Deficiency of invariant Vα14 natural killer T cells decreases atherosclerosis in LDL receptor null mice

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1. Introduction

Atherosclerosis is an inflammatory vascular disease that is now known to involve components of both the innate and acquired immune systems.1–4 Studies aimed at defining the role of distinct populations of lymphocytes in the development of atherosclerosis have indicated that natural killer (NK) cells and T helper 1 (Th1) cells are proatherogenic,5–9 whereas Th2 cells and B cells are antiatherogenic.7,8,10,11 These findings are further supported by research which has found that cytokines released from NK cells and Th1 cells [e.g. interferon (IFN)-γ and interleukin (IL)-6] are proatherogenic,9,12–16 and that among cytokines released by Th2 cells, IL-10 is antiatherogenic.17–21

Lipid antigens are presented to unique groups of T cells as part of a complex with the non-classical, non-polymorphic MHC class I b molecule CD1d displayed on certain antigen presenting cells such as dendritic cells and monocyte-derived macrophages.22 The ability of one unique subset of T cells to interact with CD1d results from the expression on these cells of a highly biased, evolutionarily conserved T cell receptor (TCR) consisting of an invariant α-chain (a Vα14 segment joined to a Jα18 segment) that pairs preferentially with one of three β-chains (Vβ8.2, Vβ-7, and Vβ-2).23,24 This unique subset of T cells also expresses the classical NK cell marker NK1.1 (CD161) and, as such, these T cells are known as invariant NKT cells25 or Vα14 NKT cells.26

Upon stimulation, all NKT cell populations have the capacity to exert immunoregulatory functions by releasing large amounts of inflammatory cytokines, including the
proatherogenic cytokine IFN-γ. This release of cytokines will in turn cause the activation of adjacent NK cells. B cells, conventional CD4+ and CD8+ T cells, as well as adjacent antigen presenting cells. Although the natural ligand(s) for CD1d-restricted NKT cells (including Vα14 NKT cells) still remain to be characterized, NKT cells have a strong response to and are selectively activated by the exogenous synthetic glycolipid α-galactosylceramide (α-GalCer), which binds specifically to CD1d. A number of recent studies have shown that α-GalCer specifically enhances atherosclerosis in apolipoprotein E null (ApoE−/−) mice and has a significant impact on the cytokine profile (including, but not limited to, IFN-γ) of these mice. Interestingly, not only have NKT cells been shown to present in the atherosclerotic lesions of recent studies have shown that CD1d-restricted NKT cells might play a participatory role in the atherogenic process. In this current study, we use Ldlr−/− mice to further examine the potential proatherogenic role of one unique population of NKT cells that recognizes CD1d; these cells are the Vα14 NKT and are reported to represent the largest population of NKT cells in the disease process. These findings, and the capacity of NKT cells to produce IFN-γ, suggest that CD1d-restricted NKT cells might play a participatory role in the atherogenic process. In this current study, we use Ldlr−/− mice to further examine the potential proatherogenic role of one unique population of NKT cells that recognizes CD1d; these cells are the Vα14 NKT cells and are reported to represent the largest population of CD1d-restricted NKT cells.

2. Methods

2.1 Animals and diet

Male Jα18−/− (formally called Jα281−/−) mice were a generous gift from Drs Malcolm S. Duthie and Stuart J. Kahn, Infectious Disease Research Institute, Seattle, WA, USA. Jα18−/− mice were found to reproduce normally and otherwise are healthy in appearance. As cited by Cui et al., the development of the lymphoid organs in Jα18−/− mice is macroscopically normal, and the numbers of total lymphocytes are not significantly different from Jα18+/+ mice with the exception of a complete loss of the Vα14 NKT subpopulation of NKT cells. Male Jα18−/− mice were bred with female Ldlr−/− mice originally obtained from The Jackson Laboratory, USA, and maintained in the Animal Care Facility at the University of Ottawa Heart Institute. Both strains of mice were previously shown to develop early-stage atherosclerotic lesions. Both genders of mice were used in this study, and all mice started the atherogenic diet just after being weaned at 4 weeks of age. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.2 Blood collection

Terminal blood samples were collected by puncture of the right ventricle. Blood was allowed to clot at room temperature for 30 min and then centrifuged at 1000 g for 25 min at 4°C.

2.3 Plasma cholesterol and lipoprotein profiles

See legend to Figure 1, and Supplementary material online.

2.4 Tissue collection

Mice were perfused with PBS via a cannula placed in the left ventricle, with perfusate drained from a severed right atrium. Hearts were separated from the aorta at the base, embedded in optimum cutting temperature medium, and snap-frozen on a metal plate that was cooled with liquid nitrogen.

2.5 Quantification of atherosclerotic lesions in tissue sections

The size of atherosclerotic lesions in the ascending aorta was determined from four Sudan IV stained serial sections, cut 10 μm thick and separated by 100 μm. Lesion analysis began with the first section of tissue that contained the ostia for the coronary arteries; region defining the boundary between the aortic sinus and ascending aorta. Using the Sudan IV staining as a guide, lesion area defined as intimal tissue within the internal elastic lamina was determined using Image-Pro Plus software (V6.2, Media Cybernetics, Silver Springs, MD, USA) on images that were created using a digital CoolSnap cf camera (Roper Scientific Inc., Duluth, GA, USA). The mean lesion area derived from the four serial sections was taken as the average lesion size for each animal as described previously.

![Figure 1](image-url)  
**Figure 1** Serum (60 μL) from male (A) and female (B) Ldlr−/− mice that were either Jα18+/+ (circular symbol) or Jα18−/− (square symbol) was resolved by size exclusion chromatography using a Superose 6 column. Total cholesterol concentrations were determined in fraction numbers 11–40, with each fraction having a total volume of 500 μL. Symbols represent the means and bars the SEM of values obtained from the serum of 5 mice/curve.
2.6 Quantification of atherosclerotic lesions in the aortic arch

The percentage of atherosclerotic lesion area covering the aortic arch in an en face preparation of the vessel was quantified as described previously. In this process, no lipophylic dye was used to aid in the visualization of discernable lesions.

2.7 Histological staining

It was performed as described previously, using sequential sections of the ascending aorta to detect neutral lipid, macrophages, CD3+ T cells, MHC class II, IFN-γ, and extracellular collagen (for more detail, see Supplementary material online). Since immunostaining for CD3 and MHC class II leads to discrete staining of definable cells, lesion-associated cells expressing either antigen were counted and the mean number of cells reported as described previously.

2.8 Quantification of the per cent lesion-associated lipid within macrophages

The lesion area staining positive for macrophages and for neutral lipid was quantified using Image-Pro Plus. An imprint was then made for each area and superimposed using Adobe Photoshop CS2 (Version 9.0.2). Both imprints are still distinguishable at this point, only the overlapping areas appeared darker. The superimposed image is then imported to Image-Pro Plus to measure the amount of overlap and the percentage of lipid associated within macrophages is calculated by dividing the overlapping area with the total area of lesion-associated lipid. It is important to note that each section used for the overlay of lesion-associated lipid and macrophage areas was only separated by 10 μm.

2.9 Laser capture microdissection and RNA extraction

Using laser capture microdissection (LCM), we were able to extract the atherosclerotic lesions from our control and experimental mice. The PixCell Ile LCM system was set at the following parameters: 30 μm laser spot size, 50 μm power, and 10 ms duration. Total RNA was extracted from dissected tissue using the Qiagen RNeasy Micro Kit (Qiagen) as per manufacturer’s instructions.

2.10 Quantitative real-time RT–PCR

Real-time RT–PCR was used to quantify transcription levels of IL-4, 2.10 Quantitative real-time RT–PCR was used to quantify transcription levels of IL-4, IL-10, IFN-γ, and extracellular collagen (for more detail, see Supplementary material online). Since immunostaining for CD3 and MHC class II leads to discrete staining of definable cells, lesion-associated cells expressing either antigen were counted and the mean number of cells reported as described previously.

2.11 Cytokine production assay

Splenocytes (2 x 10⁶) isolated from Ldlr⁻/⁻ mice that were either Jα18⁺/⁺ or Ldlr⁻/⁻ were incubated for 72 h in the presence or absence of 50 ng/mL of α-GalCer (Cedarside Laboratories, Burlington, ON, Canada) in RPMI 1640 medium supplemented with 10% FCS, 50 μM 2-mercaptoethanol, 2 mM glutamine, and antibiotics. Levels of IFN-γ and IL-4 in the supernatant were determined by ELISA (R&D Systems).

2.12 Statistics

Data analysis was performed using SigmaStat 2.03 software (SPSS Inc., Chicago, IL, USA). For each parameter, the mean and standard error of mean (SEM) were calculated. Statistical analysis between groups was by a One Way Analysis of Variance on Ranks with all pairwise multiple comparison procedures performed using the Dunn Method, after testing that the data complied with the constraints of parametric analysis. Values with P ≤ 0.05 were considered statistically significant.

3. Results

Compared with Jα18⁺/⁺ × Ldlr⁻/⁻ mice, deficiency of Vα14 NKT cells in Ldlr⁻/⁻ mice did not affect total serum-cholesterol concentrations (Table 1) or -cholesterol distribution between various fractions of lipoproteins (Figure 1). When compared with Jα18⁺/⁺ mice, loss of Vα14 NKT cells decreased lesion size by ~20 and 28% within the ascending aorta of both male (0.103 ± 0.009 mm² vs. 0.078 ± 0.007 mm², respectively, n = 9 per group; P = 0.021) and female (0.110 ± 0.008 mm² (n = 9) vs. 0.087 ± 0.006 mm² (n = 11), respectively; P = 0.026) mice (Figure 2A), and by 28 and 37% in the aortic arch of both males (5.9 ± 0.5% vs. 4.2 ± 0.6%, respectively, n = 9 per group; P = 0.037) and females (7.1 ± 1.0% (n = 11) vs. 4.4 ± 0.6% (n = 9), respectively; P = 0.038) mice (Figure 2B). When lesions of the ascending aorta were measured for the area occupied by Sudan IV staining and the area occupied by macrophage staining, loss of Vα14 NKT cells resulted in a significant reduction in the size of lipid staining and macrophage staining of said lesions (Table 2).

As a marker of lesion-associated foam cell development, we have devised a computer-assisted way of calculating reverse set of cytokine-specific PCR primers as described by Giulietti et al., and for IFN-γ, IL-4, and IL-10 and as provided by Qiagen Life Sciences for β-actin (Quantitect Mm β-actin Assay). Resulting values were normalized to the β-actin values.

| Table 1 | Serum cholesterol values, quantification of lesion-associated cells expressing CD3, MHC class II, and lesion-associated IFN-γ, IL-4, and IL-10 mRNA concentrations |
|---------|-------------------------------------------------------------------------------------|
| Gender  | Jα18 genotype of Ldlr⁻/⁻ mice | Lesion-associated CD3 positively stained cells (n) | Lesion-associated MHC class II positively stained cells (n) | Final serum cholesterol values (n) | IL-4/β-actin mRNA ratio (n) x 10⁻³ | IL-10/β-actin mRNA ratio (n) x 10⁻³ | IFN-γ/β-actin mRNA ratio (n) x 10⁻³ |
| Male    | +/+  | 13.3 ± 1.6 (8) | 16.1 ± 1.8 (8) | 1748 ± 169 (9) | ND (6) | ND (6) | 15.1 ± 1.4 (6) |
|         | −/−  | 15.8 ± 2.0 (8) | 12.4 ± 1.3 (8) | 2202 ± 204 (9) | ND (7) | ND (7) | 2.0 ± 1.3* (7) |
| Female  | +/+  | 9.0 ± 1.3 (9)  | 15.6 ± 2.2 (9) | 1742 ± 60 (9)  | ND (5) | ND (5) | 28.3 ± 1.6 (5)  |
|         | −/−  | 10.0 ± 2.0 (11)| 16.5 ± 2.3 (11)| 1981 ± 112 (11)| ND (8) | ND (8) | 2.0 ± 0.6† (8)  |

ND, not detected.

*P = 0.05 vs. Jα18 +/+ males.
†P = 0.05 vs. Jα18 +/+ females.
the percentage of lesion-associated lipid (Sudan IV positive) contained within the corresponding area that stained positive for macrophages. By performing this type of analysis, we observed no significant difference in the percentage of neutral lipid associated within macrophages located in the ascending aortic lesions of both male and female Jα18+/+ vs. −/− mice (Table 2).

Detailed histological analysis indicated that all lesions from both male and female Jα18+/+ and −/− mice were at the early-stage of development, consisting mainly of macrophage-derived foam cells (Figure 3). Absent from these lesions was the presence of a definable necrotic core or fibrous cap, which would indicate the existence of complex atherosclerotic plaques.

By coupling the techniques of LCM and quantitative real-time RT–PCR, we noted a significant reduction (85–93%) in complex atherosclerotic plaques or fibrous cap, which would indicate the existence of these lesions was the presence of a definable necrotic core represented as circles (Jα18+/+), whereas the mean lesion size of each group of mice is presented as a single horizontal line (to the right of each symbol grouping) with error bars denoting SEM. *P-values showing significant differences between groups are noted.

![Figure 2](image)

**Figure 2** Figures show (A) mean lesion size and (B) per cent lesion area. The extent of atherosclerotic lesion development in the (A) ascending aorta and the (B) aortic arch of male and female Ldlr−/− mice either +/+ or −/− for the Jα18 gene was determined as described under Section 2. Values of individual mice are represented as circles (Jα18+/+) and triangles (Jα18−/−), whereas the mean lesion size of each group of mice is presented as a single horizontal line (to the right of each symbol grouping) with error bars denoting SEM. *P-values showing significant differences between groups are noted.

| Gender | Jα18 genotype of Ldlr−/− mice | Lipid-positive stained area (mean value mm²) (n) | Macrophage-positive stained area (mean value mm²) (n) | % overlap of lipid and macrophage stained areas (n) |
|--------|--------------------------------|-----------------------------------------------|--------------------------------------------------|-----------------------------------------------------|
| Male   | +/+                            | 0.0997 ± 0.0014 (9)                           | 0.1028 ± 0.0127 (9)                              | 93.3 ± 3.2 (9)                                      |
|        | −/−                            | 0.0722 ± 0.0045* (9)                           | 0.0750 ± 0.0048* (9)                             | 95.9 ± 1.2 (9)                                      |
| Female | +/+                            | 0.0997 ± 0.0109 (9)                           | 0.1012 ± 0.0116 (9)                              | 93.4 ± 4.2 (9)                                      |
|        | −/−                            | 0.0707 ± 0.0047** (11)                         | 0.0733 ± 0.0049 (11)                             | 96.2 ± 1.7 (11)                                    |

Table 2 Lipid-positive stained area values, macrophage-positive stained area, and % overlap of lipid and macrophage stained areas

**Table 1**

Treatment of splenocytes isolated from Jα18+/+ × Ldlr−/− mice with α-GalCer caused a significant increase in the secretion of both IFN-γ and IL-4 by these cells (Figure 5). However, α-GalCer treatment of splenocytes from Jα18−/− mice did not cause a significant increase in the secretion of either IFN-γ or IL-4, and that the levels of both cytokines in the supernatant of splenocytes from Jα18−/− mice were not significantly different from control cells (spleenocytes isolated from Jα18+/+ mice) not exposed to α-GalCer.

**4. Discussion**

Invariant CD1d-restricted NKT cells function as a ‘bridge’ between innate and adaptive immunity. In this capacity, NKT cells have been shown to regulate numerous immune responses involving conditions such as autoimmune disease, tumour surveillance, and infection caused by microbial pathogens. Evidence gathered from the analysis of both human and mouse atherosclerotic lesions suggests the presence of NKT cells in this disease as well. First, nested RT–PCR has detected the invariant Vα14Jα18 TCR α-chain rearrangement in atherosclerotic lesions from Apoe−/− mice. Secondly, all four CD1 proteins (a, b, c, and d) have been detected in human atherosclerotic...
Figure 3 Representative histological sections from a region where the aortic sinus becomes the ascending aorta of a female ($A$, $C$, $E$, $G$, $I$) $J_{a}18^+/+$ $\times$ Ldir$^{-/-}$ mouse and a female ($B$, $D$, $F$, $H$, $J$) $J_{a}18^+/+$ $\times$ Ldir$^{-/-}$ mouse. ($A$, $B$) Sudan IV for neutral lipids, ($C$, $D$) monoclonal antimouse MHC class II, ($E$, $F$) rabbit antisera to mouse macrophages, ($G$, $H$) purified hamster antimouse CD3$^+$ E molecular complex antibody, and ($I$, $J$) Gomori trichrome to detect collagen. Magnification: ($A$, $B$) $\times 40$; ($C$–$M$) $\times 200$. ($A$, inset) and ($B$, inset) area contained in $C$, $E$, $G$, $I$, and $D$, $F$, $H$, $J$, respectively. Arrows indicate individual cells that are staining positive for: ($C$, $D$) MHC class II; and ($G$, $H$) CD3$^+$.

Figure 4 Representative immunohistological sections staining specifically for IFN-$\gamma$ using atherosclerotic lesions of a female ($A$) $J_{a}18^+/+$ $\times$ Ldir$^{-/-}$ mouse and a female ($B$) $J_{a}18^+/+$ $\times$ Ldir$^{-/-}$ mouse. ($C$) Representative control lesion, where staining was performed in the absence of the primary antibody against IFN-$\gamma$ and the presence of the biotinylated secondary goat anti-rat antibody. Black arrows indicate areas of positive staining for IFN-$\gamma$. White arrows indicate areas of minor background staining which appear to be held to the internal elastic lamina.
lesions associated with both macrophage-derived foam cells and dendritic cells. Finally, a number of groups have recently shown that CD1d deficiency reduces lesion development in both Apoe-/- and Ldlr-/- mice. Together, this evidence strongly implicates the participation of NKT cells in the disease process, but it does not identify the specific subpopulation of NKT cell(s) that directly promote lesion formation. By superimposing the deficiency of the Ja18 gene onto mice that are already susceptible to atherosclerosis, we show in this current study that loss of functionally active Va14 NKT cells significantly reduces the formation of early-stage lesions in both genders of mice, without affecting either serum total-cholesterol values or lipoprotein-cholesterol distributions. Furthermore, by coupling the techniques of LCM with quantitative real-time RT-PCR and by performing immunohistochemistry, we also made the important finding that expression of the proatherogenic cytokine IFN-γ is significantly reduced in lesions from Ja18-/- mice.

By using CD1d-/- mice, which effectively depletes the mouse of all functional CD1d-restricted NKT cells, Nakai et al., Major et al., and Aslanian et al., explored the collective role of CD1d-restricted NKT cells in the development of atherosclerosis in both the Apoe-/- and the Ldlr-/- murine backgrounds. The Va14Ja18 NKT cells are not the only CD1d-restricted NKT cells that are rendered functionally deficient in the CD1d-/- mouse. However, in C57BL/6 mice, targeted deletion of the Ja18 gene results in the selective depletion of Va14 NKT cells without affecting the population of other CD1d-dependent and CD1d-independent NKT cells, or the populations of NK cells and conventional T and B cells.

By making Ldlr-/- mice deficient of Va14Ja18 NKT cells, we saw a significant reduction in lesion formation in this model of atherosclerosis, which highlights the significant role this subpopulation of NKT cells plays in lesion formation. If we focus our attention on the study by Aslanian et al., who chose to study CD1d deficiency on the Ldlr-/- background, we find that the results of our study differ from those of Aslanian et al. where we found that compared with CD1d deficiency, deficiency of Ja18 resulted in a significant, yet much smaller reduction in atherosclerosis (20–28% in our study vs. 47% in the study by Aslanian et al., based on analysis within the ascending aorta). These are very important differences between our study and that of Aslanian et al. and, as such, point out that Va14Ja18 NKT cells are most likely not the only CD1d-restricted NKT cells contributing to lesion formation as at least one other cell type, which has yet to be identified, is contributing an equal share to the promotion of lesion development. This comparison highlights that our current study has shown a specific subpopulation of NKT cells to be proatherogenic, and it is precisely this unique finding that sets our current work apart from these earlier cited studies. As other subpopulations of NKT cells are identified, such as that described by Skold et al., targeted deletion of these unique NKT cells within a mouse model of atherosclerosis will allow for the examination of other subpopulations of NKT cell with respect to their own contribution to lesion progression. Although this type of systematic determination of the per cent contribution of other CD1d-restricted NKT cells to lesion development is greatly encouraged, especially given our findings compared with those of Aslanian et al., such a task still remains infinitely too complex since the existing criteria for the populations of other NKT cells are still only broadly defined and therefore the possible number of specific NKT cell types too great. For example, other CD1d-dependent NKT cells, the non-Va14 CD1d-dependent NKT, includes Va3.2-Ja9/Vb8, Va8/Vb8, and other species, whereas the CD1d-independent NKT cells include those that are CD49b₁ or those that are NK1.1- and have diverse TCRs and can either be CD8+ or CD4+.

In order to truly define the participatory role of Va14 NKT cells in promoting atherosclerosis, it is important to know whether this particular immune cell resides within atherosclerotic lesions. Two independent groups have in fact confirmed the presence of Va14-Ja18 transcripts within atherosclerotic lesions of two different mouse models of the disease. Nakai et al. showed that the development of atherosclerosis in wild type or Apoe-/- mice fed an atherogenic diet was associated with the presence of Va14-Ja18 transcripts within the developing atheroma. In the second study, Aslanian et al. detected Va14-Ja18 TCR mRNA in lesion-containing segments of the aortic arch from Ldlr-/- mice. Taken together, these independent findings are able to place Va14 NKT cells at the site of lesion formation.

The noted reduction in both splenocyte-secreted and lesion-associated IFN-γ concentrations in this study upon Va14 NKT cell deficiency provides strong mechanistic evidence that activated Va14 NKT cells promote atherosclerosis by producing and/or by mediating the production of IFN-γ by other immune cells, such as NK cells at the site of a developing lesion. In vitro, IFN-γ has been shown to be proatherogenic by promoting the processes of lipoprotein oxidation,
macrophage-derived foam cell formation, and immune cell activation. We and others have previously shown that administration of exogenous IFN-$\gamma$ will accelerate early-stage atherosclerotic lesion development in ApoE$^{-/-}$ mice and that endogenous IFN-$\gamma$ promotes lesion development in both ApoE$^{-/-}$ mice and Ldlr$^{-/-}$ mice. The identification of Vx14 NKT cells within atherosclerotic lesions of mice also validates our IFN-$\gamma$ data as it relates to Vx14 NKT cells as being the most likely mediator for the production of this pro-inflammatory cytokine and that IFN-$\gamma$, produced locally at the site of lesion development, is a key contributor to lesion formation as noted in earlier studies that used IFN-$\gamma$ null mice placed on both the Ldlr$^{-/-}$ and ApoE$^{-/-}$ backgrounds. Future studies will also need to examine whether Vx14 NKT cells promote not only early-stage, but also late-stage lesion development, and whether Vx14 NKT cells accelerate disease by affecting the activation status of additional populations of lesion-associated immune cells such as conventional T cells, NK cells, and/or the process of macrophage-derived foam cell formation by interrupting the ability of foam cells to efflux cholesterol to HDL via ABCA1. Interestingly, we discovered no significant difference in the percentage of neutral lipid associated within macrophages located in the ascending aortic lesions of Jα18$^{+/+}$ vs. $^{-/-}$ mice, despite the fact that the overall size of atherosclerotic lesions in the same area of the ascending aorta of the Jα18$^{-/-}$ mice were significantly smaller than their Jα18$^{+/+}$ littermates. These findings could possibly suggest that Vx14 NKT cell deficiency reduced atherosclerotic burden by causing a net decrease in the population of lesion-associated macrophages, rather than a decrease in the ability of macrophages to undergo lipid-loading. The cellular target of IFN-$\gamma$ expressed by Vx14 NKT cells is currently an area of investigation in our laboratory as the identification of these target cells would be seen as an important step in the elucidation of this particular proatherogenic pathway.

Supplementary material

Supplementary material is available at Cardiovascular Research online.

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