Regulation of T cell responses in atherosclerosis

Puijvelde, G.H.M. van

Chapter 5

The effect of NKT cell activation on atherosclerosis depends on apoE and lipid loading

G.H.M. van Puijvelde*, A.D. Hauer*, P. de Vos*, R. van den Heuvel*, M.J.C. van Herwijnen*, R. van der Zee#, W. van Eden#, T.J.C van Berkel* and J. Kuiper*

* Division of Biopharmaceutics, LACDR, Leiden University, Leiden, The Netherlands

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Abstract

It has been shown that NKT cell activation via the administration of α-GalCer accelerates atherosclerosis in apoE$^{-/-}$ mice. ApoE is however an important mediator in the presentation of exogenous lipids via CD1 molecules to NKT cells which may complicate conclusions on the role of NKT cell activation in atherosclerosis. Treatment of LDLr$^{-/-}$ mice with α-GalCer during Western-type diet feeding is therefore of interest to investigate the role of NKT cells in atherosclerosis. Atherosclerosis was induced by the combination of 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 for 7 weeks with α-GalCer. This resulted in a 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 strongly, while apoE$^{-/-}$ splenocytes show only a minor proliferative response. This is reflected in a larger increase in production of cytokines and especially IL-10 after *in vitro* stimulation of LDLr$^{-/-}$ splenocytes with α-GalCer compared with apoE$^{-/-}$ splenocytes. Additionally, feeding a Western-type diet for 1.5 weeks induced a strong increase in the number of NKT cells within the liver and spleen of LDLr$^{-/-}$ mice. This increase was slower and less prominent in apoE$^{-/-}$ mice which only showed an increased level of NKT cells in the liver after 4.5 weeks of diet. To conclude, 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 CD1 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.

Introduction

Atherosclerosis is a chronic inflammatory disease of the vasculature in which both the innate and the adaptive immune system play an important role. T cells, B cells, monocytes and dendritic cells (DCs) are detected in atherosclerotic plaques of mice and humans.[1,2] The inflammatory response in atherosclerosis is mainly driven by Th1 cells, producing pro-atherogenic cytokines such as IL-12 and IFN-γ.[3–5] The counteracting Th2 cells produce anti-atherogenic cytokines such as L-5, IL-10 and IL-13. IL-4, which is a Th2 cytokine, has however pro-atherogenic properties in initial stages of atherosclerosis.[6–8] 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. This NK1.1 is only detected on NKT cells in C57Bl/6 mice, and not in Balb/c mice. The majority of NKT cells express a semi-invariant T cell receptor (TCR) composed of a Vα14-Jα18 α-chain paired with a Vβ8 or Vβ2 β-chain. This TCR is unique because of it's 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, IL-12 and TNF-α) and Th2 cytokines (IL-4, IL-5, IL-10 and IL-13). This make NKT cells a unique T cell population with potentially both pro- and anti-inflammatory properties.[9]
NKT cells are found in atherosclerotic plaques of both humans[10,11] and atherosclerosis-prone (LDLr$^{-/-}$ and apoE$^{-/-}$) mice.[12–15] In human lesions, NKT cells colocalize with CD1d-expressing dendritic cells (DCs) in the shoulder regions and this contributes to plaque destabilization. The NKT cells represent 2% of the total lymphocyte population within the lesion.[10,11] Recent studies show that depletion of NKT cells by crossing apoE$^{-/-}$ and LDLr$^{-/-}$ mice with CD1d$^{-/-}$ mice results in an attenuation of atherosclerosis.[12–14,16] More recently an adoptive transfer of NKT cells into RAG1$^{-/-}$LDLr$^{-/-}$ mice accelerated atherosclerosis.[17] These studies indicate that a deficiency in CD1d-dependent NKT cells accelerates atherosclerosis, and this may suggest that endogenous activation of NKT cells is a pro-atherogenic process. However, many studies on NKT cells use the synthetic glycolipid α-galactosylceramide (α-GalCer) as a ligand. Studies on a number of Th1-mediated autoimmune diseases showed that activation of NKT cells via repeated injections with α-GalCer can polarize the adaptive immune response towards a Th2 like response[18,19] Treatment with α-GalCer resulted in protection from autoimmune diabetes,[20] experimental autoimmune encephalomyelitis[21] and colitis[22] in mice. Studies on Th1-mediated atherosclerosis showed however that α-GalCer treatment, significantly increases the disease in apoE$^{-/-}$ mice.[12–14] The administration of α-GalCer (single and multiple i.p. and i.v. injections) to apoE$^{-/-}$ mice caused an increase in pro-atherogenic (IFN-γ, IL-4) cytokines within the lesion.[13,14] However, in one study, in addition to the pro-atherogenic cytokines, an increase in anti-atherogenic IL-10 was observed within the lesion[14]. In serum of apoE$^{-/-}$ mice, injected once with α-GalCer, an increase in IFN-γ, TNFα, IL-2, IL-4 and IL-5 was observed, whereas no cytokines were detected after multiple injections.[13] A recent publication shows that apoE is an important

mediator of lipid antigen presentation on CD1 molecules, which may complicate conclusions on the effect of α-GalCer activation of NKT cells on atherosclerosis in apoE$^{-/-}$ mice[23]. Therefore it would be of major interest to determine what the effect of α-GalCer activation of NKT cells is in atherosclerosis induced in LDLr$^{-/-}$ mice in combination with endogenous activation.
In this study, we show that multiple injections with α-GalCer reduced atherosclerosis in LDLr$^{-/-}$ mice but was not protective in apoE$^{-/-}$ mice. ApoE$^{-/-}$ splenocytes showed a lower proliferative response towards α-GalCer and showed a dampened cytokine production, both *in vitro* and *in vivo*. Both effects may be caused by the lack of apoE. In addition, Western-type diet feeding of LDLr$^{-/-}$ mice increased NKT cell numbers in liver and spleen. Our findings suggest that in combination with endogenous activation of NKT cells, treatment with α-GalCer can be protective against atherosclerosis.

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 as mating pairs and bred at the Gorlaeus Laboratories, Leiden, The Netherlands. Jα281$^{-/-}$ mice on a C57BL/6 background were obtained from Dr M. Taniguchi. 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 at the start of the experiment. 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 2 weeks prior to surgery. After 2 weeks, atherosclerosis was induced by placement of perivascular collars prepared from elastic tubing (0.3 mm inside diameter; Dow Corning, Midland, Michigan), around both carotid arteries (method described by von der Thüsen et al.[24]). For this surgery the mice were anaesthetized 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, Germany). Immediately after collar placement, the mice were injected twice a week with 2 μg of β-GalCer or α-GalCer. All injections were performed half i.v./half i.p. and continued for 6 weeks. Subsequently, the mice were anaesthetized by a s.c. injection with ketamine-hypnorm and exsanguinated by femoral artery transsection. The mice were perfused and fixated through the left cardiac ventricle with PBS for 15 min. and subsequently with FormalFixx for about 30 min. Common carotid arteries and both carotid bifurcations were removed for analysis as described by von der Thüsen et al.[24] The arteries were embedded in OCT compound (TissueTek; Sakura Finetek, The Netherlands) and 5μm sections were made on a Leica CM 3050S Cryostat (Leica Instruments, UK) proximally of the place of collar occlusion. These cryosections were stained with hematoxylin (Sigma Diagnostics, MO) and eosin (Merck Diagnostica, Germany). Plaque areas were measured using a Leica DM-RE microscope and LeicaQwin software (Leica Imaging Systems, UK).

Spleen Cell Proliferation Assay

To test the responsiveness of splenocytes to α-GalCer, spleens from LDLr$^{-/-}$, apoE$^{-/-}$ and Jα281$^{-/-}$ mice were dissected and single cell suspensions were obtained by squeezing the spleen through a 70 μm cell strainer (Falcon, The Netherlands). The erythrocytes were eliminated by incubating the cells with erythrocyte lysis buffer (0.15 M NH_4Cl, 10 mM $NaHCO_3$, 0.1 mM EDTA, pH 7.3). Subsequently, the splenocytes were cultured in triplicate at $2{\cdot}10^5$ cells per well of a 96-wells 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, and 100 μg/ml streptomycin (all from BioWhittaker Europe) was used as culture medium. The splenocytes were incubated for 48 hours in a humidified atmosphere (37°C; 5% CO_2). Cultures were pulsed for the final 16 hours with [6-^{3}H]-thymidine (1 μCi/well, sp. act. 24 Ci/mmol; Amersham Biosciences, The Netherlands). The amount of [6-^{3}H]-thymidine incorporation was measured using a liquid scintillation analyzer (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 of triplicate cultures with α-GalCer to the mean counts per minute in culture medium without α-GalCer.

Cytokine assays

To determine the cytokine production by splenocytes upon stimulation with α-GalCer, splenocytes were isolated from LDLr$^{-/-}$ and apoE$^{-/-}$ mice and incubated with 100 ng/ml α-GalCer in RPMI-1640 medium (supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin and 50 μM 2-mercaptoethanol). After 0, 24 and 48 hours culture supernatants were collected for ELISA assays to measure the IFN-γ, IL-10 and IL-4 production conform the manufacturers protocols (eBioscience, 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. (50%/50%) twice a week during 7 weeks. 72 hours 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, Ontario, Canada) conform the manufacturers protocol. The cells were stimulated o/n with anti-CD3 and anti-CD28, coated onto a 96-wells plate. Cells were subsequently stained with APC-conjugated anti-CD4 or anti-CD3 (0.125 μg/sample) for 30 min and subsequently fixated and permeabilized 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, Belgium) for 30 min. Cells were washed two times and analyzed immediately by flow cytometry on a FACSCalibur. All data were analyzed with CELLQuest software (BD Biosciences, The Netherlands).

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, Ontario, Canada) conform the manufacturers protocol. Subsequently, the cells were stained with PerCP-conjugated CD3 and FITC-conjugated NK1.1 antibodies (eBioscience, Belgium) for 30 min. Cells were washed two times and analyzed immediately by flow cytometry on a FACSCalibur. All data were analyzed with CELLQuest software (BD Biosciences, The Netherlands).

Statistical analysis

All data are expressed as mean$\pm$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 injection of α-GalCer to activate NKT cells 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 the mice were treated by combined i.p. and i.v. injections (50%/50%) of 2 μg α-GalCer or β-GalCer per treatment. Both ligands were administered twice a week for 7 weeks. Representative examples of plaques of β-GalCer and α-GalCer treated LDLr$^{-/-}$ mice, stained with hematoxylin and eosin, are shown in figure 5.1A and 5.1B, respectively. Treatment with α-GalCer led to an 84.1% reduction in plaque size in LDLr$^{-/-}$ mice, when compared with β-GalCer treated LDLr$^{-/-}$ mice (Figure 5.1C; 16488$\pm$6286

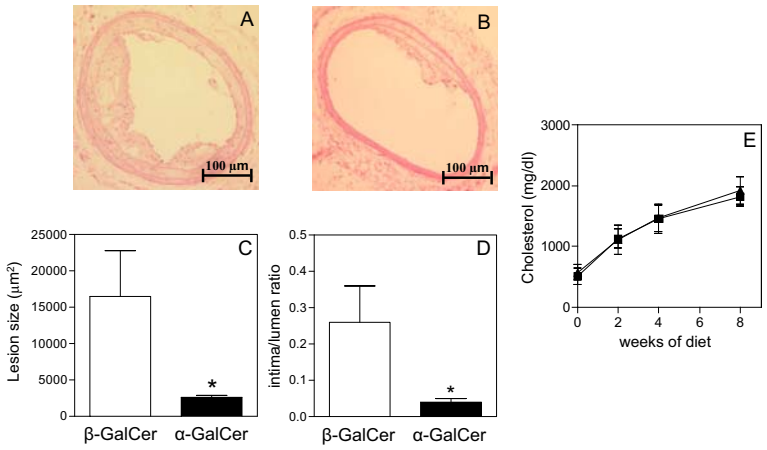

Figure 5.1: Multiple injections with α-GalCer reduces plaque formation in LDLr$^{-/-}$ mice. LDLr$^{-/-}$ mice were treated twice a week with 2 μg of α-GalCer or β-GalCer after collars were placed around the carotid arteries. Representative cryosections of plaques from β-GalCer (A) and α-GalCer (B) treated 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). $^{*}P<0.05$.

μm^2 versus 2621$\pm$263 μm^2, respectively; $P<0.05$). α-GalCer treatment also resulted in an 85.7% reduction in intima/lumen ratio (Figure 5.1D; 0.26$\pm$0.10 versus 0.04$\pm$0.01 ; $P<0.05$) and a 77.3% reduction in intima/media ratio (data not shown; 0.63$\pm$0.23 versus 0.14$\pm$0.02; $P<0.05$). Figure 5.2A and 5.2B represent examples of plaques of β-GalCer and α-GalCer treated apoE$^{-/-}$ mice, respectively. In apoE$^{-/-}$ mice a non-significant reduction on plaque size was observed (Figure 5.2C; 21700$\pm$2644 μm^2 versus 14922$\pm$3060 μm^2; P=0.30). In addition, the intima/lumen ratio (Figure 5.2D; 0.37$\pm$0.08 versus 0.25$\pm$0.04; P=0.18), and intima/media ratio (data not shown; 0.83$\pm$0.14 versus 0.64$\pm$0.07; P=0.23) were not significantly affected in apoE$^{-/-}$ mice. In both studies no effect of α-GalCer on total plasma cholesterol levels (Figure 5.1E and 5.2E) and body weight (data not shown) was observed.

In vitro effects of α-GalCer

To investigate the effect of α-GalCer on proliferation of spleen cells, splenocytes were isolated from LDLr$^{-/-}$, apoE$^{-/-}$ and Jα281$^{-/-}$ 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 (Figure 5.3, left graph, $P<0.01$). On the other hand, the response of splenocytes of apoE$^{-/-}$ mice 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 (Figure 5.3, middle graph, $P<0.05$). As a control, splenocytes were isolated from Jα281$^{-/-}$ mice, lacking CD1d-restricted NKT cells, and incubation of these cells with α-GalCer did not induce any proliferation of splenocytes using both concentrations of α-GalCer (Figure 5.3, right graph). Additionally, the cytokine production of splenocytes from LDLr$^{-/-}$ and apoE$^{-/-}$

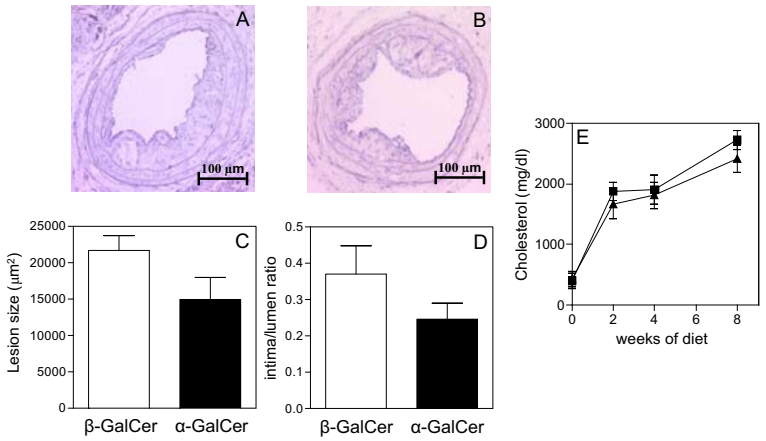

Figure 5.2: Multiple injections with α-GalCer have no significant effect on atherosclerosis in apoE$^{-/-}$ mice. ApoE$^{-/-}$ mice were treated with 2 μg of α-GalCer or β-GalCer just like the LDLr$^{-/-}$ mice in Figure 5.1. Representative cryosections of plaques from β-GalCer (A) and α-GalCer (B) treated 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).

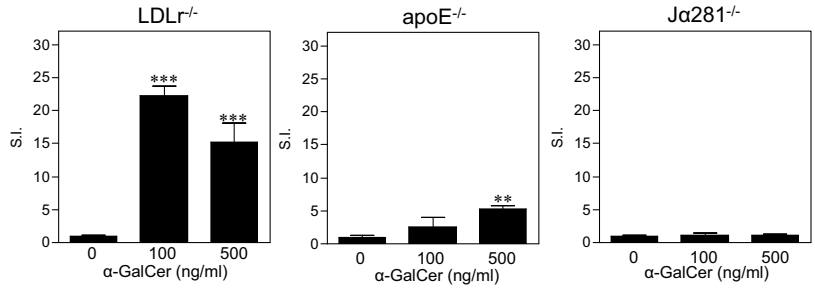

Figure 5.3: Proliferative response of splenocytes from different mouse strains on α-GalCer. Splenocytes were isolated from LDLr$^{-/-}$, apoE$^{-/-}$ and Jα281$^{-/-}$ mice and were incubated for 48 hrs with 100 or 500 ng/ml of α-GalCer. As a control, non-stimulated cells were used. Cells were pulsed with [6-^{3}H]-thymidine for the final 16 hrs 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. $^{**}P<0.01$, $^{***}P<0.001$

mice in response to α-GalCer was determined. Stimulation of LDLr$^{-/-}$ splenocytes with 100 ng/ml of α-GalCer for 48 hours resulted in an increase in the production of IL-10 (101$\pm$13 pg/ml vs. 1559$\pm$133 pg/ml), IL-4 (36$\pm$9 pg/ml vs. 585$\pm$100 pg/ml) and IFN-γ (39$\pm$13 pg/ml vs. 381$\pm$80 pg/ml) when compared with cells cultured without α-GalCer (Figure 5.4, upper panel). Splenocytes of apoE$^{-/-}$ mice cultured with 100 ng/ml of α-GalCer for 48 hrs produced much lower amounts of IL-10 (16$\pm$1 pg/ml), IL-4 (92$\pm$22 pg/ml) and IFN-γ (9$\pm$1 pg/ml) (Figure 5.4, lower panel). When compared with splenocytes of apoE$^{-/-}$ mice cultured without α-GalCer stimulation, the relative increase in IL-4 and IFN-γ is rather high but the relative production of IL-10 is much lower when compared with the cytokine production by LDLr$^{-/-}$ splenocytes.

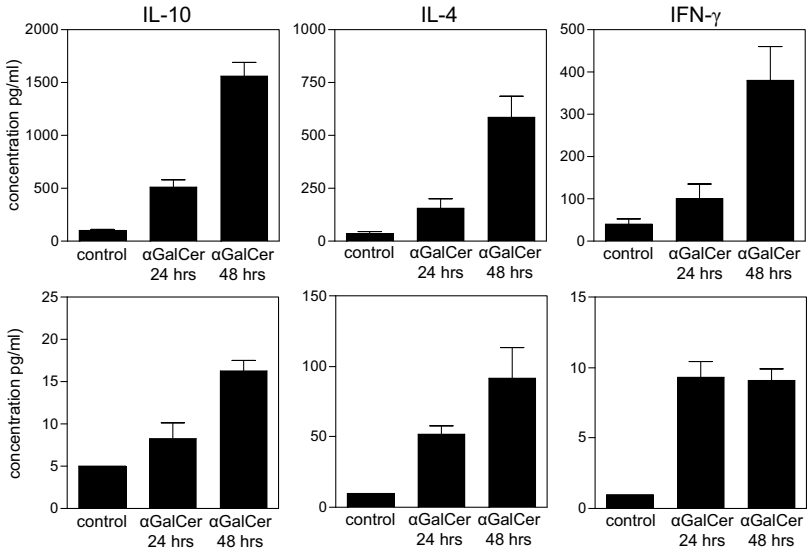

Figure 5.4: Cytokine production by splenocytes of LDLr$^{-/-}$ and apoE$^{-/-}$ mice after *in vitro* stimulation with α-GalCer. Splenocytes were isolated from LDLr$^{-/-}$ (upper panel) and apoE$^{-/-}$ (lower panel) mice and incubated with 100 ng/ml of α-GalCer or without α-GalCer (control). After 24 and 48 hrs of incubation the production of IL-10, IL-4 and IFN-γ was monitored by an ELISA on the supernatant of the cells. Values are mean cytokine concentration $\pm$ SEM.

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 of CD3$^+$ and CD4$^+$ cells in spleen and mediastinal lymph nodes was determined after multiple injections with α-GalCer. FACS analysis showed that only a small percentage of the CD4$^+$ cells in both the spleen and the lymph nodes produce Th1 and Th2 cytokines. In β-GalCer-treated LDLr$^{-/-}$ mice, 1.22$\pm$0.22%, 0,76$\pm$0.13% and 1.20$\pm$0.13%, of the CD4$^+$ cells in the spleen produce IL-4, IL-10 and IFN-γ, respectively. There was no effect on CD4$^+$IL-4$^+$ splenocytes (1.81$\pm$0.50%; P=0.35) and CD4$^+$IFN-γ^+ splenocytes (1.66$\pm$0.28%; P=0.20) after multiple treatment of LDLr$^{-/-}$ mice with α-GalCer but a significant increase in CD4$^+$IL-10$^+$ splenocytes (2.12$\pm$0.32%; $P<$0.01) was observed (Figure 5.5A, upper panel). In the mediastinal lymph nodes, the CD4$^+$IL-4$^+$ and CD4$^+$IL-10$^+$ lymphocytes increased after multiple α-GalCer injections (0.38$\pm$0.09% vs. 1.33$\pm$0.12% and 0.46$\pm$0.12% vs. 1.42$\pm$0.11%, respectively; $P<$0.05). No effect on CD4$^+$IFN-γ^+ lymphocytes (0.52$\pm$0.06% vs. 0.56$\pm$0.12%; P=0.79) was observed (Figure 5.5A, lower panel). In the spleen of β-GalCer-treated apoE$^{-/-}$ mice, 0.84$\pm$0.21%, 1.90$\pm$0.29% and 1.31$\pm$0.16% of the lymphocytes are CD3$^+$IL-4$^+$, CD3$^+$IFN-γ^+ and CD3$^+$IL-10$^+$, respectively. There was no effect on these percentages after multiple injections with α-GalCer (1.18$\pm$0.16%, 2.50$\pm$0.18% and 1.64$\pm$0.12%, respectively) (Figure 5.5B, upper panel). In the mediastinal lymph nodes of these mice, no effect on CD3$^+$IL-4$^+$ (0.41$\pm$0.09% vs. 1.09$\pm$0.12%), CD3$^+$IL-10$^+$ (0.55$\pm$0.03% vs. 1.05$\pm$0.23%) and CD3$^+$IFN-γ^+ (2.71$\pm$0.41% to 2.28$\pm$0.29%) lymphocytes was observed after

multiple injections with α-GalCer (Figure 5.5B, lower panel).

Effect of Western-type diet feeding on NKT cell numbers

LDLr$^{-/-}$ and apoE$^{-/-}$ mice were fed a Western-type diet and sacrificed at different time points. After diet feeding, the number of NKT cells in both the liver and the spleen was analyzed. 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$1.21% to 42.37$\pm$2.21% ($P<0.05$) in the liver of LDLr$^{-/-}$ mice, when compared with LDLr$^{-/-}$ mice sacrificed before feeding a Western-type diet. This effect was at this time point absent in the apoE$^{-/-}$ mice. In these mice there was no increase of NKT cell numbers in the spleen and a minor increase in the 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%; $P<0.05$). In the liver of LDLr$^{-/-}$ mice, the percentage returned to control levels. In apoE$^{-/-}$ mice still no effect of Western-type diet on NKT cell numbers in the spleen was observed, whereas in the liver a 2.4-fold increase in NKT cells was observed (11.10$\pm$1.42% versus 27.18$\pm$1.50%; $P<0.05$)(Figure 5.6).

Discussion

In this study we demonstrate for the first time that α-GalCer activation of NKT cells may, depending on the experimental conditions, act in an atheroprotective manner. Activation of NKT cells via a combination of intraperitoneal and intravenous injections of α-GalCer, resulted in a 84% reduction of lesion formation in LDLr$^{-/-}$ mice in which shear-stress induced atherosclerosis was induced via collar placement around both carotid arteries and by feeding a Western-type diet. No significant effect of α-GalCer treatment was observed in the carotid arteries of apoE$^{-/-}$ mice that were also fed a Western-type diet (31% reduction, not significant). 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 NKT cells.[25] Several other studies showed that administration of α-GalCer to mice modulates the disease process in atherosclerosis and accelerates lesion formation. Nakai et al. showed that repeated i.p. administration of α-GalCer to apoE$^{-/-}$ mice increased atherosclerotic lesions with 67%.[12] Major et al. and Tupin et al. 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.[13,14] It was hypothesized that the effect on atherosclerosis resulted from an increase in IL-4 and IFN-γ production. In a number of other Th1-mediated diseases, multiple injections of α-GalCer were shown to be protective because of the induction of Th2 cytokines IL-4, IL-5, IL-10 and IL-13 and this was beneficial in mouse models for autoimmune diseases such as diabetes[20], colitis[22] and multiple sclerosis[21], for several infectious diseases such as malaria[26] and hepatitis B[27], and for tumor treatment. Therefore it was surprising that in atherosclerosis studies, repeated injections of α-GalCer led to an increase in plaque development in atherosclerosis in apoE$^{-/-}$ mice. We observed a protective effect of α-GalCer in LDLr$^{-/-}$ mice on a Western-type

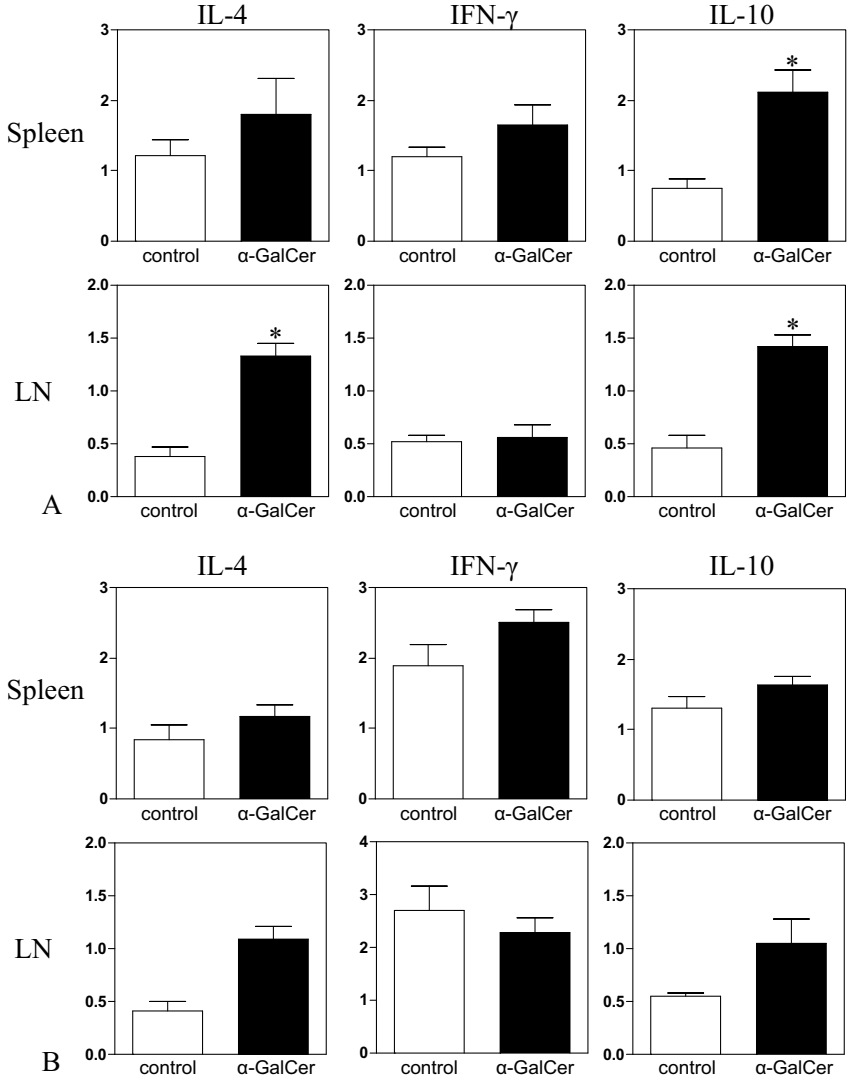

Figure 5.5: Cytokine production after *in vivo* treatment of LDLr$^{-/-}$ and apoE$^{-/-}$ mice with α-GalCer. LDLr$^{-/-}$ and apoE$^{-/-}$ mice were fed a high fat diet and were treated by multiple injections of α-GalCer. After the last injection, the mice were sacrificed and the spleen and mediastinal lymph nodes were isolated. Using the FACS the percentage of CD3^{+} and CD4^{+} cells producing IL-4, IL-10 and IFN-γ within the lymphocyte population was determined. Figure A represents the LDLr$^{-/-}$ mice with on the upper side the graphs for the spleen and on the lower side for the lymph nodes. Figure B represents the apoE$^{-/-}$ mice. $^{*}P<0.05$

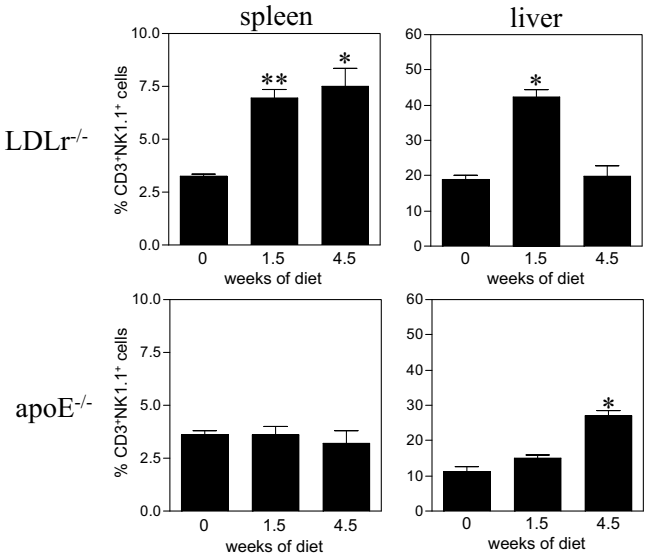

Figure 5.6: Effect of Western-type diet feeding on the number of NKT cells in LDLr$^{-/-}$ and apoE$^{-/-}$ mice. LDLr$^{-/-}$ and apoE$^{-/-}$ mice were fed a Western-type diet. After 0, 1.5 and 4.5 weeks mice were sacrificed and the number of CD3^{+}NK1.1^{+} cells in the spleen and liver was measured using FACS analysis. Values are mean $\pm$ SEM. $^{*}P<0.05$, $^{**}P<0.01$

diet and no effect in apoE$^{-/-}$ mice on 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 species is their response to α-GalCer: NKT cells from apoE$^{-/-}$ mice showed less proliferation in response to α-GalCer than those from LDLr$^{-/-}$ mice. This is explained by the study of van den Elzen et al. that shows that apoE is an important mediator in presentation of lipid antigens via CD1 molecules.[23] They proposed that apoE binds exogenous lipid antigens and efficiently targets them for receptor mediated uptake by DCs. Furthermore, intracellular apoE may be important in the delivery of the lipid to compartments containing CD1d, leading to presentation of α-GalCer on CD1d. In line with the defect in lipid presentation, apoE$^{-/-}$ mice show exacerbated experimental allergic encephalomyelitis (EAE),[28] 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.[29] The lack of apoE thus explains the lower response to α-GalCer in apoE$^{-/-}$ mice compared to splenocytes from LDLr$^{-/-}$ mice which is in line with Major et al. who showed a reduced NKT cell proliferation upon α-GalCer stimulation in apoE$^{-/-}$ mice, compared with C57Bl/6 mice.[14] However, since CD1d-restricted NKT cells are still present in apoE$^{-/-}$ mice it can be concluded that apoE is not a prerequisite for NKT cell activation and other pathways can 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.[30] This is confirmed by the low, but significant response of apoE$^{-/-}$ splenocytes to α-GalCer in our experiment and that of Major et al.[14] 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, one publication showed that aged apoE$^{-/-}$ mice have fewer CD1d-restricted NKT cells in the spleen than young apoE$^{-/-}$ mice.[14]

In our current study we observed 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 smaller in splenocytes from apoE$^{-/-}$ mice when compared with LDLr$^{-/-}$ mice, while the relative increase in production of IL-4 and IFN-γ was almost the same.

In addition, it is surprising that we did not observe an aggravating effect of α-GalCer in apoE$^{-/-}$ mice in contrast to previous publications.[12–14] This result may be caused by the fact that our mice were fed a high fat diet. As we now show in this study, 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 2-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 9 weeks of diet feeding all NKT cell levels were back at basal levels (data not shown). This may be in line with the study of Aslanian et al. who showed an effect of CD1d deficiency in LDLr$^{-/-}$ mice on initial stages of atherosclerosis only. After 4 weeks of diet feeding, lesions were smaller in the CD1d$^{-/-}$LDLr$^{-/-}$ mice[16]. This is the time point at which we observed the largest increase in NKT cells. After 8 and 12 weeks of diet no effect of CD1d deficiency on atherosclerosis was observed. In apoE$^{-/-}$ mice the increase in NKT cells in the liver was delayed and in the spleen no increase was observed. This may of course result from the reduced lipid antigen presentation in apoE$^{-/-}$ mice.[30,31]

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. This is confirmed by our data on the in vivo cytokine profile after high fat diet feeding and multiple injections of α-GalCer. We observed a significant increase in both IL-4 and IL-10 producing T cells in the spleen and mediastinal lymph nodes of the LDLr$^{-/-}$ mice. No effect on the production of IFN-γ was observed. In apoE$^{-/-}$ mice there was however no significant effect on IL-4, IL-10 and IFN-γ production by T cells after high diet feeding and multiple injections of α-GalCer. After two weeks

of diet feeding and at the beginning of α-GalCer administration, the hepatic and splenic NKT cells of apoE$^{-/-}$ mice are not endogenously triggered by the diet yet. Administration of α-GalCer at that time point may have activated the NKT cells but was not protective. We however did not see an increased lesion size and increased levels of IL-4 and IFN-γ as was observed in former studies.[12–14] It is therefore concluded that the endogenous activation of NKT cells which is delayed in apoE$^{-/-}$ mice still affects the previously described 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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