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Natural killer T cells in lipoprotein metabolism and atherosclerosis

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Abstract

Cells of both the innate and adaptive immune system participate in the development of atherosclerosis, a chronic inflammatory disorder of medium and large arteries. Natural killer T (NKT) cells express surface markers characteristic of natural killer cells and conventional T cells and bridge the innate and adaptive immune systems. The development and activation of NKT cells is dependent upon CD1d, a MHC-class I-type molecule that presents lipids, especially glycolipids to the TCR on NKT cells. There are two classes of NKT cells; invariant NKT cells that express a semi-invariant T cell receptor and variant NKT cells. This review summarizes studies in murine models in which the effect of the activation, overexpression or deletion of NKT cells or only invariant NKT cells on atherosclerosis has been examined.

Keywords

atherosclerosis; CD1d; NKT cells

Atherosclerosis, the underlying cause of most cardiovascular diseases, is a chronic inflammatory condition that involves the participation of both the innate and adaptive immune systems (1–3). While it is clear from deficiency states in mice at least that the adaptive immune system is not obligatory for atherogenesis, there is also a large body of experimental and clinical evidence that T cells and B cells powerfully modulate the development of atherosclerotic lesions. An important, though minor, subset of T cells are the natural killer T (NKT) cells. NKT cells are so named because they coexpress cell surface molecules of both natural killer cells (e.g. NK1.1/CD161 and Ly49) and T cell receptors (TCR) characteristic of T cells (4–6). NKT cells respond rapidly to antigenic stimulation which for the most part are glycolipids presented in the context of a MHC class I-like antigen presenting molecule CD1d in association with β_2 -microglobulin. This CD1d molecule is essentially non-polymorphic in contrast to its highly polymorphic MHC class I cousin which presents peptide antigens to conventional T cells. The rapid NKT cell response to antigenic stimulation is associated with the production of a complement of both Th1 cytokines (IFN γ and TNF α) and Th2 cytokines (IL-4, IL-13, IL-10). These cytokines interact with other cellular elements of both the adaptive and innate immune systems. Their antigen recognition, rapid response and cytokine profile uniquely positions them to serve as a bridge between the two immune systems. The implied involvement of NKT cells in atherosclerosis derives primarily from the finding of these cells in human atherosclerotic lesions (7) and that they respond to glycolipids that may be present in atherosclerotic lesions (8). This review will focus on mouse models that have been used for the experimental study of the involvement of NKT cells in atherogenesis.

While this review focuses on the role of NKT cells in atherosclerosis, this subset of T cells has also been implicated in a variety of other diseases (9, 10). These cells respond to a variety of microbes, including bacteria, parasites and viruses (4, 5, 11, 12). This may be by direct recognition of a microbial antigen or an upregulated endogenous glycolipid antigen or by Toll-like receptor (TLR) dependent production of cytokines (6, 11). NKT cells have been implicated in tumor surveillance and prevention of tumor metastasis (9, 10). They may exacerbate asthma when pulmonary NKT cells produce IL-4 and IL-13 (6). They also influence tissue transplantation outcomes and autoimmunity such as type 1 diabetes, multiple sclerosis and systemic lupus erythematosus, which may be triggered by bacterial NKT cell activation involving mimicry of tissue antigens (6, 10). As NKT cells may produce GM-CSF they can regulate hematopoiesis particularly affecting monocyte formation, which is of great relevance to atherosclerosis. NKT cell stimulation may elicit a “network of cellular activation events” (5) affecting dendritic cell maturation and NK cell proliferation and IFN γ production, as well as B and T cell function. The production of antibodies to glycolipid antigens is greatly helped by cognate NKT cells in large part by increased expression of co-stimulatory molecules such as CD40L and CD28 (13). These costimulatory molecules have also been implicated in atherosclerosis (14, 15). In this respect, glycolipid ligands of NKT cells may serve as valuable vaccine adjuvants (16). In addition, due to secretion of cytokines such as IL-10, IL-4, and IL-13, they may function as regulatory cells. This catalogue of possible actions indicates that the role of NKT cells may be quite complex in a variety of disease situations. Their relatively small numbers belies their possible biological actions.

CD1d expression

In humans there are two major classes of CD1 antigen presenting molecules— the class I molecules CD1a, CD1b, and CD1c and the class II molecules CD1d and CD1e (17). All CD1 molecules bind and present lipid antigens to TCRs. Unlike humans, only the class II CD1d molecule is expressed in mice. In addition, there are two murine CD1d genes; CD1d1 and CD1d2 (18). The development and activation of NKT cells is dependent upon the interaction of their TCR with antigen-loaded CD1d molecules (4– 6). CD1d molecules are expressed in by a variety of professional antigen presenting cells such as dendritic cells, macrophages and B cells. Among the B cells expressing CD1d are a small subset of IL-10 producing B regulatory cells that express high levels of CD1d and function as a suppressor of inflammation (19). CD1d is also expressed in the liver on hepatocytes and Kupffer cells (20, 21) where it may play a role in the homing and sequestering of NKT cells in the liver, the organ in which NKT cells are notably enriched, along with the interaction of CXCR6 on NKT cells with CXCL16 on endothelial cells (22). The NKT cells in the liver probably play a critical role in the clearance of microbial pathogens. CD1d is also expressed in Paneth cells in the intestine (23), which are a major source of defensins, and perhaps also on intestinal epithelial cells (24). In these latter sites they recognize gut microbes. Indeed in CD1d deficient mice, there is increased colonization of the small intestine with commensal bacteria (25). This wide distribution of CD1d suggests that not all features of CD1d deficiency can be attributable to the absence of NKT cells.

NKT cell subsets¹

NKT cells are not nearly as diverse as conventional T cells. There are two major classes of CD1 restricted NKT cells (4–6). The majority of the NKT cells express a semi-invariant T cell receptor and are referred to as class I NKT cells or invariant NKT (iNKT) cells. In humans, the semi-invariant T cell receptor on the iNKT cells is composed of a V α 24-J α 18 TCR α chain coupled mostly with the V β 11 TCR β chain. The semi-invariant T cell receptor in mice is the V α 14-J α 18 chain coupled with the V β 8.2, V β 7, or V β 2 chain. More recently an iNKT-like cell has been found in the intestine (mNKT). It has a different invariant T cell receptor (V α 7.2 in humans and V α 19 in mice) and is responsive to antigen presented by the MHC-related molecule 1 (MR1) (24). While iNKT cells constitute the majority of the NKT cells in most tissues, there are smaller numbers of NKT cells that express more diverse T cell receptors. These are referred to as class II NKT cells or variable NKT (vNKT) cells. Because of the diversity of the T cell receptors and lower frequency much less is known about the function of the vNKT cells.

NKT cell antigens

The limited diversity of T cell receptors in the iNKT cell subset suggests a limited set of CD1d presented lipid antigens. However unlike conventional T cell receptors, which have a very high specificity for the recognition of specific peptide antigens, the recognition of glycolipids is somewhat more promiscuous (18). A reagent that has been heavily employed for the exogenous activation of these cells is the marine sponge glycolipid α -galactosylceramide (α GalCer). Indeed iNKT cells may be stained in FACS analysis by CD1d tetramer containing α GalCer or one of its derivatives. Several other glycolipids derived from pathogens have been found to be effective exogenous iNKT cell ligands, including a diacylglycerol derivative associated with the Lyme disease causing bacteria *Borrelia burgdorferi* (17, 21). The rapid response of NKT cells to antigen suggests that in vivo the cells have been activated by an endogenous antigen. However the nature of this endogenous or self antigen is still uncertain. It has been suggested that isoglobotrihexosyl ceramide (iGb3) might be an endogenous agonist (26) though this has recently been disputed (27, 28). We have reported that the LDL from atherosclerosis susceptible LDL receptor deficient (LDLR $^{-/-}$) mice fed a high fat, high cholesterol Western-type diet is able to activate a V α 14J α 18 expressing hybridoma and this activity is destroyed by copper oxidation of the LDL (29). This LDL may include minimally modified LDL (mmLDL), a potentially important initiator of atherosclerosis (30). While it has generally been supposed that NKT cells respond to glycolipid or phospholipid antigen, an important recent report points to the possibility that self antigenic peptides may be presented to and activate NKT cells. In this case a mouse type II collagen peptide (corresponding to residues 707–721) directly activates NKT cells in a CD1d dependent reaction to suppress collagen induced arthritis but also other inflammatory responses, including delayed type hypersensitivity, antigen induced airway inflammation, and experimental allergic encephalitis (31).

The acquisition of antigen by the CD1d molecule is a complex process. CD1d is synthesized in the endoplasmic reticulum where its ligand binding site is occupied by endogenous phospholipids which are ultimately exchanged for the effector lipid antigen derived from processing in endocytic compartments (17). The delivery of these lipids to CD1d is mediated by the lipid transfer proteins microsomal triglyceride transfer protein, saposin and

¹In what follows NKT cell without prefix refers to the total pool of CD1d restricted T cells, i.e. includes iNKT cells and vNKT cells or relates to studies in which these subset distinctions have not been explored. iNKT cell (class I NKT cells) refers to the major subset of this pool that expresses the semi-invariant NKT cell receptor and are specifically stimulated by α GalCer presented by CD1; indeed the α GalCer CD1d tetramer positive T cells is the best characterization of iNKT cells. vNKT cells (class II NKT cells) refers to a much smaller subset of NKT cells restricted by CD1d but carrying a more variable T cell receptor.

Niemann Pick type C 1 and 2 proteins. The lipid loaded CD1 molecule is then transported through the secretory apparatus to the plasma membrane for antigen presentation.

The delivery of serum borne lipid antigen to the endocytic compartment of antigen presenting cells may be mediated by engagement of the LDL receptor by lipoproteins carrying apoE, particularly VLDL (32). ApoE itself is capable of binding α GalCer based molecules, but the VLDL also may serve as a carrier of as yet unidentified additional lipid antigens. While both dendritic cells and B cells express CD1d, the apoE mediated delivery of antigen via the LDL receptor is much more pronounced in B cells than in dendritic cells (33). The apoE mediated enhancement of antigen presentation leads to activation of NKT cells and subsequent activation of B cells and represents a pathway by which NKT cells provide help for B cells.

NKT cell activation

iNKT cells have an activated/memory phenotype, responding immediately to an activating antigen presented by CD1d by gaining cytotoxic activity (secretion of perforin or FasL) and secreting Th1 or Th2 cytokines (6, 11). An alternative pathway for the activation of iNKT cells has been recently described, especially for microorganisms that activate these cells (6, 11). Antigen presenting cells such as macrophages or dendritic cells may be activated by ligands for the TLRs resulting in increased production of endogenous glycolipids. Alternatively, TLR-mediated activation of the antigen presenting cells may increase their production of IL-12, IL-18 or type 1 IFN, which can activate iNKT cells via CD1d independent pathways. Several TLR pathways have been implicated in this indirect activation of NKT cells. These latter pathways of activation of iNKT cells are potentially important for the endogenous activation of iNKT cells in relation to atherogenesis. For example, TLR4 can be activated by mmLDL to induce phenotypic changes in the macrophage that may contribute to atherogenesis including stimulation of the uptake of oxidized LDL and cytokine production that may promote the activation of NKT cells (34). Therefore, there are at least three potential pathways for activation of iNKT cells in the context of atherogenesis, two of which depend upon the iNKT cell TCR and endogenous antigens and one which may be independent of this receptor.

As mentioned above the activation of iNKT cells leads to the secretion of Th1 cytokines and Th2 cytokines and perhaps also IL-17. The cytokines produced by the iNKT cells may modulate the immune response by other cells of the immune system e.g. neutrophils, B cells, dendritic cells and conventional T cells, thus polarizing the immune response so that their effects may not always be directly the result of the action of the iNKT cells themselves. Upon continuous exposure to the hyperlipidemia in the atherogenic susceptible apoE^{-/-} mouse model, the NKT cells are persistently anergic (i.e. hyporesponsive to exogenous stimulation) (35). Thus the characteristic cytokine responses may be blunted with longer duration of antigenic stimulation. This is obviously of considerable importance in the case of atherosclerosis in which the atherogenic stimuli, by their nature, occur over an extended time period.

NKT cells in atherogenesis

α GalCer activation of NKT cells and atherosclerosis

In order to study the involvement of NKT cells in atherosclerosis several murine models have been employed (Table). Absent clear information about the nature of the endogenous activating glycosphingolipid, the exogenous activator α GalCer has been employed in several studies. α GalCer is a very robust agonist that elicits major increases in the plasma levels or splenocyte secretion of IFN γ and IL-4 in vivo (36–38) and in vitro (38, 39).

Augmentation of atherosclerosis was noted in most studies in which this agent was administered intraperitoneally into wild type mice and the atherosclerosis susceptible LDLR^{-/-} mice and apoE^{-/-} mice, especially if early atherosclerosis was examined (36, 37, 39). α GalCer had no effect on plasma lipid and lipoprotein levels. Taking account of the anergy of iNKT cells with continuous stimulation (35) it is not surprising that the effects of manipulating NKT cells is most manifest in early lesions, perhaps before the anergy to continuous exposure to endogenous lipid antigen has taken effect. The action of α GalCer is dependent on the presence of NKT cells in that no response is noted in the absence of NKT cells as a result of the elimination of CD1d, the restriction element necessary for NKT cell development (37, 39). In only one study has α GalCer appeared to be atheroprotective (38). In this case the effect of the agonist on perivascular collar induced carotid artery atherosclerosis was studied in LDLR^{-/-} mice and apoE^{-/-} mice fed a Western type diet for a total of 9 weeks. The agonist was administered both intraperitoneally and intravenously. Unlike other investigations, atherosclerosis was markedly reduced in the LDLR^{-/-} model but had no effect in the apoE^{-/-} model. The observations in the LDLR^{-/-} mice were correlated with an increase in splenocyte proliferation and a substantial increment in splenic IL-10 production. Presumably the use of a different atherosclerosis induction model and differences in the method of administration of α GalCer in this latter study accounts for the different outcome compared to the other studies.

V α 14 transgenic mice and atherosclerosis

Somewhat analogous to this is the use of the V α 14 transgenic (V α 14tg) mouse with an overabundance of iNKT cells (40). We used this model in adoptive transfer experiments in which splenocytes from V α 14 transgenic mice were adoptively transferred into immune deficient mice (LDLR^{-/-}RAG^{-/-}) resulting in an increase in aortic root atherosclerosis after 12 weeks of Western type diet feeding (29). Upon crossing the V α 14 transgenic mice with LDLR^{-/-} mice we noted an increase in innominate artery atherosclerosis only in female mice after feeding the Western-type diet for 12 weeks (submitted for publication). There was no change in atherosclerosis in either the aortic root or the aortic arch. We have noted site selective atherosclerosis responses in other immune deficient models (41, 42). The site selective differences between these two models do not have an obvious explanation. The expression of the V α 14 transgene had no effect on plasma lipids. However the two models differ in the extent to which the immune system is intact, perhaps implicating the interaction between iNKT cells and other cells of the immune system. In these systems the atherosclerotic responses are probably attributable to the activation of the abundant iNKT cells by endogenous glycolipid effectors present at least in part in the plasma LDL fraction or in the atherosclerotic plaque or activated by TLR ligands. Overall, the increase in number or activity of iNKT cells suggests that the cells are pro-atherogenic.

CD1d deficiency and atherosclerosis

CD1d is the restriction element for the development of both iNKT and vNKT cells. Thus CD1d deficiency is associated with an absence of both classes of NKT cells. As indicated in the table several investigators have examined atherosclerosis in CD1d^{-/-} mice. In the apoE^{-/-} model, atherosclerosis is notably reduced e.g. by 68% in the aortic root in the double knockout mice (39) and also as indicated above these mice do not respond to α GalCer stimulation. Three studies have been performed using the LDLR^{-/-} model. Nakai et al transplanted CD1d^{-/-} bone marrow into LDLR^{-/-} recipients and noted the expected reduction in lesion formation based on the presumed pro-atherogenic nature of NKT cells (36). Aslanian and colleagues studied CD1d^{-/-} LDLR^{-/-} mice of both genders fed a high fat, high cholesterol diet (43). A reduction of aortic root atherosclerosis was noted in both genders only at an early time point after diet initiation, namely 4 weeks, without any change in plasma lipids, although there was a slight decrease in VLDL levels. However, this

reduction was abolished with longer diet feeding. No difference was observed in cytokine mRNA levels in the aortas. Our own results diverge from these results (submitted for publication). We noted an increase in aortic root atherosclerosis in both genders of CD1d^{-/-}LDLR^{-/-} mice after 4 weeks of diet feeding but the lesions were reduced after 12 weeks of feeding. In the ascending aorta there was no difference in lesion area after 4 weeks of diet, but lesion area was reduced by 12 weeks. These two observations suggest that NKT cells may influence lesion progression as the growth of lesions in the deficient mice was substantially slower (comparison of 4 and 12 week results).

How does one account for the very different results in the two sets of experiments with CD1d^{-/-}LDLR^{-/-} mice? The diets employed in these two studies were slightly different. A diet containing 15% fat and 1.25% cholesterol was used in the Aslanian study and the more usual Western type diet (21% fat, 0.15% cholesterol) in our experiments. We have repeated the 4-week feeding experiment using the Aslanian diet but did not replicate their observations. It is also worth noting that two different laboratories generated CD1d^{-/-} mice. The Aslanian study employed animals from Luc Van Kaer in which only the CD1d1 gene is eliminated (44) while our studies were performed with animals from Chyung-Ru Wang (45) in which both genes were knocked out. It remains to be seen whether there are differences among these two independently derived CD1d^{-/-} mice and whether the two CD1d genes function as restriction elements for different subsets of NKT cells.

Specific loss of iNKT cells and atherosclerosis

Jα18 deficiency eliminates only the iNKT cells. Rogers and colleagues examined female Jα18^{-/-}LDLR^{-/-} double knockout mice at 8 weeks of Western type diet feeding (46). They observed no change in plasma lipids and decreases in both aortic root and aortic arch atherosclerosis. In our studies the double knockout animals were examined at 4 and 12 weeks of diet feeding. Plasma total cholesterol was increased in both genders of these mice while aortic arch lesions were reduced only in female mice after 4 weeks of diet (submitted for publication). Lesions at 12 weeks were indistinguishable from the control LDLR^{-/-} mice. Thus between 4 and 12 weeks the lesions in the aortic arch grew more rapidly than in the control animals, again suggesting a role of iNKT cells in lesion progression. The modest differences between our studies and those of Rogers and colleagues are probably a function of the timing of the observation after the initiation of the diet.

Our study is the first study in which Jα18 and CD1d knockout mice have been examined in the same laboratory and in the same atherosclerotic susceptible background. Differences in the response of the atherosclerotic lesions to a deficiency of Jα18 and CD1d in the LDLR^{-/-} background could be attributable to the activity of the vNKT cells in the Jα18^{-/-} mice. The balance between iNKT and vNKT may also influence lipid and lipoprotein metabolism. Unfortunately no model is available for the selective deletion of only the vNKT cells and thus this latter supposition cannot currently be directly examined. There is data in the cancer literature suggesting that the balance between iNKT cell activity and vNKT cell activity may be operative in determining the phenotype of experimental animals (47). There are perhaps alternative explanations for these differences. For example, we measured the level of antibodies to oxidized LDL in the plasma of the mice and noted a selective increase in the level of the IgM antibody that recognizes phosphorylcholine of oxidized lecithin (diacylphosphatidylcholine) (i.e. the EO6/T15 antibody) (48) in CD1d^{-/-}LDLR^{-/-} mice (manuscript in preparation). There was no increase in antibody titer in the Jα18^{-/-} mice suggesting this increase is not a function of iNKT cell activity. No changes were seen in IgM or IgG antibodies recognizing MDA-LDL or CuOx LDL. CD1d is expressed on B1 cells and on the so called B regulatory cells whose products include IL-10 and TGFβ (49). We have preliminary evidence that these IL-10 producing B cells are reduced in number in our CD1d^{-/-}LDLR^{-/-} mice. The extent to which this change accounts for the increment in

early aortic root atherosclerotic lesions and also for the increment in IgM antibody production remains to be established. It is worth recalling that LDLR^{-/-} mice receiving α GalCer demonstrate a notable increment in splenic IL-10 levels (38).

In summary, the outcome of the manipulation of the NKT cell populations in atherosusceptible mouse models appears to be more complex than was first thought. The NKT cell subsets may variably affect initiation as well as progression of lesions and lipid metabolism and alterations in the balance between these subsets may have implications for atherogenesis. These bridge cells produce at least three major cytokines known to influence atherogenesis, IFN γ , IL-4 and IL-10. The relative contribution of these cytokines secreted by the NKT cells to the development of atherosclerosis remains to be established. Transgenic mice expressing human class I CD1 molecules that present lipid antigen to non-NKT cells have been generated (50), but their role in atherogenesis has not yet been examined. There is clearly much room for further investigation of the role of activation of T cells by lipid antigens on atherogenesis.

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Table

Effect of activation of NKT cells by α GalCer or overexpression or deficiency of NKT cells or NKT cell subsets on atherosclerosis.

<i>αGalCer Treatment</i>	Diet	Gender	Strain	Aortic Root	En face	Other Sites
Nakai et al. (36)	Chow	Female	apoE ^{-/-} (13 wks old) apoE ^{-/-} (19 wks old)	$\uparrow$ (60%) —	—	
Tupin et al. (37)	Chow	Female	apoE ^{-/-} apoE ^{-/-} CD1d ^{-/-}	$\uparrow$ (50%) —		
Major et al. (39)	Chow	Male	apoE ^{-/-} apoE ^{-/-} CD1d ^{-/-}	$\uparrow$ (2 fold, 16 wks) $\uparrow$ (20%, 24 wks) —	$\uparrow$ (50%, 24 wks) —	
van Puijvelde et al. (38)	15% fat 0.25% chol	Male	LDLR ^{-/-}			perivascular carotid collar $\downarrow$ (85%)
		Male	ApoE ^{-/-}			—
<i>No αGalCer</i>						
Nakai et al. (36)	15% fat 1.2% chol 0.5% cholate	Female	CD1d ^{-/-} LDLR ^{-/-} recipient of CD1d ^{-/-} bone marrow	$\downarrow$ (60%) $\downarrow$ (40%)		
Major et al. (39)	Chow	Male	CD1d ^{-/-} apoE ^{-/-}	$\downarrow$ (68%)		
Tupin et al. (37)	Chow	Female	CD1d ^{-/-} LDLR ^{-/-}	$\downarrow$ (25%)		
Aslanian et al. (43)	1.25% chol 15% fat	Both	CD1d ^{-/-} LDLR ^{-/-}	$\downarrow$ (4 wks) $\downarrow$ (4 wks) — (8–12 wks)		
Rogers et al. (46)	21% fat 0.15% chol	Both	Ja.18 ^{-/-} LDLR ^{-/-}	$\downarrow$ (8 wks)		$\downarrow$ (ascending aorta, 8 wks)
VanderLaan et al. (29)	21% fat 0.15% chol	Female	LDLR ^{-/-} RAG ^{-/-} recipients of V α 14tg splenocytes or CD1d ^{-/-} splenocytes	$\uparrow$ (62%) —		
VanderLaan et al. (submitted)	21% fat 0.15% chol	Female	V α 14tg LDLR ^{-/-}			$\uparrow$ 12 wks (innominate)

<i>αGalCer Treatment</i>	Diet	Gender	Strain	Aortic Root	<i>En face</i>	Other Sites
			CD1d ^{-/-} LDLR ^{-/-}	↑ (4 wks) — (12 wks)		↓ (ascending aorta, 12 wks)
			Ja.18 ^{-/-} LDLR ^{-/-}			↓ (ascending aorta, 4 wks)
	Male		CD1d ^{-/-} LDLR ^{-/-}	↑ 4 wks — (12 wks)		↓ (ascending aorta, 12 wks)

↑ increased atherosclerosis; ↓ decreased atherosclerosis, — no difference in atherosclerosis relative to control mice.

Abbreviations: wk – weeks; chol- cholesterol