

CD1d-dependent Activation of NKT Cells Aggravates Atherosclerosis

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Abstract

Adaptive and innate immunity have been implicated in the pathogenesis of atherosclerosis. Given their abundance in the lesion, lipids might be targets of the atherosclerosis-associated immune response. Natural killer T (NKT) cells can recognize lipid antigens presented by CD1 molecules. We have explored the role of CD1d-restricted NKT cells in atherosclerosis by using apolipoprotein E-deficient (apoE^{-/-}) mice, a hypercholesterolemic mouse model that develops atherosclerosis. ApoE^{-/-} mice crossed with CD1d^{-/-} (CD1d^{-/-}apoE^{-/-}) mice exhibited a 25% decrease in lesion size compared with apoE^{-/-} mice. Administration of α -galactosylceramide, a synthetic glycolipid that activates NKT cells via CD1d, induced a 50% increase in lesion size in apoE^{-/-} mice, whereas it did not affect lesion size in apoE^{-/-}CD1d^{-/-} mice. Treatment was accompanied by an early burst of cytokines (IFN γ , MCP-1, TNF α , IL-2, IL-4, IL-5, and IL-6) followed by sustained increases in IFN γ and IL-4 transcripts in the spleen and aorta. Early activation of both T and B cells was followed by recruitment of T and NKT cells to the aorta and activation of inflammatory genes. These results show that activation of CD1d-restricted NKT cells exacerbates atherosclerosis.

Key words: α -galactosylceramide • cytokines • inflammation • apolipoprotein E • mice

Introduction

Atherosclerosis is a slowly progressive disease that develops at sites of lipid accumulation in large- and medium-sized arteries, which can ultimately lead to infarction of the heart and brain. Studies in man and experimental models have shown an involvement of innate and adaptive immune mechanisms in the disease process (1, 2). Indeed, atherosclerotic lesions are infiltrated by activated T cells and professional APCs. It has been proposed that the cell-mediated immune response in atherosclerosis is directed against peptide antigens (2). However, given their abundance in the atherosclerotic plaque, lipid antigens may also be targets of disease-associated immune responses. The apolipoprotein E-deficient (apoE^{-/-}) mouse suffers from hypercholesterolemia and develops atherosclerosis spontaneously (3). The distribution

of the atherosclerotic lesions and their composition is similar to those observed in humans, and lesion formation proceeds from fatty streak to advanced atheroma with infiltrating inflammatory cells (2, 4).

Lipid antigens can be presented to T cells as part of a complex with the CD1 molecule, which is displayed on certain APCs (5). CD1d-restricted NKT cells constitute a unique subpopulation of T cells with surface expression of proteins commonly expressed by NK cells, such as the NK1.1 receptor (CD161) (6). NKT cells in mice express TCRs with a semiinvariant V α 14-J α 281 α -chain associated with either V β 2, β 7, or β 8 β -chains. Upon stimulation, these cells have the capacity to rapidly secrete large amounts of cytokines, including IL-4 and IFN γ and to cause bystander activation of NK cells, B cells, DCs (7), and CD4⁺ and CD8⁺ T cells (8). The natural ligand(s) for CD1d-restricted NKT cells remains to be characterized, but the synthetic glycolipid α -galactosylceramide (α GalCer) (9) has been shown to specifically activate these cells, influence the Th effector response (6), and modulate T cell-dependent

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autoimmune diseases (10–12). Atherosclerosis bears several features of such diseases and is affected by Th modulation (2, 13). An oligoclonal expansion of V α 14⁺ (also called V α 34s) cells has been observed in the lesions of apoE^{-/-} mice (14), and both CD1⁺ and NKT cells have been detected in human lesions (15, 16), underlining their possible involvement in the disease. These findings, and the capacity of NKT cells to produce large amount of cytokines, suggest that inflammation in the atherosclerotic plaque, and thus atherogenesis, might be modulated by CD1d-restricted NKT cells.

In this report, we studied the effect of CD1d-dependent stimulation of NKT cells on atherosclerosis by using α GalCer treatment in apoE^{-/-} and apoE^{-/-}CD1d^{-/-} mice. The present results show that CD1d deficiency reduces atherosclerosis, whereas α GalCer treatment significantly increases disease in apoE^{-/-} mice. Together, these data imply that CD1d-restricted NKT cell activation promotes development of atherosclerosis and suggests that lipid antigen presentation may be an important contributor to the pathogenesis of the disease.

Materials and Methods

Animals. Double deficient apoE^{-/-}CD1d^{-/-} mice were generated by crossing CD1d^{-/-} mice (17), backcrossed at least six times to the C57BL/6J background, with apoE^{-/-} mice (Taconic M&B). The offspring was intercrossed to produce mice with homozygous deletion in both apoE and CD1d genes (apoE^{-/-}CD1d^{-/-}). For experiments, 5-wk-old female apoE^{-/-} and apoE^{-/-}CD1d^{-/-} mice were injected twice a week with PBS (controls) or α GalCer (Kirin Brewery Company). The first injection was performed i.v. and the following i.p. For the long-term protocol (20 injections), 12 mice per group were used. For shorter protocols (one, three, or five injections), groups of five to six mice received α GalCer and groups of three to five mice received PBS. Animals were kept on chow diet, and all experiments were approved by the institutional ethical committee for animal welfare.

Sample Preparation and Lesion Analysis. Animals were anesthetized with carbon dioxide and killed by exsanguination through cardiac puncture. After vascular perfusion with sterile RNase-free PBS, the heart, spleen, and liver were removed. The descending aorta was immediately and carefully dissected free from surrounding tissue under a dissection microscope and snap frozen for mRNA analysis. Lipoprotein profiles were determined in serum by fast protein liquid chromatography (18). The heart and proximal aorta were embedded in OCT compound (Tissue-tek) and frozen. 10- μ m cryosections of the aortic root were analyzed for lesion size. Lesion size was defined as the cross section surface area of Oil Red O staining within the aortic intima plus media. Mean lesion size for each mouse was calculated from measurement of cryosections taken from every 100 μ m of the first 600 μ m in the ascending aorta, starting from the aortic cusps. Sections were also used for immunohistochemistry to identify I-A^b (KH74; BD Biosciences), VCAM-1 (429; BD Biosciences), and α SM-actin (A5691; Sigma-Aldrich). The expression of VCAM-1 was quantitated by dividing the stained surface area by the total surface area of the vessel. The expression of I-A^b was quantitated by dividing the number of I-A^b⁺ cells by the total number of hematoxylin-stained cells per lesion. All histological analyses were done in a blinded fashion.

Flow Cytometry. Spleen and hepatic mononuclear cells were prepared as described previously (13, 19). NKT cells were detected either by immunofluorescent double staining with PE-anti-NK1.1 (PK136) and FITC-anti-TCR β (H57–597) antibodies or double staining with α GalCer-loaded CD1d-dimer (PE-labeled) (Becton Dickinson) and FITC-anti-TCR β . Activated T cells were detected by staining spleen mononuclear cells with PE-anti-CD69 (H1–2F3) and FITC-anti-TCR β antibodies, whereas activated B cells were detected by PE-anti-CD19 (1D3) and FITC-anti-B7.2 (GL1) antibodies. All antibodies were from BD Biosciences, and the analyses performed on a FACSCalibur[®] (Becton Dickinson).

Cytometric Bead Array. IFN γ , MCP-1, TNF α , IL-2, IL-4, IL-5, IL-6, IL-10, and IL-12 concentrations in sera were analyzed with the Mouse Th1/Th2 Cytokine (no. 551287) and the Mouse Inflammation (no. 552364) Cytometric Beads Array kits from BD Biosciences according to the manufacturer's instructions. Analyses were run on a FACSCalibur[®].

RNA Preparation and RT-PCR. RNA was prepared from spleen and aorta after homogenizing tissue in FastRNA[®] Pro Green (Q-Biogene) with equal volumes of RLT lysis buffer (QIAGEN) and phenol-CHISAM (Sigma-Aldrich). Purification of total RNA was performed using the RNeasy system (QIAGEN) including a DNase step. RNA concentration and quality were assessed on a Bioanalyzer capillary electrophoresis system (Agilent). Real-time PCR was performed in a TaqMan 7700 (Applied Biosystems) according to the manufacturer's instructions. cDNA was synthesized from total RNA using Superscript II (Invitrogen).

Statistics. Data are presented as mean \pm SEM. Differences between the means were evaluated by using the Mann-Whitney test and were considered significant when $P < 0.05$.

Online Supplemental Material. The supplemental Materials and Methods (available at <http://www.jem.org/cgi/content/full/jem.20030997/DC1>) provides details on the real-time RT-PCR analyses.

Results and Discussion

Two approaches were used in order to determine the role of the CD1d-NKT cells in atherosclerosis: generation of double deficient apoE^{-/-}CD1d^{-/-} mice and specific NKT cell stimulation by α GalCer treatment. Total cholesterol and triglyceride levels did not differ between apoE^{-/-}CD1d^{-/-} double deficient and apoE^{-/-} single deficient mice, treated or not with α GalCer, nor did plasma lipoprotein profiles determined by fast protein liquid chromatography (not depicted).

ApoE^{-/-}CD1d^{-/-} mice exhibited significantly smaller atherosclerotic lesions in the aortic root than apoE^{-/-} single knockout mice (74,638 \pm 5.9 μ m² versus 100,548 \pm 5.6 μ m²; $P = 0.0087$) (Fig. 1, a–c). This was apparent throughout the aortic root (Fig. 1 b). When CD1d-restricted NKT cells were activated by administration of α GalCer over a 10-wk period, lesion size increased by 50% in apoE^{-/-} mice compared with PBS-treated control mice (151,276 \pm 9,377 μ m² versus 100,548 \pm 5,572 μ m²; $P < 0.0001$). In apoE^{-/-}CD1d^{-/-} mice, α GalCer did not affect lesion size (Fig. 1, a and c), demonstrating that the effect of α GalCer on atherosclerosis depends on CD1d. Together, these findings indicate that CD1d-dependent activation of NKT cells aggravates

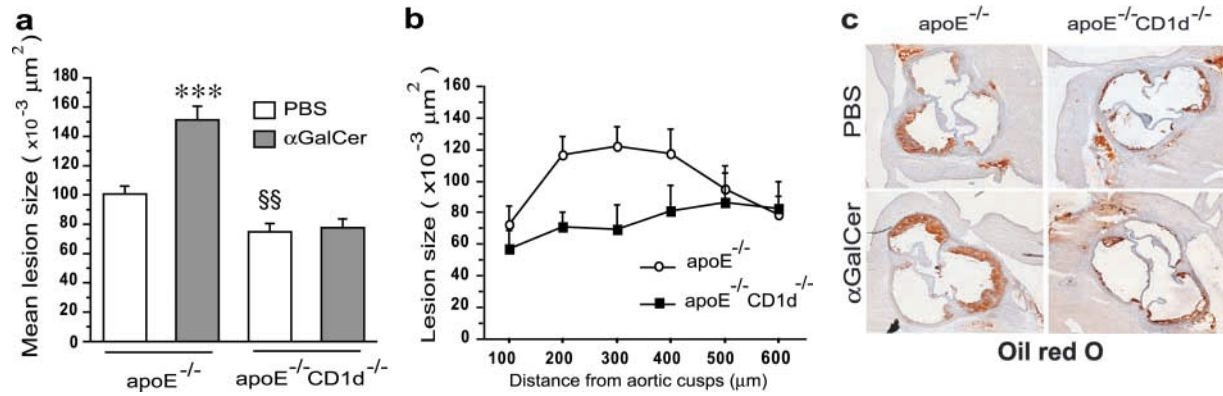


Figure 1. Effects of CD1d deficiency and α GalCer treatment on atherosclerosis. 5-wk-old female apoE^{-/-} and apoE^{-/-}CD1d^{-/-} mice were injected twice a week for 10 wk with α GalCer or PBS and killed 48 h after the last injection ($n = 12$ for each group). (a) Mean lesion size in Oil Red O–stained aortic root sections. Mean \pm SEM (** $P < 0.001$ versus apoE^{-/-}-PBS and all apoE^{-/-}CD1d^{-/-}; §§ $P < 0.01$ versus apoE^{-/-}-PBS mice). (b) Lesion size at every 100 μ m for the first 600 μ m of the aortic root in apoE^{-/-} and apoE^{-/-}CD1d^{-/-} mice. (c) Representative Oil Red O–stained cryosections of aortic roots (magnification $\times 50$).

atherosclerosis and that lack of CD1d, the restriction element for presentation of lipid antigens to NKT cells, leads to reduced lesions in a mouse model of human atherosclerosis.

To assess inflammatory activation, lesions in the aortic root were stained for the adhesion molecule VCAM-1 and the MHC class II protein I-A^b. VCAM-1 was expressed in the lesion and in the media underneath the lesion (Fig. 2, a–c). α SM-actin staining of adjacent aortic root sections confirmed that VCAM-1 was mainly expressed by smooth muscle cells (not depicted). I-A^b was expressed by inflam-

matory cells in the lesions (Fig. 2, d and e). ApoE^{-/-}CD1d^{-/-} mice expressed significantly less VCAM-1 than apoE^{-/-} mice ($P = 0.027$) (Fig. 2 a). The proportion of I-A^b-expressing cells did not differ between apoE^{-/-} and apoE^{-/-}CD1d^{-/-} mice (Fig. 2 d); however, the decrease in lesion area in the CD1d^{-/-} mice resulted in a reduced number of I-A^b cells per section when compared with apoE^{-/-} mice. α GalCer treatment increased VCAM-1 and I-A^b expression in apoE^{-/-} mice ($P = 0.036$) but not in apoE^{-/-}CD1d^{-/-} mice (Fig. 2, a–e), implying that α Gal-

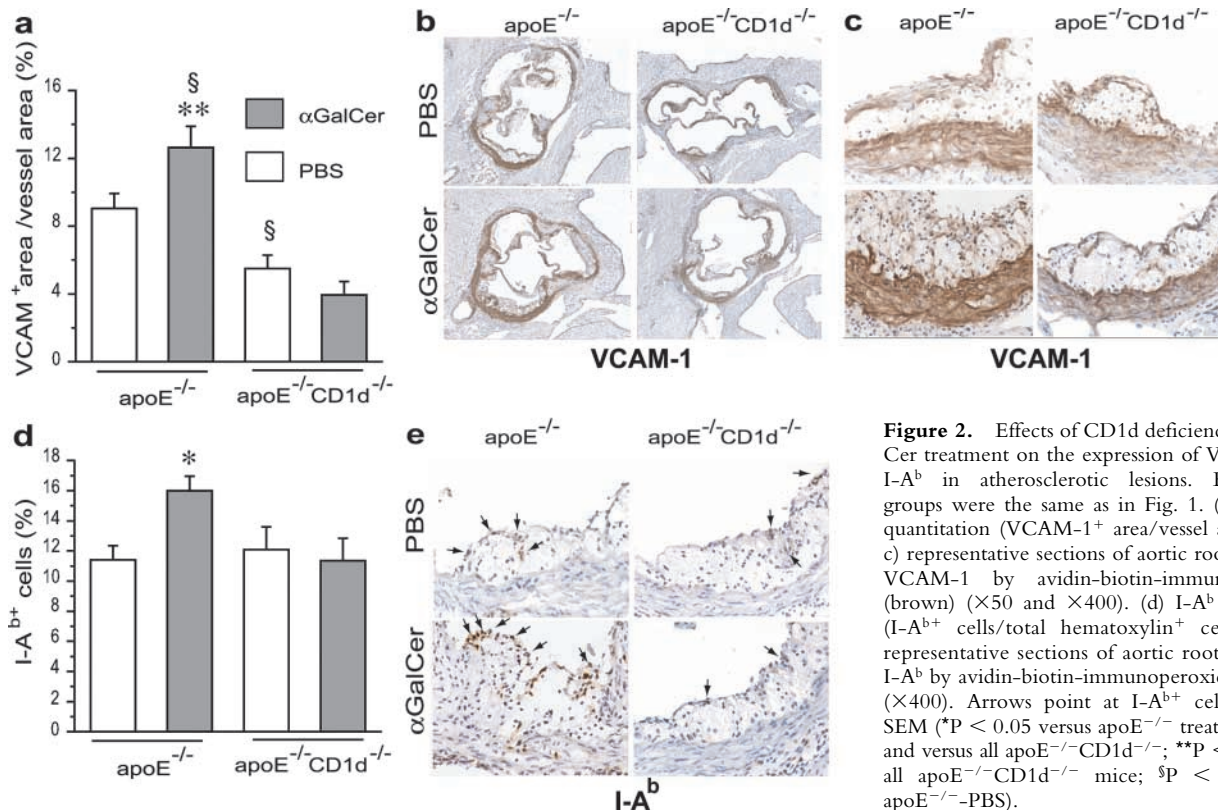


Figure 2. Effects of CD1d deficiency and α GalCer treatment on the expression of VCAM-1 and I-A^b in atherosclerotic lesions. Experimental groups were the same as in Fig. 1. (a) VCAM-1 quantitation (VCAM-1⁺ area/vessel area); (b and c) representative sections of aortic root stained for VCAM-1 by avidin-biotin-immunoperoxidase (brown) ($\times 50$ and $\times 400$). (d) I-A^b quantitation (I-A^b⁺ cells/total hematoxylin⁺ cells) and (e) representative sections of aortic roots stained for I-A^b by avidin-biotin-immunoperoxidase (brown) ($\times 400$). Arrows point at I-A^b⁺ cells. Mean \pm SEM (* $P < 0.05$ versus apoE^{-/-} treated with PBS and versus all apoE^{-/-}CD1d^{-/-}; ** $P < 0.01$ versus all apoE^{-/-}CD1d^{-/-} mice; § $P < 0.05$ versus apoE^{-/-}-PBS).

Cer induction of these genes was dependent on CD1d-restricted NKT cells.

NKT cell activation is likely to enhance activation of macrophages, endothelial cells, and other cells capable of secreting proinflammatory cytokines. Such a cascade may be responsible for the increased expression of VCAM-1 and I-A^b observed in α GalCer-treated mice. VCAM-1 expression by vascular smooth muscle cells is a characteristic feature of atherosclerosis, where it reflects inflammatory activation of lesion cells; however, its role in the recruitment and activation of inflammatory cells remains unclear. Endothelial VCAM-1 expression contributes to atherosclerosis by promoting recruitment of mononuclear cells to forming lesions (20).

An early burst of inflammatory cytokines was detected in sera after an injection of α GalCer; both typical Th1 (IFN γ , TNF α , IL-2) and Th2 (IL-4, IL-5) cytokines were increased as well as IL-6 and MCP-1 (Fig. 3 a). However, neither IL-10, which has antiinflammatory and atheroprotective properties (18, 25), nor IL-12 was detected in any of the groups (not depicted). The increase was remarkable, e.g., 5,000-fold for IFN γ and 250-fold for MCP-1. By-stander activation of T and B cells was registered by an increased number of cells double positive for CD69/TCR β and B7.2/CD19, respectively (Fig. 3 b). This early burst of cytokines might explain the increased expression of VCAM-1, which can be induced by proinflammatory cytokines, and I-A^b, which is induced by IFN γ . The increased levels of circulating MCP-1 might be significant for the exacerbated lesion development in α GalCer-treated mice, since this chemokine has important proatherogenic effects (21, 22). High levels of IL-6 could also have effects on atherosclerosis, since early lesions in apoE^{-/-} mice are exacerbated by recombinant IL-6 (23). Elevated circulating IL-6 concentrations is correlated with increased risk of coronary and peripheral atherosclerosis in man (24). After repeated injections, serum levels of cytokines fell below the detection limit, and no signs of increased T or B cell activation could be detected anymore. Real-time RT-PCR analysis (see Supplemental Materials and Methods, available at <http://www.jem.org/cgi/content/full/jem.20030997/DC1>) of spleen mRNA confirmed the increase in IFN γ (85-fold) and IL-4 (25-fold) mRNA in mice injected once with α GalCer (Fig. 3 c) and demonstrated that increased IFN γ and IL-4 expression in the spleen was still detectable after repeated injections (Fig. 3 c).

Direct evidence for a proatherogenic action of IFN γ has been provided by several studies in gene-targeted mice (2, 26), and analysis of human lesions has revealed expression of IFN γ by activated immune cells (2). Therefore, it is likely that the early burst of IFN γ upon α GalCer treatment contributed to the proatherogenic effect observed in our study. The increased expression of IFN γ -inducible I-A^b protein in aortic lesions and the demonstration of elevated aortic IFN γ mRNA (see below) in α GalCer-treated mice support this notion. The effects of the sustained production of IL-4 are more difficult to interpret since the role of IL-4

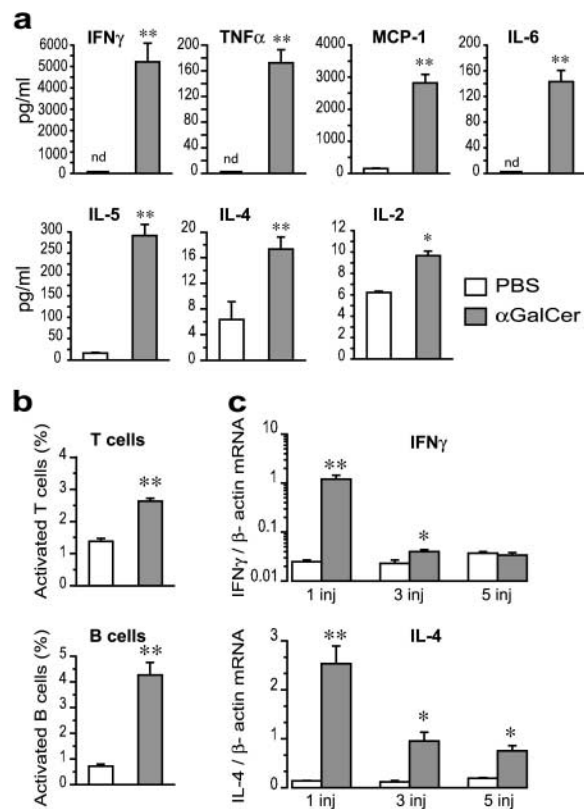


Figure 3. Systemic effects of α GalCer in apoE^{-/-} mice. 5-wk-old apoE^{-/-} mice were injected one, three, or five times, twice a week, with α GalCer or PBS. The first injection was i.v. ($n = 6$ for α GalCer and $n = 5$ for PBS) and the following ones i.p. ($n = 5$ for α GalCer and $n = 3$ for PBS). Mice were killed 12 h after the last injection. (a) Serum levels of cytokines after one injection of α GalCer or PBS. In control (PBS) mice, levels of IFN γ , TNF α , and IL-6 were not detectable (nd). (b) Activated spleen T (CD69⁺TCR $\alpha\beta$ ⁺) and B (CD19⁺B7.2⁺) cells determined by flow cytometry. (c) IFN γ and IL-4 mRNA in spleens measured by quantitative real-time RT-PCR and normalized to β -actin mRNA after one, three, and five injections. The values for IFN γ are represented in a logarithmic scale. Mean \pm SEM (* $P < 0.05$; ** $P < 0.01$ versus PBS-injected mice).

in atherosclerosis is still under debate (24). However, our data show a correlation between increased IL-4 secretion and increased lesion size. These findings are in line with those in a recent study of atherosclerosis in double-deficient apoE^{-/-}IL-4^{-/-} mice (27).

α GalCer injections led to dramatic systemic changes in the NKT cell population. When comparing apoE^{-/-} mice injected once or repeatedly with α GalCer, a transient decrease followed by recovery was observed for TCR⁺NK1.1⁺ cells in the spleen (Fig. 4 a) and liver (not depicted). This could either be due to activation-induced apoptosis or down-regulation of the NK1.1 receptor. To address this issue, NKT cells were also detected using CD1d-dimer staining in mice injected once i.v. with α GalCer and killed 12 or 72 h later. In the spleen, activation through α GalCer injection led to an initial reduction of NKT cells, but after 72 h the spleen had five times more NKT cells than spleens from PBS-injected controls (Fig. 4 b). In the liver, the NKT cell

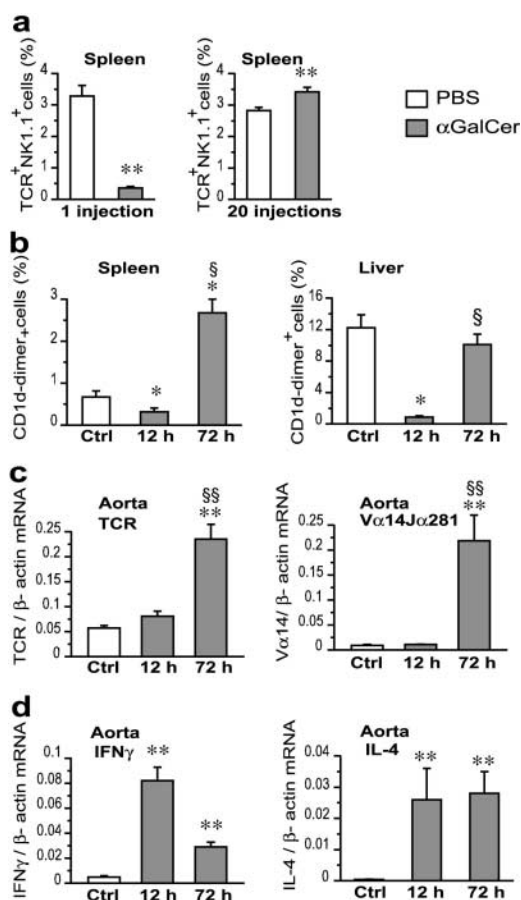


Figure 4. Effect of α GalCer injection on cytokine and TCR transcripts in spleen and aorta of apoE^{-/-} mice. (a) Proportion of spleen cells double positive for TCR β and NK1.1 in mice injected once and killed at 12 h and in mice injected 20 times, starting at 5 wk of age, and killed 48 h after the last injection. FACS[®] data expressed as the percentage of forward/side scatter-gated lymphocytes. (b) CD1d-dimer⁺ NKT cells in spleen and liver of 5-wk-old mice injected once i.v. with PBS (Ctrl) ($n = 6$) or α GalCer and killed 12 h ($n = 5$) or 72 h ($n = 6$) later. FACS[®] data expressed as the percentage of forward /side scatter-gated lymphocytes. (c and d) Levels of total TCR β and V α 14J α 281- specific TCR mRNA (c) and of IFN γ and IL-4 mRNA (d) in the aorta of mice treated with PBS or α GalCer as described for panel b. Real-time RT-PCR data are expressed as relative mRNA units normalized to β -actin mRNA. Mean \pm SEM (* $P < 0.05$; ** $P < 0.01$ versus PBS-injected mice; § $P < 0.05$; §§ $P < 0.01$ versus α GalCer-injected mice, killed at 12 h).

population decreased 12 h after the injection but returned to normal levels after 72 h (Fig. 4 b).

The local NKT cell response in the aorta was detected by TCR mRNA analysis (see Supplemental Materials and Methods, available at <http://www.jem.org/cgi/content/full/jem.20030997/DC1>). Both total TCR mRNA and NKT cell-specific V α 14-J α 281 transcripts were already present in the aorta of PBS-injected apoE^{-/-} control mice (Fig. 4 c). This is in line with our previous finding of V α 14 (also called V α 34s) when analyzing TCR transcripts in advanced lesions of apoE^{-/-} mice (14). After one i.v. injection of α GalCer, an increase in TCR mRNA in the aorta could be detected as early as 12 h after injection and continued to in-

crease at 72 h. NKT cell-specific V α 14-J α 281 mRNA increased dramatically 72 h after treatment also. IFN γ and IL-4 mRNA increased in the aorta of α GalCer-injected mice (Fig. 4 d). This rise peaked at 12 h after injection, and transcripts remained elevated after 72 h. The early increases in IFN γ and IL-4 mRNA in the aorta is likely due to the NKT cells already present in the lesion, which may in turn activate other cytokine-producing cells such as classical T cells and macrophages. Our data also suggest that systemic NKT cell activation induced by α GalCer injection leads to increased recruitment and, probably, activation of NKT cells in lesions. Such a scenario is also supported by the increased I-A^b and VCAM-1 expression in lesions of mice with α GalCer-activated NKT cells.

The finding of a proatherogenic role for NKT cells suggests that antigen-specific activation of these cells could contribute to disease development. The natural antigens for CD1d-restricted NKT cells are still unknown, despite numerous studies, but are thought to include lipid antigens. Atherosclerosis is strongly linked to lipid metabolic disturbances, and therefore our results suggest that lipid antigens may be involved in the disease process (2, 28). Further studies will be needed to identify such disease-related lipid antigens, clarify the precise role of NKT cells in the different phases of disease development, and determine the role of the CD1d-NKT cells in human atherosclerosis. In conclusion, controlling the activation state of NKT cells may represent a new therapeutic approach for atherosclerosis.

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