Regulation of T cell responses in atherosclerosis

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Chapter 7

Effect of deficiency in the natural killer T cell specific Vα14-Jα281 receptor on atherosclerosis in LDL receptor deficient mice

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Abstract

Natural killer T (NKT) cells, unique T cells with characteristics of NK cells, are activated by lipid antigens presented by CD1d. Thus far a few CD1d-binding plant- and bacteria-derived glycolipids are found to be natural ligands for NKT cells but their exact role is unclear. Since atherosclerosis is a disease in which lipids play a predominant role and since CD1d deficiency reduces atherosclerosis, one could imagine that one of those lipids might be an endogenous ligand for NKT cells. LDLr$^{-/-}$ mice were fed a Western-type diet for 0-24 weeks and during this period the frequency of NKT cells firstly increased in the liver and subsequently in spleen and lymph nodes. LDLr$^{-/-}$ mice were crossbred with Jα281$^{-/-}$ mice, deficient in NKT cells, and fed a Western-type diet for 12 weeks. Lesion size at the aortic root was unaffected in Jα281$^{-/-}$LDLr$^{-/-}$ mice when compared to LDLr$^{-/-}$ mice. Interestingly, splenocytes from Jα281$^{-/-}$ mice, which do not respond to α-GalCer stimulation, showed an impaired proliferation upon stimulation with oxLDL as compared to LDLr$^{-/-}$ mice. To conclude, NKT cells do not play a role in later stages of atherosclerosis. OxLDL, which is one of the most important (modified) lipids in atherosclerosis, or one of its components, may be an endogenous ligand for NKT cells but does not utilize the activation of NKT cells to affect advanced lesions in atherosclerosis.

Introduction

Natural killer T (NKT) cells represent a small population of T cells, which share characteristics with NK cells. A majority of the NKT cells express NK1.1 and a TCR composed of the Vα14-Jα18 α-chain paired with a Vβ8 or Vβ2 β-chain.[1,2] This TCR is different from the normal TCRs because it recognizes glycolipid antigens, which are presented to NKT cells by CD1d, an MHC class I related molecule present on most antigen presenting cells (APCs). α-Galactosylceramide (α-GalCer) was the first described glycolipid presented by CD1d to NKT cells and able to induce their proliferation.[3–8] Most studies on NKT cells use this ligand or one of its analogues to activate NKT cells. α-GalCer stimulation of NKT cells results in an enormous secretion of both Th1 and Th2 cytokines.[5–9] Repetitive administration of α-GalCer is shown to protect against several autoimmune diseases,[10–12] however several studies in apoE$^{-/-}$ mice showed that α-GalCer activated NKT cells accelerate atherosclerotic plaque formation.[13–15] Deficiency in CD1d, which leads to a lack of CD1d dependent NKT cells, also resulted in an amelioration of atherosclerosis in both apoE$^{-/-}$ and LDLr$^{-/-}$ mice.[13–16] These data suggested that NKT cells aggravate atherosclerosis and it also indicates that NKT cells are activated by endogenous ligands during the onset of atherosclerosis. In these studies, experiments were performed with CD1d$^{-/-}$ mice. These mice lack CD1d-restriced NKT cells but are also deficient in other T cells that are activated via the presentation of glycolipids or hydrophobic peptides presented by CD1d.[17–19] Another problem is that CD1d-independent NKT cells are still present in these mice. To avoid this problem, Jα281$^{-/-}$ mice are used in this study. These mice are deficient in both CD1d-restricted and CD1d-non-restricted NKT cells, while other CD1d-dependent T cells are unaffected.

X-ray data revealed that the lipid portion of ligands for NKT cells fits in a CD1d-binding groove whereas the carbohydrate part is exposed for recognition by the TCR of NKT cells.[20,21] Synthetic variants of α-GalCer showed that also sulfatides bind to CD1d and effectively stimulate NKT cells.[22] A few natural ligands for NKT cells are known nowadays. Recently some microbial ligands were found. Glycosphingolipids present in the outer membrane of *Sphingomonas* strongly activate NKT cells and NKT cells are important for the clearance of this infection.[23–25] In addition, more recently a self-ligand for NKT cells was found. The endogenous lysosomal glycosphingolipid isoglobotrihexosylceramide (iGb3) may activate mouse Vα14 and human Vα24 NKT cells and seems to be important for the intra-thymic development of NKT cells,[26,27] while a role for iGb3 in activation of peripheral NKT cells has also been described.[23] Others showed that plant-derived glycolipids may be natural CD1d-binding ligands for NKT cells.[28] The active plant- and bacteria-derived glycolipids are especially phosphatidyl choline (PC) and phosphatidyl ethanloamin (PE) or glycolipids that have lipid moieties which are almost identical to PC and PE.[28,29] Whether these ligands affect the Th1/Th2 cytokine secretion of NKT cells and whether these ligands can be used as treatment for diseases needs further investigation.[30]

Atherosclerosis is a disorder in which enhanced plasma lipid levels play a central role. Because the TCR of NKT cells recognizes lipid antigens presented on CD1d it is plausible that one of the lipids in atherosclerosis may be an endogenous

ligand for NKT cells. Among these lipids, phospholipids and triglycerides are mainly bound to proteins for transport: the lipoproteins.
In this study we observed an increased frequency of NKT cells in several organs after Western-type diet feeding of LDLr$^{-/-}$ mice. In addition we showed that oxLDL may be an endogenous ligand for NKT cells since NKT cell deficient splenocytes did not proliferate in response to oxLDL when compared to LDLr$^{-/-}$ splenocytes. Deficiency in NKT cells at advanced stages of atherosclerosis did not affect lesion size and therefore we conclude that NKT cells play a pro-atherogenic role only in initial stages of 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$^{-/-}$ mice on a total C57BL/6 background were obtained from Jackson Laboratory, Bar Harbor, Me. Jα281$^{-/-}$ mice on a C57BL/6 background were obtained from Dr M. Taniguchi. Double deficient LDLr$^{-/-}$Jα281$^{-/-}$ mice were generated by crossing Jα281$^{-/-}$ mice with the LDLr$^{-/-}$ mice. The offspring was intercrossed to produce mice with homozygous deletion in both LDLr and Jα281 genes (LDLr$^{-/-}$Jα281$^{-/-}$). For experiments, 10-12 week old male LDLr$^{-/-}$, Jα281$^{-/-}$ and LDLr$^{-/-}$Jα281$^{-/-}$ mice were used. All mice were kept under standard laboratory conditions and bred in-house. The mice 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). Water and food were administered *ad libitum*.

Media and reagents

The complete cell culture medium used for splenocytes was RPMI 1640 (with L-Glutamine) (Cambrex, Belgium) 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. LDL was isolated from serum of a healthy volunteer after centrifugation of the serum according to Redgrave et al.[31] The isolated LDL was dialyzed against phosphate buffered saline (PBS) with 10 μM EDTA (pH 7.4) for 24 hours at 4°C and oxidized by exposure to 10 μM $CuSO_4$ at 37°C for 20 hours as previously described.[32]

Effect of Western type diet feeding on NKT cells

To investigate the effect of Western-type diet on the number of NKT cells, LDLr$^{-/-}$ mice were fed a Western-type diet for 0, 1.5, 4.5, 9 and 24 weeks. After diet feeding, the mice were sacrificed and the liver, spleen, mediastinal lymph nodes and iliac lymph nodes were dissected. Also blood was collected from these mice. Mononuclear cells were isolated from spleen, liver and blood using Lympholyte (Cedarlane, Ontario, Canada) conform the manufacturers protocol. The lymph nodes were squeezed through a 70 μm cell strainer and a single cell suspension

was used. Subsequently, the mononuclear cells were stained with PerCP-conjugated CD3 and FITC-conjugated NK1.1 antibodies (eBioscience, Belgium) for 30 min. To block a-specific binding of cytokines, samples were incubated with normal mouse serum for 30 min. before staining. After staining, the 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).

Splenocyte proliferation

To test the responsiveness of splenocytes to α-GalCer, spleens from LDLr$^{-/-}$, Jα281$^{-/-}$ and LDLr$^{-/-}$Jα281$^{-/-}$ mice were dissected and single cell suspensions were obtained by squeezing the spleen through a 70μm 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 complete medium with or without 100 ng/ml or 500 ng/ml of α-GalCer. 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. To investigate the response of different splenocytes to oxLDL, spleens were dissected from LDLr$^{-/-}$ and Jα281$^{-/-}$ mice. Splenocytes were isolated as above and cultured with 0, 0.5 or 1 μg/ml of oxLDL for 24 hours.

FACS analysis

To verify the deficiency in NKT cells in the LDLr$^{-/-}$Jα281$^{-/-}$ mice, livers were isolated from both LDLr$^{-/-}$Jα281$^{-/-}$ and LDLr$^{-/-}$ mice. Mononuclear cells were isolated from the non-parenchymal cell population in the liver using Lympholyte (Cedarlane, Ontario, Canada) conform the manufacturers protocol. Subsequently, the cells were stained with APC-conjugated CD3 and PE-conjugated α-GalCer/CD1d tetramer (NIH tetramer core facility, GA) 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).

Induction of atherosclerosis

To determine the effect of a deficiency in NKT cells on atherosclerosis, atherosclerosis was induced in LDLr$^{-/-}$ and LDLr$^{-/-}$Jα281$^{-/-}$ mice. The mice were fed a Western-type diet for 12 weeks. The diet response is followed by measuring the cholesterol levels in serum of these mice. Total cholesterol levels were quantified spectrophotometrically using an enzymatic procedure (Roche Diagnostics,

Germany). After 12 weeks, 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. The hearts with the aortic root were removed and were embedded in OCT compound (TissueTek; Sakura Finetek, The Netherlands). 10μm cryosections of the aortic root were made on a Leica CM 3050S Cryostat (Leica Instruments, UK). These cryosections were stained with hematoxylin (Sigma Diagnostics, MO) and Oil-red O. Plaque areas were measured using a Leica DM-RE microscope and LeicaQwin software (Leica Imaging Systems, UK).

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

Increased NKT cell frequency after Western-type feeding

LDLr$^{-/-}$ mice were fed a Western-type diet for 0, 1.5, 4.5, 9 or 24 weeks. Subsequently, the mice were euthanized and the frequency of NKT cells was determined in several organs. After 1.5 weeks of high fat diet, an increase in CD3^{+}NK1.1^{+} cells was observed in the liver (19.05$\pm$1.21% versus 42.37$\pm$2.21%; $P<0.05$) and the spleen (3.24$\pm$0.14% versus 6.95$\pm$0.42%; $P<0.01$), compared to mice sacrificed before high fat diet feeding was started. This increase persisted in the spleen after 4.5 weeks of diet feeding (7.50$\pm$0.84%; $P<0.05$), whereas the frequency of CD3^{+}NK1.1^{+} cells decreased to basal level again in the liver. This remained for an additional 20 weeks of high fat diet feeding (Figure 7.1, upper part). After 9 weeks, an increase in CD3^{+}NK1.1^{+} cells was also observed in the mediastinal lymph nodes, located near the ascending aorta and the heart (5.85$\pm$0.98% versus 8.97$\pm$0.60%; $P<0.05$). After 24 weeks of high fat diet feeding, the frequency of CD3^{+}NK1.1^{+} cells in all organs returned to basal level like before the mice were fed a high fat diet. During these 24 weeks there was no significant effect on the frequency of CD3^{+}NK1.1^{+} cells in blood and iliac lymph nodes (Figure 7.1, lower part).

NKT cell response to α-GalCer and oxLDL

To investigate whether oxLDL could be a ligand for NKT cells, splenocytes were isolated from LDLr$^{-/-}$, Jα281$^{-/-}$ and LDLr$^{-/-}$Jα281$^{-/-}$ mice. As a control, the responsiveness of splenocytes from these mice to α-GalCer was tested first. Splenocytes from LDLr$^{-/-}$ mice showed a large response to 100 and 500 ng/ml of α-GalCer; stimulation indexes of 22.2$\pm$1.4 and 15.2$\pm$2.9 ($P<0.001$), respectively. Splenocytes from the NKT cell deficient Jα281$^{-/-}$ and LDLr$^{-/-}$Jα281$^{-/-}$ mice were inert to α-GalCer. Splenocyte populations lacking NKT cells do not show

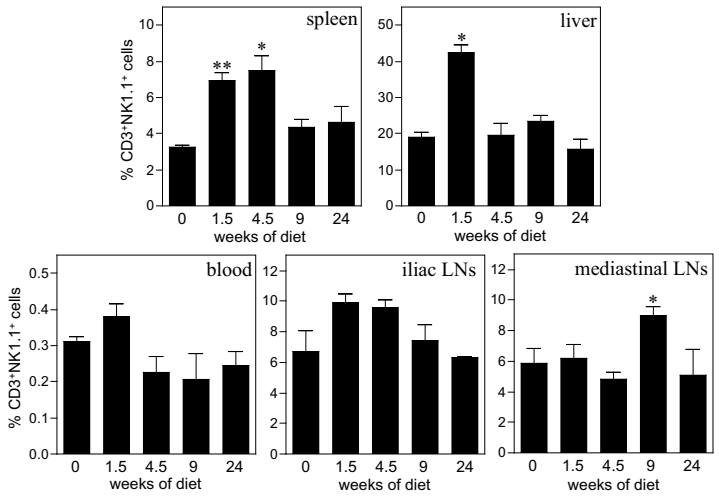

Figure 7.1: NKT cell frequency in different organs after high fat diet feeding. LDLr$^{-/-}$ mice were fed a Western-type diet for 1.5, 4.5, 9 or 24 weeks. Subsequently, the mice were sacrificed and the number of $CD3^{+}NK1.1^{+}$ cells in the spleen, liver, blood, iliac lymph nodes and mediastinal lymph nodes was measured using FACS analysis. As a control, mice were sacrificed before high fat diet feeding was started (indicated as 0). Values are mean $\pm$ SEM. $^{*}P<0.05$, $^{**}P<0.01$

any significant proliferative response to α-GalCer (Figure 7.2). To investigate whether oxLDL could be a ligand for NKT cells, splenocytes were isolated from LDLr$^{-/-}$ mice and Jα281$^{-/-}$ mice and were incubated with different concentrations of oxLDL. Low concentrations of oxLDL induced a 9.8 to 18.7-fold increase ($P<0.01$) in proliferation of splenocytes from LDLr$^{-/-}$ mice when compared to splenocytes incubated without oxLDL. The proliferative response of splenocytes from Jα281$^{-/-}$ mice was much lower. Only a non-significant 0.8 to 2.8-fold increase was observed and a large proliferative response was only observed at higher concentrations of oxLDL (Figure 7.3).

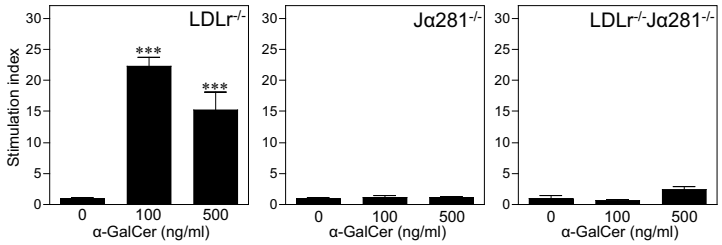

Figure 7.2: Spleen cell proliferation in response to α-GalCer. Splenocytes were isolated from LDLr$^{-/-}$, Jα281$^{-/-}$, and LDLr$^{-/-}$Jα281$^{-/-}$ mice. The splenocytes were incubated *in vitro* with or without 100 ng/ml or 500 ng/ml of α-GalCer for 48 hours. Proliferation was measured by incorporation of ^{3}H-thymidine. Data are shown as the stimulation index $\pm$ SEM. This stimulation index is the ratio of the mean cpm of cultures with α-GalCer to the mean cpm of cultures without α-GalCer. $^{***}P<0.001$.

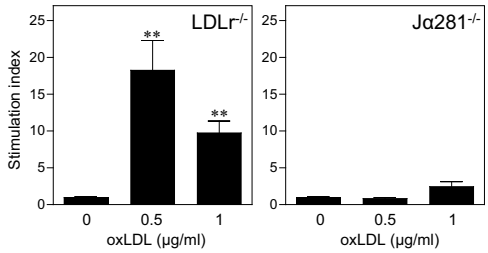

Figure 7.3: Spleen cell proliferation in response to oxLDL. Splenocytes were isolated from LDLr$^{-/-}$ and Jα281$^{-/-}$ mice. These splenocytes were incubated *in vitro* with or without 0.5 and 1 μg/ml of oxLDL for 24 hours. Proliferation was measured by incorporation of ^{3}H-thymidine. Data are shown again as the stimulation index $\pm$ SEM. The stimulation index in this experiment is the ratio of the mean cpm of cultures with oxLDL to the mean cpm of cultures without oxLDL. $^{**}P<0.01$.

Effect of NKT cell depletion on atherosclerosis

To investigate the role of NKT cells in atherosclerosis, LDLr$^{-/-}$ mice were cross-bred with Jα281$^{-/-}$ mice. These LDLr$^{-/-}$Jα281$^{-/-}$ mice were tested for the presence of NKT cells in the liver using the α-GalCer/CD1d tetramer and anti-CD3 antibodies. In the liver of LDLr$^{-/-}$ mice, 10.9$\pm$1.2% of the lymphocytes are CD3^{+}Tetramer^{+} (Figure 7.4D). These CD3^{+}Tetramer^{+} cells were absent in the LDLr$^{-/-}$Jα281$^{-/-}$ (Figure 7.4E; 1.5$\pm$0.2%). To determine whether the deficiency in NKT cells has an effect on atherosclerosis, LDLr$^{-/-}$ and LDLr$^{-/-}$Jα281$^{-/-}$ mice were fed a Western-type diet for 12 weeks. In figure 7.4A and 7.4B representative examples of plaques at the aortic root of LDLr$^{-/-}$ and LDLr$^{-/-}$Jα281$^{-/-}$ mice are shown, respectively. An equal level of atherosclerosis was observed at the aortic root of the LDLr$^{-/-}$ mice (623925$\pm$38750 μm^2) and LDLr$^{-/-}$Jα281$^{-/-}$ mice (Figure 7.4C; 662186 $\pm$ 49988 μm^2; P=0.58) and the lesions were rather advanced in size. In addition there was no effect on total plasma cholesterol levels and body weight during the experiment (data not shown).

Discussion

The fact that NKT cells are a unique subset of T cells recognizing lipid antigens instead of peptide antigens may provide them a unique position in atherosclerosis research, since atherosclerosis is a disease in which both natural and modified lipids play a pivotal role. Most studies on NKT cells are however performed with synthetic ligands, because only a few natural ligands are known. Recently, some bacterial, plant-derived and lysosomal glycosphingolipids which strongly activate NKT cells were found, but their role in NKT cell activation in correlation with diseases remains unclear.[23–27,30] It is of great interest to investigate the possibility whether one of the lipids important in atherosclerosis may form a natural ligand for NKT cells. In the present study we observed an increased frequency in NKT cells in the liver and spleen after 1.5 weeks of Western-type diet feeding to LDLr$^{-/-}$ mice. This increased frequency is still seen in the spleen after 4.5 weeks, whereas in the liver this frequency returned to basal level again. This can be due to a migration of NKT cells out of the liver to the spleen. There was

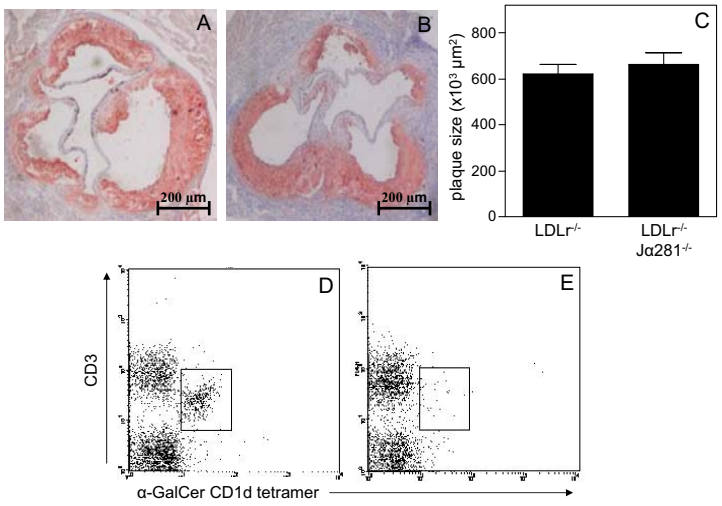

Figure 7.4: The effect of a deficiency in Jα281 on atherosclerosis. LDLr$^{-/-}$ mice were crossbred with Jα281$^{-/-}$ mice to get LDLr$^{-/-}$Jα281$^{-/-}$ mice. These LDLr$^{-/-}$Jα281$^{-/-}$ mice and LDLr$^{-/-}$ mice were fed a Western-type diet for 12 weeks. Subsequently, the mice were euthanized and sectioned aortic roots of LDLr$^{-/-}$ (A) and LDLr$^{-/-}$Jα281$^{-/-}$ (B) mice were stained with hematoxylin and Oil-red O. Lesions were quantified by computer-assisted morphometric analysis and plaque size was determined (C). To verify that the LDLr$^{-/-}$Jα281$^{-/-}$ mice lack NKT cells, a FACS analysis was performed. Lymphocytes from the liver of LDLr$^{-/-}$ (D) and LDLr$^{-/-}$Jα281$^{-/-}$ (E) mice were stained for CD3 and the α-GalCer/CD1d tetramer. Values are mean $\pm$ SEM.

however no increase in the frequency of NKT cells in blood. Whether NKT cells can migrate to the spleen via lymphatic vessels needs further investigation. The migration theory is however further supported by the observation of an increased NKT cell frequency in the mediastinal lymph nodes after 9 weeks of diet. After 24 weeks of diet the levels were the same as without diet feeding. It is however possible that the number of athero-specific NKT cells, specific for one of the lipids in the Western type diet, increased after this long period of diet feeding, but that this was not detectable because of the large decline in total number of NKT cells. The data on prolonged diet feeding (24 weeks) are in contrast with observations by Nakai et al.[15] They observed a significant decrease in both total NKT cells and α-GalCer/CD1d tetramer^{+} cells in the hepatic mononuclear cell population after 20 weeks of atherogenic diet feeding to wild type mice. They suggested that this decrease might be due to activation-induced cell death caused by for example oxLDL, due to a continuous down-modulation of NK1.1 and the TCR or due to a migration of NKT cells to other locations. Our data support this last suggestion. In addition we can confirm that NKT cells may play a role in the initiation of atherosclerosis since NKT cells seem to respond to diet feeding within the first weeks.

Since high fat diet seems to induce NKT cell proliferation and probably stimulate NKT cell migration, the next interesting question was whether oxLDL may be able to activate NKT cells. It is known that oxLDL-reactive T cells exist in the vessel wall and plaques of atherosclerosis patients.[33,34] In a previous study we observed that splenocytes from LDLr$^{-/-}$ mice respond to oxLDL in terms of an increased proliferation.[29] This indicates that a certain cell type responds to one

of the components of oxLDL. We showed that these cells were $CD3^+$ cells and not macrophages or B cells. Since oxLDL contains both proteins and lipids, epitopes of oxLDL could be both peptidic and lipidic. The macrophages and dendritic cells that ingest and process oxLDL, express different types of antigen presenting molecules such as MHC molecules and CD1d. These molecules can respectively become loaded with peptidic and lipidic antigens. It was therefore of interest to investigate if a part of the $CD3^+$ T cells proliferating in response to oxLDL could be NKT cells. Splenocytes isolated from J$\alpha281^{-/-}$ mice showed a much lower proliferative response to oxLDL when compared to splenocytes from $LDLr^{-/-}$ mice. This may indicate that NKT cells represent at least a fraction of the proliferating splenocytes when cultured with oxLDL. The observed proliferation of J$\alpha281^{-/-}$ splenocytes may be due to the proliferation of T cells responding to peptidic epitopes of oxLDL. In a recent study, VanderLaan et al. showed that LDL, isolated from the serum of $LDLr^{-/-}$ mice fed a Western-type diet, was able to induce IL-2 production by a NKT cell hybridoma when loaded on C57BL/6 DCs. This effect was absent when $CD1d^{-/-}$ DCs were used.[35] This confirms our hypothesis that NKT cells may be activated by an endogenous ligand present in the mice after Western-type diet feeding. However, they did not observe an effect of cupper-oxidized LDL on NKT cell activation. They postulated that the putative antigen in LDL was destroyed by extensive oxidation, but our results show that this may vary with isolation and the grade of oxidation of LDL. It is however clear that further investigation is needed to detect the specific structure in oxLDL that is recognized by the specific TCR on NKT cells. A few studies showed that phosphatidyl-choline (PC) may be a CD1d-binding ligand for NKT cells.[28,36] PCs are also present in oxLDL such as 1-(Palmitoyl)-2-(5-oxovaleroyl)-phosphatidyl-choline (POV-PC) and oxidized 1-palmitoyl-2-arachidonoyl-phosphatidyl-choline (oxPAPC). Both PCs trigger inflammation and promote atherosclerosis.[37–39] In future studies we will test these ligands for their possible NKT activating capacities and the effect thereof on atherosclerosis. Because NKT cells seem to play a role in atherosclerosis the effect of a deficiency in NKT cells on lesion formation was investigated. $LDLr^{-/-}$ mice were crossbred with J$\alpha281^{-/-}$ mice. These mice are deficient in both CD1d-dependent and CD1d-independent NKT cells and are different from $CD1d^{-/-}$ mice. $CD1d^{-/-}$ mice lack only CD1d-dependent NKT cells and in addition also T cells that are activated via the presentation of glycolipids or hydrophobic peptides presented by CD1d. Therefore, J$\alpha281^{-/-}$ mice are really different from $CD1d^{-/-}$ mice and J$\alpha281^{-/-}$ mice are an interesting model to investigate the exact role of NKT cells in atherosclerosis. We however observed that $LDLr^{-/-}$J$\alpha281^{-/-}$ mice fed a Western type diet for 12 weeks had similar lesion sizes as $LDLr^{-/-}$ mice. In an earlier publication,[16] it was shown that $CD1d^{-/-}LDLr^{-/-}$ mice fed an atherogenic diet for 8 and 12 weeks also have similar lesion size as $LDLr^{-/-}$ mice. In that study it was concluded that CD1d-dependent NKT cells only play a role in the initial stage of lesion development because a deficiency in CD1d reduced lesion formation in mice fed the diet for 4 weeks. In conclusion we describe in this study that the initial effect of NKT cells on atherosclerosis may be due to an activation of NKT cells by a ligand present in $LDLr^{-/-}$ mice after Western-type diet feeding. One of the candidate ligands may be oxLDL, or at least one of its components.

The exact role of activation of NKT cells by these lipids in atherosclerosis needs further investigation but we assume that these ligands activate the NKT cells which consequently accelerate atherosclerosis.

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