A CD1d-dependent lipid antagonist to NKT cells ameliorates atherosclerosis in ApoE\(^{-/-}\) mice by reducing lesion necrosis and inflammation

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Aims

Atherosclerosis-related deaths from heart attacks and strokes remain leading causes of global mortality, despite the use of lipid-lowering statins. Thus, there is an urgent need to develop additional therapies.

Methods and results

Reports that NKT cells promote atherosclerosis and an NKT cell CD1d-dependent lipid antagonist (DPPE-PEG\(_{350}\)-1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N[methoxy(polyethylene glycol)-350]) reduces allergen-induced inflammation led us to investigate its therapeutic potential in preventing the development and progression of experimental atherosclerosis. DPPE-PEG\(_{350}\) was administered to hyperlipidaemic ApoE\(^{-/-}\) mice with/without established atherosclerosis. Atherosclerosis and immune cells were assessed in the aortic sinus lesions. Lesion expression of monocyte chemoattractant protein-1 (MCP-1) and vascular cell adhesion protein-1 (VCAM-1) responsible for inflammatory immune cell recruitment as well as mRNA expression of IFNgamma and its plasma levels were investigated. Necrotic cores and lesion smooth muscle and collagen contents important in plaque stability were determined as were plasma lipid levels. DPPE-PEG\(_{350}\) reduced atherosclerosis development and delayed progression of established atherosclerosis without affecting plasma lipids. CD4 and CD8 T cells and B cells in atherosclerotic lesions were decreased in DPPE-PEG\(_{350}\)-treated mice. Lesion MCP-1 and VCAM-1 protein expression and necrotic core size were reduced without affecting lesion smooth muscle and collagen content. IFNgamma and lymphocytes were unaffected by the treatment.

Conclusion

The attenuation of progression of established atherosclerosis together with reduced development of atherosclerosis in hyperlipidaemic mice by the NKT antagonist, without affecting NKT cell or other lymphocyte numbers, suggests that targeting lesion inflammation via CD1d-dependent activation of NKT cells using DPPE-PEG\(_{350}\) has a therapeutic potential in treating atherosclerosis.

Keywords

NKT cells • Atherosclerosis • ApoE\(^{-/-}\) mice • DPPE-PEG • NKT antagonist

1. Introduction

Atherosclerosis is a chronic inflammatory disease initiated by lipid entry into the arterial wall. Immune cells drive the development, acceleration, and propagation of atherosclerotic lesions that lead to formation of vulnerable plaques by complex interactions involving both adaptive and innate immune mechanisms, with lymphocytes activated by antigen-presenting dendritic cells, secreting pro-atherogenic factors including inflammatory cytokines and cytotoxins\(^{1}\) and macrophages activated via innate mechanisms or cytokines such as IL-4 increasing their expression of MMPs.\(^{2}\) Vulnerable plaques are characterized by large necrotic cores.\(^{3}\) Rupture of vulnerable plaques causes thrombotic...
occlusion of coronary and carotid arteries resulting in myocardial infarctions and strokes that together remain leading causes of global mortality. Current atherosclerosis management primarily aims to lower plasma LDL levels through statin medication and lifestyle changes with secondary measures to prevent platelet aggregation and hypertension without targeting inflammation. The observations that cholesterol-lowering drugs, statins, reduce the risk of cardiovascular events by only one-third of patients with atherosclerosis, and evidence that inflammation is crucial for the development of cardiovascular events have stimulated the development of new therapeutic strategies that target inflammation and the immune system in atherosclerosis management. Experimental therapeutic studies that target inflammation in atherosclerosis include the development of vaccines, depletion of pathogenic immune cells, and expansion of atheroprotective immune cells. NKT cells express NK markers and TCR that recognize lipid antigens presented by the MHC class I-like molecule CD1d on antigen-presenting cells. CD1d-restricted NKT cells are classified into two types: Type I, expressing invariant TCR and Type II, expressing diverse TCR. The TCR of invariant NKT cells has TCRα chain (Vα14-Jα18) paired with TCRβ chain of Vβ8.2, Vβ7, or Vβ2. Two major subsets of Type I invariant NKT cells are distinguished by CD4 and CD8 expression into CD4+ and CD4/CD8 double negative (DN) subsets. In mice, NKT cells are found at highest frequency in the liver (10–30% of liver lymphocytes), and at lower frequencies in lymphoid organs. Activated NKT cells modulate immune responses in transplantation, oncology, autoimmunity, infection, and inflammation. CD1d-restricted Type I and Type II NKT cells have differential recognition of lipid antigens. Type I NKT cells are potently stimulated by α-galactosylceramide (αGalCer) derived from marine sponge. In contrast, Type II NKT cells respond to sulfatide, a sulfated form of β-galactosylceramide (βGalCer). NKT cells are present in human and mouse atherosclerotic lesions. Mice rendered NKT cell deficient by knocking out CD1d gene have reduced atherosclerosis, indicating that NKT cells promote atherosclerosis development. Type 1 NKT cells are pro-atherogenic as NK cell activation by injecting αGalCer-aggregated atherosclerosis. It has been further confirmed as atherosclerosis was reduced in Jα18−/− mice selectively deficient in invariant NKT (iNKT) cells while adoptive transfer of iNKT cells from Jα18 TCR transgenic mice to Rag1−/−/Ldlr−/− mice aggravated atherosclerosis. We reported that NKT cell deficiency induced by neonatal thymectomy reduced atherosclerosis lesions, and that this was reversed by transfer of total NKT cells. Our data suggest that NKT cell antagonists, such as DPPE-PEG350, have therapeutic potential to reduce inflammation in atherosclerosis and its combination therapy with lipid-lowering agents may further reduce atherosclerosis-associated mortality.

2. Methods

2.1 Animals, diet, and maintenance

ApoE−/− mice obtained from Jackson Laboratory were bred at Precinct Animal Centre (PAC), Almeda Medical Research and Education Precinct (AMREP). The mice were maintained under a 12-h light/dark cycle in standard animal facility at PAC. A high-fat diet (HFD) containing 21% fat and 0.15% cholesterol (Speciality Food, Western Australia) was used to generate lipid-induced atherosclerosis. HFD and autoclaved sterile water were provided ad libitum throughout experiments. A total of 64 male 6- to 8-week-old mice were used in this study. All mice were killed by using slowfill carbon dioxide asphyxiation. Animal experiment protocols and procedures were approved by the AMREP animal ethics committee (E/1230/2012/B) and conform to the Guide of Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 2011).

2.2 Experimental design

To study the effect of DPPE-PEG350 in atherosclerosis development (prevention experiment), mice were treated with DPPE-PEG350 (250 μg i.v. via tail vein twice weekly) at the beginning of 8-week HFD. To investigate whether DPPE-PEG350 can reduce established atherosclerosis (intervention experiment), mice fed a HFD for 6 weeks were treated with DPPE-PEG350 in a similar fashion for 6 weeks while fed a HFD. Control mice were given vehicle control (phosphate buffer saline, PBS, containing 0.05% DMSO). In some experiment, mice were treated first with DPPE-PEG350 (500 μg i.v. via tail vein once weekly) at the beginning of HFD. One week later NKT ligand, αGalCer (2 μg i.p. fortnightly) was given to some mice. Control mice were injected with PBS alone. All mice receiving αGalCer injection were killed at the completion of 7-week HFD.

2.3 Preparation of DPPE-PEG350 and αGalCer

DPPE coupled with polyethylene glycol mono-methyl-ether with an molecular weight of 350 (DPPE-PEG350; cat #880400P) was purchased from Avanti Polar Lipids (Alabaster, AL, USA) and dissolved in DMSO at a stock concentration of 1 mg/mL (final 0.05% DMSO concentration when injected into mice) as instructed by the manufacturer. Marine sponge-derived synthetic αGalCer (cat #KRN7000, Funakoshi) was dissolved at a stock concentration of 200 μg/mL as suggested.

2.4 Endpoint tissue collection

At the end of experiment, livers were collected for NKT and non-NKT lymphocyte analysis. Aortic sinuses were dissected, embedded in OCT, and kept at −80°C. Spleen and aortic arches were snap-frozen and provided by the US National Institutes of Health (NIH Publication No. 85-23, revised 2011).
kept at −80°C. Plasmas separated from whole blood collection were kept at −80°C.

2.5 Liver lymphocyte isolation
Livers collected in 1× PBS containing 2% fetal bovine serum (FBS) and 2 mM ethylenediaminetetraacetic acid (EDTA) were washed using a plunger and metal sieve (200 μm wire mesh) and washed in 50 mL of 1× PBS containing 2% FBS and 2 mM EDTA twice with centrifugation at 500 g for 5 min at 4°C. Cell pellets resuspended in 25 mL of isotonic Percoll (#17-0891-01, GE Healthcare, Piscataway, NJ, USA) were centrifuged at 750 g for 20 min at room temperature. Floating hepatocytes were removed and cell pellets containing lymphocytes and red blood cells were treated with red cell lysis buffer (0.155 M NH₄Cl, 0.01 M KHCO₃, and 0.1 mM EDTA). Liver lymphocytes were then collected after centrifugation at 300 g for 10 min at 4°C.

2.6 Assessment of liver lymphocytes
All liver lymphocytes were stained with phycoerythrin (PE)-conjugated CD19 (BD Biosciences), allophycocyanin (APC)-conjugated CD4 antibody (BD Biosciences, San Diego, CA, USA), and FITC-conjugated anti-CD8 antibody (BD Biosciences). R-PE with cyanine dye (Cy7; PE-Cy7)-conjugated anti-NK1.1 antibody (BD Biosciences), and FITC-conjugated anti-TCRβ antibody (BD Biosciences) as described. In some experiment, PE-conjugated CD1d tetramer (a gift from Dale Godfrey, Melbourne University) and FITC-conjugated anti-CD3 antibody (BD Biosciences) were utilized. Data were acquired in FACSCanto™ II flow cytometer (BD Biosciences) and analysed by the FACS Diva software (BD Biosciences).

2.7 Section of aortic sinus and analysis of stained sections
Cryostat sections (6 μm) of aortic sinuses were collected on 14 sequential slides containing three sections per slide. One slide was used for each immunological or histological staining and were analysed by different laboratory personnel.

2.8 Assessment of atherosclerosis
Frozen sections of 6 μm thick aortic sinuses were stained with Oil Red-O (Sigma, St Louis, MO, USA). Total lesion areas defined as intimal atherosclerotic lesions and lesion lipid accumulation areas identified by ORO-staining areas were measured using the Optimas software as described.

2.9 Assessment of macrophage and lymphocyte accumulation
Immunohistochemical analysis was carried out on frozen sections of aortic atherosclerotic lesions using anti-CD68, anti-CD4, and anti-CD8 antibodies (BD Biosciences). Macrophages were identified and counted as haematoxylin-stained nuclei associated with CD68 staining. CD4+ and CD8+ T cells were also similarly counted under the microscope and adjusted according to total intimal lesion area as described. Aortic sinus lesions were also immunohistologically stained for B cells using anti-CD19 antibody (BD Biosciences), counted, and presented as total cells per lesion areas as described above.

2.10 Assessment of necrotic cores, smooth muscles, and collagen
Necrotic core areas, content of smooth muscle and collagen in atherosclerotic lesions, were measured as described. Briefly, Optimas software was used to measure necrotic core areas defined as acellular areas of H&E-stained sections. Smooth muscle content in atherosclerotic lesions was assessed by staining with anti-α-smooth muscle antibody (BD Biosciences). To assess collagen contents, a polarized microscope was used to identify birefringent collagen from picrosiris red-stained atherosclerotic sections.

2.11 Total RNA extraction
Snap-frozen samples of spleens and aortic arches were processed to extract total RNAs using the RNeasy Mini kit (#74104, Qiagen, Hilden, Germany) and the RNeasy fibrous tissue Mini kit (#74704, Qiagen), respectively, as instructed by the manufacturer. One-column DNAase treatment in the RNA extraction procedure was performed as per manufacturer’s recommendation to eliminate genomic DNA contamination. Quantity and quality of extracted RNA were determined by Nanodrop 2000C spectrophotometer (Thermo Scientific, Waltham, MA, USA).

2.12 Assessment of mRNA IFNγ expression
One-step RT-PCR kit (#210215, Qiagen) was utilized to determine splenic and arterial mRNA expression of IFNγ as described. IFNγ mRNA expression levels were analysed using the comparative cycle threshold method with 18S rRNA primers (Applied Biosystems Foster City, CA, USA).

2.13 Assessment of plasma IFNγ
Plasma level of IFNγ was determined by a commercial ELISA kit (H0002, Elisokit.com) as per manufacturer’s instruction. Briefly, four-fold diluted plasma samples were incubated in wells pre-coated with mouse IFNγ capture antibody. Subsequent incubation with biotin-labelled detection antibody was followed by freshly prepared streptavidin–HRP conjugate incubation. Colour developed by TMB substrate was stopped and optical density at 450 nm wavelength determined using a microplate reader. Plasma IFNγ concentration was calculated using a standard curve provided and corrected by a dilution factor.

2.14 Plasma lipid analysis
Plasma lipids and cholesterol were determined as described.

2.15 Statistical analysis
GraphPad Prism (ver. 5.0) was used to analyse the data. Two-tailed unpaired Student’s t-test (to compare two groups) and one-way analysis of variance with Dunnett’s post hoc test comparing with the PBS-injected group (to compare three groups) were used to calculate statistical significance and a value of P < 0.05 was statistically considered significant.

3. Results
3.1 Chronic DPPE-PEG₃₅₀ treatment does not alter NKT and other lymphocytes
Mice were injected with DPPE-PEG₃₅₀ for a total of 16 times over 8 weeks. First, we asked whether chronic treatment of DPPE-PEG₃₅₀ affects NKT cell population in lipid-induced atherosclerosis. As NKT cells are predominantly found in liver parenchyma,17 we determined liver-resident NKT cell population in mice that received DPPE-PEG₃₅₀ treatment at the end of 8-week HFD. FACS-assisted analysis of liver lymphocytes showed that NK1.1+ TCRβ+ NKT cells did not differ between treated and control mice (Figure 1A). DPPE-PEG₃₅₀ treatment also did not affect populations of CD4+ and CD8+ T cells, B cells, and NK cells (Figure 1B).

3.2 DPPE-PEG₃₅₀ treatment reduces the development of atherosclerosis
Next, we investigated whether DPPE-PEG₃₅₀ affects plasma lipid levels and atherosclerosis development. DPPE-PEG₃₅₀-treated mice showed reduced body weight at the end of 8-week HFD (Figure 1C). Despite ~10% reduction of body weight, DPPE-PEG₃₅₀ did not affect plasma lipids (Figure 1D). However, the development of lipid-induced atherosclerosis was significantly reduced in treated mice compared with the
control group. Total intimal atherosclerotic lesion size and ORO-stained lipid accumulation were reduced by 45 and 52%, respectively, in ApoE^{-/-} mice that received DPPE-PEG350 treatment compared with the PBS group (Figure 1E–G). But, the ratio of lipid accumulation relative to intima area was similar (see Supplementary material online, Figure S1A).
3.3 T and B cells are reduced in atherosclerotic lesions following DPPE-PEG350 treatment

DPPE-PEG350 has been shown to reduce immune cells in alveolar tissues of OVA-injected mice and in hypersensitized area. We investigated whether DPPE-PEG350 affects macrophages and T and B cells in atherosclerotic lesions. Immunohistochemical analysis showed that DPPE-PEG350 tended to reduce CD68+ macrophages, but the reduction did not reach a statistical significance (Figure 2A and B). CD4+ and CD8+ T cells by 54% (Figure 2C and D) and 94% (Figure 2E and F), respectively, and CD19+ B cell accumulation by 80% (Figure 2G and H) in atherosclerotic lesions. The findings suggest that DPPE-PEG350 affects recruitment of T and B cells into atherosclerotic lesions.

3.4 DPPE-PEG350 treatment reduces monocyte chemotactic protein-1 and vascular cell adhesion protein-1 in developing atherosclerotic lesions

The finding of reduced T and B cells in atherosclerotic lesions prompted us to investigate the expression of leucocyte-attractant proteins, such as monocyte chemotactic protein-1 (MCP-1) and vascular cell adhesion protein-1 (VCAM-1), because their expression is important for immune cell recruitment. Atherosclerotic lesion stained with anti-MCP-1 and anti-VCAM-1 antibodies showed that protein expression of MCP-1 and VCAM-1 was reduced by 30% (Figure 3A and B) and 57% (Figure 3C and D), respectively, in mice that received DPPE-PEG350 compared with the control group. Collectively, our results suggest that reduced MCP-1 and VCAM-1 in atherosclerotic lesions may contribute to the reduced recruitment of macrophages, T cells, and B cells into lesion areas.

3.5 Reduced necrotic core accompanies reduced lesions of developing atherosclerotic lesions

As formation of necrotic cores in atherosclerotic lesions indicates progressive inflammation of atherosclerosis and is a key feature of vulnerable atherosclerotic plaques and is at least in part mediated by iNKT cells, we next investigated the size of necrotic cores in atherosclerotic lesions of mice that received DPPE-PEG350 compared with the vehicle control group. Defined by acellular areas in HE-stained atherosclerotic lesions, DPPE-PEG350 reduced necrotic core areas by 64% in hyperlipidaemic ApoE/−− mice (Figure 3E and F). In contrast, DPPE-PEG350 did not affect smooth muscle and collagen content in atherosclerotic lesions in mice with established atherosclerosis when aortic atherosclerotic lesions were immunohistochemically stained with anti-smooth muscle antibody and histologically stained with Picrosirus red (Figure 3G–J).

3.6 DPPE-PEG350 treatment retards progression of established atherosclerosis

After establishing that DPPE-PEG350 decreases the development of atherosclerosis, we investigated whether DPPE-PEG350 will also similarly retard established atherosclerotic lesions, because this approach will test the therapeutic potential of chronically inhibiting iNKT cell activation to treat patients with established atherosclerosis. ApoE/−− mice were first fed a HFD for 6 weeks to generate lipid-induced atherosclerosis, followed by DPPE-PEG350 treatment as described above for a further 6 weeks while the mice were continued to be fed a HFD. With comparable body weight and plasma lipid profiles (data not shown), DPPE-PEG350 treatment reduced atherosclerosis by 49% when assessed by total intimal lesion areas (Figure 4A and B). Mice that received DPPE-PEG350 treatment also reduced lipid accumulation in aortic sinus lesions by 27% (Figure 4C), demonstrating the potent pharmacological action of DPPE-PEG350 in treating established atherosclerosis, but the ratio of lipid accumulation to intima area was unaffected (see Supplementary material online, Figure S1B). Immunohistochemical staining of atherosclerotic lesions also showed that the numbers of CD68+ macrophages, CD4+, CD8+ T cells, and CD19+ B cells in atherosclerotic lesions were reduced in mice with established atherosclerosis after treatment with DPPE-PEG350 (Figure 4D–G). Similar to findings in the development of atherosclerosis, DPPE-PEG350 reduced necrotic cores by 59% in established atherosclerotic lesions (Figure 4H), but it did not affect smooth muscle and collagen content (Figure 4I and J).

3.7 DPPE-PEG350 treatment reduces apoptotic cells in atherosclerotic lesions

NKT cells promote apoptosis in atherosclerotic lesions via perforin- and granzyme B-dependent mechanism. Our data in the present study show that DPPE-PEG350 treatment reduces necrotic core areas in developing and established lesions, suggesting reduced NKT-generated apoptosis. Assessment of apoptotic cells in developed atherosclerotic lesions by TUNEL assay confirms that DPPE-PEG350 treatment decreases apoptotic cells in established atherosclerosis (Figure 5A and B). Next, we investigated the cellular targets of apoptosis using IF staining of the lesion. We found that macrophages, not smooth muscle or endothelial cells, were a major target of apoptosis that were reduced by DPPE-PEG350 treatment (Figure 5C and Supplementary material online, Figure S2A and B). Continuity of plaque endothelial cells assessed by IF staining was not affected by DPPE-PEG350 (Figure 5D), indicating that endothelial cells are not targeted by NKT cell-generated apoptosis.

3.8 DPPE-PEG350 does not affect mRNA expression and plasma level of inflammatory cytokine IFNγ

To determine the effect of DPPE-PEG350 on production of IFNγ as activated NKT cells secrete a large amount of IFNγ, we first assessed IFNγ mRNA expression in arterial arches and spleens using quantitative real-time PCR and also determined plasma IFNγ level by ELISA. Control and treated ApoE/−− mice did not show any difference in IFNγ mRNA expression in arterial and spleen tissues (Figure 6A and B). DPPE-PEG350-treated mice also showed similar levels of plasma IFNγ compared with control mice (Figure 6C and D).

3.9 DPPE-PEG350 ameliorates αGalCer-aggravated atherosclerosis

DPPE-PEG350 competes with αGalCer for lipid antigen binding to CD1d to potently prevent NKT cell effector functions in allergic models. To determine whether DPPE-PEG350 will compete with αGalCer to prevent NKT cell activation in atherosclerosis, we designed an experiment where DPPE-PEG350-treated ApoE/−− mice were subsequently treated with αGalCer to activate NKT cells. In mice without DPPE-PEG350 treatment, αGalCer injection increased total intimal lesion by 112% in hyperlipidaemic ApoE/−− mice, consistent with a previous report (Figure 7A and B). In contrast, αGalCer-aggravated
Atherosclerosis was completely abrogated by DPPE-PEG350 treatment (Figure 7A and B). A similar finding was observed with ORO-stained lipid accumulation (Figure 7C); however, lipid contents adjusted to lesion area were similar (data not shown). FACS analysis of liver lymphocytes showed that αGalCer injection reduced CD1d-tetramer+ iNKT cells (Figure 7D and E).
Figure 3: MCP-1 and VCAM-1 expression and necrotic cores are reduced in atherosclerotic lesions of DPPE-PEG350-treated ApoE−/− mice. Immunohistochemical staining shows reduced expression of both MCP-1 (A and B) and VCAM-1 (C and D) proteins in atherosclerotic lesions in treated mice compared with the vehicle control group. Necrotic core areas identified as acellular areas of H&E-stained atherosclerotic lesions were also reduced (E and F) by DPPE-PEG350 treatment. However, smooth muscle assessed by immunohistochemical staining with α-actin smooth muscle antibody (G and H) and collagen assessed by Picrosirius-red staining (I and J) of atherosclerotic lesions were unaffected by DPPE-PEG350 treatment. Data presented as scatter circle-plots with the mean value. Each circle (white—control group and black—treated group) represent an individual mouse. *P < 0.05. Representative microimages show staining of MCP-1 (B), VCAM-1 (D), necrotic cores (F), smooth muscle (H), and collagen (J) in atherosclerotic lesions. Scale bar—100 μm.
Established atherosclerosis is retarded by DPPE-PEG350 treatment. ApoE<sup>−/−</sup> mice were fed a HFD for 6 weeks to generate lipid-induced atherosclerosis, and then treated with DPPE-PEG350 injection (250 μg i.v. twice weekly) for a further 6 weeks of HFD. DPPE-PEG350 treatment reduced established atherosclerosis assessed at the aortic sinus by total intimal areas and ORO-stained lipid accumulation (A–C) in ApoE<sup>−/−</sup> mice. CD68<sup>+</sup> macrophages (D), CD4<sup>+</sup> and CD8<sup>+</sup> T cells (E and F), and CD19<sup>+</sup> B cells (G) were also reduced in atherosclerotic lesions. While DPPE-PEG350 treatment reduced necrotic core area (H), lesion content of smooth muscle (I) and collagen (J) was not affected. Data presented as scatter circle-plots with the mean value. Each circle (triangle—6-week HFD fed, white—control group, and black—treated group) represent an individual mouse. *P < 0.05. Representative microimages show ORO staining (B) in atherosclerotic lesions. Scale bar—100 μm.
4. Discussion

Our results showed that the CD1d-dependent lipid NKT antagonist, DPPE-PEG<sub>350</sub>, reduces both the development and progression of atherosclerosis induced by hyperlipidaemia. Our finding of significant reductions in atherosclerotic necrotic cores following DPPE-PEG<sub>350</sub> treatment is consistent with the cytotoxic actions of NKT cells augmenting inflammation as well as the development/progression of atherosclerosis. 28

In a pulmonary alveolitis mouse model, a single dose of DPPE-PEG<sub>350</sub> is sufficient to prevent αGalCer-augmented inflammation, indicating a
potent anti-inflammatory effect of DPPE-PEG350 in NKT-aggravated immune responses. Here, we showed that long-term treatment with the CD1d-dependent lipid antagonist DPPE-PEG350 over 8 weeks reduced atherosclerosis development. Importantly, 6-week treatment of DPPE-PEG350 also delayed the progression of atherosclerosis established by feeding a HFD for 6 weeks. While plasma lipids were unaffected in both experiments, DPPE-PEG350-treated mice showed a small reduction in body weight in atherosclerosis development (prevention experiment). It is possible that the small reduction in body weight is due to DPPE being partially metabolized in vivo by phosphatidylethanolamine N-acyltransferase to generate N-arachidonyl-DPPE and N-acylphosphatidylethanolamines; that are also produced by the small intestine can suppress food intake.

Our findings suggest a therapeutic potential of NKT-targeted CD1d-dependent antagonist approach to control inflammation in the management of patients with atherosclerosis. The reduced atherosclerosis in both development and progression was not associated with plasma lipid levels, suggesting a direct effect on iNKT cells, most likely by DPPE-PEG350 competing with lipid antigen for binding to the CD1d molecule on antigen-presenting cells. The suggestion is consistent with the observation that PEG-conjugated DPPE maximally inhibited oxazolone-induced dermatitis when included during priming and challenge. Therefore, molecules, such as DPPE-PEG350, may be suitable to prevent lipid antigen presentation by CD1d to NKT cells in the treatment of NKT cell-mediated inflammatory disorders including atherosclerosis.

NKT cells representing at 0.5–2.5% of T cells in blood, spleens, and lymph nodes are found mainly in liver where they comprise up to 30% of T cells in liver sinusoids. Analysis of liver lymphocytes showed no difference in both NKT and other lymphocyte profiles between treated and untreated groups, suggesting that DPPE-PEG350 treatment have no effect on lymphocyte populations. Our results are in accordance with earlier observation where expression of Vα14Jα18 (NKT TCR) transcripts were uniformly found before and many days after chemical challenge, indicative of the importance in NKT cell activation, in NKT cell-augmented inflammation.

When NKT agonist, αGalCer, was given to ApoE−/− mice, CD1d-competent mice recruited more T cells (either CD3+ or TCRβ+) into atherosclerotic lesions. Recruitment of immune cells in allergen-induced alveolitis and dermatitis was dramatically ameliorated by the treatment of PEG-conjugated DPPE. These findings suggest an important role of activated NKT cells in recruitment of immune cells into inflammatory sites. In our study, the numbers of lesion CD4+ and CD8+ T cells were decreased in atherosclerotic lesions when NKT cell activation was prevented by DPPE-PEG350, confirming that prevention of NKT cell activation by NKT cell antagonist reduces immune cells in atherosclerotic lesions. Our finding that lesion B cell numbers were also reduced is consistent with iNKT cells enhancing B cell responses.
Recruitment of immune cells is associated with expression of adhesion molecules and leucocyte-attractant proteins. In addition to stimulating homing of monocytes, MCP-1 can also direct migration of B and T cells to inflammatory sites. VCAM-1 is also important for induction of T cell antigen receptor-dependent activation of CD4⁺ T cells. MCP-1 and VCAM-1 proteins play an important role in recruitment of monocytes and lymphocytes into atherosclerotic lesions. Our results showing reduced expression of MCP-1 and VCAM-1 in atherosclerotic lesions of mice treated with the NKT antagonist, DPPE-PEG₃₅₀, are consistent with CD1d-dependent activation of iNKT cells by αGalCer in atherosclerosis and most likely to contribute to the observed reductions in macrophage accumulation within atherosclerotic lesions.

Previously, we have shown that iNKT cells influence lesion collagen levels, but in the present study lesion collagen levels were unaffected by DPPE-PEG₃₅₀ treatment. One possible explanation for this difference is that collagen levels are more influenced by mechanisms other than iNKT cell activation.
than lipid antigen presentation to iNKT cells during the development of atherosclerosis. Recently, it has been shown that peptides may also activate CD1d-restricted NKT cells. It is possible that such activation is not prevented by DPPE-PEG150. The mechanisms by which iNKT cells influence lesion collagen are yet to be determined, but appear independent on lesion vascular smooth muscle cell numbers.

In early development of atherosclerosis, macrophage apoptosis and efficient effecytosis suppress fatty streak development/progression. As atherosclerosis progresses to atheromatous (Type IV) lesions and beyond, more cells, including smooth muscles and/or immune cells mostly macrophages, undergo apoptosis and clearance of apoptotic cells becomes impaired, leading to post-apoptotic necrosis and necrotic core development and accelerated sterile inflammation in atherosclerotic lesions. Enlargement of necrotic cores is a key feature of vulnerable plaques. Our finding that lesion smooth muscle content was unaffected in CD1d-dependent NKT antagonist-treated mice suggests that reduced lesion necrosis is mainly due to reduced macrophage post-apoptotic necrosis. The data are consistent with our recent report that NKT cells are able to promote atherosclerosis by perforin- and granzyme B-dependent cytotoxicity that results in apoptosis and generation of necrotic cores in atherosclerotic lesions.

IFNγ expression in DPPE-PEG150-treated mice that did not differ from the control group indicates that the NKT cell antagonist does not affect IFNγ expression after 8 weeks of treatment. The reduction in inflammation induced by DPPE-PEG150 is most likely, at least in part, the consequence of the reduced post-apoptotic necrosis, given that cell necrosis initiates inflammatory responses including inflammasome activation that leads to production of molecules such as MCP-1. Necrotic cells also release damage-associated molecular patterns (DAMPs) that drive inflammation. Endogenous DAMPs, such as high mobility group box 1 protein (HMGB1), biglycans, heat shock proteins, heparan sulfate, SA100A8/A9, and fibrinogen, are responsible for sterile inflammation in atherosclerosis. We have previously shown that one such DAMP, HMGB1, augments the development of atherosclerosis. Apoptosis- and post-apoptotic necrosis-derived proteins are present at every stage during atherosclerosis pathogenesis. The important roles of necrotic cells in atherosclerosis, targeting post-apoptotic cell necrosis with an agent such as the CD1d-dependent lipid agonist might be a more efficacious pharmacological approach to control inflammation in atherosclerosis than targeting individual DAMPs.

DPPE-PEG150 prevents iNKT cell effector function by blocking CD1d-dependent lipid antigen presentation, and inhibits glycolipid-induced CD1d-dependent cytokine production from monocytes. The observation that αGalCer increased atherosclerosis was totally prevented by prior treatment with DPPE-PEG150 confirms CD1d-dependent atheroprotective effect of DPPE-PEG150 treatment. However, iNKT cells detected in liver were very low in a GalCer-injected mice and our finding is consistent with down-regulated TCR expression in iNKT cells that requires several days to reach normal expression.

Several lines of evidence indicate that NKT cell/CD1d+ antigen-presenting cell interactions are also important for progression of atherosclerotic lesions in humans. NKT cells and CD1d-expressing antigen-presenting cells are present in human atherosclerotic plaques. CD1d+ cells are abundant in advanced human neovascularized lesions, suggesting an important role for NKT cells activated by CD1d-mediated lipid antigen presentation in promoting vulnerable plaque formation. NKT cells isolated from human plaques showed increased inflammatory responses when stimulated with αGalCer in culture. Proinflammatory lysophosphatidylphospholipids, lysophosphatidylcholine, and plasmanogen lysophosphatidylethanolamine are known lipid antigens for human NKT cells. The observation that DPPE-PEG150 completely abrogates αGalCer-induced aggravated atherosclerosis suggests a possible therapeutic approach to limit NKT cell activation in human atherosclerosis.

In summary, we have shown that targeting NKT cells during the development of atherosclerosis as well as the progression of established atherosclerosis with CD1d-dependent lipid antagonist effectively ameliorates atherosclerosis in hyperlipidemic ApoE−/− mice by preventing accumulation of necrotic cells/debris and associated inflammation. Our findings suggest that such targeting using agents, such as DPPE-PEG, in combination with anti-hyperlipidemic agents may further improve clinical outcomes in atherosclerosis.

Supplementary material
Supplementary material is available at Cardiovascular Research online.

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