CD4⁺ Natural Killer T Cells Potently Augment Aortic Root Atherosclerosis by Perforin- and Granzyme B-Dependent Cytotoxicity

Yi Li,* Kelly To,* Peter Kanellakis, Hamid Hosseini, Virginie Deswaerte, Peter Tipping, Mark J. Smyth, Ban-Hock Toh,† Alexander Bobik,† Tin Kyaw†

Rationale: CD4⁺ natural killer T (NKT) cells augment atherosclerosis in apolipoprotein E–deficient (ApoE)−/− mice but their mechanisms of action are unknown.

Objectives: We investigated the roles of bystander T, B, and NK cells; NKT cell–derived interferon-γ, interleukin (IL)-4, and IL-21 cytokines; and NKT cell–derived perforin and granzyme B cytotoxins in promoting CD4⁺ NKT cell atherogenicity.

Methods and Results: Transfer of CD4⁺ NKT cells into T- and B-cell–deficient ApoE−/−Rag2−/− mice augmented aortic root atherosclerosis by ≈75% that was ≈30% of lesions in ApoE−/− mice; macrophage accumulation similarly increased. Transferred NKT cells were identified in the liver and atherosclerotic lesions of recipient mice. Transfer of CD4⁺ NKT cells into T-, B-cell–deficient, and NK cell–deficient ApoE−/−Rag2−/−γC−/− mice also augmented atherosclerosis. These data indicate that CD4⁺ NKT cells can exert proatherogenic effects independent of other lymphocytes. To investigate the role of NKT cell–derived interferon-γ, IL-4, and IL-21 cytokines and perforin and granzyme B cytotoxins, CD4⁺ NKT cells from mice deficient in these molecules were transferred into NKT cell–deficient ApoE−/−Ja18−/− mice. CD4⁺ NKT cells deficient in IL-4, interferon-γ, or IL-21 augmented atherosclerosis in ApoE−/−Ja18−/− mice by ≈95%, ≈80%, and ≈70%, respectively. Transfer of CD4⁺ NKT cells deficient in perforin or granzyme B failed to augment atherosclerosis. Apoptotic cells, necrotic cores, and proinflammatory VCAM-1 (vascular cell adhesion molecule) and MCP-1 (monocyte chemotactic protein) were reduced in mice receiving perforin-deficient NKT cells. CD4⁺ NKT cells are twice as potent as CD4⁺ T cells in promoting atherosclerosis.

Conclusions: CD4⁺ NKT cells potently promote atherosclerosis by perforin and granzyme B–dependent apoptosis that increases postapoptotic necrosis and inflammation. (Circ Res. 2015;116:245-254. DOI: 10.1161/CIRCRESAHA.116.304734.)

Key Words: atherosclerosis ■ cell death ■ granzymes ■ inflammation ■ natural killer T cells ■ perforin

Atherosclerosis is a chronic inflammatory arterial disease driven by the immune system. Rupture of advanced vulnerable atherosclerotic lesions displaying abundant apoptosis and necrosis is responsible for heart attacks and strokes,1 which remain leading causes of global mortality. The adaptive immune system makes critical contributions to atherosclerosis development.2 Atherosclerotic lesions are reduced in apolipoprotein E–deficient (ApoE)−/−scid/scid mice lacking T and B cells.3 Adoptive transfer of CD4⁺ T cells4 or CD8 T cells5 aggravate atherosclerosis. Proatherogenic CD4⁺ T cells are included within the Th1 lineage; those related to the Th2 lineage can also exert proatherogenic effects,6 whereas regulatory T cells suppress atherosclerosis.6 Conventional B2 B cells are proatherogenic,7,8 whereas peritoneal B1a cells are atheroprotective.9

Natural killer T (NKT) cells are minor lymphocyte populations that accumulate in rupture-prone shoulder regions of advanced human atherosclerotic lesions.10,11 NKT cells are T
NKT cells secrete large amounts of cytokines, including interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), and IL-4, 17 and initiate bystander activation of γδ T cells. Conversely, NKT cells augment atherosclerotic development.21 Independently of T, B, and NK cells, NKT cells can secrete proinflammatory cytokines such as IFN-γ, IL-4, and IL-21 but are dependent on perforin and granzyme B–mediated cytotoxicity.

### Methods

#### Experiment Design and Animal Ethics

Male atherogenic mice were fed a high-fat diet (HFD) consisting of 21% butter fat and 0.15% cholesterol (specialty feeds) for 8 weeks, commencing at 6 weeks of age. At the end of the study mice were killed with an overdose of pentobarbitone (120 mg/kg IP), blood collected by cardiac puncture, and aortic sinus collected for histology and immunohistochemistry.

In adoptive transfer experiments, NKT cells (3×10^5) were purified by flow cytometric sorting and intravenously injected via the tail vein into 6-week-old B- and T-cell–deficient ApoE−/−Rag2−/− and lymphocyte-deficient ApoE−/−Rag2−/−γc−/− mice; CD4+ NKT cells from wild-type (WT), IFN-γ−/−, IL-4−/−, IL-21−/−, perforin, and granzyme B–deficient mice were intravenously injected into 6-week-old NKT cell–deficient ApoE−/−Jα181−/− mice. Controls received equivalent intravenous injections of PBS.

All animal experiments were approved by AMREP (Alfred Medical Research and Education Precinct) Animal Ethics Committee.

For detailed Materials and Methods, see the Online Data Supplement.

### Results

#### NKT Cells Can Promote Atherosclerosis Independently of T, B, and NK Cells

NKT cells may exert their effects by activating bystander CD4+ T cells, 33 CD8+ T cells, 20 and B cells34 that have been implicated in atherosclerotic development.3,7,35 To determine whether these cells are required for the proatherogenic action of NKT cells, we adoptively transferred 3×10^5 liver NKT cells into T- and B-cell–deficient ApoE−/−Rag2−/− mice followed by 8 weeks of a HFD. NKT cell transfer augmented atherosclerotic development by nearly doubling the size of atherosclerotic lesions assessed by intimal lesion area (P<0.05; Figure 1A). Accumulation of macrophages (1.5-fold) and lipid (0.8-fold) was increased (P<0.05; Figure 1A), indicating that neither T cells nor B cells were necessary for NKT cells to augment the development of atherosclerosis.

Because NKT cells can also activate NK cells, we next investigated whether proatherogenic effects of NKT cells are dependent on NK cell activation.36 Adoptive transfer of NKT cells into lymphocyte-deficient ApoE−/−Rag2−/− mice also augmented development of atherosclerosis by increasing intimal lesion area and macrophage and lipid accumulation (P<0.05; Figure 1B). NKT cell transfer into the ApoE−/−Rag2−/− or ApoE−/−Rag2−/−γc−/− mice did not affect very-low-density lipoprotein /low-density lipoprotein-lipid levels. A slight, but not statistically significant decreasing trend was observed in all cholesterols measured (P>0.05) apart from a significantly reduced HDL in ApoE−/−Rag2−/−γc−/− mice (P<0.05); however, the HDL reduction in ApoE−/−Rag2−/− mice did not reach statistically significance (P>0.05; Online Table I).

#### CD4+ NKT Cells Can Promote Atherosclerosis Independently of IFN-γ, IL-4, and IL-21 Production

NKT cells can secrete proinflammatory cytokines such as IFN-γ,36 which augment atherosclerotic development.37 Because we previously demonstrated that CD4+, and not double-negative NKT, cells augment atherosclerosis,38 we focused solely on CD4+ NKT cells. To determine whether CD4+ NKT cell–derived IFN-γ is required for their proatherogenic action, we transferred CD4+ NKT cells isolated from WT and

### Nonstandard Abbreviations and Acronyms

<table>
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<th>Abbreviation</th>
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<tr>
<td>ApoE</td>
<td>apolipoprotein E</td>
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<td>HFD</td>
<td>high-fat diet</td>
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<td>IFN</td>
<td>interferon</td>
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<td>IL</td>
<td>interleukin</td>
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<td>NKT cells</td>
<td>natural killer T cells</td>
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<td>TCRα</td>
<td>T-cell receptor α chain</td>
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<td>WT</td>
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For detailed Materials and Methods, see the Online Data Supplement.
IFN-γ−/− mice into NKT cell–deficient ApoE−/−Jα18−/− mice followed by feeding a HFD for 8 weeks. Similar to the effects of WT CD4+ NKT cell transfer to ApoE−/−Jα18−/− mice, transfer IFN-γ−/−–deficient CD4+ NKT cells increased lesion size (assessed by total lesion area) to nearly double and also macrophage and lipid accumulation (P<0.05; Figure 2). These data indicate that IFN-γ is not required for CD4+ NKT cells to exert their proatherogenic effects. As IL-4 has also been shown to be proatherogenic and NKT cells secrete IL-4, we next assessed the role of IL-4 derived from NKT cells; IL-21 augments cytokine secretion by NKT cells and is expressed in atherosclerotic lesions. IL-21–deficient CD4+ NKT cells also augmented atherosclerotic development, by ≈60% increase the size of atherosclerotic lesions (P<0.05; Figure 2). Plasma cholesterol levels were slightly elevated (15%) in mice transferred with granzyme B–deficient CD4+ NKT cells did not significantly increase lesion size (P>0.05; Figure 3). Similarly, transfer of perforin and granzyme B–deficient NKT cells did not significantly increase macrophage accumulation (P>0.05; Figure 3) and apparent increases in lipid accumulation were also not statistically significant (P>0.05; Figure 3); Together, these studies indicate that perforin and granzyme B are required for the proatherogenic action of CD4+ NKT cells. Plasma cholesterol levels were slightly elevated (15%) in mice transferred with granzyme B–deficient CD4+ NKT cells (P<0.05), but this did not significantly increase atherosclerosis (Online Table II).

Adoptive Transfer of Perforin-Deficient CD4+ NKT Cells Results in Smaller Necrotic Cores and Reduced Apoptosis in Developing Lesions

In atherosclerotic lesions defective clearance of apoptotic cells leads to postapoptotic necrosis and development of larger necrotic cores. To determine whether CD4+ NKT cell–derived perforin contributed to the development of necrotic cores, we compared acellular regions of atherosclerotic lesions from ApoE−/−Jα18−/− mice receiving either vehicle (control), WT CD4+ NKT cells, or perforin-deficient CD4+ NKT cells. After adoptive transfer of WT CD4+ NKT, the

Figure 1. Atherosclerotic lesions after adoptive transfer of natural killer T (NKT) cells to apolipoprotein E–deficient (ApoE)−/− mice deficient in (A) B and T cells (Rag DKO) and (B) B, T, and NK cells (TKO) and fed a high-fat diet (HFD) for 8 weeks. Atherosclerosis assessment was done via intimal lesion areas, CD68+ macrophage, and Oil Red O–stained lipid accumulation. Atherosclerosis assessment done on 8-week HFD-fed ApoE−/− mice was included for comparison in (A). Bar graphs represent intimal lesion areas, macrophage (MΦ) accumulation, and lipid accumulation. Data present mean±SEM. Bar represents 250 µm; *P<0.05 (Student t test); n=8 to 15 per group.

CD4+ NKT Cells Require Perforin and Granzyme B for Atherosclerotic Development

An increase in the frequency of apoptotic cells in developing lesions is associated with increases in atherosclerotic lesion size. Because perforin and granzyme B induce apoptosis and these are major cytotoxins by which NKT cells induce cell death, we assessed the effects of perforin and granzyme B deficiency in CD4+ NKT cells on atherosclerotic development. Wild-type CD4+ NKT cells increased lesion size by ≈70% (P<0.05; Figure 3) as well as increasing both macrophage and lipid accumulation (P<0.05), whereas transfer of perforin or granzyme B–deficient CD4+ NKT cells did not significantly increase lesion size (P>0.05; Figure 3). Together, these studies indicate that perforin and granzyme B are required for the proatherogenic action of CD4+ NKT cells. Plasma cholesterol levels were slightly elevated (15%) in mice transferred with granzyme B–deficient CD4+ NKT cells (P<0.05), but this did not significantly increase atherosclerosis (Online Table II).
necrotic core defined as percent lesion area occupied by acellular material increased in size compared with control (*P<0.05; Figure 4A). In contrast, necrotic cores within lesions of mice receiving perforin-deficient CD4+ NKT cells were similar to control (P>0.05; Figure 4A). A similar profile of effects was observed on apoptotic cells defined by terminal deoxynucleotidyl transferase dUTP nick end labeling assay (TUNEL) and active caspase 3 staining within lesions (Figure 4B and 4C). Besides accumulation of apoptotic cells, defective apoptotic cell clearance by phagocytes (efferocytosis) also contributes to necrotic core generation.44 Therefore, we investigated efferocytosis by assessing macrophage clearance of apoptotic cells after transfer of WT and perforin-deficient CD4+ NKT cells into ApoE−/− Jx18−/− mice. Both WT and perforin-deficient NKT cells enhanced macrophage efferocytosis indicating that efferocytosis does not contribute to the action of perforin on necrotic core formation (Figure 4D).

CD4+ NKT Cells Stimulate Proinflammatory Mediators

Necrotic cells trigger sterile inflammatory responses by releasing the alarmin HMGB145 and activating Nlrp3 inflammasomes, which in turn activate IL-1β and IL-18,12,46 proinflammatory mediators, which augment the development of atherosclerosis.45,47-49 We examined whether VCAM-1 (vascular cell adhesion molecule) and MCP-1 (monocyte chemotactic protein) elevated by these proinflammatory mediators47,50,51 were altered in lesions of WT and perforin-deficient NKT cells from WT, perforin-deficient, and granzyme B–deficient mice. Eight weeks after feeding a HFD, VCAM-1 expression in lesions of mice that received CD4+ NKT cells deficient in perforin or granzyme B was reduced compared with mice that received CD4+ NKT cells from WT mice (P<0.05; Figure 5A); similarly, lesion MCP-1 expression was also attenuated (P<0.05; Figure 5B). Similar increased expression of VCAM-1 and MCP-1 was observed in T- and B-cell–deficient ApoE−/−Rag2−/− mice and lymphocyte-deficient ApoE−/−Rag2−/−gc−/− mice that received WT perforin- or granzyme-competent NKT cells (P<0.05; Figure 5D and 5E).

Donor NKT Cells Do Not Express ApoE

To investigate whether donor CD4+ NKT cells express ApoE, we determined the expression of ApoE mRNA in NKT cells and macrophages from WT and ApoE−/− mice. We found that although macrophages from WT mice express ApoE, WT NKT cells from both chow diet and 8-week HFD-fed mice did not (Figure 6A), suggesting that NKT cells from ApoE-competent donor mice do not influence result outcomes by producing ApoE.

Donor NKT Cells Are Found in the Liver and Atherosclerotic Lesions of Recipient Mice

We performed 2 experiments. First, we tracked CD4+ NKT cells after their adoptive transfer from congenic Ly5.1 mice. We identified Ly5.1+ NKT cells identified by flow cytometry 48 hours after transfer, where they comprised ≈20% of liver NKT cells compared with WT mice (Figure 6B). Immunofluorescence microscopy performed on aortic sinus of lymphocyte-deficient mice that received Ly5.1 CD4+ NKT cells at the beginning of 8-week HFD showed Ly5.1 NKT cells in close proximity.
to lesion macrophages (Figure 6C). Next, we performed quantitative reverse transcription polymerase chain reaction for expression of NKT cell–specific Vα 14-Jα18 TCRα chain after NKT cell transfer that was 40% to 50% of that in ApoE−/− mice, findings supported by representative reverse transcription polymerase chain reaction products are visualized on gel electrophoresis (Online Figure I).

Figure 3. Adoptive transfer of CD4+ natural killer T (NKT) cells deficient in perforin or granzyme B into apolipoprotein E–deficient (ApoE)−/− Jα18−/− mice. Atherosclerotic lesions from ApoE−/− Jα18−/− mice stained with (A) Oil Red O or (B) anti-CD68 antibody after injecting vehicle (Jα18 DKO control), wild-type CD4+ NKT cells (WT NKT), perforin-deficient CD4+ NKT cells (Pfp−/− NKT), or granzyme B–deficient CD4+ NKT cells (GrzB−/− NKT) and fed a high-fat diet for 8 weeks.

C, Bar graphs show intimal lesion areas, CD68+ macrophage accumulation, and Oil Red O–stained lipid accumulation. Data present means±SEM. Bars represent 250 μm. *P<0.05 compared with Jα18 DKO control (1-way ANOVA with Dunnett test); n=4 to 7 per group. DAPI indicates 4′,6-diamidino-2-phenylindole; and H&E, hematoxylin–eosin.

Figure 4. Acellular necrotic cores and apoptotic cells in atherosclerotic lesions. A, Photomicrographs showing hematoxylin-stained nuclei in atherosclerotic lesions from apolipoprotein E–deficient (ApoE)−/− Jα18−/− mice–administered vehicle (Jα18 DKO control), wild-type CD4+ natural killer T cells (WT NKT), or perforin-deficient CD4+ NKT cells (Pfp−/− NKT) and fed a high-fat diet. Bar graph shows necrotic core (nuclei free region) expressed as percentage of lesion area. B, Terminal deoxynucleotidyl transferase dUTP nick end labeling assay (TUNEL)-positive cells in lesions of ApoE−/− Jα18−/− mice–administered vehicle, WT NKT, and Pfp−/− NKT cells and treated as in A. Bar graph shows average percentage of TUNEL-positive cells in atherosclerotic lesions. C, Acellular 3+–positive nuclei (brown color) in atherosclerotic lesions of ApoE−/− Jα18−/− mice that received vehicle, WT NKT, and Pfp−/− NKT cells and treated as in A. Bar graph shows average percentage of caspase 3+–positive cells in atherosclerotic lesions. Data present means±SEM. *P<0.05 compared with WT NKT (1-way ANOVA with Dunnett test); group, n=8 to 9 per group. D, Macrophage effecrocytosis in atherosclerosis lesions of similar groups as above; white arrow indicates TUNEL-positive cells associated with macrophages; yellow arrow, TUNEL-positive cells free from macrophages. Bar graph shows quantification of macrophage effecrocytosis. Data present mean. *P<0.05 from control (1-way ANOVA with Dunnett test); n=4 to 7 per group. DAPI indicates 4′,6-diamidino-2-phenylindole; and H&E, hematoxylin–eosin.
CD4+ NKT cells Are More Potent Than Total CD4 T Cells in a Lymphocyte-Deficient Environment.

We compared the atherogenicity of liver-derived CD4+NKT cells with total spleen-derived CD4+CD25− T cells, by the transfer of 3×10⁵ of these cells to lymphocyte-deficient ApoE−/− mice. We selected CD4+CD25− T cells for transfer to exclude the presence of CD25+ regulatory CD4+ T cells that are atheroprotective. Atherosclerosis at the end of 8-week HFD showed a significant doubling in lesion size after transfer of CD4+ NKT cells compared with transfer of CD4+CD25− T cells, consistent with a potent atherogenic action of CD4+ NKT cells (Online Figure II). We found similar expression of perforin and granzyme B in NKT cell–transferred and PBS-transferred mice (Online Figure III).

Discussion

The present study has identified unexpected and novel mechanisms by which NKT cells augment atherosclerosis. The mechanisms did not require activation of bystander B cells, T cells, or NK cells nor the secretion of proinflammatory cytokines IFN-γ, IL-4, and IL-21. Rather, actions of perforin and granzyme B are essential for their proatherogenic effects; perforin and granzyme B−deficient CD4+ NKT cells did not increase lesion size, lesion macrophage accumulation, or lesion lipid accumulation. CD4+ NKT cells producing perforin and granzyme B seem to augment atherosclerosis by inducing apoptosis resulting in augmented postapoptotic necrosis, reflected by increased necrotic core size within lesions. Arterial mRNA expression of perforin and granzyme B was not different in recipients that received CD4+ NKT cells compared with PBS, suggesting that NKT-derived cytotoxins likely mediate their cytoxicity by polarized secretion at immunologic synapses on target cells. The suggestion is supported by immunofluorescent colocalization of NKT cells with macrophages in atherosclerotic lesions.

Expression of the atherosclerosis-promoting molecules, MCP-1 and VCAM-1, in atherosclerotic lesions of DKO (B- and T-cell–deficient ApoE−/− mice [DKO]) and of TKO (lymphocyte-deficient ApoE−/−Rag2−/−γc−/− mice [TKO]) after injecting vehicle or WT NKT cells and fed a HFD for 8 weeks. Data present mean±SEM. *P<0.05 compared with WT NKT (1-way ANOVA with Dunnett test); n=8 to 9 per group.

The role of lymphocytes in atherosclerosis was thought to be minor in early reports. It is difficult to interpret these studies as unlike our studies where congenic ApoE−/− and Rag2−/− mice (and γc−/− mice) were used to generate lymphocyte-deficient mice on the C57BL/6 genetic background, experimental mice in the earlier studies were generated from mice on mixed genetic backgrounds. Furthermore, our recent studies using lymphocyte-deficient mice on C57BL/6 background and the 2001 report using T- and B-cell-deficient ApoE−/−Rag2−/− mice on C57BL/6 genetic background exhibited markedly smaller lesions than WT mice fed a HFD for 8 weeks. Our model of lymphocyte-deficient mice fed a HFD is a sensitive model to demonstrate that conventional B cells and CD8 T cells are proatherogenic and we have again used this model in the present study.

NKT cells are potent immune cells because transfers of small numbers (2.5–3×10⁵ cells) exert potent effects on tumors, diabetes mellitus, and atherosclerosis. To determine the presence of adoptively transferred NKT cells in recipient mice, we harvested liver lymphocytes 48 hours after transfer of 3×10⁵ NKT cells. FACS (fluorescence-activated cell sorting) data showed that the transferred liver NKT cells...
comprised ≈20% of WT mice (mean±SEM 5.00×10^4±0.49 versus 25.86×10^4±4.06). Furthermore, we showed that transferred Ly5.1 NKT cells colocalized with macrophages after 8 weeks on a HFD. Although not investigated here, our previous study suggests that the atherogenic action of NKT cells may be dose dependent.

We assessed the presence of NKT cells in atherosclerotic lesions after transfer of NKT cells from congenic Ly5.1 mice and confirmed our previous report that adoptively transferred NKT cells are home to atherosclerotic lesions. These findings are supported by the demonstration of NKT cells in lesions by reverse transcription polymerase chain reaction after transfer of NKT cells into recipient mice that corresponded to ≈40% of NKT cells in lesions of ApoE^−/− mice fed a HFD for 8 weeks. Potency of the CD4^+ NKT cell population is supported by the doubling of atherosclerotic lesion size after transfer of this population compared with transfer of total CD4^+CD25^− T cells free of CD25^+ regulatory T cells. Collectively, we conclude that atherogenic CD4^+ NKT cells potently promote atherosclerotic development by perforin- and granzyme-dependent mechanisms in lesions.

Initially, we investigated whether NKT cells could augment atherosclerotic development indirectly, by activating bystander B, T, and NK cells. NKT cells are known to activate B and T cells. B cells express CD1d, suggesting that direct interactions between NKT and B cells can influence B-cell function; conventional B cells promote atherosclerotic development. CD4^+ NKT cells have been shown to induce naive and memory B cells to proliferate and secrete IgG1 and IgM. Similarly, NKT cells enhance the proliferation of CD8^+ T cells and the proliferation and inflammatory activity of CD4^+ T cells. Our findings in ApoE^−/− mice deficient in B and T cells indicate that NKT cells can exert proatherogenic effects independently of B and T cells. Although NKT cells can also mediate their effects indirectly by activating NK cells, adoptive transfer of NKT cells into lymphocyte-deficient ApoE^−/−Rag2^−/−γc^−/− mice also augmented atherosclerosis development by ≈75% that was ≈30% of the size of lesions of ApoE^−/− mice. Together these studies indicate that NKT cells can augment atherosclerosis independently of other lymphocyte populations, although it is possible that interactions with other lymphocyte populations might further amplify their proatherogenic effects.

Activated CD4^+ NKT cells secrete copious amounts of IFN-γ, IL-4, and IL-21. IFN-γ and IL-4 augment development of atherosclerosis, whereas IL-21 augments cytokine secretion by NKT cells and is expressed in atherosclerotic lesions. Our findings indicate that the ability of CD4^+ NKT cells to augment atherosclerosis is not dependent on secretion of these cytokines. Although our studies show that NKT cells use perforin and granzyme B to mediate caspase 3-dependent apoptosis, Granzyme B and perforin form multimeric complexes with the proteoglycan serglycin in cytotoxic granules and perforin–serglycin complexes mediate cytosolic delivery of macromolecular granzyme B–serglycin at immunologic synapses without producing detectable plasma membrane pores.

NKT cell–derived cytotoxins are potent stimulators of apoptosis and in atherosclerotic lesions where removal of apoptotic cells is impaired, this leads to postapoptotic necrosis. Necrotic cells are highly inflammatory, releasing the proatherogenic alarmin HMGB1 and metabolic products such as ATP, a potent activator of the NLRP3 (NOD-like receptor family, pyrin domain containing 3) inflammasome, factors that together can augment atherosclerosis. Our findings that augmented atherosclerosis is dependent on CD4^+ NKT cells secreting perforin and granzyme B are consistent with such a mechanism. HMGB1 action initiates caspase 3-dependent apoptosis.
released by necrotic cells likely promotes atherosclerosis by enhancing macrophage recruitment and attenuating apoptotic cell removal by inhibiting efferocytosis, thereby further increasing postapoptotic necrosis. However, efferocytosis is a dynamic process that can be positively regulated by many other factors such as cyclin-dependent kinase inhibitor 2B and milk fat globule-epidermal growth factor 8. NLRP3 inflammasomes are necessary for processing the proatherogenic cytokines pro–IL-1β and pro–IL-18 to their active forms. Thus, our findings that MCP-1 and VCAM-1 expression in atherosclerotic lesions is dependent on NKT cell–derived perforin and granzyme B are consistent with HMGB1 and NLRP3 inflammasome-dependent proatherogenic downstream mechanisms.

Our findings that NKT cell–derived perforin and granzyme B lead to accumulation of apoptotic lesions within developing lesions suggest that NKT cells may target macrophages and macrophage-derived foam cells. Macrophages, including macrophage-derived foam cells, express CD1d in atherosclerotic lesions and NKT cells selectively target CD1d-expressing cells for killing primarily via perforin/granzyme B mechanisms. Because vascular smooth muscle cells associated with lesions do not express CD1d, these cells are likely to be spared. This suggestion is consistent with our previous observation that vascular smooth muscle cell numbers associated with lesions are unaffected by NKT cells. We have also recently reported that CD8 T cells and NK cells promote atherosclerosis by perforin-dependent mechanisms. Our present findings suggest that NKT cells may act in concert with these cytotoxic CD8 T cells and NK cells to promote the development of vulnerable, rupture-prone atherosclerotic lesions characterized by abundant apoptosis and large necrotic cores.

VanderLaan et al. showed that atherosclerosis augmented by NKT cells adoptively transferred into lymphocyte-deficient LDLR−/−/Ldlr−/− mice was limited to aortic sinuses without affecting the ascending aortas. Our data also showed that transferred NKT cells into NKT cell–deficient Apoe−/−/ldlr−/− mice increased atherosclerosis at the aortic sinuses. Although we did not investigate other arterial sites, it is possible that NKT cell–driven atherosclerosis may differ in different mouse models and in different anatomic areas.

In conclusion, our adoptive transfer studies have shown that neither bystander lymphocytes nor cytokine secretion is essential for NKT cells to exert their proatherogenic effects. Rather, their proatherogenic effects are critically dependent on secretion of the cytotoxins perforin and granzyme B, thereby increasing apoptotic and necrotic cells in lesions which in turn augments inflammation. Targeting downstream NKT cell apoptotic cell mediators may be useful in attenuating atherosclerosis.

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Disclosures
None.

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cyclin-dependent kinase inhibitor 2B regulates effec

Novelty and Significance

What Is Known?

• Natural killer T (NKT) lymphocytes were identified in rupture-prone atherosclerotic lesions.
• CD4+ but not CD4−CD8− invariant NKT (iNKT) lymphocytes promote atherosclerosis development.
• Cytotoxins and cytokines produced by iNKT lymphocytes mediate their biological function.

What New Information Does This Article Contribute?

• CD4+ NKT lymphocytes promote atherosclerosis independently of other lymphocytes.
• CD4+ iNKT lymphocytes promote atherosclerosis by production of cyto
toxins, perforin, and granzyme that mediate apoptosis and secondary necrosis in atherosclerotic lesions.
• Cytokines produced by iNKT lymphocytes do not contribute to their atherogenicity.

The mechanism by which CD4+ iNKT cells promote atherosclerosis is not known. Using adoptive transfer of CD4+ iNKT cells deficient in cytotoxins or cytokines into lymphocyte-deficient and NKT cell–deficient atherogenic mice, we report that iNKT cells promote atherosclerosis by cytokotoxin-dependent and not by cytokine-dependent mechanisms. Cytotoxicity of CD4+ iNKT cells in atherosclerosis is based on the following observations: (1) atherosclerosis in lymphocyte-deficient mice was increased after wild-type NKT cell transfer; (2) perforin-deficient and granzyme B−deficient iNKT cells failed to increase atherosclerosis, whereas NKT cells deficient in interleukin-4, interferon-γ, and interleukin-21 increased atherosclerosis to the same extent as wild-type NKT cells after transfer into iNKT cell–deficient mice; (3) the increased apoptosis and necrosis produced by wild-type NKT cell transfer was reduced after transfer of cytokotxin-deficient NKT cells; and (4) transferred NKT cells were identified in athero
sclerotic lesions. Our data indicate that NKT cells promote cell death via cytokotoxin-dependent mechanism in atherosclerotic le
sions, leading to necrosis and accelerated atherosclerosis. The findings highlight potential therapeutic strategies targeting CD4+ iNKT cells for the treatment of atherosclerosis.
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Materials and Methods

Mice
ApoE⁻/⁻ mice backcrossed to C57BL/6J background for 10 generations were obtained from Alfred Medical Research Education Precinct (AMREP) Animal center. We generated NKT cell-deficient ApoE⁻/⁻Jα18⁻/⁻ mice by crossing ApoE⁻/⁻ with Jα18⁻/⁻ mice on a C57BL/6J background. B and T cell deficient-ApoE⁻/⁻Rag2⁻/⁻ mice were generated by crossing ApoE⁻/⁻ with Rag2⁻/⁻ on a C57Bl/6J background whilst lymphocyte deficient ApoE⁻/⁻Rag2⁻/⁻γc⁻/⁻ mice were generated by crossing ApoE⁻/⁻ Rag2⁻/⁻ mice with ApoE⁻/⁻γc⁻/⁻ mice on a C57Bl/6J background.¹ Genotyping confirmed the deletion status of the double and triple knockout mice (data not shown). Donor IFN-γ⁻/⁻, IL-21⁻/⁻, IL-4⁻/⁻, granzyme B⁻/⁻ and perforin⁻/⁻ mice, all on a C57BL/6J background and wild type C57BL/6J mice, were obtained from Peter MacCallum Cancer Center, Melbourne, Australia; Congenic Ly5.1 mice and IL-21⁻/⁻ mice were from Walter and Eliza Hall Institute of Medical Research and Zymogenetics respectively.

Isolation of donor NKT cells and CD4 T cells
Unfractionated and CD4⁺ NKT cells were obtained from livers of donor mice. Livers were perfused with ice-cold PBS before removal from mice, finely minced and passed through a 200µm stainless steel mesh into FACS buffer (PBS pH7.4 containing 2%FCS and 2mM EDTA). After two washes, lymphocytes were isolated by centrifugation through a 33% isotonic Percoll density gradient (GE Healthcare, followed by lysis of any contaminating red blood cells with 0.156M ammonium chloride (pH 7.2). Then lymphocytes were pre-incubated with anti-mouse CD16/32 (2.4G2) antibody to prevent non-specific binding of antibodies to FcRγ, followed by staining with fluorescein isothiocyanate-conjugated anti-CD4 (RM4-5) and phycoerythrin-conjugated CD1d/α-GalCer tetramer. CD4⁺ NKT cells were sorted using a FACSAria (Becton Dickinson). CD4⁺CD25⁺ Regulatory T Cell Isolation Kit (Miltenyi Biotec) was used to enrich CD4⁺ T cells from spleen-derived cell suspension as instructed by manufacturer, followed by staining with allophycocyanin-conjugated anti-CD25 (PC61) and fluorescein isothiocyanate-conjugated anti-CD4 (GK1.5) antibodies (BD Bioscience). FACSAria (Becton Dickinson) was used to purify CD4⁺ CD25⁻ T cells. All purified cells were >99% pure and >95% viable.

Flow cytometry
Cells prepared from livers and lymph nodes were stained with fluorescein isothiocyanate-conjugated anti-TCR-β, phycoerythrin Cy7-conjugated anti-NK1.1 and allophycocyanin-conjugated anti-CD4 antibodies (BD Bioscience). Data collection and NKT cell analysis was performed as described.¹²

Assessment of atherosclerotic lesions
Heart and proximal aorta were dissected from mice, embedded in OCT compound (Tissue-tek) and frozen. Six µm sections of aortic sinus were analysed for lesion size, defined as the cross section surface area of intimal lesion area and Oil Red O staining within the aortic intima. Mean lesion size for each mouse was calculated from cross sections from every 60µm of the first 180µm in the ascending aorta, as described, starting at the aortic cusp.³
**Immunohistochemistry**

Six µm cryosections from similar parts of the aortic sinus of the different groups of mice were used for immunohistochemistry to assess macrophage accumulation, VCAM-1 and MCP-1. Briefly, sections were fixed in cold (-20°C) acetone for 20 min. Then sections were incubated in 3% hydrogen peroxide in PBS, normal horse serum and biotin/avidin blocking reagents (Vector Laboratories). Sections were then incubated with primary antibodies in serum, rat anti-mouse CD68 (1-100; Serotec:cat#MCA1957), rat anti-mouse VCAM-1 (1-50; BD Pharmingen:cat#550546) or rabbit ant-rat MCP-1 (1-50; Abcam:cat#ab7202). Subsequently sections were treated with appropriate biotinylated secondary antibodies, followed by streptavidin horseradish peroxidise complex and 3,3-diaminobenzidine and counterstaining with hematoxylin, as described. 4

**Immunoflorescent staining**

Six µm aortic sinus cryosections blocked by 10% normal horse serum were stained with rat anti-mouse CD68 (1-100; Serotec). After incubation with goat anti-rat Alexa Fluor 488 (Molecular Probe), the slides were washed three times with PBS. Subsequent staining with allophycocyanin-conjugated Ly5.1 (in-house) and counter-staining with 6-diamidino-2-phenylindole (DAPI), images were visualised under Olympus BX61 fluorescence microscope and images captured using FVII Olympus camera.

**Necrotic Core and Apoptosis Assessment**

To analyse necrotic core areas, aortic sinus atherosclerotic lesions were counter stained with hematoxylin and acellular (non-stained) areas were measured using Optimus software and expressed as percentage of total lesion area.2 Apoptotic cells were identified using deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) system [in situ cell death kit-AP assay (Roche)] as described.2 Purified rabbit anti-active caspase-3 antibody (BD Bioscience) was used to detect anti-caspase 3-positive cells in atherosclerotic lesions.

**Expression of ApoE gene in NKT cells**

NKT cells were isolated using FACS sorting from livers as described earlier and plastic attached macrophages were purified from spleens. Total NKT and macrophage mRNAs extracted by RNeasy fibrous tissue mini kit (Qiagen) were used to determine ApoE gene expression with sense (S) primer 5'-CCTGAACCGCTTCTGGATT-3 and anti-sense (AS) primer 5'-GCTCTTCCTGGACCTGGTCA-3, using single-step QuantiFast SYBR Green RT-PCR kit (Qiagen) on 7500 Fast Real-Time PCR system (Applied Biosystem). The target gene expression levels were analyzed using comparative cycle threshold method with 18S rRNA primers (Applied Biosystems).1,2

**Realtime PCR analysis of perforin, granzyme B, and NKT cell-specific TCR chain**

RNA extracted from aortic arches and thoracic aorta as described above were utilised to determine mRNA expression of perforin5, granzyme5 and V α14Jα18-TCR α chain3;primers for house-keeping gene, 18S (Applied Biosystem) were included for comparative cycle threshold method. Sequences of primers used were as follows.
Pfp (S) 5'-GAGAAGACCTATCAGGACCA -3',
Pfp (AS) 5'-AGCCTGTGGTAAGCATG -3';
GnzB (S) 5'-CCTCCTGCTACTGCTGAC -3',
GnzB (AS) 5'-GTCAGCACAAAGTCCTCTC-3';
Jα18 (S) : 5'-TGGATGACACTGCCACCTACAT-3',
Jα18 (AS) : 5'- TCCAAATGCAGCCTCCCTA-3';

Plasma Cholesterol and Triglycerides
Plasma VLDL/LDL- and HDL-cholesterol were determined enzymatically using a Cobas Mira Plus Autoanalyzer and a HDL and VLDL/LDL-cholesterol quantification kit (BioVision Mountain View, CA). Plasma triglycerides were determined using a triglyceride quantification kit (BioVision, Mountain View, CA).

Statistical Analyses
For multiple comparisons statistical analyses were performed using one way ANOVA followed by post hoc analysis (Dunnett’s test). For single comparisons Student’s t-test was used after confirming a normal distribution using Kolmogorov–Smirnov test. All analyses were performed using GraphPad Prism. P < 0.05 was considered statistically significant.

References
Online Table I Plasma cholesterol, VLDL/LDL-cholesterol and HDL-cholesterol and body weights of lymphocyte-deficient mice receiving vehicle or CD4+ NKT cells.

<table>
<thead>
<tr>
<th>Group</th>
<th>Weight</th>
<th>Total cholesterol</th>
<th>Triglyceride</th>
<th>HDL</th>
<th>VLDL/LDL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rag2(^{-/-}) x ApoE(^{-/-}) control</td>
<td>35.6 ± 0.6 (8)</td>
<td>22.0 ± 1.6 (8)</td>
<td>4.9 ± 0.9 (8)</td>
<td>3.8 ± 0.3 (8)</td>
<td>16.1 ± 1.2 (8)</td>
</tr>
<tr>
<td>Rag2(^{-/-}) x ApoE(^{-/-}) + NKT cell transfer</td>
<td>32.4 ± 0.7** (8)</td>
<td>20.0 ± 1.5 (9)</td>
<td>6.1 ± 0.9 (9)</td>
<td>3.2 ± 0.2 (9)</td>
<td>14.1 ± 0.9 (9)</td>
</tr>
<tr>
<td>TKO control</td>
<td>34.2 ± 0.6 (6)</td>
<td>22.2 ± 1.5 (7)</td>
<td>5.9 ± 0.8 (7)</td>
<td>3.7 ± 0.2 (7)</td>
<td>15.8 ± 1.2 (7)</td>
</tr>
<tr>
<td>TKO + NKT cell transfer</td>
<td>30.9 ± 0.6' (7)</td>
<td>19.1 ± 2.4 (7)</td>
<td>5.4 ± 0.9 (7)</td>
<td>2.8 ± 0.3* (7)</td>
<td>14.0 ± 1.7 (7)</td>
</tr>
</tbody>
</table>

**p < 0.01 compared to Rag2\(^{-/-}\) x ApoE\(^{-/-}\) control. *p < 0.05, 'p < 0.01 compared to TKO control.
Total cholesterol, triglyceride, HDL and VLDL/LDL measurements are in mmol/L.
Number of mice in each group is indicated in parentheses.
Data represent the mean ± SEM.
**Online Table II** Plasma cholesterol, VLDL/LDL-cholesterol and HDL-cholesterol and body weights of ApoE−/−Ja18−/− mice receiving vehicle or CD4+ NKT cells

<table>
<thead>
<tr>
<th>Group</th>
<th>Weight (g)</th>
<th>Total cholesterol</th>
<th>Triglyceride</th>
<th>VLDL/LDL</th>
<th>HDL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jα18 DKO control</td>
<td>37.0 ± 1.0 (16)</td>
<td>24.0 ± 1.1 (15)</td>
<td>3.0 ± 0.4 (15)</td>
<td>18.9 ± 0.8 (15)</td>
<td>3.4 ± 0.3 (15)</td>
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<tr>
<td>WT NKT</td>
<td>34.1 ± 0.8 (11)</td>
<td>19.8 ± 1.8 (13)</td>
<td>2.8 ± 0.6 (13)</td>
<td>15.8 ± 1.4 (13)</td>
<td>3.0 ± 0.4 (13)</td>
</tr>
<tr>
<td>IL-4−/− NKT</td>
<td>34.7 ± 0.9 (10)</td>
<td>25.6 ± 1.7 (10)</td>
<td>2.5 ± 0.3 (10)</td>
<td>20.0 ± 1.2 (10)</td>
<td>4.4 ± 0.4 (10)</td>
</tr>
<tr>
<td>IFN-γ−/− NKT</td>
<td>36.3 ± 1.5 (10)</td>
<td>26.9 ± 1.7* (9)</td>
<td>2.6 ± 0.4 (9)</td>
<td>21.6 ± 1.3* (9)</td>
<td>4.1 ± 0.4 (9)</td>
</tr>
<tr>
<td>IL-21−/− NKT</td>
<td>37.4 ± 1.5 (6)</td>
<td>23.2 ± 2.1 (6)</td>
<td>4.2 ±1.0 (6)</td>
<td>17.0 ± 1.5 (6)</td>
<td>4.3 ± 0.4 (6)</td>
</tr>
<tr>
<td>GrzB−/− NKT</td>
<td>37.2 ± 1.4 (11)</td>
<td>27.7 ± 1.6* (7)</td>
<td>3.2 ±0.5 (7)</td>
<td>22.0 ± 1.2* (7)</td>
<td>4.2 ± 0.4 (7)</td>
</tr>
<tr>
<td>Pfp−/− NKT</td>
<td>38.4 ± 1.3 (9)</td>
<td>26.4 ± 2.2 (7)</td>
<td>3.0 ± 0.4 (7)</td>
<td>20.7 ± 1.8 (7)</td>
<td>4.4 ± 0.4 (7)</td>
</tr>
</tbody>
</table>

*p < 0.05 compared to WT NKT group

Total cholesterol, triglyceride, HDL and VLDL/LDL measurements are in mmol/L.
Number of mice in each group is indicated in parentheses.
Data represent the mean ± SEM.
Online Figure I. Expression of NKT cell-specific TCR α chain in atherosclerotic lesion.
Quantitative assessment of Va14-Jα18 TCRα chain expression in arterial RNAs extracted from Rag DKO (Rag2−/− ApoE−/−) mice that received either PBS or NKT cells and from ApoE−/− mice showed (A) expression of NKT cell-specific TCR α chain following NKT cell transfer that was ~40-50% of that in ApoE−/− mice. (B) Representative RT-PCR product visualised on gel electrophoresis showing expression of Va14-Jα18 TCRα chain in lesions of ApoE−/− and Rag DKO mice that received NKT cells and no expression in Rag DKO with PBS transfer. Data present mean±SEM. (one way ANOVA); n =4-7 per group.
Online Figure II. **CD4+ iNKT cells are potent atherogenic cells.** Adoptive transfer of 0.3x10⁶ CD4⁺ NKT into lymphocyte-deficient ApoE⁻/⁻ mice showed approximate doubling of atherosclerotic lesion size compared to transfer of CD4⁺CD25⁻ T cells at the end of 8 week HFD after cell transfer. Representative image of each group presented. Oil Red-O stained atherosclerotic lesions used to determine total intimal lesion area and lipid accumulation. Data present mean±SEM. * P < 0.05 (student's T test). n=4 per group.
Online Figure III. Expression of perforin and granzyme B following CD^+ NKT cell transfer.
Quantitative assessment of perforin and granzyme B in arterial tissues by RT-PCR following CD4^+ NKT cell transfer into ApoE^-/- mice deficient in B and T cells (Rag DKO) or lymphocytes (TKO) and NKT cells (Jα18DKO). No difference in mRNA expression of (A) perforin and (B) Granzyme B was detected. Data present mean±SEM. one way ANOVA. n=8-12 per group.