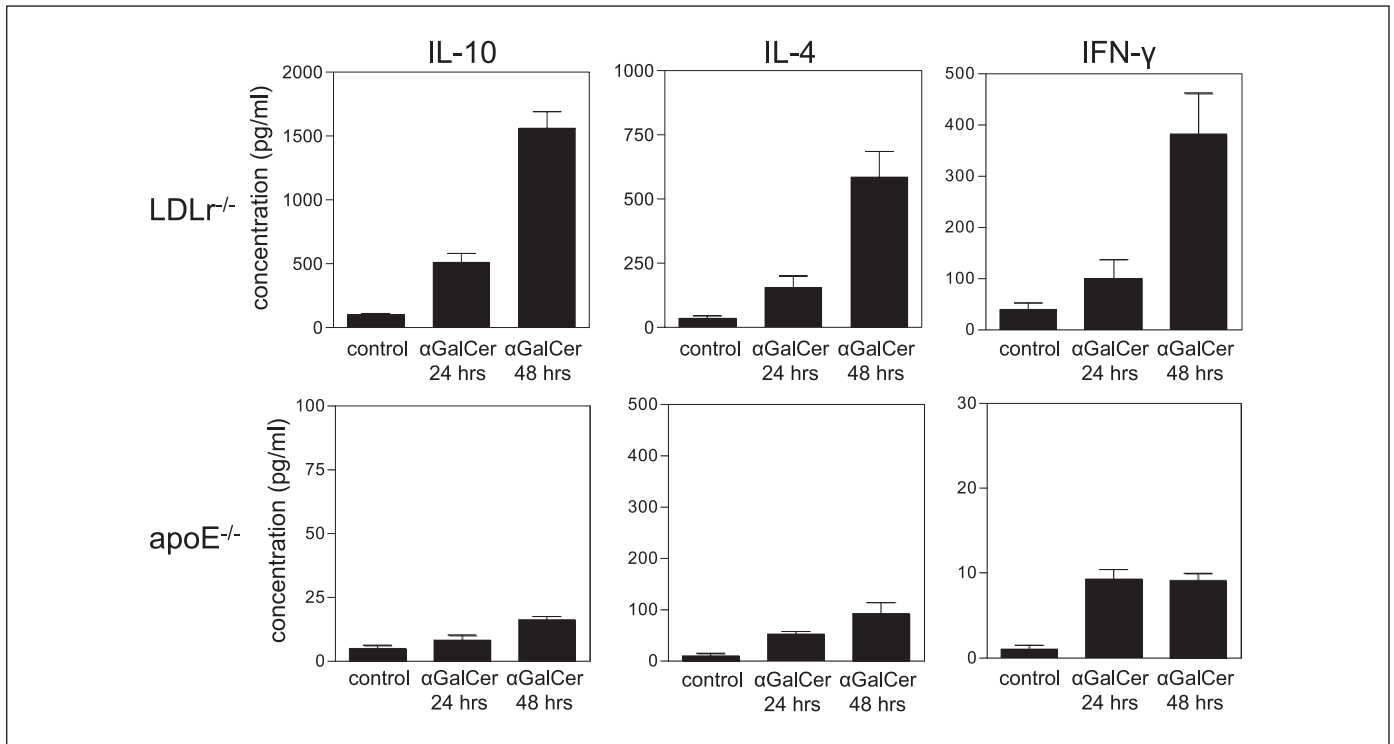


Figure 4: Cytokine production of splenocytes of $LDLr^{-/-}$ and $apoE^{-/-}$ mice after stimulation with α -GalCer *in vitro*. Splenocytes were isolated from $LDLr^{-/-}$ mice and $apoE^{-/-}$ mice and incubated with 100 ng/ml of α -GalCer or without α -GalCer (control). After 24 and 48 h of incubation the production of IL-10, IL-4 and IFN- γ was monitored by an ELISA on the supernatant of the splenocytes. Values are mean cytokine concentration \pm SEM.

crease in IL-10 producing $CD3^{+}T$ cells, we also determined the IL-10 levels in serum. We observed that multiple injections of α -GalCer in $LDLr^{-/-}$ resulted in a significant 2.4-fold increase in serum IL-10 levels of α -GalCer-treated mice versus control-treated mice (114.3 ± 22.1 pg/ml vs. 47.1 ± 4.2 pg/ml, $p < 0.02$), while no increase in IL-10 was determined in the serum of $apoE^{-/-}$ mice.

Effect of Western-type diet feeding on NKT cell numbers (Fig. 6)

Since NKT cells respond to lipids and mice were hyperlipidemic during the experiments, we sacrificed $LDLr^{-/-}$ and $apoE^{-/-}$ mice that were fed a Western-type diet at different time points. Diet feeding led to an increase in the number of NKT cells in both the liver and the spleen of $LDLr^{-/-}$ mice. After 1.5 weeks of diet feeding, the percentage of $CD3^{+}NK1.1^{+}$ cells increased significantly from $3.24 \pm 0.14\%$ to $6.95 \pm 0.42\%$ ($p < 0.01$) in the spleen and from $19.05 \pm 1.21\%$ to 42.37 ± 2.21 ($p < 0.05$) in the liver of $LDLr^{-/-}$ mice, when compared with $LDLr^{-/-}$ mice sacrificed before feeding a Western-type diet. In $apoE^{-/-}$ mice, no increase in NKT cell numbers was observed in spleen and liver. After 4.5 weeks of diet, the percentage of NKT cells increased even more in the spleen of $LDLr^{-/-}$ mice ($7.50 \pm 0.84\%$; $p < 0.05$), while the percentage in the liver returned to control levels. In $apoE^{-/-}$ mice still no effect of Western-type diet on NKT cell numbers was observed in the spleen, whereas in the liver a 2.4-fold increase in NKT cells was observed (11.10 ± 1.42 vs. 27.18 ± 1.50 ; $p < 0.05$). All data are represented as percentages of the lymphocyte popu-

lation in the specific organs and the change in percentages reflect the change in absolute numbers of NKT cells.

Discussion

In this study we demonstrate for the first time that activation of NKT cells via α -GalCer may, depending on the route of administration and the combination of feeding the mice a Western-type diet, induce an atheroprotective effect. Activation of NKT cells in a combination of i.p. and i.v. injections of α -GalCer resulted in a reduction of lesion formation in $LDLr^{-/-}$ mice. No significant effect of α -GalCer treatment was observed on lesion formation in the carotid arteries of $apoE^{-/-}$ mice that were also fed a Western-type diet. In both experiments, the β -variant of α -GalCer (β -GalCer) was used as a control ligand. This glycolipid is known to bind to CD1d but is not able to induce any proliferative response and does not affect cytokine production by NKT cells (28, 29). Several other studies showed that administration of α -GalCer to atherosclerosis-prone mice accelerates lesion formation. Nakai et al. showed that repeated i.p. administration of α -GalCer to $apoE^{-/-}$ mice increased atherosclerotic lesions (15). Major et al. (16) and Tupin et al. (17) found a similar increased plaque size when $apoE^{-/-}$ mice, fed a normal chow diet, were treated with α -GalCer twice a week for 10 weeks. It was hypothesised that the effect on atherosclerosis resulted from an increase in IL-4 and IFN- γ production. In a number of other Th1-mediated diseases, however, multiple injections of α -GalCer were shown to be protective because of the induction

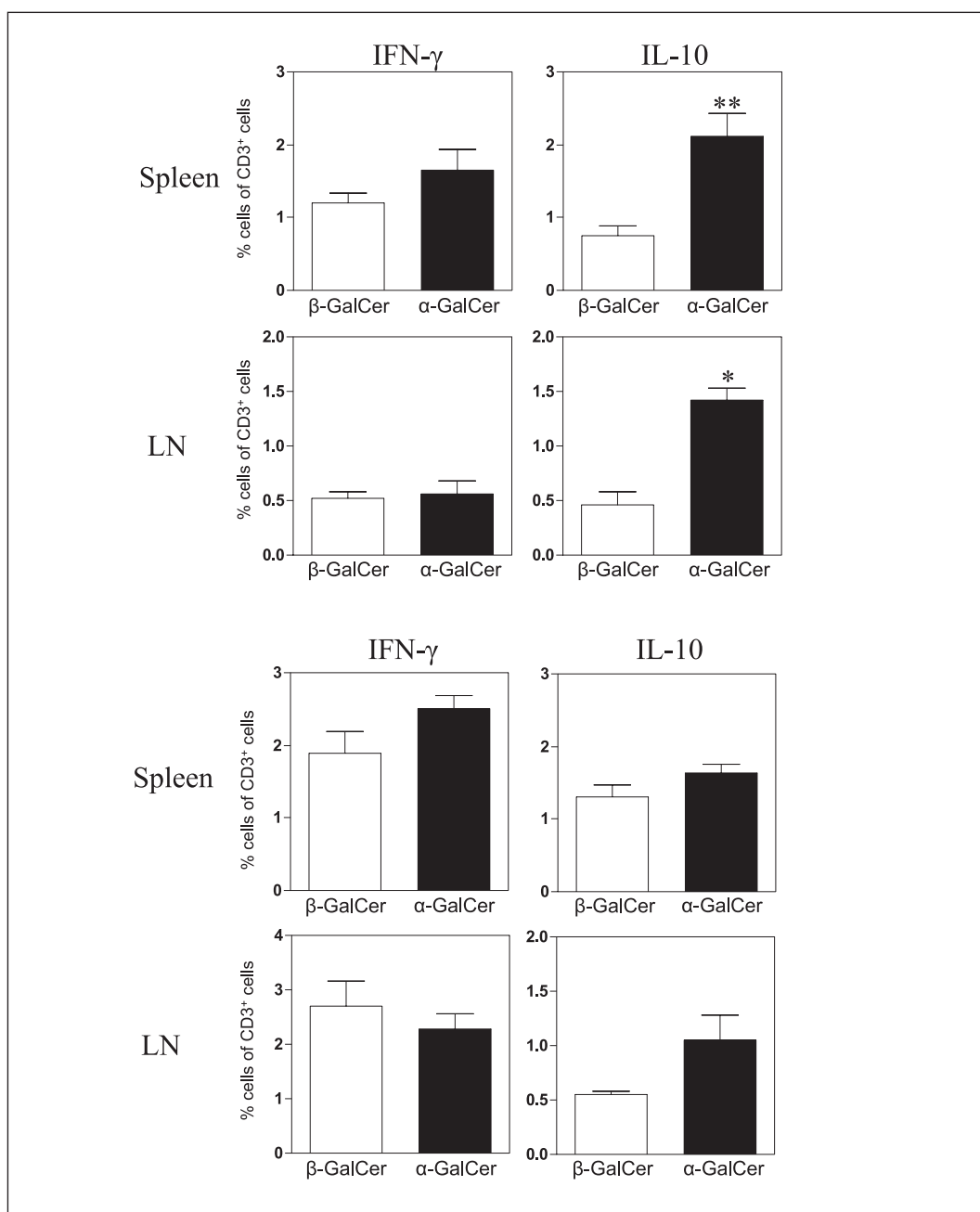


Figure 5: Cytokine production after in-vivo treatment with α -GalCer.

LDLr^{-/-} and apoE^{-/-} mice were fed a high-fat diet and were treated by multiple injections of α -GalCer. After the last injection, the mice were sacrificed and the spleen and mediastinal lymph nodes were isolated. Using the FACS the percentage CD3⁺ T cells producing IFN- γ and IL-10 within the lymphocyte population was determined. A) LDLr^{-/-} mice with the graphs for the spleen on the upper side and for the lymph nodes on the lower side. B) Data from the apoE^{-/-} mice. *p<0.05

of Th2 cytokines IL-4, IL-5, IL-10 and IL-13 (21–23). Therefore it was somewhat surprising that in atherosclerosis studies, repeated injections with α -GalCer led to an increase in plaque development in atherosclerosis in apoE^{-/-} mice. We, however, observed a protective effect of α -GalCer in LDLr^{-/-} mice and no significant effect in apoE^{-/-} mice, both fed a Western-type diet, whereas previous studies showed an aggravation of atherosclerosis in apoE^{-/-} mice fed a normal chow diet. A major difference between the two mouse species is their response to Western-type diet and to α -GalCer: splenocytes from apoE^{-/-} mice showed less proliferation in response to α -GalCer and than those from LDLr^{-/-} mice. This is in line with the study of van den Elzen et al. that shows that apoE is an important mediator in presentation of

lipid antigens via CD1 molecules (26). They proposed that apoE binds exogenous lipid antigens and efficiently targets them for receptor mediated uptake by DCs. It may be added that apoE also binds to the cellular membrane via heparan sulfate proteoglycans, which may enhance the delivery of α -GalCer on CD1d. In line with the defect in lipid presentation, apoE^{-/-} mice show exacerbated experimental allergic encephalomyelitis (EAE) (30), and it may be speculated that this results from a reduced activation of sulfatide-specific CD1d-restricted NKT cells in apoE^{-/-} mice, which normally inhibit EAE (31). The lack of apoE, especially membrane-bound apoE, thus explains the lower response to α -GalCer in apoE^{-/-} mice which is in line with Major et al. who showed a reduced NKT cell proliferation upon α -GalCer

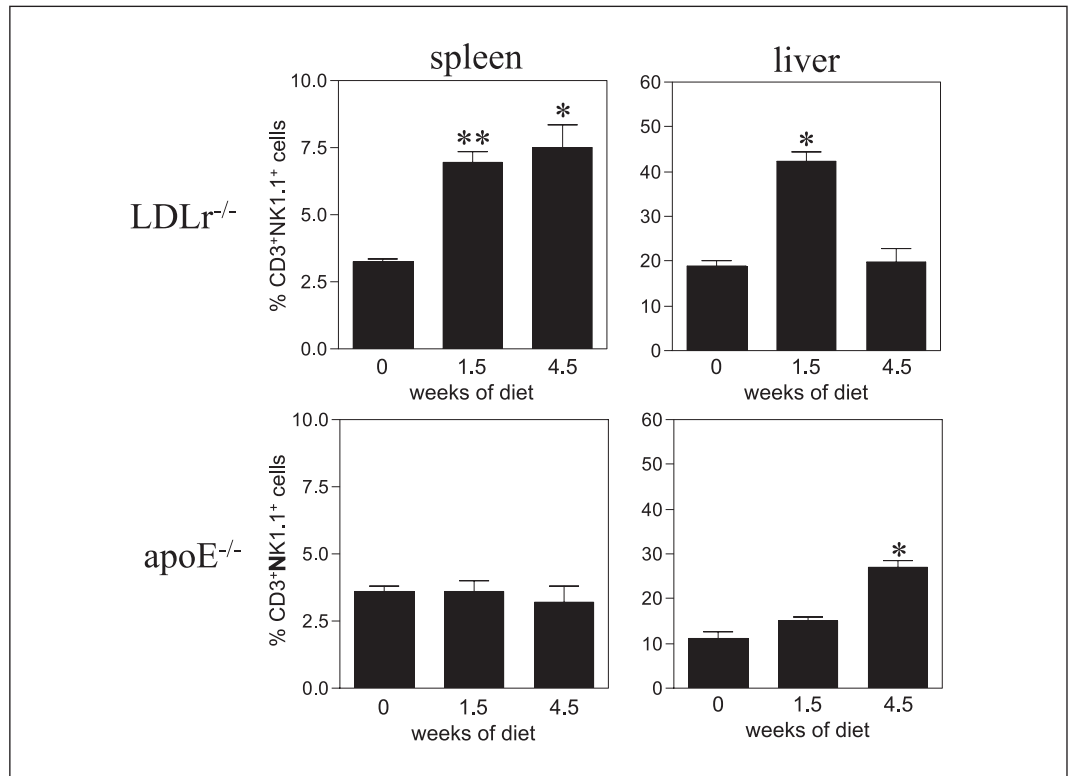


Figure 6: Effect of a Western-type diet on the number of NKT cells. LDLr^{-/-} and apoE^{-/-} mice were fed a Western-type diet. After 0, 1.5 and 4.5 weeks mice were sacrificed and the number of CD3⁺NK1.1⁺ cells in the spleen and liver was measured using FACS analysis. Values are mean \pm SEM. * $p < 0.05$, ** $p < 0.01$.

stimulation in apoE^{-/-} mice, compared with C57Bl/6 mice (17). However, since CD1d-restricted NKT cells are still present in apoE^{-/-} mice it can be concluded that apoE is only enhancing NKT cell activation, and other pathways can still result in CD1d antigen presentation. Lipid transporters, apolipoproteins and lipoprotein receptors are also likely to participate in lipid antigen uptake and subsequent presentation via CD1 molecules (32). This is confirmed by the low, but significant response of apoE^{-/-} splenocytes to α -GalCer in our experiment and that of Major et al. (17). The lower degree of activation of NKT cells in apoE^{-/-} mice is confirmed by the fact that in our study, apoE^{-/-} mice had 50% less NKT cells in the liver when compared with age-matched LDLr^{-/-} mice. In addition, we observed in our current study that splenocytes from apoE^{-/-} mice produce lower amounts of cytokines in response to α -GalCer when compared with LDLr^{-/-} mice. These data correlate with the lower splenocyte proliferation observed in apoE^{-/-} mice and may also explain the effect of α -GalCer on atherosclerosis. Especially the relative increase in production of IL-10 after α -GalCer stimulation was much higher in splenocytes from LDLr^{-/-} mice while the relative increase in production of IL-4 and IFN- γ was more or less the same. In addition we show that this is accompanied by an increase in serum IL-10 levels. Since it is known that an increase in circulating IL-10 levels results in a reduced development of atherosclerotic lesions (33, 34), we suggest that the increase in IL-10 producing CD3⁺ cells in the spleen and the increase in serum IL-1 may have a large impact on atherosclerosis and may be responsible for the reduction in lesion size observed in the LDLr^{-/-} mice after α -GalCer treatment.

It is, however, also surprising that we did not observe an ag-

gravating effect of α -GalCer in apoE^{-/-} mice which is in contrast to previous publications (15–17). This result may be affected by the fact that our mice were fed a high-fat diet. As we now show, Western-type diet feeding of LDLr^{-/-} mice caused a rapid 2.2-fold increase in NKT cells in the liver, which returned to control levels after 4.5 weeks of diet. In spleen a two-fold increase was observed for up to 4.5 weeks of diet, which may be explained by migration of NKT cells from the liver to the spleen. After nine weeks of diet feeding the number of NKT cells were back at basal levels (data not shown). This may be in line with the study of Aslanian et al. (19) who showed an effect of CD1d deficiency on initial stages of atherosclerosis in LDLr^{-/-} mice. After four weeks of diet feeding, lesions were smaller in the CD1d^{-/-}LDLr^{-/-} mice (19). This is the time point at which we observed the largest increase in NKT cells. In apoE^{-/-} mice the increase in NKT cells in the liver was delayed, and in the spleen no increase was observed. This again may result from the reduced lipid antigen presentation in apoE^{-/-} mice (32, 35).

Although the natural ligand for NKT cells is still not known, our data strongly suggest that a high-fat diet induces a proliferation and probably an activation of NKT cells in liver and spleen. Whether this endogenous activation of NKT cells is harmful in atherosclerosis needs further investigation, but from data on CD1d^{-/-}LDLr^{-/-} mice we conclude that endogenous activation may accelerate atherosclerosis. In our current study the LDLr^{-/-} and apoE^{-/-} mice were treated with a combination of endogenous activation (diet feeding) and a synthetic ligand (α -GalCer). When α -GalCer was injected for the first time, the mice were already fed the high-fat diet for two weeks. At that time point the NKT cells are triggered endogenously and present in increased

levels in both liver and spleen of the LDLr^{-/-} mice. We hypothesize that α -GalCer turns these “triggered” NKT cells into Th2-cytokine producing cells that ameliorate atherosclerosis. We observed a significant increase in IL-10 producing T cells in the spleen and mediastinal lymph nodes of the LDLr^{-/-} mice while no effect was seen in apoE^{-/-} mice. After two weeks of diet feeding and at the beginning of α -GalCer administration to apoE^{-/-} mice, the hepatic and splenic NKT cells are not yet endogenously triggered by the diet. Administration of α -GalCer at that time point may have activated the NKT cells but was not protective. It is therefore concluded that the endogenous activation of NKT cells which is delayed in apoE^{-/-} mice still affects the previously de-

scribed negative effect of α -GalCer activation of NKT cells in a beneficial way, but not sufficiently enough to significantly reduce atherosclerosis.

In conclusion we describe in this study that in hyperlipidemic conditions, NKT cell activation by α -GalCer may have a protective role in atherosclerosis. However, this protective effect is only found in LDLr^{-/-} mice, and not in apoE^{-/-} mice since these mice have a retarded lipid antigen presentation. The proposed negative role of NKT cells in atherosclerosis may need reconsideration and further investigation into the endogenous ligands will be necessary.

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