EFFECTS OF DYSLIPIDEMIA ON INVARIANT NATURAL KILLER T CELL ACTIVATION

By

Nicole A. Braun

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Approved:

W. Gray (Jay) Jerome, PhD

Larry Swift, PhD

Gregory Sephel, PhD

Anne Kenworthy, PhD

Luc van Kaer, PhD

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CHAPTER I

INTRODUCTION

Atherosclerosis

Atherosclerosis is a chronic inflammatory disease characterized by dyslipidemia and accumulation of lipids in the arterial intima, with activation of both innate and adaptive immunity. Reciprocally, dyslipidemia associated with atherosclerosis can perturb normal immune function. Natural killer T (NKT) cells are a specialized group of immune cells that share characteristics with both conventional T cells and natural killer cells. However, unlike these cells, NKT cells recognize glycolipid antigens and produce both pro- and anti-inflammatory cytokines upon activation. Because of these unique characteristics, NKT cells have recently been ascribed a role in the regulation of immunity and inflammation, including cardiovascular disease. In addition, NKT cells represent a bridge between dyslipidemia and immune regulation. This chapter summarizes the current knowledge of NKT cells and discusses the interplay between dyslipidemia and the normal functions of NKT cells and how this might modulate inflammation and atherosclerosis.

Over the past 2 decades, atherosclerosis has gained recognition as an immune-mediated process. Many investigators have elegantly demonstrated that not only does cholesterol homeostasis play a role in the pathogenesis of atherosclerosis, but that innate and adaptive immunity are important in this

disease. Atherosclerosis is modulated by infection (1), immunodeficiency (2) and autoimmunity (3). Perhaps one of the most interesting recent discoveries is that circulating lipids and immunity are closely linked and have a great deal of influence on each other. For example, it has been demonstrated that apolipoproteins, such as apoliprotein (apo)E and apo-AI, i.e. molecules that have extensively been studied for their role in lipoprotein metabolism and clearance, also have important roles in the development of normal immune responses and inhibiting inflammation (4, 5). Conversely, it has been shown that modified lipoproteins, such as oxidized low-density lipoprotein (oxLDL), can have detrimental effects on inflammation by promoting chemotaxis and cytokine secretion by macrophages (6). In fact, oxLDL is one of the most prevalent antigens associated with atherosclerosis eliciting specific responses from both B and T cells. Almost every aspect of immunity, including apoptosis and efficient clearance of apoptotic cells by macrophages, has been highlighted as either being protective or pathogenic in the atherosclerotic process. In this chapter, we will highlight the importance of an interesting member of the innate immune system that shares qualities with cells of both innate and adaptive immune responses: natural killer T (NKT) cells. As detailed in the following sections, NKT cells are unique effector cells that share identity with traditional natural killer (NK) cells and classical T cells (Figure 1). Numerous investigators have demonstrated regulatory roles of NKT cells and have found that, although few in number, their presence and responses can have a catalytic impact on disease progression. Their importance in atherosclerosis was emphasized in 2004, when three studies

were published demonstrating that activation of NKT cells by specific ligand resulted in enhanced aortic lesion formation (7-9).

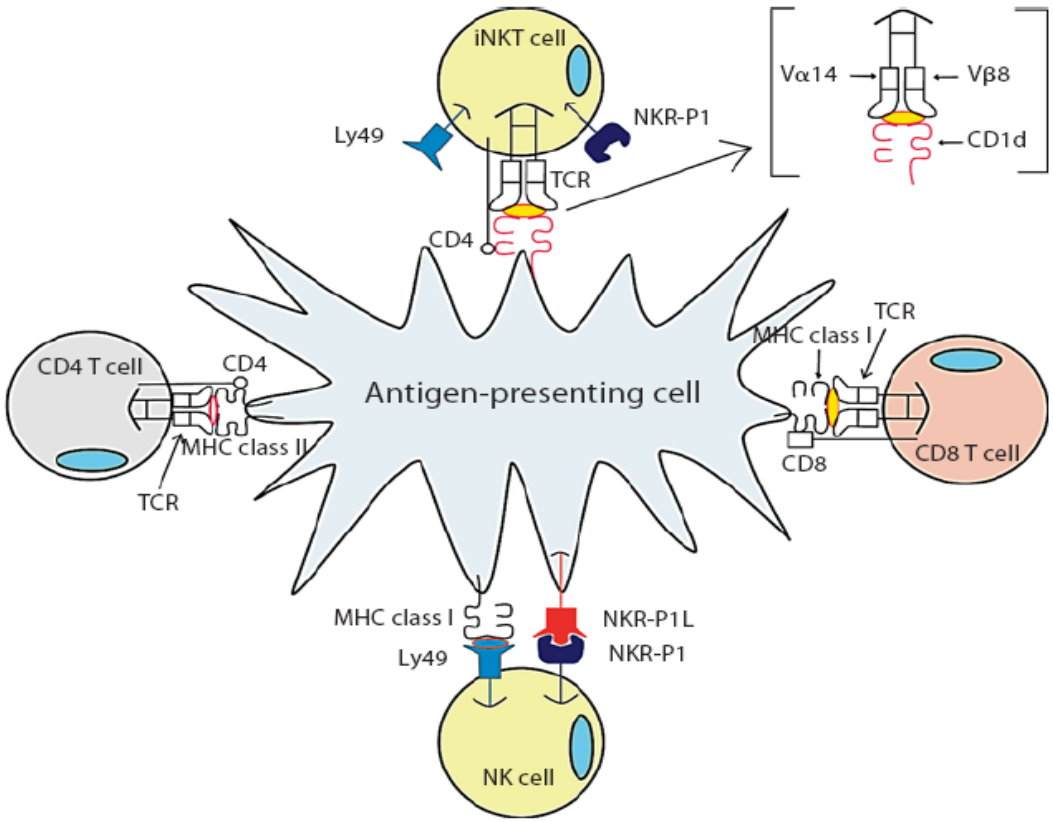

Figure 1. **iNKT cell receptor expression in comparison to NK cells and conventional CD4$^+$ and CD8$^+$ T cells.** iNKT cells express surface receptors similar to conventional T cells (TCR), as well as NK cells (Ly49 and NKR-P1); however, unlike conventional T cells which are activated by peptide antigen presented by MHC molecules, iNKT cells recognize glycolipid antigen in the context of CD1d molecules on antigen presenting cells.

Atherosclerosis and Immunity

Atherosclerosis is a disease involving many cellular processes, and has long been associated with hypercholesterolemia. A growing body of evidence

also supports the role of inflammation and immunity in the pathogenesis of atherosclerosis. Studies in CVD have suggested both anti- and pro-atherogenic roles for immunity. In general, it has been demonstrated that macrophages and T cells make up the largest percentage of immune cells present in the atherosclerotic plaque and they appear to contribute to the inflammatory process by producing cytokines that attract smooth muscle cells and other lymphocytes, and compromise plaque stability (10). $CD4^+$ T cells reactive against oxLDL can be isolated from human atherosclerotic lesions (11), and studies in T-cell-deficient mice have demonstrated a pro-atherogenic role for conventional $CD4^+$ T cells. Conversely, $CD4^+CD25^+$ regulatory T cells have been shown to protect against atherosclerosis (12, 13).

Although it was thought that B cells were present in few numbers in lesions, Galkina *et al*. (14) recently demonstrated that B cells are a significant population in both normal and atherosclerotic mouse aortic adventitia. The effects of B cells and the antibodies they produce seem to depend on their antigen specificity. For example, although titers of oxLDL antibodies are shown to correlate directly with severity of disease and are often used as markers of CVD risk (15-18), antibodies generated against modified phospholipid (such as phosphorylcholine found in oxLDL and apoptotic cells) have been shown to be protective (19-22). In fact, immunization of atherosclerosis-susceptible rabbits and mice with oxLDL and malondialdehyde (MDA)-LDL results in protection against atherosclerosis (22, 23), perhaps via a strong Th2 (anti-inflammatory) polarization which seems to rely heavily on IL-5-mediated stimulation of B-1 B

cells (20). Conversely, antibodies against β2 -glycoprotein I (β2GPI; identified in both humans suffering from CVD and in animal models of atherosclerosis (24-28) are thought to promote atherosclerosis (29, 30), and immunization of animals with HSP-60/65 results in increased atherosclerosis (30-32) . Finally, we have shown that specific deletion of B cells in LDLr$^{-/-}$ mice results in enhanced atherosclerosis compared to control animals (33). Therefore, it is possible that the 'quality' of the antibody response (i.e. isotype or antigen specificity) may influence the atherosclerosis outcome. For more in depth discussion regarding adaptive immunity and atherosclerosis see recent reviews on this topic (34, 35).

Innate Immunity and Atherosclerosis

It is no secret that innate immunity plays a major role in the atherosclerotic process. Macrophages, NK cells, mast cells and dendritic cells have all been studied and shown to play a role in plaque progression (35, 36). Macrophages are the initial and primary cell type that make up lesions both in humans and mice. Numerous studies have focused on the role of macrophages in CVD from the aspect of cells important for cholesterol homeostasis and scavenging modified lipoproteins and apoptotic cells (37) to their role as mediators of basic inflammatory responses. More recent studies have demonstrated that macrophages have a unique ability to regulate inflammation in atherosclerosis via the expression of Toll-like receptors (TLRs). These receptors, specifically TLR-4 (38), TLR-2 (39) and most recently TLR-9 (40), have been shown to play integral roles in the development of atherogenic plaques. Mice that are deficient

in MyD-88, a shared adapter molecule for TLR-4, TLR-2 and TLR-9, have been shown to have decreased atherosclerosis on the apoE$^{-/-}$ and LDLr$^{-/-}$ background (38). These studies demonstrated that receptors and molecules once thought to only function in immunity against infection have specific roles in the atherogenic process. This further emphasizes that atherosclerosis is more than simply a disease associated with lipid metabolism and that the intricate interplay between immunity, inflammation and dyslipidemia is a significant area of further investigation.

Invariant NKT Cells

In addition to the more mainstream members of the innate and acquired immune system, recent studies have highlighted the role of a less traditional cell type, NKT cells, and their role in chronic inflammatory diseases. NKT cells are a unique subset of T lymphocytes that share surface receptors with both conventional T cells (TCR and CD4) and natural killer (NK) cells (NK1.1 and Ly49) and are found in both humans and mice. NKT cells are abundant in the liver and most lymphoid tissues, and type I NKT cells, or invariant NKT (iNKT) cells, have a restricted T-cell receptor expression (Vα14-Jα18/Vβ8 in mice and α24-Jα18/Vβ11 in humans) (41). However, unlike conventional T cells which recognize peptide antigens presented by major histocompatibility complex (MHC) I or MHC II molecules, iNKT cells recognize glycolipid antigen presented by the non-classical antigen presenting molecule CD1d on antigen-presenting cells (APCs). Upon activation, iNKT cells rapidly secrete large amounts of anti-

inflammatory cytokines such as IL-4, IL-10 and IL-13, and pro-inflammatory cytokines such as IFN-γ, which allows for a wide range of regulatory potential (42). Activated iNKT cells can also promote dendritic cell maturation and monocyte activation by signaling through CD1d (43), and are capable of inducing tolerance by communicating with regulatory T cells (44). In addition to displaying immune-regulating properties, iNKT cells have been implicated in a variety of disease conditions, thus modulation of the functions of these cells may lead to potential therapies. For example, studies involving autoimmune diseases (highlighted below) have shown that iNKT cells suppress inflammation (45-47). iNKT cells have also been shown to increase anti-tumor immunity (48) and protect against infections (49) . In contrast, our laboratory (7) and others (8, 9) have demonstrated that iNKT cells are pro-atherogenic in both apoE$^{-/-}$ and LDLr$^{-/-}$ mice, as well as C57BL/6 mice on a high-fat diet.

Although few physiological ligand(s) are known, iNKT cells strongly respond to and are specifically activated by α-galactosylceramide (α-GalCer), a glycosphingolipid originally isolated from a marine sponge *(Agelas mauritanus)*. First identified for its anti-metastatic properties, α-GalCer specifically binds to CD1d on APCs and selectively activates iNKT cells (50). Activation of iNKT cells by α-GalCer (synthetic homologue KRN7000) *in vivo* has been shown to suppress inflammation in autoimmune diseases such as type-1 diabetes in mice (51); however it has also been shown to exacerbate atherosclerosis (7–9). iNKT cells are also activated by other 'natural' glycolipid ligands, albeit much less effectively. For example, several bacterial glycolipids have been shown to

activate iNKT cells and may possibly play a role in clearing of infections by iNKT cells. Among these are α-galactosyldiacylglycerols expressed by Gram-negative, LPS-negative *Borrelia burgdorferi* and α-galacturonosylceramide and α-glucuronosylceramide derived from Gram-negative, LPS-negative *Sphingomonas* (52).

iNKT Cell Ontogeny

iNKT cells undergo thymic development similar to conventional T cells, however they diverge during the double-positive thymocyte stage of development (53). In contrast to the selection of conventional T cells by peptide antigens presented by MHC molecules, iNKT cell selection requires glycolipid antigen presented by CD1d on double-positive cortical thymocytes (54). Once positively selected, iNKT cells undergo expansion within the thymus. Most iNKT cells leave the thymus as immature ($NK1.1^{-}$) cells and further mature in the periphery (55, 56), however, recent studies have shown that a smaller portion of mature iNKT cells remain $NK1.1^{-}$ in peripheral tissues and are functionally distinct from the $NK1.1^{+}$ population (57). Mature iNKT cells have the capability to rapidly produce large amounts of cytokines upon activation. Although a variety of lipid antigens have been shown to activate iNKT cells, each of these molecules must be presented to iNKT cells by CD1d. CD1d is an MHC class I-like molecule constitutively expressed by APCs, such as macrophages, dendritic cells and B cells (58). Shortly after biosynthesis in the endoplasmic reticulum, CD1d is loaded with endogenous lipid by microsomal triglyceride transfer protein and

trafficked to the plasma membrane. CD1d then undergoes extensive recycling between the plasma membrane and lysosome, where saposins facilitate lipid exchange.

Saposins are a group of four lipid transfer proteins derived from a common precursor, termed prosaposin (59). A genetic link between iNKT cells and lipid metabolism was demonstrated in studies using prosaposin-deficient mice, which lack iNKT cells and display impaired ability to present iNKT ligand (60). The deep hydrophobic antigen- binding pocket of CD1d allows for glycolipid antigen binding (61) and, after trafficking to the plasma membrane, lipid antigen-loaded CD1d engages the invariant TCR on the iNKT cell leading to subsequent activation and rapid cytokine production.

In addition to this direct pathway of activation by glycolipid antigens presented on CD1d, iNKT cell activation can also occur via an indirect mechanism first involving APC activation (Figure 2). Briefly, TLR-mediated activation of APCs leads to proinflammatory cytokine production which, along with weak interaction of iNKT cells with endogenous antigens, can activate iNKT cells (62).

iNKT Cells and Infection

Cells of the iNKT lineage are essential components in the fight against infectious agents. Much like the cells of the innate immune system, iNKT cells are capable of recognizing pathogenic structures of similar patterns. An infectious agent capable of eliciting an iNKT cell response is the Gram-negative

spirochete bacterium causative of Lyme disease: *B. burgdorferi* (63). In a study performed to elucidate the *B. burgdorferi*-mediated mechanism of iNKT cell activation, it was reported that BbGL-IIc, a galactosyl diacylglycerol comprising 12% of the lipid content of *B. burgdorferi*, is responsible for proliferation and activation of splenic and hepatic iNKT cells derived from C57BL/6 mice. BbGL-IIc associated with CD1d and variants of this lipid were ineffective at eliciting similar activated phenotypes on iNKT cells (64). These data indicate that iNKT cells can respond to specific pathogen lipid components through direct engagement of their TCR. In a recent study, C57BL/6 mice lacking iNKT cells exhibited severe heart inflammation and bacterial burden in response to *B. burgdorferi* challenge. In contrast, it was found that C57BL/6 mice with functional iNKT cells were found to have reduced bacterial loads and mitigated symptoms of disease (65). In this infectious model, iNKT cells were found to migrate to infected sites of the heart and provide protection via secreted IFN-γ and macrophage activation. A different, well-studied example of iNKT cell activation via microbial glycolipids includes GSL-1 derived from *Sphingomona paucimobilis* (66). Just like BbGL-IIc, GSL-1 was found to load on CD1d and produce an iNKT-cell-mediated response. The only characteristic shared by these two iNKT cell ligands is an α-anomeric sugar attached to the different lipid groups (63). These examples of glycolipid recognition illustrate the ability of iNKT cells to behave like cells of the innate immune system by responding to similar molecular patterns associated with different pathogens.

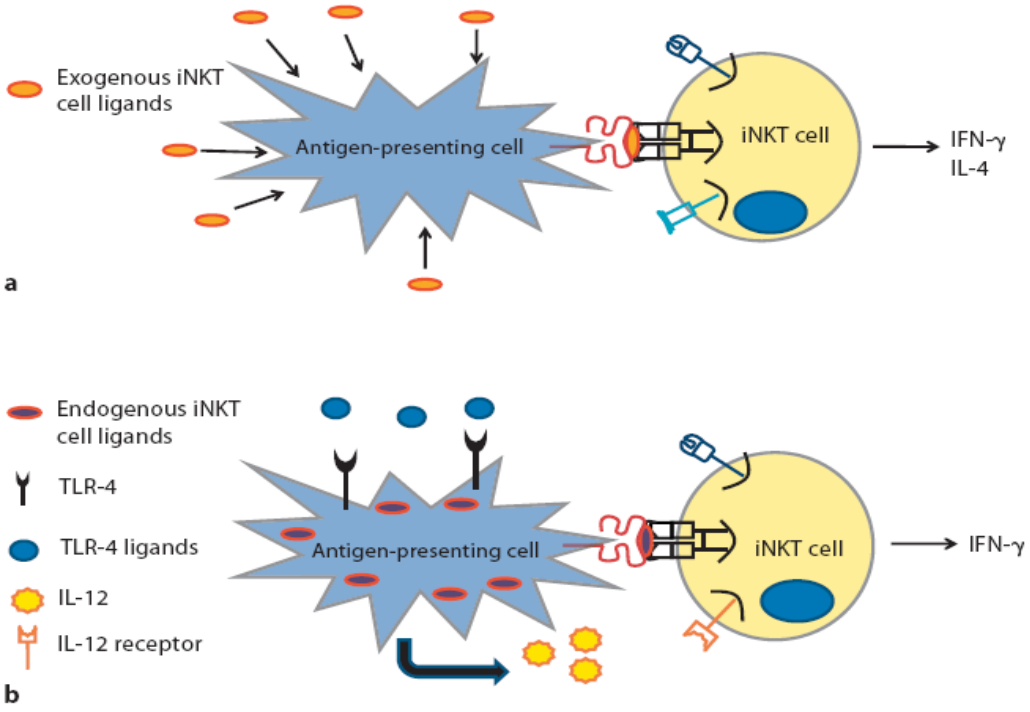

Figure 2. Potential mechanisms of iNKT cell activation. A, Direct activation of iNKT cells via exogenous gylcolipids, such as α-GalCer, taken up by APCs and presented on CD1d. **B,** Indirect activation of iNKT cells via TLR activation of APCs. Proinflammatory cytokines, such as IL-12, produced during APC activation, activate iNKT cells in combination with weak interactions of the iNKT cells with endogenous antigens.

iNKT Cells and Cancer

Harnessing cytotoxic effects of iNKT cells to targets responsible for the formation of cancer has been one of the main drivers of research for this cell type. The marine- sponge-derived synthetic iNKT cell ligand α-GalCer was discovered by its ability to prevent metastatic tumor formation (50, 67). When iNKT cells were discovered to be the main cell type responsible for recognition of α-GalCer, great excitement followed research to understand their mechanism of

tumor suppression (68).This excitement has been met with difficulty to detect the endogenous lipid ligands present in cancer cells responsible for iNKT cell activation (69). Early studies of mice deficient in iNKT cells provided an indirect measure to study resistance to tumor formation. In murine models of tumor induction mediated by methylcholanthrene- induced fibrosarcomas, it was shown that passive transfer of iNKT cells provided protection against sarcoma formation (70). Initial findings of iNKT cell immunogenicity have driven scientific groups to produce iNKT cell vaccines that provide immunity against tumor formation. In an iNKT cell vaccine study (71), it was reported that loading of a non-immunogenic murine lymphoma cell line, A20, with α-GalCer produced tumor-specific protective immunity. Mice re-challenged with A20 cells exhibited a memory phenotype protecting mice against lymphoma formation. These studies have shown the adaptive immune system becomes activated following innoculation with iNKT-cell-specific vaccine.

Adaptive immunity involvement is an interesting outcome and elucidating this mechanism would be a breakthrough in cancer research; it would provide a system that bridges innate and adaptive immunity and targets tumor cells. Translation of this protection to humans has proven difficult, as iNKT cell frequency is lower in human spleen, thymus and blood than in mice (53), and use of the anti-metastatic compound α-GalCer can cause side effects affecting the cardiovascular system (see atherosclerosis section). Whether this protection translates to humans is a feat to be accomplished.

iNKT Cells and Autoimmunity

There is an intricate relationship between iNKT cell function and defects in the modulation of autoimmune disease. The majority of the information known about iNKT cell modulation of disease comes from murine models of autoimmune disorders such as: experimental autoimmune encephalomyelitis (EAE), non-obese diabetic mice, graft transplantation rejection model representing human type I diabetes (T1D) and human graft-versus-host disease (72). A clue to the immunomodulatory ability of iNKT cells came from EAE studies where synthetic analogues of α-GalCer were utilized to treat a peptide-induced model of EAE (73). In this particular study, a peptide derived from myelin oligodendrocyte was used to induce CNS disease in C57BL/6 mice and iNKT-cell-deficient (Jα281$^{-/-}$) mice. C57BL/6 mice were protected from EAE by injection of α-GalCer analogues while in iNKT-cell-deficient mice EAE progression was not altered.

Additionally, iNKT cells have been studied in the context of T1D. In studies of monozygotic twins suffering from T1D, lower numbers of iNKT cells and abnormal polarization of Th1 responses have been observed (74). This phenotype is similar to the non-obese diabetic mouse model (75), where iNKT cell number and function are also abnormal. Similar to the EAE model, restoration of functional Vα14^{+} T cells provides protection against T1D progression. These studies indicate that iNKT cells can be modulators of autoimmune pathogenesis by affecting responses mediated by cells of the adaptive immune system. These examples illustrate how iNKT cell function alters

progression of autoimmune disease. Although human studies of iNKT cell function in autoimmune diseases are limited in number and quality of controls, the presence of functional iNKT cells in humans makes this cell lineage a great target for therapy to modulate autoimmune disorders.

iNKT Cells in Atherosclerosis

Given that lipid accumulation is a hallmark of atherosclerosis and the fact that iNKT cells are activated by glycolipid antigens, it is not surprising that iNKT cells were hypothesized to play a role in this inflammatory disease. In fact, iNKT cells have been found in human carotid artery plaques, specifically in the shoulder region of these lesions (76), as well as in human atherosclerotic tissue derived from abdominal aortic aneurysms (77). In addition, several different approaches have been taken to define the role of these unique cells in atherosclerosis using mouse models of the disease.

Given their strong association with decreasing inflammatory response, a reasonable hypothesis would be that iNKT cells are protective against progression of atherosclerosis. However, since 2004, several studies using both CD1d-deficient mice, which lack iNKT cells, have demonstrated that iNKT cells are pro-atherogenic. For example, when $CD1d^{-/-}$ mice were crossed onto the $apoE^{-/-}$ background, atherosclerosis was decreased (7–9) . In addition, wild-type $CD1d^{-/-}$ mice fed an atherogenic diet had decreased atherosclerotic lesions (8) compared to controls. It was also shown by our laboratory (7) and others (9) that repeated exogenous activation of iNKT cells by α-GalCer (or the related

glycolipid OCH (8)) increases atherosclerosis in apoE$^{-/-}$ mice. Reciprocally, using an adoptive transfer model of atherosclerosis in which iNKT cells were transferred to RAG1$^{-/-}$LDLr$^{-/-}$ mice, VanderLaan *et al*. (78) demonstrated that iNKT cells are pro-atherogenic in the absence of exogenous stimulation by α-GalCer. Studies in CD1d-deficient mice were further confirmed and refined when Rogers *et al*. (79) observed that loss of functionally active iNKT cells by using a targeted deletion of the Jα18 gene in LDLr$^{-/-}$ mice reduced the formation of early atherosclerotic lesions. These latter experiments demonstrated that the invariant type I NKT cells and not the type II NKT cells (which have variant but limited TCR usage) were responsible for increasing lesion formation in the mouse models of atherosclerosis.

Interestingly, as with many effects of immunity on atherosclerosis, the effects of iNKT cells appear to be most influential during early lesion progression. This was reported by Aslanian *et al*. (80) who demonstrated that absence of iNKT cells in LDLr$^{-/-}$ mice leads to decreased early atherosclerosis but did not influence later, more advanced lesions, and that the iNKT cell contribution to lesion progression is transient. More recently, an adoptive transfer study using various subsets of iNKT cells transferred to apoE$^{-/-}$ mice fed high-fat diet showed that CD4^{+} iNKT cells are the subset responsible for pro-atherogenic activity of iNKT cells (81). This appeared to be related to the decreased expression of the inhibitory surface receptor Ly49 on CD4^{+} iNKT cells and the increased ability of CD4^{+} iNKT cells to secrete proinflammatory cytokines such as IL-2, TNF-α and IFN-γ upon stimulation with α-GalCer. These studies illustrate the complexity of

the iNKT cell population and highlight the importance of considering their intricate functions in the design of lipid-mediated therapeutics for human inflammatory diseases.

Although most studies assign a pro-atherogenic role for iNKT cells, one study suggests the opposite effect of iNKT cells and therefore deserves discussion. van Puijvelde *et al*. (82) observed an atheroprotective role for iNKT cells in one model of atherosclerosis. Specifically, exogenous activation of iNKT cells by α-GalCer injections protected LDLr$^{-/-}$ mice, but not apoE$^{-/-}$ mice, from carotid atherosclerosis. However, in contrast to the previous studies which examined aortic sinus atherosclerotic lesions in response to α-GalCer, this study used a model of carotid atherosclerosis induced by placement of perivascular collars around the carotid arteries. These findings suggest that discrepancies in the roles of iNKT cells in atherosclerosis may be a result of the models used or that iNKT cells play different roles in different anatomic sites of the vasculature. At any rate, these diametrically opposed results indicate that further investigations are needed to fully determine the capabilities of iNKT cells to promote or protect against atherosclerosis in different settings.

Effects of Circulating Lipids on iNKT Cell Function

It is well established that increased lipid accumulation is associated with atherosclerosis in both humans and genetically altered mice. Interestingly, van den Elzen *et al*. (83) have shown that circulating lipoproteins, such as very low density lipoproteins and, more specifically, those associated with apoE, can

enhance iNKT cell responses to glycolipid antigen presented by human dendritic cells. The authors suggest that perhaps lipoprotein mediated uptake of bacterial lipid antigens, such as those associated with *Mycobacterium* spp., and the subsequent activation of NKT cells is an important means to enhance immunity. In addition, it has recently been shown that B cells, which can present lipid antigen to iNKT cells, use an apoE-dependent pathway of lipid antigen presentation, and that these APCs are more dependent upon this pathway than dendritic cells (84). Studies from our laboratory (7) have shown that iNKT cell numbers in the spleen and liver, as well as iNKT-cell-induced IFN-γ production, are decreased in apoE$^{-/-}$ mice in an age-dependent manner, which correlates with increased levels of circulating lipids in these mice. Consistent with these results, B6 mice fed a high-fat diet have suppressed iNKTcell-derived IFN- γ production in response to α-GalCer (85). To further demonstrate the importance of lipids in iNKT cell responses, LDLr$^{-/-}$ mice fed Western-type diet accumulate a CD1d-dependent antigen in the serum, and the increased level of this antigen is consistent with the presence of LDL (78). Collectively, these studies support the relationship between iNKT cells and lipoproteins and substantiate the possible link between infection, immunity and the progression of atherosclerosis.

Given that uptake and lysosomal processing of glycolipid is essential to iNKT cell activation, determining the key players in this pathway is essential. The study by van den Elzen *et al.* mentioned above, showed that apoE and one of its receptors, the LDLr, are involved in the uptake of glycolipid antigen and subsequent activation of iNKT cells. Another member of the LDLr gene family,

the low density lipoprotein receptor-related protein (LRP) has vital structural resemblance to the LDLr and recognizes a wide array of diverse ligands, including apoE-enriched lipoproteins. LRP is a ubiquitously expressed, multi-ligand receptor with many functions which include lipoprotein transport, cell surface protease activity, and cellular uptake and lysosomal delivery of secreted prosaposins, saposin precursors (86). Importantly, saposins are required for the loading and unloading of CD1d within the lysosomes (see iNKT cell ontogeny section). In addition to its role in lipoprotein metabolism, LRP has been shown to enhance antigen uptake by monocytes and macrophages (87) and is involved in control of cellular entry of bacterial toxins and viruses (88). Molecules bound to LRP are internalized and delivered to the lysosomes for subsequent degradation. Given that LRP is involved in the capture of saposin precursors, important for the CD1d pathway, and that LRP has been shown to play a role in the immune response, further studies are warranted to identify a role for this receptor in iNKT cell activation.

Summary

In summary, iNKT cells are a specialized subset of T cells which recognize glycolipid antigens. These unique cells have been studied for their involvement in several inflammatory and autoimmune diseases. Because of their ability to quickly secrete large amounts of pro- and anti-inflammatory cytokines, these cells present an attractive target for potential therapies in human disease. Given that manipulation of iNKT cells has been proposed in human disease

therapy, further investigation into how iNKT cells are influenced by their environment and how this might lead to unwanted side effects, such as an increased risk of CVD, could have obvious effects on potential therapeutic strategies. Here, we have highlighted the importance of these unique cells of the immune system within several human pathologies, including those associated with increased circulating lipids. In the following chapters, we provide evidence for the influence of a dyslipidemic environment on iNKT cell function, and that molecules involved in lipid metabolism may also play a role in iNKT cell activation.

CHAPTER II

SPONTANEOUS INKT CELL ANERGY IN A MOUSE MODEL OF DYSLIPIDEMIA

Introduction

Although few “natural” ligands of iNKT cells have been identified, it is well established that iNKT cells respond strongly to, and are specifically activated by α-galactosylceramide (α-GalCer) (50). Animal studies have demonstrated that activation of iNKT cells by *in vivo* administration of α-GalCer can mediate both protective and pathological immune responses. For example, α-GalCer has been shown to suppress inflammation in mouse models of autoimmune diseases such as type 1 diabetes and multiple sclerosis (45, 46). Our laboratory (7), as well as others (8, 9, 78), have shown that activation of iNKT cells with α-GalCer is pro-atherogenic. Because of the strong immunoregulatory potential of iNKT cells, α-GalCer has been proposed as a viable human therapy for autoimmune and inflammatory diseases, as well as cancer (89). Therefore, understanding how environmental factors can influence or skew the iNKT cell response to glycolipids is extremely important.

Nakai *et al*, demonstrated that C57BL/6 mice fed a high fat diet (8) have decreased iNKT cells numbers and functions, as measured by cytokine secretion. Our laboratory has reported similar changes in iNKT cell dynamics in

spontaneously dyslipidemic apolipoprotein E-deficient (apoE$^{-/-}$) mice (7). These studies demonstrate, perhaps not surprisingly, that iNKT cells are extremely sensitive to their lipid environment. It is not known, however, whether diet and lipid-mediated changes in iNKT cell functions are due to intrinstic changes in iNKT cells or defects in the ability of antigen presenting cells, such as dendritic cells, to activate iNKT cells.

In the current study we investigated the mechanism(s) by which spontaneous dyslipidemia decreases the responsiveness of iNKT cells to exogenous glycolipid antigen. We demonstrate that hyperlipidemia chronically activates iNKT cells, placing them in an irreversible anergic-like state, and that these changes in the iNKT cell phenotype are irreversible once established, and independent of the source of antigen presenting cell.

Methods

Animals. Male C57BL/6J (B6), B6.129P2-Apoe^{tm1Unc}/J (apoE$^{-/-}$), and LDLr$^{-/-}$ mice were originally purchased from Jackson Laboratories (Bar Harbor) and maintained in our colonies under pathogen-free conditions. ApoE$^{-/-}$ mice have been backcrossed to B6 mice for greater than 10 generations. Animals were fed standard chow diet *ad libitum*. All animal studies were approved by the Institutional Animal Care and Use Committee of Vanderbilt University (Nashville, TN).

Reagents. α-GalCer (KRN7000) was obtained from Kirin Brewing Company (Japan) and reconstituted in PBS containing 0.5% polysorbate-20 (Sigma Aldrich). GGC (PBS-18) was described previously (90). Fluorescently labeled tetrameric CD1d molecules loaded with α-GalCer (CD1d tetramers) were obtained from the National Institute of Allergy and Infectious Diseases (NIH tetramer facility). For blocking experiments, anti-mouse PD-1 (91) and rat anti-mouse PD-L2 (TY25) (92) monoclonal antibodies were described previously. Anti-TCRβ-FITC, anti-PD-1-PE, anti-PD-L1-PE, anti-PD-L2-PE, anti-B220-PerCP, and anti-Ly49-PE were obtained from BD Pharmingen. Anti-CD11c-PerCP-Cy5.5 was obtained from eBioscience. For recognition of CD1d:α-GalCer complexes, L363 monoclonal antibody was used as previously described (93). For the *in vitro* iNKT cell hybridoma assay, the DN32.D3 and N38-2C12 hybridomas were kindly provided by Dr. Albert Bendelac (University of Chicago, IL). The noncanonical iNKT cell hybridoma N37-1A12 has been previously described (94). CFSE labeling kit was purchased from Invitrogen.

Flow Cytometry. Single-cell suspensions of the spleen and liver were prepared, and stained with fluorescently-labeled monoclonal antibodies as described previously (95). Flow cytometry was performed using a FACS Calibur instrument (BD Pharmingen) and the data were analyzed using FCS Express software (De Novo Software). The iNKT cell population was defined as $B220^{-}$ $TCR\beta^{int}$ $tetramer^{+}$.

Intracellular cytokine staining. For intracellular cytokine staining, splenocytes were stimulated with 25-100 ng/ml α-GalCer in the presence of GolgiStop for 4 hours. Cells were fixed and permeabilized with Cytofix/Cytoperm (BD Pharmingen) reagents according to the manufacturer's protocol.

ELISA. Mouse IL-4 and IFN-γ were measured by standard sandwich ELISA according to the manufacturer's protocol (BD Pharmingen).

Measurement of in vitro and in vivo responses to α-GalCer. 4 μg α-GalCer reconstituted in 0.5% polysorbate (vehicle) was i.p. injected into mice, splenocytes stained and analyzed by flow cytometry, and serum IL-4 and IFN-γ measured by ELISA. *In vitro*, splenocytes were plated in 96-well plates at $2.5x10^5$ cells per well in RPMI-1640 (Hyclone) containing 10% fetal bovine serum (Sigma), penicillin-streptomycin with 50 μmol/L L-glutamine (Gibco), and 50 μmol/L β-mercaptoethanol (Sigma) in the presence of varying concentrations of α-GalCer. Supernatants were collected after 72 hours of culture and cytokine levels were determined by ELISA. For proliferation studies, splenocytes were labeled with CFSE and cultured in the presence of 50 U/ml recombinant mouse IL-2 (BD Biosciences) as described previously (96).

Isolation of splenic DCs. Splenic DCs were isolated as described previously (95). Briefly, spleens were digested with 1 mg/ml collagenase type II (Sigma) in Hank's

Balanced Salt Solution (Mediatech) for 30 minutes. DCs were enriched by magnetic sorting using anti-CD11c microbeads (Miltenyi Biotech) according to the manufacturer's protocol. The purity of the enriched $CD11c^+$ population was 80-85% (data not shown). Purified DC were pulsed with 50 ng/ml α-GalCer for 30 min, washed twice with RPMI medium, and then cultured ($5x10^4$ cell/well of a 96-well plate) with iNKT cells. Bone marrow derived DCs were isolated and cultured as previously described (97).

Enrichment of iNKT cells. Liver iNKT cells were enriched as previously described (95). Briefly, livers were perfused with PBS, digested with type II collagenase (Sigma), and then pressed through a 70 μm cell strainer. To remove adherent APCs, two rounds of panning, 1 hour each, were performed. The enriched iNKT cells were co-cultured for 48 hours with α-GalCer-loaded DCs as described above.

ConA-induced hepatitis. Mice were injected i.v. with 350 μg ConA (Sigma) in 200 μl PBS, and sacrificed 24 hours post-injection. Hematoxylin and eosin staining of paraffin-embedded livers sections was performed as previously described (98).

Serum lipid and free fatty acid analyses. Total serum cholesterol and triglyceride were measured in fasting mice as previously described (99). Free fatty analysis was conducted by gas chromatography by the Vanderbilt Mouse Metabolic Phenotyping Center.

Statistics. Statistical significance between two groups was determined using a Student's *t* test, and significance between multiple groups was determined using one-way ANOVA with Newman-Keuls multiple comparison test for post hoc analysis. A value of $p<0.05$ was considered statistically significant and all statistical analyses were performed using GraphPad Prism software.

Results

ApoE$^{-/-}$ mice have decreased percentages and absolute numbers of peripheral iNKT cells.

Previously, our lab demonstrated that apoE$^{-/-}$ mice have an age-associated decrease in iNKT cell numbers (7); however it was not determined whether this apparent decrease was due to systemic changes in iNKT cells or was the result of impaired iNKT cell development in the thymus. To address this question, we compared iNKT cell numbers in the spleen, liver, and thymus of older (≥ 16 week-old) age matched B6 and apoE$^{-/-}$ mice. As previously reported (7), we observed a significant decrease in the percentages of iNKT cells (TCRβ^{int}tetramer^{+}) in livers and spleens of apoE$^{-/-}$ mice compared to B6 mice (Figure 3A). Given that there was no difference in the numbers of total lymphocytes in liver and spleen, the decrease in iNKT cell percentages translates into a decrease in absolute numbers of iNKT cells (Figure 3B). Thymic iNKT cell percentages and absolute numbers did not differ between apoE$^{-/-}$ and B6 mice

(Figure 3A and B). In addition, surface markers of thymic iNKT cell differentiation, CD44 and NK1.1, were not different between the two strains (Figure 3C). The data suggest that defects in iNKT cell numbers occur in the periphery and are not due to decreased thymic differentiation.

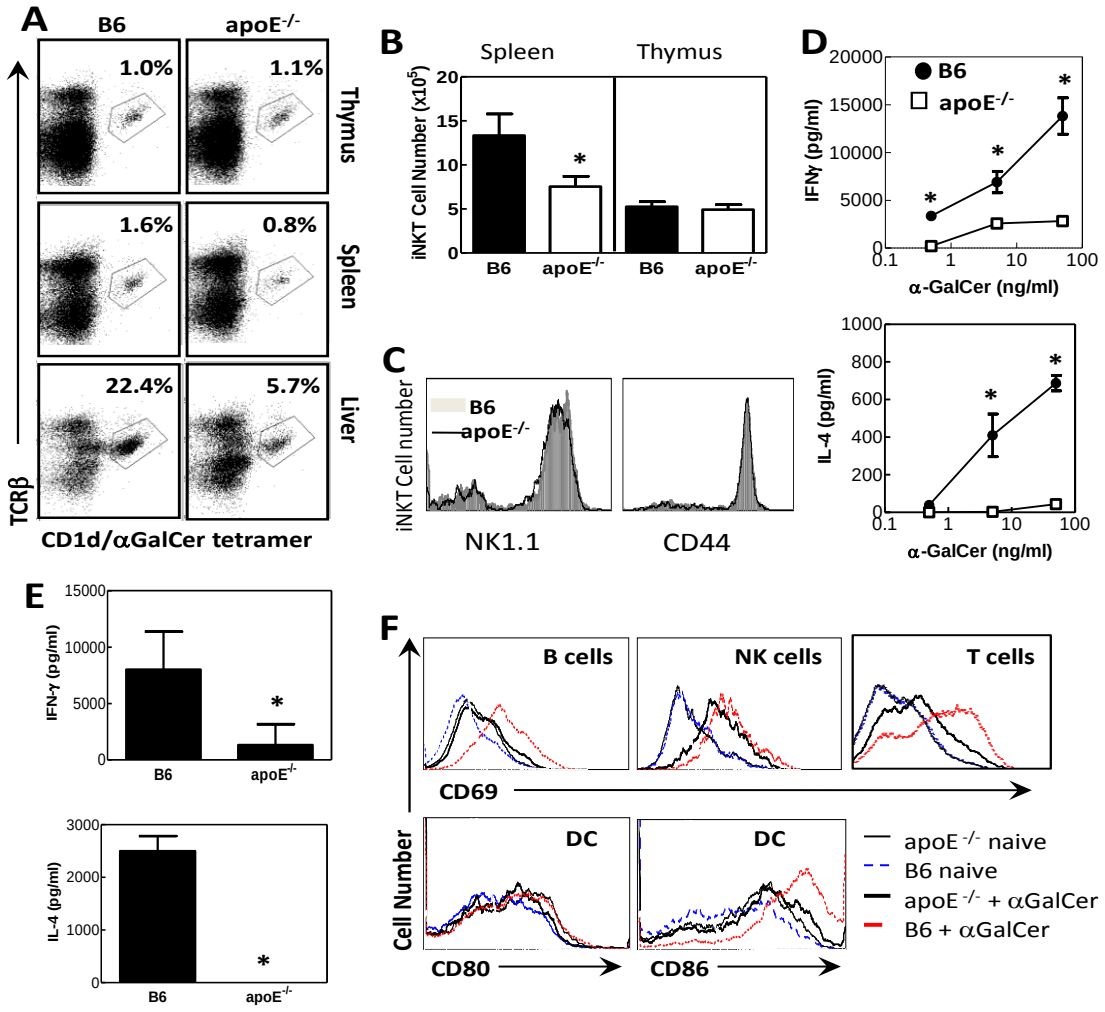

Figure 3. Decreased iNKT cell numbers and functions in apoE$^{-/-}$ mice. A, iNKT cell percentages in the thymus, spleen, and liver of B6 and apoE$^{-/-}$ mice. Shown are representative dot plots of 3 experiments with 3 mice per group. **B**, Absolute iNKT cell numbers in the spleen and thymus. Bars represent the mean and standard error. Data are representative of 3 experiments. **C**, Flow cytometry analyses of NK1.1 and CD44 on iNKT cells from the thymus of B6 and apoE$^{-/-}$ mice. **D**, Splenocytes from B6 and apoE$^{-/-}$ mice were cultured for 72 hours in the presence of the indicated concentrations of α-GalCer. IFN-γ and IL-4 were determined by ELISA. Data points represent the mean and standard error of 3 mice in each group. Shown is one representative experiment of 3. **E**, Mice were injected i.p. with 4 µg α-GalCer and cytokine production was determined by ELISA. Serum IFN-γ was measured 24h post-injection and IL-4 was measured 2h post-injection. Shown are the mean and SD of 3 mice per group. **F**, Mice were injected with 4 µg α-GalCer, and 24h post-injection, activation of splenic B cells, NK cells, T cells and dendritic cells was determined by flow cytometry. Shown are representative histograms of 5 mice in each group. *p<0.05 as determined by Student's *t* test.

Because of this, we hypothesized that spontaneous hyperlipidemia and/or spontaneous accumulation of circulating inflammatory free fatty acids (FFA), as seen in apoE-deficiency, could affect iNKT cell responsiveness. Not surprisingly, when we stimulated whole splenocytes with α-GalCer *in vitro*, we observed blunted IL-4 and IFN-γ production by apoE$^{-/-}$ splenocytes compared to age-matched controls (Figure 3D). Notably, the decrease in cytokine production in apoE$^{-/-}$ cultures was greater than what would be expected if it was due only to decreased absolute iNKT cell numbers. To determine if this degree of iNKT cell hyporesponsiveness also occurred *in vivo*, we injected apoE$^{-/-}$ and B6 mice with 4 μg/mouse of α-GalCer or vehicle intraperitoneally. At 2 and 24 hours following injection (times associated with peak iNKT cell-mediated IL-4 and IFN-γ production, respectively), we observed that, similar to *in vitro* analyses, the *in vivo* response to α-GalCer was also blunted in apoE$^{-/-}$ mice, as indicated by serum IFN-γ and IL-4 (Figure 3E). This was associated with a decreased ability to transactivate other immune cells downstream of iNKT cell activation, such as B cells, NK cells, T cells, and DCs (Figure 3F).

These data show that iNKT cells from apoE$^{-/-}$ mice are hyporesponsive to exogenous stimulation; however it is unclear whether this decreased function is due to genetic deficiency of the APOE gene. Although secretion of apoE protein by iNKT cells has not been previously reported, we did not anticipate this to be the case given that the primary function of iNKT cells is to rapidly secrete large amounts of cytokines to aid in regulation of immune responses. However, if iNKT cells produce apoE, this finding could affect our interpretations of the results

using an apoE-deficient mouse model. To determine whether iNKT cells synthesize and secrete apoE *in vivo*, we isolated wild-type primary iNKT cells from the spleen. Peritoneal macrophages from B6 and apoE$^{-/-}$ mice were used as positive and negative controls, respectively. As expected, primary iNKT cells, in addition to iNKT cell hybridomas, do not express apoE protein, as determined by Western blot analyses (Figure 4).

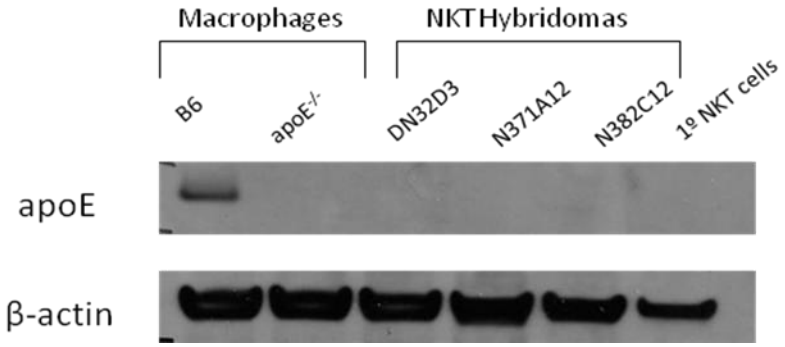

Figure 4. iNKT cells do not express apoE. ApoE expression, as determined by Western blot, in 3 iNKT cell hybridoma cell lines (DN32D3, N371A12, and N382C12) and primary iNKT cells isolated from B6 spleens. Peritoneal macrophages elicited from B6 and apoE$^{-/-}$ mice were used as positive and negative controls, respectively.

Decreased cytokine production in apoE$^{-/-}$ mice following α-GalCer stimulation is iNKT cell specific.

Although the apoE$^{-/-}$ mice had a 2-fold decrease in the absolute numbers of iNKT cells in the spleen and liver, the cytokine response to α-GalCer stimulation *in vitro* and *in vivo* was disproportionately suppressed and could not

be completely attributed to decreased iNKT cell numbers. Because *in vivo* activation or *in vitro* stimulation of splenocytes with α-GalCer allows for the potential of multiple cellular cytokine sources, we conducted intracellular cytokine staining on iNKT cells isolated from apoE$^{-/-}$ and B6 mice. Following a 4 hour *in vitro* stimulation with α-GalCer, we observed a significant decrease in IFN-γ positive splenic iNKT cells in apoE$^{-/-}$ mice compared to controls (Figure 5A). The percentage of IL-4 positive iNKT cells was reduced in apoE$^{-/-}$ mice compared to B6 mice but did not reach statistical significance (Figure 5B).

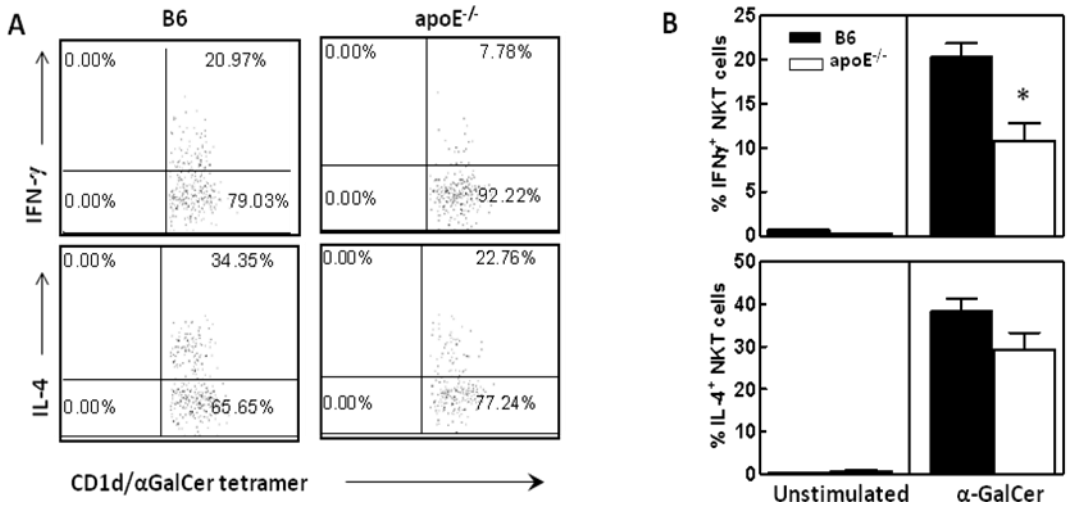

Figure 5. Decreased cytokine production is iNKT cell specific. Splenocytes from B6 and apoE$^{-/-}$ mice were stimulated for 4 h *in vitro* with 25 ng/ml of α-GalCer and intracellular IFN-γ and IL-4 were determined by flow cytometry. **A,** Representative dot plots from 3 experiments. **B,** Percentages of IFN-γ $^{+}$ (left panel) and IL-4^{+} (right panel) iNKT cells. *p<0.05 as determined by Student's *t* test.

iNKT from apoE$^{-/-}$ mice cells resemble exhausted, chronically activated cells.

Because we observed a decrease in the ability of apoE$^{-/-}$ splenic iNKT cells to produce cytokines upon stimulation with α-GalCer, we examined the ability of iNKT cells to expand *in vivo* following specific activation. Previous studies have demonstrated that upon *in vivo* activation with α-GalCer, iNKT cells respond by initially contracting at 24 hours and then expanding 3 days following α-GalCer injection (96). iNKT cells that have been rendered anergic due to a previous antigen exposure, however, fail to respond in this manner (96). Therefore, to test the hypothesis that iNKT cells in hyperlipidemic apoE$^{-/-}$ mice display an anergic phenotype, we injected B6 or apoE$^{-/-}$ mice with 4 μg/mouse of α-GalCer (or vehicle) intraperitoneally, and tracked changes in the splenic iNKT cell population over time. As shown in Figure 6A, the B6 iNKT cells responded as expected following α-GalCer injection. Although iNKT cells from apoE$^{-/-}$ mice initially contracted 24 hours following injection, these cells showed blunted expansion at three days. Quantitatively, this resulted in a significant decrease in the ability of iNKT cells from apoE$^{-/-}$ mice to proliferate in response to α-GalCer stimulation (Figure 6B). This difference was also significant following normalization to the baseline absolute numbers of iNKT cells (Figure 6C). To determine whether these changes in iNKT cell responses had physiologic consequences, we compared apoE$^{-/-}$ and B6 mice in iNKT cell-dependent ConA-induced hepatitis. As expected, livers from apoE$^{-/-}$ mice showed decreased inflammatory infiltration compared to B6 mice (Figure 6D). Therefore, iNKT cells

from apoE$^{-/-}$ mice respond to antigen stimulation *in vivo* similar to those from wild-type mice rendered anergic by repeated activation, and this abrogation of iNKT cell function is relevant to modulation of disease pathology.

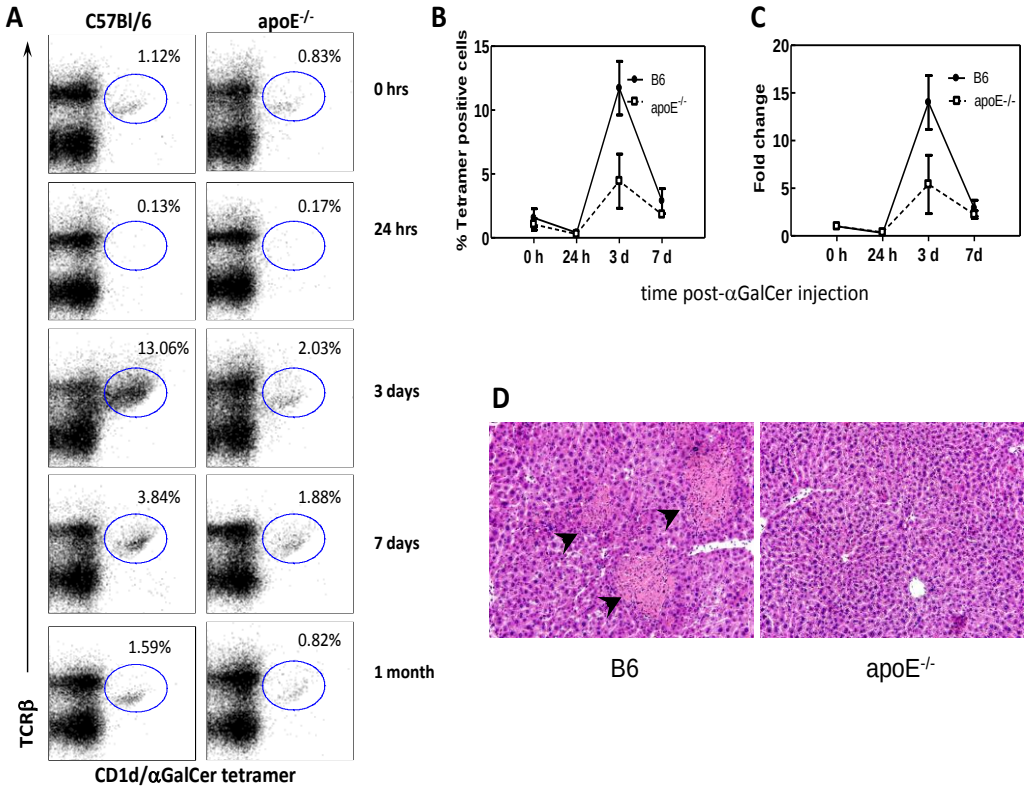

Figure 6. iNKT cells from apoE$^{-/-}$ mice display an anergic phenotype. A, B6 and apoE$^{-/-}$ mice were injected with 4 µg α-GalCer and splenic iNKT cells were analyzed by flow cytometry at 0, 24 hours, 3 days, 7 days, and 1 month following injection. Shown are representative dot plots of 3 mice per group gating on B220^{-} cells. **B**, Flow cytometry data as shown by percent tetramer^{+} cells (gated on B220^{-}TCRβ^{int}). **C**, Fold change over baseline in percent tetramer^{+} cells. **D**, B6 and apoE$^{-/-}$ mice were injected i.v. with 350 µg ConA in 200 µl PBS and sacrificed 24 hours later. ConA-induced hepatitis (black arrowheads show areas of lymphocyte infiltration) was assessed by hematoxylin and eosin staining of paraffin-embedded liver sections. Shown are representative sections from 4 mice per group.

Changes in iNKT cell activation are cell autonomous and not due to defective antigen presentation.

Given that CD1d on antigen presenting cells is required for presentation of glycolipid antigen to iNKT cells, and previous reports suggested that apoE increases uptake and presentation of glycolipid antigens by DCs (83), we isolated splenic DCs from B6 and apoE$^{-/-}$ mice and compared CD1d expression. Flow cytometry analyses demonstrated that CD1d expression was not decreased on apoE$^{-/-}$ DCs compared to B6 DCs (Figure 7A). To examine differences in glycolipid antigen presentation, splenocytes from B6 and apoE$^{-/-}$ mice were loaded with the α-GalCer precursor galactosyl(α1-2)galactosylceramide (GGC) *in vitro,* and then incubated with a monoclonal antibody specific to CD1d:α-GalCer complexes (L363) (93). GGC must undergo lysosomal processing to be loaded on CD1d molecules for surface presentation. Flow cytometry analyses showed that apoE$^{-/-}$ DCs exhibited increased CD1d loading and presentation of processed GGC at the cell surface. (Figure 7B).

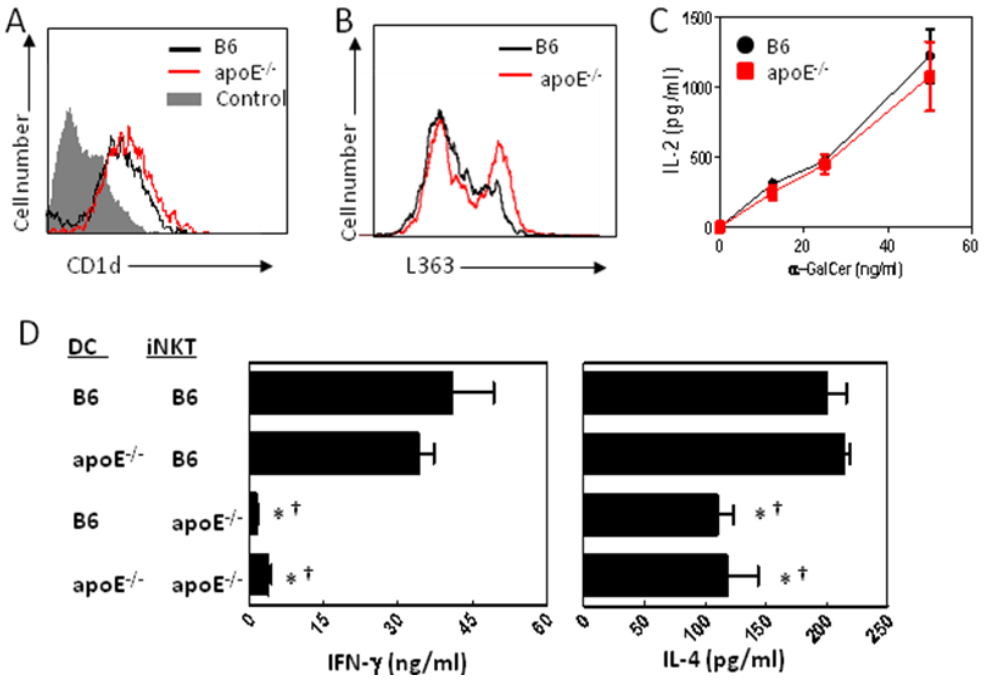

Figure 7. Dendritic cells from apoE$^{-/-}$ mice retain the ability to activate iNKT cells. **A**, CD11c^{+} splenic dendritic cell expression of CD1d as determined by flow cytometry. **B,** Splenocytes from B6 and apoE$^{-/-}$ mice were incubated 18 hours with 50 ng/ml GGC, then stained with 1 µg/ml L363 mAb to detect surface CD1d:α-GalCer complexes. Flow cytometry analyses were performed by gating on CD11c^{+} cells. Shown are representative histograms of 2 experiments using 3 mice per group. **C**, CD11c^{+} dendritic cells were purified from spleens of B6 and apoE$^{-/-}$ mice, loaded with 50 ng/ml α-GalCer for 30 min, then incubated with the iNKT cell hybridoma DN32.D3 for 48 hours. Activation of the hybridoma, as measured by IL-2 production in culture supernatants, was determined by ELISA. α-GalCer alone (with no DC) did not result in IL-2 production. **D,** Purified splenic DCs were incubated with 50 ng/ml α-GalCer for 30 min, then added to purified iNKT cells and co-cultured for 72 hours. IFNγ (left panel) and IL-4 (right panel) were determined by ELISA. *p<0.05 when compared to B6 DCs cultured with B6 iNKT cells, and †p<0.05 when compared to apoE$^{-/-}$ DCs cultured with B6 iNKT cells, as determined by 1way ANOVA.

In addition, to demonstrate that the *in vivo* changes we observed in iNKT cells from apoE$^{-/-}$ mice were not due to defective ability of apoE$^{-/-}$ DCs to process and present antigen, we grew bone marrow-derived DCs from B6 and apoE$^{-/-}$ mice. DCs were pulsed *in vitro* with α-GalCer, and then incubated with the Vα14^{+} iNKT cell hybridoma DN32.D3. We observed that DCs from both strains of mice

were able to activate the iNKT cell hybridoma with equal efficiency (Figure 7C). Finally, when lethally irradiated LDLr$^{-/-}$ mice were reconstituted with B6 bone marrow (to restore the apoE-LDLr axis on hematopoietic cells, yet retain dyslipidemia), similar decreases in iNKT cell functions were observed when mice were fed chow diet (Figure 8).

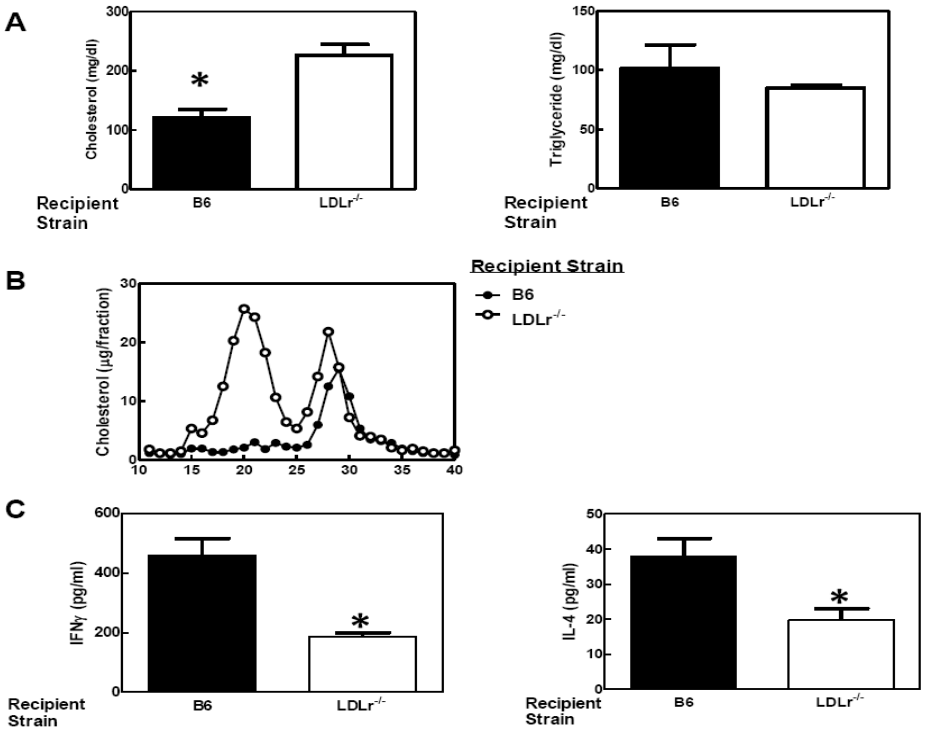

Figure 8. LDLr$^{-/-}$ mice transplanted with B6 bone marrow are dyslipidemic and have decreased iNKT cell activation. B6 and LDLr$^{-/-}$ mice were lethally irradiated and transplanted with B6 bone marrow at six weeks of age. Mice were fed standard chow diet and sacrificed at 6 weeks post-transplant. **A**, Serum cholesterol (left panel) and triglyceride (right panel) was determined by standard colorimetric assay at time of sacrifice. **B**, Serum lipoprotein profile was determined by fast-performance liquid chromatography. C, Splenocytes were cultured for 72 hours in the presence of 50 ng/ml of α-GalCer, and *in vitro* IFNγ (left panel) and IL-4 (right panel) were determined by ELISA. *p<0.05 as determined by Student's *t* test.

To determine whether iNKT cells from apoE$^{-/-}$ mice can respond to wild type APCs, splenic DCs were isolated from B6 and apoE$^{-/-}$ mice, pulsed with α-GalCer, and mixed with iNKT cells from B6 or apoE$^{-/-}$ mice. Analysis of cultured supernatants demonstrated that, although the apoE$^{-/-}$ DCs were able to stimulate cytokine production by B6 iNKT cells, the apoE$^{-/-}$ iNKT cells had a reduced ability to respond to B6 DCs (Figure 7D). Collectively, these data demonstrate that, in our system, absence of apoE does not directly affect antigen presentation or iNKT cell responsiveness but that changes are more likely due to a dyslipidemic environment.

iNKT cells from apoE$^{-/-}$ mice appear to be spontaneously activated.

It has been previously demonstrated that upon activation, the surface TCR expression on iNKT cells is downregulated via internalization (100). To determine whether this phenomenon was responsible for the apparent decrease in absolute numbers of iNKT cells in apoE$^{-/-}$ mice, we performed intracellular and extracellular staining on splenocytes using CD1d/α-GalCer tetramers and anti-TCRβ antibodies. We observed that spleens of B6 mice had similar percentages of iNKT cells following both intracellular and extracellular staining (Figure 9A, comparing surface to surface+internalized). Because surface receptors may be stained during the intracellular staining process, these data suggest that the majority of the control iNKT cells have not internalized their TCR and are not activated. In contrast, intracellular staining of apoE$^{-/-}$ splenocytes demonstrated an increase in the percentage of iNKT cells that had internalized their TCRβ,

supporting the hypothesis that iNKT cells from hyperlipidemic animals are spontaneously activated. Interestingly, when the numbers of iNKT cells that had internalized their TCR were accounted for, the apoE$^{-/-}$ mice had increased numbers of iNKT cells compared to B6 control mice. These data suggest that iNKT cells from hyperlipidemic apoE$^{-/-}$ mice (>12 weeks of age) are spontaneously activated and have internalized their TCR, accounting for the decreased detection by conventional surface staining. In contrast, *ex vivo* analyses of iNKT cells from young B6 and apoE$^{-/-}$ mice (5 weeks of age) revealed no differences in intracellular and extracellular TCRβ between the two groups (Figure 9B), suggesting that iNKT cells from apoE$^{-/-}$ mice are not spontaneously activated at an early age.

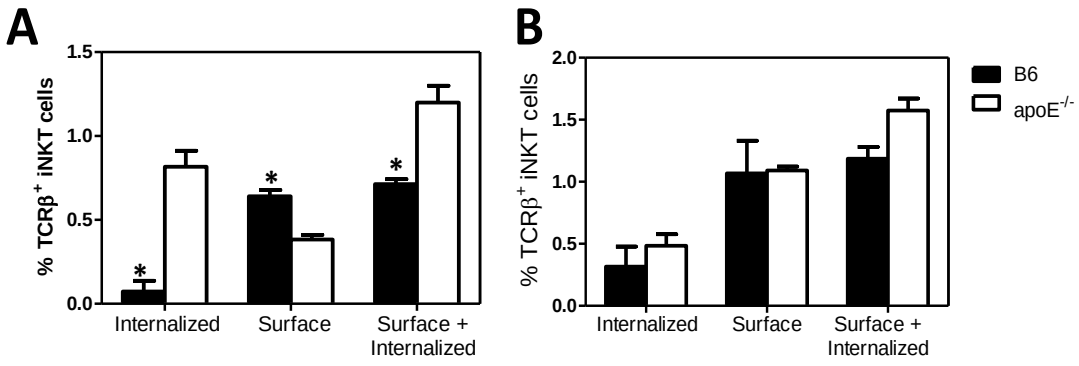

Figure 9. Increased TCRβ internalization in iNKT cells from aged apoE$^{-/-}$ mice. iNKT cells from **A,**16-20 week old and **B,** 5 week old B6 and apoE$^{-/-}$ mice and were stained for internalized and surface TCRβ expression and analyzed by flow cytometry. Internalized TCRβ was determined by subtracting surface TCRβ from surface+internalized TCRβ. Shown are representative histograms from 3 mice per group.*p<0.05 as determined by Student's *t* test

Recent studies have demonstrated that T cells from mice with chronic viral infection express increased levels of the inhibitory markers Programmed Death-1 receptor (PD-1) and PD-1 ligand-1 (PD-L1) (101). Increased expression of PD-1 and PD-L1 has also been demonstrated on iNKT cells rendered anergic by α-GalCer stimulation (102, 103). Given that iNKT cells from hyperlipidemic apoE$^{-/-}$ mice appear to be spontaneously anergic both *ex vivo* and *in vivo*, we examined the expression of the inhibitory markers PD-1, PD-L1, PD-1 ligand-2 (PD-L2), and Ly49 on iNKT cells of naïve B6 and apoE$^{-/-}$ mice. Flow cytometry analyses demonstrated that, consistent with previous reports, iNKT cells from B6 mice have some constitutive expression of PD-1, PD-L1 and PD-L2 (Figure 10A, right panels); however, the percentage of PD-1 positive iNKT cells was significantly increased in apoE$^{-/-}$ mice (Figure 10B). These increases were not observed in iNKT cells from young 5- to 6-week-old apoE$^{-/-}$ mice (Figure 10A, left panels), demonstrating that changes in iNKT cells were acquired and not directly the result of developmental effects of apoE$^{-/-}$ deficiency. These data indicate that apoE$^{-/-}$ iNKT cells exhibit an exhausted phenotype perhaps due to increased activation by accumulation of endogenous lipid antigens.

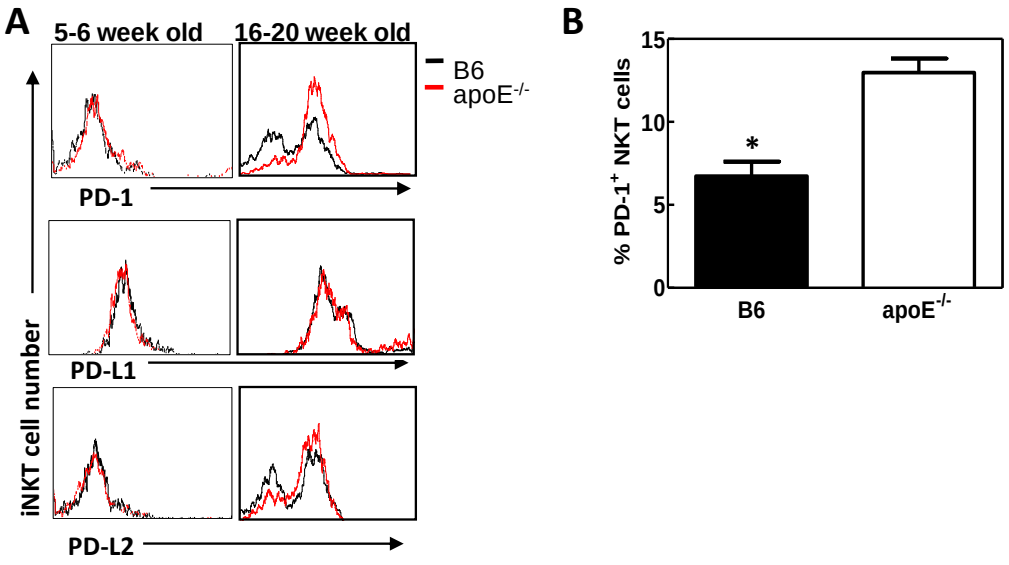

Figure 10. Increased PD-1 expression by iNKT cells from aged apoE$^{-/-}$ mice. A, *Ex vivo* PD-1, PD-L1, and PD-L2 expression on iNKT cells from 5-6 week old (left panels) and 16-20 week old (right panels) B6 and apoE$^{-/-}$ mice. Shown are representative histograms from 3 experiments. **B,** Percent PD-1^{+} iNKT cells *ex vivo* in 16-20 week old B6 and apoE$^{-/-}$ mice. *p<0.05 as determined by Student's *t* test.

PD-1 blockade can neither rescue nor prevent downregulation of iNKT cell responses in apoE$^{-/-}$ mice.

To determine whether blockade of PD-1 could restore apoE$^{-/-}$ iNKT cell responses as previously described (102), we incubated whole splenocytes from naïve B6 and apoE$^{-/-}$ mice with 50 ng/ml α-GalCer in the presence of a PD-1 blocking antibody or isotype control antibody. Analysis of 72 hour supernatants demonstrated a partial increase in cytokine secretion in apoE$^{-/-}$ mice (Figure 11A); however, PD-1 blockade did not result in normalization of cytokine levels in supernatants of apoE$^{-/-}$ mice. These data suggest that blocking PD-1 cannot rescue the anergic phenotype of apoE$^{-/-}$ iNKT cells.

Previous work by Parekh *et al,* (96) demonstrated that, although PD-1 blockade could not rescue anergic iNKT cells, it could prevent the development of anergy in this cell population if conducted before activation with α-GalCer. To determine whether this also applied to our apoE$^{-/-}$ model, we injected apoE$^{-/-}$ mice with anti-PD-1 or control rat immunoglobulin beginning at weaning (approximately 4 weeks of age). Mice received antibody for 6 weeks after which responses to α-GalCer were analyzed. We observed that PD-1 blockade could not prevent induction of iNKT cell anergy in apoE$^{-/-}$ mice (Figure 11B). These data suggest that upregulation of PD-1 is not the primary mechanism by which iNKT cells become non-responsive in apoE$^{-/-}$ mice.

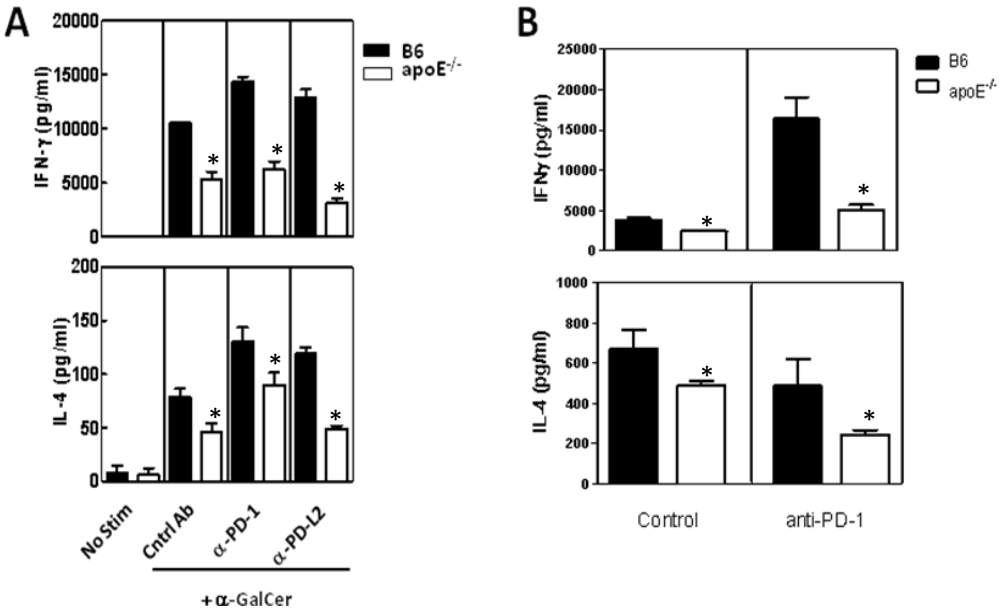

Figure 11. PD-1 blockade is not sufficient to restore or prevent iNKT cell responsiveness. A, Splenocytes were cultured *in vitro* in the presence or absence of α-GalCer, with 50 µg/ml of control Ig, anti-PD-1, or anti-PD-L2 antibodies. After 72 hours, IFN-γ (top panel) and IL-4 (bottom panel) were determined by ELISA in culture supernatants. **B,** B6 and apoE$^{-/-}$ mice were injected with control Ig or anti-PD-1 antibodies for 6 weeks, beginning at weaning. Mice were sacrificed and splenocytes were cultured with 50 ng/ml α-GalCer for 72 hours and IFN-γ (top panel) and IL-4 (bottom panel) were determined by ELISA. *p<0.05 as determined by Student's *t* test.

Exogenous IL-2 restores apoE$^{-/-}$ iNKT cell proliferation in vitro.

It is known that administration of exogenous cytokines, such as IL-2, can overcome conventional T cell exhaustion, as well as iNKT cell anergy (96). Given that iNKT cells from apoE$^{-/-}$ mice display an anergic-like phenotype, we tested the ability of exogenous IL-2 to restore iNKT cell responses *in vitro*. Splenocytes from B6 and apoE$^{-/-}$ mice were labeled with CFSE and stimulated with α-GalCer in the presence or absence of IL-2. As shown by the CFSE dilution profile of tetramer+ cells (Figure 12), iNKT cells from apoE$^{-/-}$ mice displayed decreased

proliferation in response to α-GalCer as compared to B6 iNKT cells. Consistent with previous studies (96), addition of IL-2 resulted in restoration of the ability of apoE$^{-/-}$ iNKT cells to proliferate. These data indicate that iNKT cells from hyperlipidemic apoE$^{-/-}$ mice are anergic, and that IL-2 is sufficient to overcome this anergy *in vitro*.

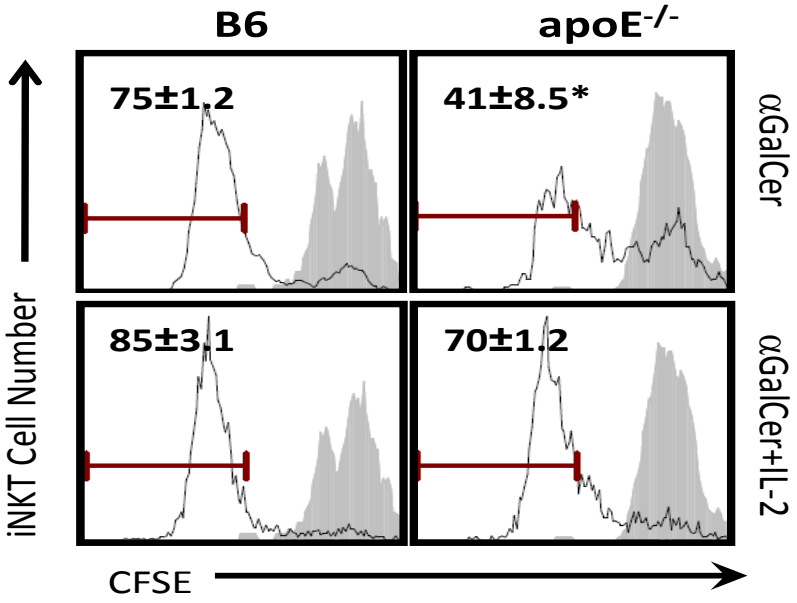

Figure 12. IL-2 overcomes iNKT cell anergy *in vitro*. Splenocytes from B6 and apoE$^{-/-}$ mice were labeled with CFSE and cultured for 24 hours with α-GalCer (100 ng/ml) with or without IL-2 (50 U/ml). The cells were then washed, and cultures were continued for an additional 72 hours without α-GalCer, in the presence or absence of IL-2. After culture, CFSE dilution was analyzed on iNKT cells. Shown are representative histograms from 3 mice per group. *$p<0.05$ as determined by Student's *t* test.

Discussion

iNKT cells are a unique subset of T cells that recognize glycolipid antigens and play a role in the regulation of inflammation and immunity. Furthermore, our laboratory (7) and others (8, 9, 78), have shown that activation of iNKT cells is proatherogenic. During conditions of spontaneous hyperlipidemia in apoE$^{-/-}$ mice (7) and diet-induced dyslipidema (8), iNKT cell numbers and functions are decreased. However, the etiology behind these changes is not yet known. Modulation of iNKT cell responses is being clinically tested as a possible therapeutic for cancer (104), diabetes (105), and HIV (106) in humans. In addition, it has been hypothesized that activating iNKT cells during immunization is a superior vaccination strategy (107). Thus, it is imperative to understand how the lipid environment might dampen or skew the iNKT cell response.

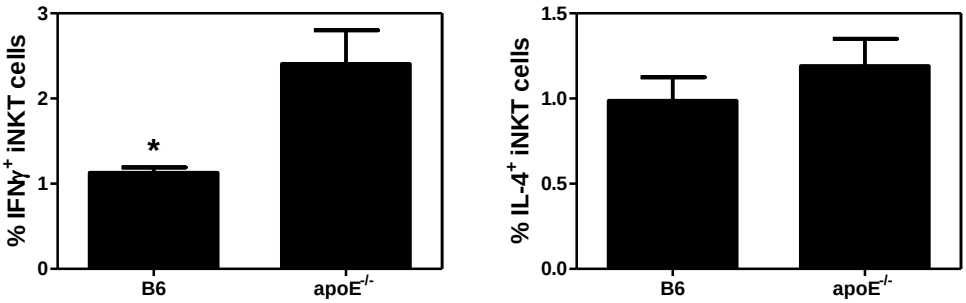

Figure 13. Liver iNKT cells from apoE$^{-/-}$ mice produce higher levels of IFN–γ compared to B6 controls. Liver lymphocytes from B6 and apoE$^{-/-}$ mice (20 weeks of age) were stimulated for 4 h *in vitro* with 100 ng/ml of α-GalCer and intracellular IFN-γ and IL-4 were determined by flow cytometry. Percentages of IFNγ $^{+}$ (left panel) and IL-4^{+} (right panel) iNKT cells. *p<0.05 as determined by Student's *t* test.

In the current study, we sought to examine the mechanisms by which chronic hyperlipidemia might decrease iNKT cell numbers and functions. We observed that apoE$^{-/-}$ mice have decreased numbers of iNKT cells in spleens and livers compared to B6 controls, and that iNKT cells present in the spleens of apoE$^{-/-}$ mice have an impaired ability to respond to exogenous stimulation by the glycolipid α-GalCer. This decreased cytokine production seemed to affect IFN-γ responses more than IL-4 suggesting that Th1 responses may be more effected than Th2. Interestingly, liver iNKT cells from apoE$^{-/-}$ mice produced higher levels of IFN-γ compared to B6 controls (Figure 13). However, both apoE$^{-/-}$ and B6 mice had a very low percentage of liver iNKT cells that was cytokine positive following stimulation. Therefore, it is difficult to determine the impact of these data, although, it is possible that liver iNKT cells are already somewhat non-responsive to *ex vivo* stimulation due to the high frequency of lipid in the liver.

It is well established that glycolipids play an important role in the development of iNKT cells within the thymus, and that normal thymic development of these iNKT cells requires the recognition of natural lipid ligands presented by CD1d. Previous studies have suggested that the endogenous glycosphingolipid isoglobotrihexosylceramide (iGb3) is important for iNKT cell development and self-recognition (108). Because a spontaneous increase in circulating lipids, as seen in apoE$^{-/-}$ mice, could affect the development of iNKT cells and subsequently lead to decreased peripheral numbers, we analyzed

thymic iNKT cells from apoE$^{-/-}$ and B6 mice by flow cytometry. Our data suggest that decreased iNKT cell numbers in apoE$^{-/-}$ mice are not the result of defects in thymic development because numbers and maturation markers of iNKT cells in the thymus remained largely unchanged. Instead, our data support the hypothesis that the iNKT cell defect results from changes in the periphery. This is in stark contrast to studies in mice with deficiencies in the Niemann-Pick type C1 and C2 proteins where reductions in iNKT cell numbers were observed in the thymus and peripheral lymphoid tissues (109-111). This was also found to be the case in mouse models of other lysosomal lipid storage diseases such as mice deficient for $\alpha-$ or $\beta-$galactosidase or hexosaminidases A and S (112). Therefore, decreased numbers of iNKT cells in the peripheral lymphoid organs of apoE$^{-/-}$ mice suggest that the endogenous glycolipids necessary to elicit normal selection of iNKT cells in the thymus are not affected by lipid accumulation.

Recent evidence has shown that B cell-mediated activation of iNKT cells is enhanced by apoE and is dependent upon the low density lipoprotein receptor (LDLr) (84). In addition, findings from Van den Elzen *et al* (83) have shown that circulating lipoproteins, such as very low density lipoproteins (VLDL), can enhance iNKT cell responses to glycolipid antigen presented by DCs. These experiments elegantly demonstrated that apoE may enhance glycolipid uptake, however, there was no evidence that glycolipid and apoE interact *in vivo* or that apoE was necessary for normal iNKT cell responses to exogenous lipid antigen. Although the absence of apoE and the effect this might have on antigen uptake is still an important consideration in our system, we have several pieces of

evidence suggesting that the absence of apoE alone cannot explain the difference in iNKT cell activation observed in hyperlipidemic mice. First, DCs from apoE$^{-/-}$ mice are not compromised in their ability to present antigen to B6 iNKT cells. Additionally, CD1d loading of GGC and presentation on the cell surface, as measured by L363 antibody staining, was not compromised in apoE$^{-/-}$ DCs. Therefore, we conclude that, although apoE may increase iNKT activation under normolipidemic conditions, the functional defects we observe in apoE$^{-/-}$ mice cannot be completely attributed to the lack of apoE.

It has been shown previously in atherosclerosis studies that lipids such as the glycosphingolipid β-glucosylceramide (β-GlcCer) (113) and the disialoganglioside GD3 (114) accumulate in the serum of apoE$^{-/-}$ mice as well as in humans. In addition, natural activation of iNKT cells during microbial infection is often dependent on both IL-12 and presentation of endogenous glycolipid antigen by DCs (115). Thus, a possible explanation for the iNKT cell hyporesponsiveness we observe in apoE$^{-/-}$ mice is that these cells are chronically activated by increased levels of endogenous glycolipid. Although, recent data from VanderLaan *et al* (78) suggest that endogenous iNKT antigen was not present in the serum of apoE$^{-/-}$ mice fed high fat diet, it is possible that presence of an endogenous ligand in tissue (such as in the liver, spleen or lymph node) and not serum is responsible for the changes in iNKT cells we observed. Alternatively, given that glycolipid antigens are processed and loaded onto CD1d within the APC after uptake, lysosomal accumulation of lipids during hyperlipidemia may play a role in the differences we observe. Supporting this

possibilty, a recent study from Bai *et al* (116), shows that lipid exchange within the lysosome is regulated by lipid structure as well as acidic lysosomal pH. However, our results indicate that DCs from apoE$^{-/-}$ mice retain their ability to activate iNKT cells, suggesting that the iNKT cell hyporesponsiveness in apoE$^{-/-}$ mice is due to chronic activation rather than defective antigen presentation.

Previous studies have shown that repeated activation of conventional T cells, such as in chronic viral infection, results in T cell exhaustion (117). Similarly, repeated activation of iNKT cells by α-GalCer also results in a functionally unresponsive anergic phenotype (96). Our data show that apoE$^{-/-}$ iNKT cells display a phenotype similar to those rendered anergic due to repeated activation. This evidence supports our hypothesis that spontaneous dyslipidemia, as observed in apoE$^{-/-}$ mice, leads to chronic activation of iNKT cells. Several recent reports have shown that the inhibitory receptor PD-1 is upregulated on exhausted T cells due to chronic activation (118, 119). In addition, PD-1 has been implicated in the induction and maintenance of iNKT cell anergy (102). In a recent study by Parekh *et al* (103), it was shown that blocking PD-1/PD-L interactions could prevent iNKT cell anergy, but could not overcome anergy once established. Our study illustrates that hyperlipidemic apoE$^{-/-}$ mice have spontaneously increased PD-1 expression on iNKT cells, although blocking PD-1 *in vitro* was not able to restore responsiveness to α-GalCer stimulation. Consistent with our data, recent clinical findings in HIV-infected humans indicated that these patients have increased PD-1 expression on iNKT cells, and PD-1 blockade did not restore iNKT cell function (36). Taken together, these data

suggest that in our system, decreased functionality of chronically activated iNKT cells is not dependent on PD-1 expression and may be irreversible once established.

It is known that iNKT cells become undetectable with tetramer soon after *in vivo* stimulation with α-GalCer due to surface TCR downregulation (99). Our flow cytometry analyses demonstrate that there is a significant increase in the intracellular TCRβ in the apoE$^{-/-}$ mice as compared to B6 mice. Importantly, these studies were performed *ex vivo* without exogenous stimulation. In addition, we have shown that hyperlipidemic apoE$^{-/-}$ mice have modest but significant increases in myristic acid (14:0) and palmitic acid (16:0) (Figure 14). Although, the physiologic relevance of this increase is not tested in the current study, the serum concentrations we observe are in excess of fatty acids concentrations that have been shown to activate APCs through Toll-like receptor activation (120). Therefore, it is possible that saturated fatty acid signaling in DCs via Toll-like receptor 4 leads to the production of IL-12, thus activating iNKT cells by the alternative or indirect pathway (62). Collectively, these data support the hypothesis that the hyperlipidemic environment of the apoE$^{-/-}$ mice spontaneously activates the iNKT cells, rendering them unresponsive to further stimulation. Further studies to test this hypothesis are warranted.

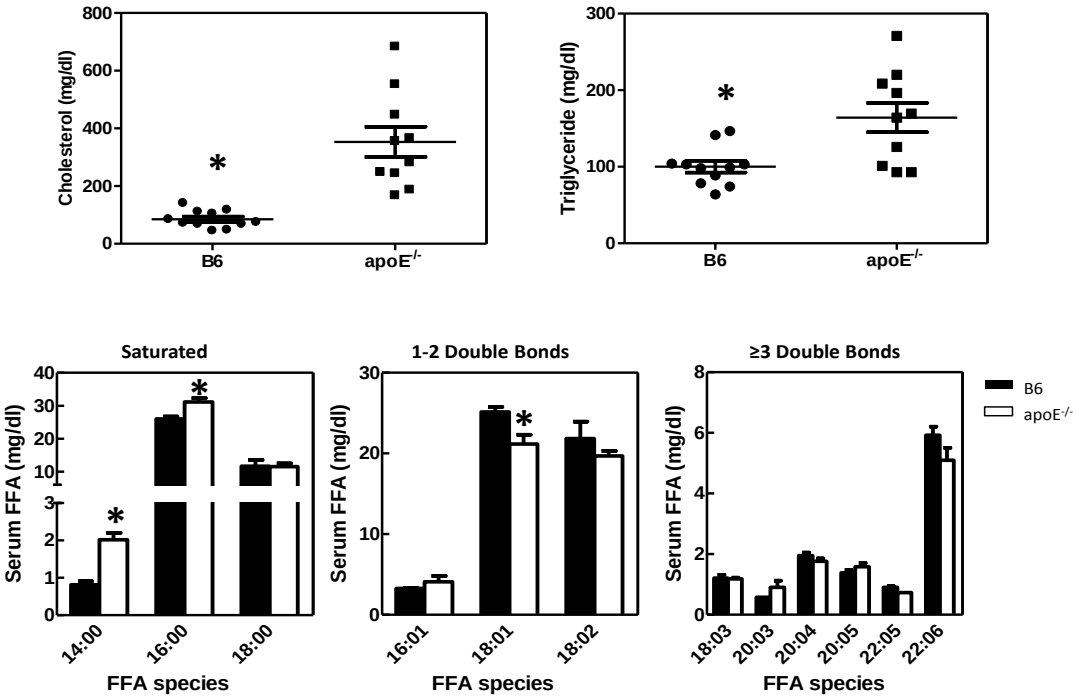

Figure 14. apoE$^{-/-}$ mice are dyslipidemic. Serum cholesterol (upper left panel) and triglyceride (upper right panel) of male B6 and apoE$^{-/-}$ mice (≥16 weeks old) was determined by standard colorimetric assay. Free fatty acid (FFA) composition (lower panels) of serum from B6 and apoE$^{-/-}$ mice (16 weeks old) was determined by gas chromatography. (n=3 mice per group). *p<0.05 as determined by Student's *t* test.

In summary, our results indicate that an increase in circulating lipids leads to decreased iNKT cell functionality and that this dyslipidemia-associated decrease is iNKT cell intrinsic. The functional characteristics of iNKT cells are important to understand not only in chronic conditions of dyslipidemia, such as atherosclerosis, but also in diseases for which therapeutic approaches involving manipulation of iNKT cells are being considered. In addition, given that similar decreases in iNKT cell numbers and functions are observed in wild-type mice fed

a high fat diet (8), caution may be warranted in immunologic studies involving the use of lipid-laden diets and therapies that give rise to dyslipidemia.

CHAPTER III

DYSLIPIDEMIA DIRECTLY AFFECTS INKT CELL ACTIVATION

Introduction

Dyslipidemia has long been associated with human diseases such as atherosclerosis. It is well established that atherosclerosis is a chronic inflammatory disease involving both innate and adaptive immunity, and several reports have investigated the effects of increased circulating lipids on cells of the immune system. The effects of cholesterol on macrophage function are well known. It has also been suggested that DC function may be reduced or inhibited during conditions of hypercholesterolemia (121, 122), however recent evidence suggests these cells retain their antigen presentation function even when cholesterol loaded (35). In addition to these studies, recent attention has focused on the role of cellular cholesterol homeostasis on conventional T cell activation and proliferation. Bensinger *et al.* have reported that liver X receptors (LXR), whose target genes are involved in cholesterol and fatty acid metabolism, play an important role in lymphocyte function (123).

Although well characterized in other immune cells, the direct influence of increased circulating lipids on iNKT cells has not been extensively studied. Given that iNKT cells are unique cells of the immune system which recognize glycolipid antigens, and that these cells are currently being considered as therapeutic

targets for several human diseases, understanding how the lipid environment directly affects their activation is imperative.

Our previous data show that iNKT cell hyporesponsiveness observed in dyslipidemic apoE$^{-/-}$ mice is iNKT cell autonomous and is not due to defective antigen presentation. Because these data suggest that increased circulating lipids in apoE$^{-/-}$ mice can affect iNKT cell function without affecting antigen presentation, we hypothesized that chronic dyslipidemia directly renders iNKT cells unresponsive to exogenous glycolipid antigen stimulation through intracellular lipid accumulation. The influence of intracellular lipid on the control of iNKT cell immune responses has yet to be determined. In this study, we sought to determine the direct effects of increased lipids on iNKT cell activation. Our results suggest that iNKT cells are directly influenced by their lipid environment and that these lipid-mediated changes in iNKT cell functions are due to direct changes in iNKT cells rather than through indirect mechanisms via APCs.

Methods

Animals. Male C57BL/6J (B6) and B6.129P2-Apoe^{tm1Unc}/J (apoE$^{-/-}$) mice were originally purchased from Jackson Laboratories (Bar Harbor) and maintained in our colonies under pathogen-free conditions. ApoE$^{-/-}$ mice have been backcrossed to B6 mice for greater than 10 generations. Vα14 transgenic mice were previously described (124). Animals were fed standard chow diet *ad libitum*.

All animal studies were approved by the Institutional Animal Care and Use Committee of Vanderbilt University (Nashville, TN).

Reagents. α-GalCer (KRN7000) was obtained from Diagnocine and reconstituted in PBS containing 5.6% sucrose, 0.75% L-histadine, and 0.5% polysorbate-20 (Sigma Aldrich). GGC (PBS-18) was described previously (90). Fluorescently labeled tetrameric CD1d molecules loaded with α-GalCer (CD1d tetramers) were obtained from the National Institute of Allergy and Infectious Diseases (NIH tetramer facility). For the *in vitro* iNKT cell hybridoma assays, the DN32.D3 hybridoma was kindly provided by Dr. Albert Bendelac (University of Chicago, IL). CFSE labeling kit was purchased from Invitrogen. Rabbit anti-ABCA1 and anti-LXR were purchased from Novus Biologicals, and filipin, LXR agonist GW3965, and mouse anti-β-actin were purchased from Sigma Aldrich. Purified LDL from human donors was kindly provided Dr. Jay Jerome (Vanderbilt University). LDL was heavily oxidized by adding 0.4% copper sulfate (Sigma) in PBS, incubated overnight, and reaction quenched by 1mM EDTA (Sigma).

Flow cytometry. Single-cell suspensions of the spleen and liver were prepared, and stained with fluorescently-labeled monoclonal antibodies as described previously (95). Flow cytometry was performed using a MACSQuant Analyzer instrument (Miltenyi Biotec) and the data were analyzed using FCS Express software (De Novo Software). The iNKT cell population was defined as $B220^{-}$ $TCR\beta^{int}$ $tetramer^{+}$.

Enrichment of iNKT cells. Liver iNKT cells were enriched as previously described (95). Briefly, livers were perfused with PBS, digested with type II collagenase (Sigma), and then pressed through a 70 µm cell strainer. To remove adherent APCs, two rounds of panning, 1 hour each, were performed. The enriched iNKT cells were co-cultured for 48 hours with α-GalCer-loaded DCs.

Adoptive transfer of purified iNKT cells. Splenic iNKT cells were isolated as previously described (95) and labeled with CFSE according to the manufacturer's protocol (Invitrogen). Recipient mice were sub-lethally irradiated (450 rads) using a cesium source and 10^6 CFSE-labeled iNKT cells were injected retro-orbitally. Mice were injected with 4 µg/mouse α-GalCer 4 days following adoptive transfers, and sacrificed 3 days post injection.

iNKT cell hybridoma assays. Cells were cultured 24 hours in complete RPMI medium containing either FBS, B6 mouse serum, or apoE$^{-/-}$ mouse serum (from mice >16 weeks of age). The following day CD11c$^+$ dendritic cells were isolated from B6 spleens. Splenic DCs were isolated as described previously (95), loaded with 50 ng/ml α-GalCer for 30 minutes at 37°, and co-cultured with the hybridomas for an additional 48 hours. iNKT cell hybridoma activation was assessed by IL-2 production in cell culture supernatants by ELISA.

ELISA. Mouse IL-4, IFN-γ, and IL-2 were measured by standard sandwich ELISA according to the manufacturer's protocol (BD Pharmingen).

Statistics. Statistical significance between two groups was determined using a Student's *t* test, and significance between multiple groups was determined using one-way ANOVA with Newman-Keuls multiple comparison test for post hoc analysis. A value of $p<0.05$ was considered statistically significant and all statistical analyses were performed using GraphPad Prism software.

Results

Increased circulating lipids in apoE$^{-/-}$ mice directly affect NKT cell activation.

To determine whether increased serum lipids in apoE$^{-/-}$ mice decreases iNKT cell activation *in vitro,* the classical Vα14^{+} iNKT hybridoma (DN32.D3) were cultured 24 hours in complete RPMI medium containing either 10% FBS, B6 mouse serum, or apoE$^{-/-}$ mouse serum. The following day, B6 splenic CD11c^{+} dendritic cells were isolated, loaded with 50 ng/ml α-GalCer, and co-cultured with the hybridomas for an additional 48 hours. iNKT cell hybridoma activation was assessed by IL-2 production in cell culture supernatants. As shown in Figure 15, incubation with apoE$^{-/-}$ mouse serum reduces the activation of the iNKT cell hybridoma. Although these experiments were repeated multiple times, they did

not yield consistent results; however, these preliminary data are suggestive of a negative effect of lipid-laden apoE$^{-/-}$ mouse serum on iNKT cell activation *in vitro*, although further studies to confirm this are warranted.

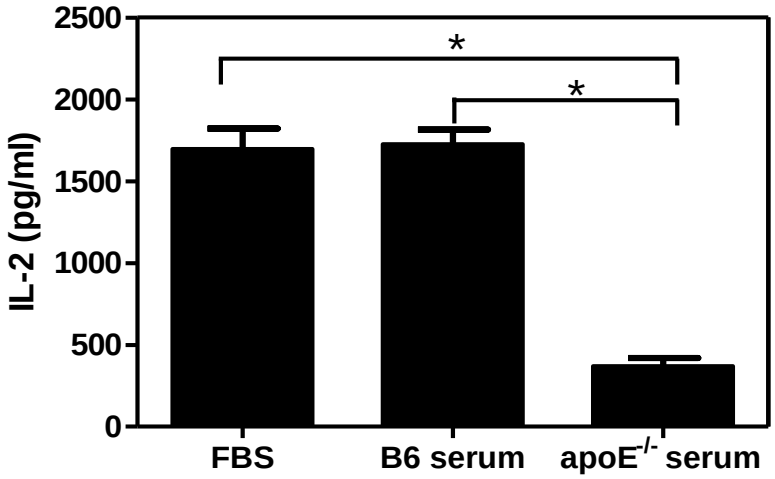

Figure 15. apoE$^{-/-}$ mouse serum decreases iNKT cell hybridoma activation *in vitro*. iNKT hybridomas (DN32.D3) were cultured in complete RPMI containing 10% FBS, B6 mouse serum, or apoE$^{-/-}$ mouse serum for 24 hours, then co-cultured with α-GalCer-loaded B6 DCs for an additional 48 hours, and hybridoma activation, as determined by IL-2 production, was measured by ELISA. *p<0.05 as determined by one-way ANOVA.

Lipid accumulation decreases iNKT cell activation.

To address whether iNKT cell responsiveness is directly affected by the increased circulating lipids, we tested iNKT cell hybridoma activation after oxLDL incubation *in vitro*. DN32.D3 hybridomas were incubated overnight in the presence or absence of 50 μg/ml oxLDL, then washed and co-cultured with α-GalCer-loaded (50 ng/ml) CD11c^{+} dendritic cells (purified from B6 spleens) for an

additional 48 hours. iNKT cell hybridoma activation, as determined by IL-2 production, was measured by ELISA. As shown in Figure 16 (left panel), iNKT cell hybridoma activation is significantly decreased in cells loaded with oxLDL for 24 hours, suggesting that lipid accumulation impairs the ability of iNKT cells to respond to glycolipid antigen. This decrease was not due to detrimental effects of oxLDL on the cells as indicated by no difference in percent viable cells (Figure 16, right panel). Cell viability was determined after the removal of supernatant at the end of culture and calculated by trypan blue exclusion.

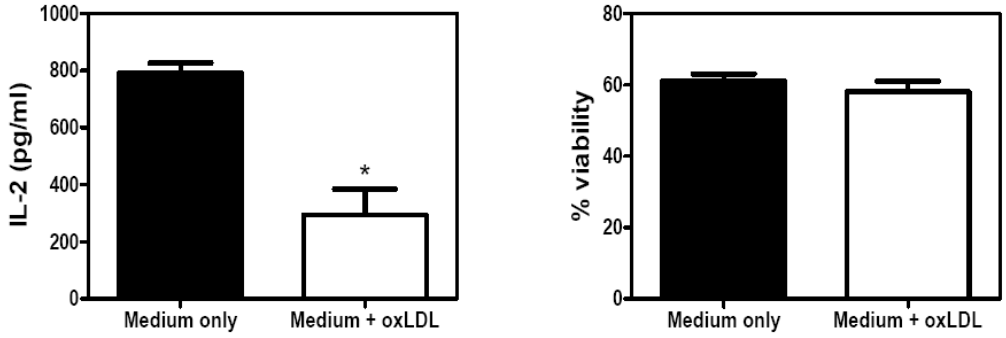

Figure 16. oxLDL decreases iNKT cell hybridoma activation *in vitro*. The iNKT cell hybridoma DN32.D3 was incubated with 50 ug/ml oxLDL for 24 hours prior to co-culture with α-GalCer-loaded B6 DCs (left panel) and the percentage of viable cells was determined by trypan blue exclusion (right panel). *p<0.05 as determined by Student's *t* test.

Given that LXRs are oxysterol sensing nuclear receptor important in reverse cholesterol transport, and have been shown to affect conventional T cell activation and proliferation (123), we tested whether activation of the LXR pathway *in vitro* affects iNKT cell activation. iNKT cell hybridomas (DN32.D3) were incubated for 24 hours in the presence of the synthetic LXR agonist

GW3965 (2μM), then washed and co-cultured with α-GalCer-loaded (50 ng/ml) $CD11c^+$ dendritic cells (purified from B6 spleens) for an additional 48 hours. iNKT cell hybridoma activation, as determined by IL-2 production, was measured by ELISA (Figure 17A). These results show that activation of the LXR pathway decreases iNKT cell hybridoma responsiveness to exogenous glycolipid stimulation *in vitro*.

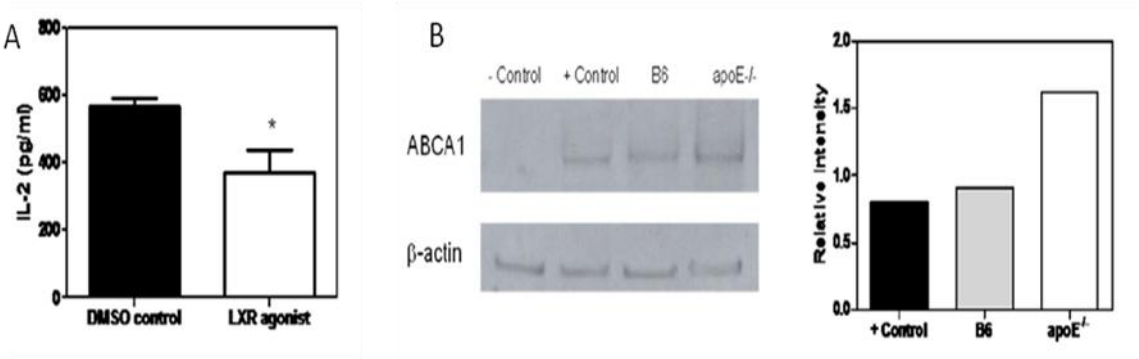

Figure 17. LXR activation in iNKT cells. A, iNKT cell hybridomas DN32.D3 were incubated with 2 μM LXR agonist GW3965 for 24 prior to co-culture with α-GalCer-loaded B6 DCs and hybridoma activation, as measured by IL-2 production, was determined after an additional 48 hours by ELISA. **B,** The expression of ABCA1 in primary iNKT cells isolated from B6 and $apoE^{-/-}$ mice was determined by Western blot analyses (left panel). Quantification of the ABCA1 Western blot (right panel). iNKT cell hybridomas cultured in the presence or absence of the LXR agonist GW3965 were used as positive and negative controls, respectively. *p<0.05 as determined by Student's *t* test.

In addition, we examined LXR activation *ex vivo* using primary iNKT cells isolated from the spleen and liver of B6 and $apoE^{-/-}$ mice. The cells were purified by magnetic bead separation, then lysed and subjected to polyacrylamide gel electrophoresis. Western blot analysis was performed for detection of ABCA1 expression, a downstream target of LXR activation. iNKT cell hybridomas incubated for 24 hours either in the presence of the LXR agonist GW3965

(positive control) or DMSO (negative control) were used as controls for the expression of ABCA1 in this experiment. As shown in Figure 17B, ABCA1 is upregulated in primary iNKT cells from apoE$^{-/-}$ mice compared to B6, suggesting that activation of the LXR pathway is occurring in these cells.

iNKT cells are capable of accumulating lipids *in vitro* and *in vivo*.

To determine whether iNKT cell lipid accumulation occurs *in vitro*, iNKT cell hybridomas were incubated 24 hours with complete medium containing BSA in the presence or absence of either 50 µg/ml oxLDL or native LDL. After incubation, the cells were washed, fixed with 2% paraformaldehyde, stained with 12.5 µg/ml filipin, and analyzed by flow cytometry. Gating on live cells, the results are shown in Figure 18A. When compared to BSA only (MFI=13.48), the native LDL cultured cells (MFI=13.12) show no difference in filipin staining, however, the oxLDL cultured cells (MFI=20.22) have increased filipin staining, suggesting an increase in free cholesterol accumulation.

To determine whether iNKT cells accumulate lipids *in vivo*, we analyzed iNKT cells from spleens and livers of B6 and apoE$^{-/-}$ mice (age 16 weeks). After staining for surface markers, the cells were washed, fixed with 2% paraformaldehyde, stained with 12.5 µg/ml filipin, and analyzed by flow cytometry. Gating on TCRβ^{+}Tetramer^{+}B220^{-} cells, the results for the filipin stain are shown in Figure 18B. These data indicate that there is no difference in splenic iNKT cell filipin staining (B6 MFI=8.48, apoE$^{-/-}$ MFI=9.57), however liver iNKT cells from apoE$^{-/-}$ mice have increased filipin staining (B6 MFI=5.59, apoE$^{-/-}$

MFI=9.61), suggesting free cholesterol accumulation within membranes of these cells.

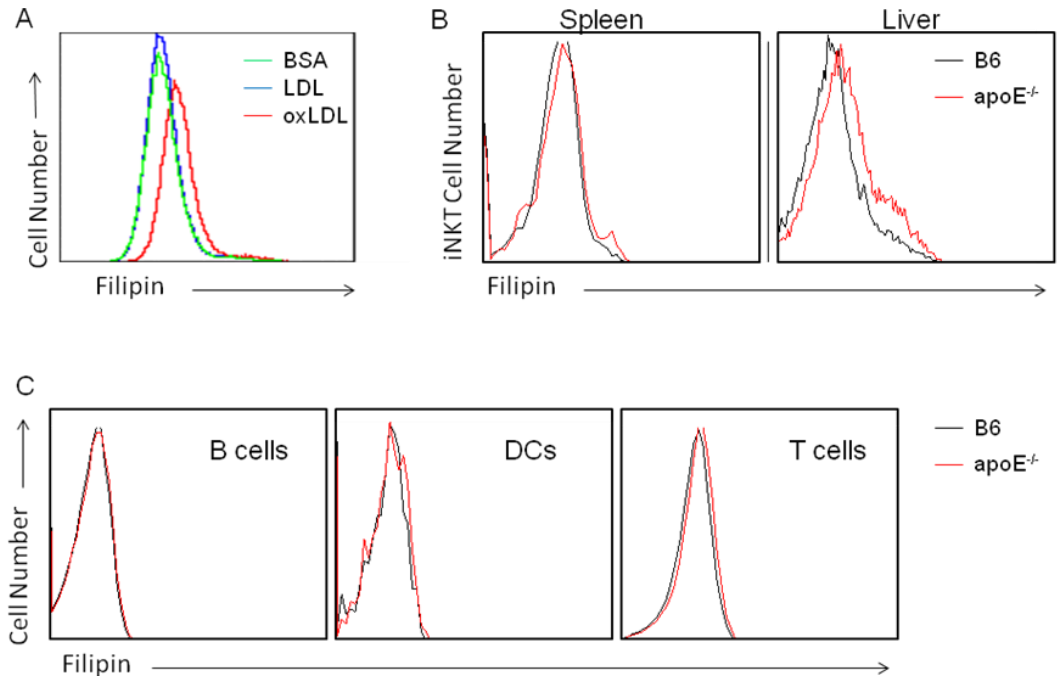

Figure18. iNKT cells are capable of free cholesterol accumulation. A, iNKT cell hybridomas were cultured in media containing BSA in the presence or absence of LDL or oxLDL for 24 hours, and membrane cholesterol, as determined by filipin staining, was analyzed by flow cytometry. **B,** Spleen and liver iNKT cells from B6 (black line) and apoE$^{-/-}$ (red line) mice were stained *ex vivo* with filipin and analyzed by flow cytometry. **C,** Splenic B cells (CD19^{+}), DCs (CD11c^{+}), and T cells (TCRβ^{+}) were stained *ex vivo* with filipin and analyzed by flow cytometry.

Furthermore, we analyzed B cells, dendritic cells, and conventional T cells from the spleens of these mice for cholesterol accumulation by filipin staining. As shown in Figure 18C, there is no difference in cholesterol staining of B cells,

DCs, or T cells from apoE$^{-/-}$ mice as compared to B6, suggesting that the hyperlipidemic environment directly affects the iNKT cell population specifically.

Changes in iNKT cell activation in hyperlipidemic apoE$^{-/-}$ mice are not due to accumulation of an endogenous ligand.

To determine whether an endogenous ligand within apoE$^{-/-}$ mice is responsible for the changes observed in iNKT cell activation, we performed adoptive transfer studies to analyze iNKT cell proliferation. Thymic iNKT cells were purified from Vα14 transgenic B6 mice by magnetic bead separation, then labeled with CFSE, and injected into sub-lethally irradiated B6 and apoE$^{-/-}$ mice (12 weeks of age). After 7 days, the mice were sacrificed and iNKT cell proliferation was determined by flow cytometry. As positive controls, B6 mice which had been adoptively transferred were injected with α-GalCer 3 days prior to sacrifice to induce iNKT cell proliferation. As shown in Figure 19, there was no difference in proliferation of B6 iNKT cells adoptively transferred into B6 and apoE$^{-/-}$ mice, as determined by CFSE dilution. These data suggest that within the hyperlipidemic environment of the apoE$^{-/-}$ mice, wild type iNKT cells are not being activated by an endogenous glycolipid antigen within the duration of these experiments. It is possible, however, that the hyperlipidemic environment may require longer than 7 days to exert pronounced effects upon iNKT cells, although this has yet to be determined.

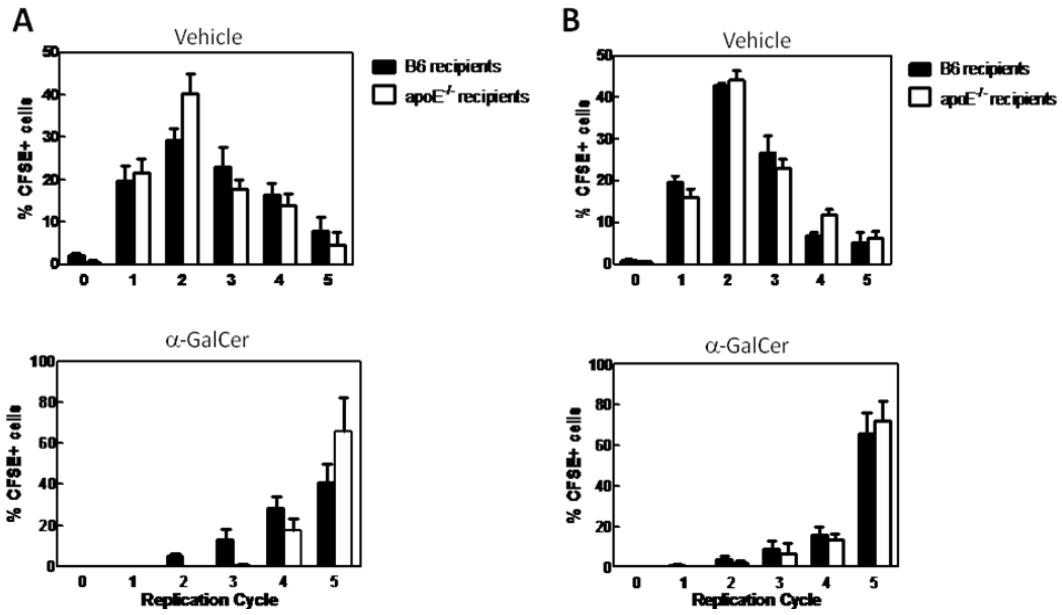

Figure 19. Adoptive transfer of B6 iNKT cells into B6 and apoE$^{-/-}$ mice. Thymic iNKT cells were purified from Vα14 transgenic B6 mice, labeled with CFSE, and adoptively transferred into B6 and apoE$^{-/-}$ mice. Four days after transfer, mice were injected with vehicle (top panels) or α-GalCer (bottom panels) and mice were sacrificed 3 days post injection. iNKT cell proliferation, as determined by CFSE dilution, was assessed by flow cytometry in the **(A)** spleen and **(B)** liver of recipient mice.

Effects of dyslipidemia on iNKT cells may be irreversible once established.

Given that dyslipidemia seems to directly affect iNKT cells by rendering them hyporesponsive, we examined the possibility that placing these cells into a normo-lipidemic environment could restore iNKT function. To address this possibility, we adoptively transferred CFSE-labeled splenic iNKT cells from apoE-deficient Vα14 transgenic mice (age 20 weeks) into age matched B6 and apoE$^{-/-}$ mice. Four days after the transfer, the mice were injected with α-GalCer to stimulate iNKT cell proliferation and sacrificed 3 days later. iNKT cell replication cycles were determined by CFSE dilution as measured by flow cytometry. As

shown in Figure 20, compared to apoE$^{-/-}$ mice, the iNKT cells within the normolipidemic B6 mice do not display an increase in proliferation. Although representative of one experiment, these preliminary data suggest that once the negative effects of hyperlipidemia are exerted on iNKT cell responsiveness, these effects may be irreversible by correcting lipid homeostasis alone.

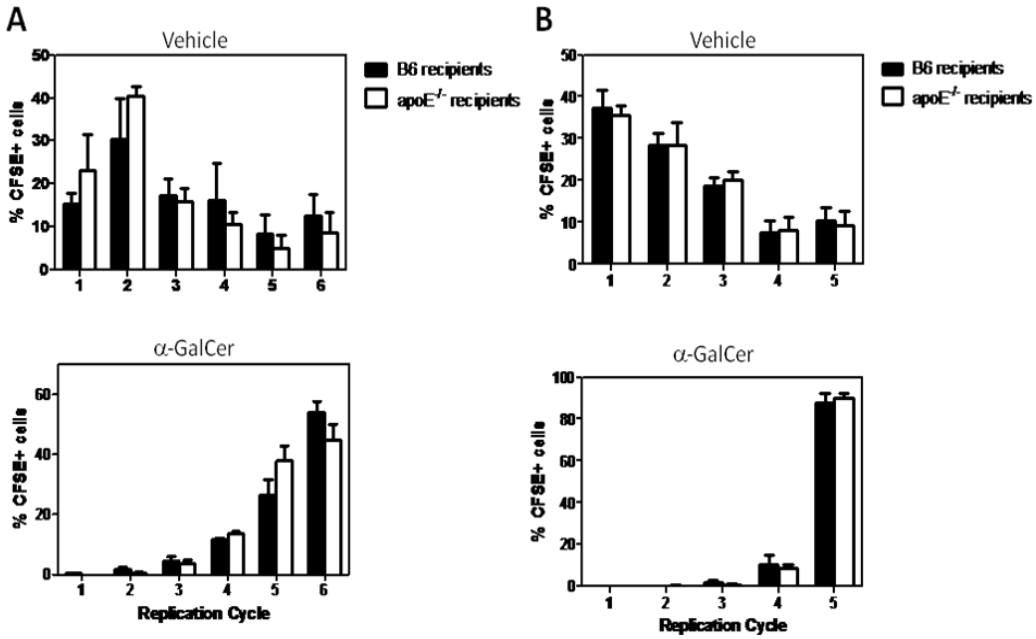

Figure 20. Transfer of iNKT cells from hyperlipidemic mice into wild-type mice does not increase proliferation. Splenic iNKT cells were purified from Vα14 transgenic apoE$^{-/-}$ mice, labeled with CFSE, and adoptively transferred into B6 and apoE$^{-/-}$ mice. Four days after transfer, mice were injected with vehicle (top panels) or α-GalCer (bottom panels) and mice were sacrificed 3 days post injection. iNKT cell proliferation, as determined by CFSE dilution, was assessed by flow cytometry in the **(A)** spleen and **(B)** liver of recipient mice.

Discussion

It is well established that increased lipid accumulation is associated with human diseases such as atherosclerosis. Increasing evidence also supports the role of inflammation and immunity in the pathogenesis of this disease. Several cells of the immune system, including macrophages, NK cells, mast cells, and dendritic cells, have been shown to play a role in atherosclerotic lesion progression (35, 36). Additionally, numerous studies have focused on macrophages in cardiovascular disease from their role as cells important in cholesterol homeostasis and scavenging modified lipoproteins and apoptotic cells (37), to their role as mediating inflammatory responses. In fact, direct effects of lipid accumulation within these cells have been well characterized. However, intrinsic effects of increased circulating lipids on iNKT cell activation are not known. Our data provide novel evidence that iNKT cells are directly affected by increased circulating lipids.

The direct effects of increased circulating lipids on particular cells of the immune system have been well characterized. It is well established that macrophage foam cells can form upon accumulation of cholesterol, a process which is primarily mediated by scavenger receptors (125). Previous studies involving atherosclerosis have shown that lipids, such as the glycosphingolipid β-glucosylceramide (β-GlcCer) (113) and the disialoganglioside GD3 (114), accumulate within the serum of both humans and apoE$^{-/-}$ mice. Therefore, a possible explanation for the iNKT cell hyporesponsiveness we observe in

hyperlipidemic apoE$^{-/-}$ mice is that these cells are spontaneously activated by increased levels of endogenous glycolipid. However, given that we did not observe an increase in iNKT cell proliferation, our adoptive transfer studies of B6 iNKT cells into apoE$^{-/-}$ mice suggest that there is not an accumulation of endogenous glycolipid antigen within the apoE$^{-/-}$ mice.

Previous studies have suggested that hyperlipidemia directly affects DCs, specifically by reducing their migratory capabilities (121), or inhibiting their activation (122). In contrast to these findings, our previously published data indicate that DCs from hyperlipidemic apoE$^{-/-}$ mice retain their ability to present antigens. Furthermore, the results from our *ex vivo* filipin staining indicate that spontaneous hyperlipidemia in apoE$^{-/-}$ mice does not result in free cholesterol accumulation within DCs in our model. In addition, a recent study by Packard *et al* (35), showed that DCs within a hypercholesterolemic environment fully retain their antigen presentation capability, even when cholesterol loaded, further supporting the our hypothesis that increased circulating lipids directly affect iNKT cells rather than through indirect mechanisms via APCs.

Recent attention has also focused on the influence of lipid metabolism on conventional T cell activation and proliferation. In a recent study by Bensinger *et al* (123), it was shown that transcriptional regulation of intracellular cholesterol homeostasis impacts the immune response by T cells. Specifically, LXRβ is an intrinsic regulator of lymphocyte proliferation, and sustained LXR signaling inhibits proliferation of T cells. In addition to LXR, another molecule involved in cholesterol efflux, apolipoprotein A-1 (apoA-1), has been shown to play a role in

T cell proliferation. ApoA-1$^{-/-}$LDLr$^{-/-}$ mice fed a high fat diet display decreased T cell proliferation and activation, as well as develop features of autoimmunity (126).

Our previous data show that iNKT cells from hyperlipidemic apoE$^{-/-}$ mice have an impaired ability to respond to exogenous antigen stimulation and this hyporesponsiveness is not due to defective antigen presentation. Here we have shown that activation of the LXR pathway *in vitro* results in decreased iNKT cell hybridoma activation. Additionally, we observed spontaneous upregulation of ABCA1 in iNKT cells from apoE$^{-/-}$ mice, suggesting activation of the LXR pathway within these cells *in vivo*. Taken together, these studies suggest that cholesterol efflux via LXR-regulated ABC transporters potentially plays a suppressive role in iNKT cell activation. These data provide novel insight into the mechanism by which iNKT cells are hyporesponsive to exogenous stimulation in our model of spontaneous hyperlipidemia. Our results indicate that iNKT cells exhibit an impaired ability to respond to exogenous glycolipid antigen stimulation within a dyslipidemic environment. Therefore, the effects of lipid accumulation should be taken into consideration when designing future therapies manipulating iNKT cells.

CHAPTER IV

ROLE OF LRP IN INKT CELL ACTIVATION

Introduction

A recent study has shown that the low density lipoprotein receptor (LDLr) may be involved in the uptake of glycolipid antigen and enhance the activation of iNKT cells (83). However, because LDLr is sterol-sensitive and is downregulated with increased cellular cholesterol levels, this seems a limiting and inefficient means of antigen presentation. Another member of the LDLr gene family, the low density lipoprotein receptor-related protein (LRP) has vital structural resemblance to the LDLr and recognizes a wide array of diverse ligands, including apoE-enriched lipoproteins and chylomicrons.

In addition to its role in lipoprotein metabolism, LRP has been shown to enhance antigen uptake within monocytes and macrophages (87) and is involved in control of cellular entry of bacterial toxins and viruses (88). The primary functions of LRP include lipoprotein transport, cell surface protease activity, and cellular uptake and lysosomal delivery of secreted prosaposins, saposin precursors (86). Importantly, saposins are required for the lysosomal loading and unloading of lipids onto CD1d (127), the molecule responsible for lipid antigen presentation to iNKT cells. Molecules bound to LRP are internalized and delivered to the lysosomes for subsequent degradation.

The role of LRP in lipoprotein metabolism has been well characterized, however its role in iNKT cell activation is not known. Given that LRP binds bacteria and viruses and is essential for recapture of saposin precursors, we hypothesized that LRP plays a role in glycolipid antigen uptake and subsequent iNKT cell activation. In the current study, we addressed the role of LRP in iNKT cell activation and found mice lacking LRP on granulocytes, such as dendritic cells and macrophages, have reduced iNKT cell-mediated cytokine production, specifically IL-4, in response to exogenous glycolipid antigen stimulation. These data suggest that not only does LRP on APCs play a role in iNKT cell activation, it may also preferentially modulate iNKT cell cytokine production.

Methods

Animals. Male C57BL/6J (B6) and B6.129P2-Apoe^{tm1Unc}/J (apoE$^{-/-}$) mice were originally purchased from Jackson Laboratories (Bar Harbor) and maintained in our colonies under pathogen-free conditions. LRP$^{fl/fl}$LyzMCre^{+} mice and human apoE-expressing transgenic (apoE2, apoE3, and apoE4) mice were kindly provided by Dr. Sergio Fazio (Vanderbilt University). Animals were fed standard chow diet *ad libitum*. All animal studies were approved by the Institutional Animal Care and Use Committee of Vanderbilt University (Nashville, TN).

Reagents. α-GalCer (KRN7000) was obtained from Diagnocine and reconstituted in PBS containing 5.6% sucrose, 0.75% L-histadine, and 0.5% polysorbate-20 (Sigma Aldrich). GGC (PBS-18) was described previously (90). Fluorescently labeled tetrameric CD1d molecules loaded with α-GalCer (CD1d tetramers) were obtained from the National Institute of Allergy and Infectious Diseases (NIH tetramer facility). Anti-rabbit LRP antibody was kindly provided by Joachim Herz (University of Texas Southwestern). For the *in vitro* iNKT cell hybridoma assays, the N38-2C12 hybridoma was kindly provided by Dr. Albert Bendelac (University of Chicago, IL)

Flow cytometry. Single-cell suspensions of the spleen and liver were prepared, and stained with fluorescently-labeled monoclonal antibodies as described previously (95). Flow cytometry was performed using a FACSCaliber machine BD Biosciences) and the data were analyzed using FCS Express software (De Novo Software). All fluorescently labeled antibodies used for flow cytometry were purchased from BD Biosciences.

iNKT cell hybridoma assays. Cells were cultured 24 hours in complete RPMI medium containing FBS (Sigma). Bone marrow-derived DCs were isolated and cultured as previously described (97), loaded with 50 ng/ml α-GalCer for 30 minutes at 37°, and co-cultured with the hybridomas for an additional 48 hours. iNKT cell hybridoma activation was assessed by IL-2 production in cell culture supernatants by ELISA.

ELISA. Mouse IL-4, IFN-γ, IL-2, and IgE were measured by standard sandwich ELISA according to the manufacturer's protocol (BD Pharmingen).

Statistics. Statistical significance between two groups was determined using a Student's *t* test, and significance between multiple groups was determined using one-way ANOVA with Newman-Keuls multiple comparison test for post hoc analysis. A value of $p<0.05$ was considered statistically significant and all statistical analyses were performed using GraphPad Prism software.

Results

Cell specific LRP deletion

To determine the importance of LRP in iNKT cell activation, we used a mouse model with a granulocyte specific LRP deficiency ($LRP^{fl/fl}LyzMCre^{+}$). This model utilizes Cre/lox recombination under the lysozyme M promoter and has previously been demonstrated to achieve >80% knockdown of LRP on granulocytes such as macrophages (128). The efficiency of LRP gene inactivation in bone marrow-derived DCs was determined by western blot (Figure 21A). We observe a 90% decrease in LRP expression on BM-DCs derived from $LRP^{fl/fl}LyzMCre^{+}$ mice compared to controls. Additionally, we examined LRP

expression on CD11c^{+} DCs in the spleen and observed decreased LRP on these cells.

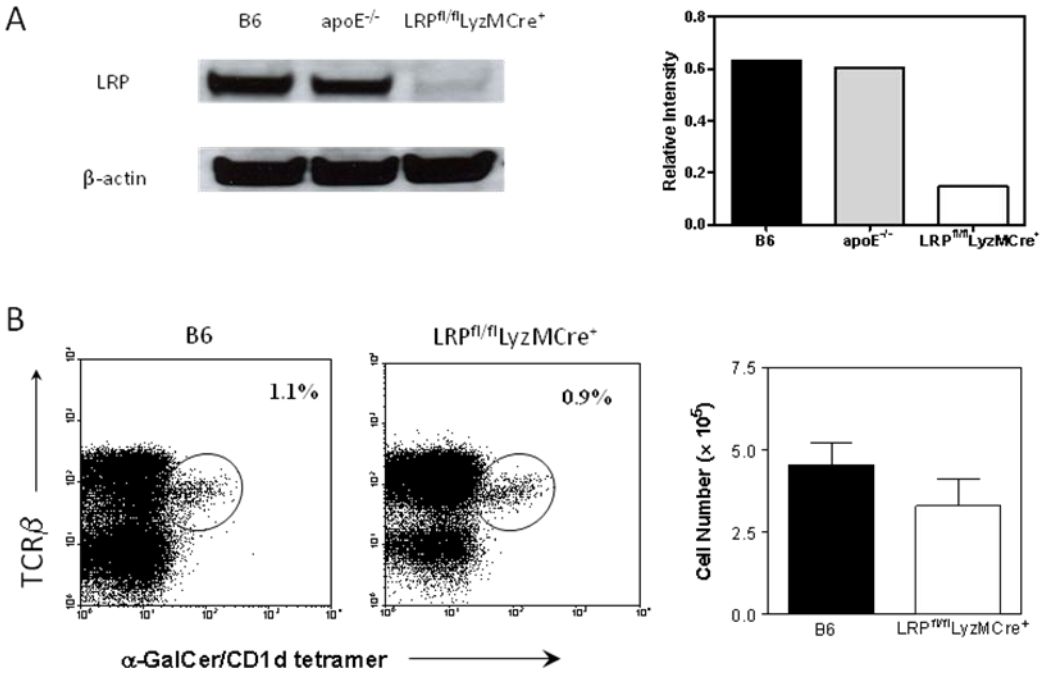

Figure 21. Analysis of BM-DCs and iNKTs in mice with a cell-specific knockdown of LRP. **A,** BM-DCs were isolated from B6, apoE$^{-/-}$, and LRP$^{fl/fl}$LyzMCre^{+} mice, and LRP expression was determined by Western blot (left panel). Western blot quantification shown in right panel. **B,** Percentages (left panel) and absolute numbers (right panel) of iNKT cells from the spleen of B6 and LRP$^{fl/fl}$LyzMCre^{+} mice.

To determine whether granulocyte specific knockdown of LRP affects iNKT cell numbers, splenocytes from age matched B6 and LRP$^{fl/fl}$LyzMCre^{+} mice were stained and analyzed by flow cytometry. There were no differences in iNKT cell percentages or absolute numbers (Figure 21B) between these groups of

mice, indicating any differences we may observe in iNKT cell activation in $LRP^{fl/fl}LyzMCre^{+}$ mice are not due to differences in iNKT cell numbers.

Effect of LRP deficiency on antigen presentation

To determine the ability of $LRP^{fl/fl}LyzMCre^{+}$ antigen presenting cells to activate iNKT cells *in vitro*, BM-DCs were isolated from these mice, pulsed with either α-GalCer or GGC (an α-GalCer precursor), and co-cultured with the autoreactive NKT hybridoma N382C12**.** After 48 hour incubation, the $LRP^{fl/fl}LyzMCre^{+}$ BM-DCs displayed a significant decrease in their ability to activate the NKT hybridoma as measured by IL-2 production (Figure 22A). Importantly, because GGC requires lysosomal trafficking to be converted to its antigenic form α-GalCer (83), these data support a possible role for LRP in lysosomal trafficking of glycolipid antigen and subsequent surface presentation for iNKT cell activation. Given that a decrease in CD1d expression could be responsible for the decrease in iNKT cell hybridoma activation we observed, we analyzed CD1d expression on BM-DCs from $LRP^{fl/fl}LyzMCre^{+}$ mice. As shown in Figure 22B, $LRP^{fl/fl}LyzMCre^{+}$ BM-DCs have comparable CD1d expression to those from age matched B6 mice.

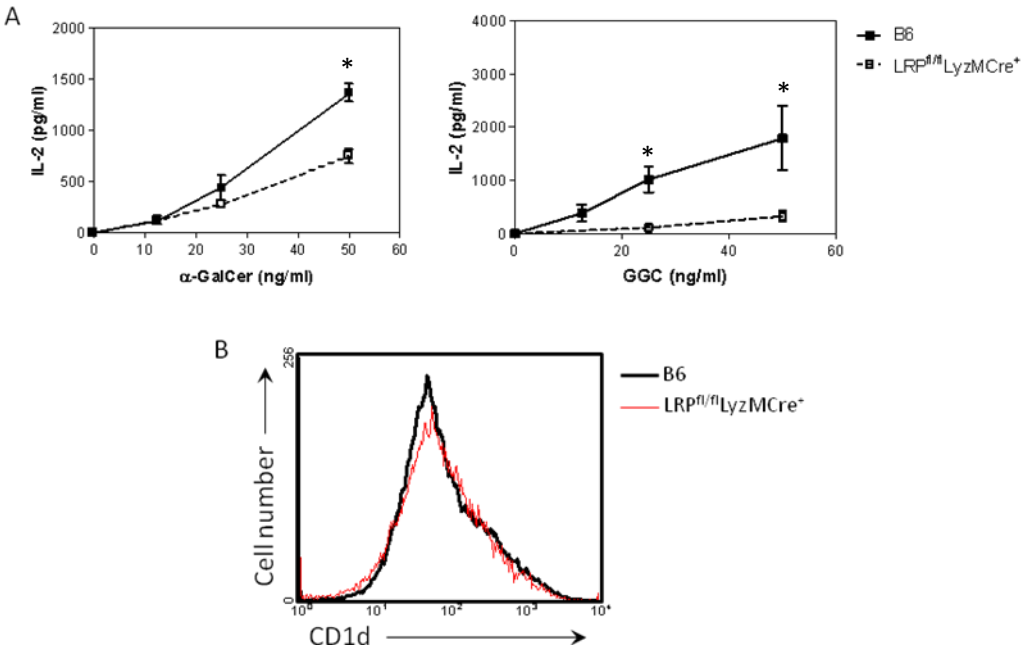

Figure 22. *In vitro* iNKT cell hybridoma activation by LRP$^{fl/fl}$LyzMCre^{+} BM-DCs. A, BM-DCs isolated from B6 and LRP$^{fl/fl}$LyzMCre^{+} mice were loaded in vitro with α-GalCer (left panel) or GGC (right panel) then co-cultured with the iNKT cell hybridoma N382C12. iNKT cell activation after 48 hours, as measured by IL-2 production, was determined by ELISA. **B,** CD1d expression on BM-DCs was determined by flow cytometry. *p<0.05 as determined by Student's *t* test.

Absence of LRP skews iNKT cell cytokine production in response to exogenous stimulation

To determine the role of LRP in iNKT cell activation *in vivo*, we injected male age-matched LRP$^{fl/fl}$LyzMCre^{+} and B6 mice with 4μg α-GalCer i.p. per mouse. Serum was collected after 2 and 24 hours, and levels of IL-4 and IFN-γ were determined by ELISA. LRP$^{fl/fl}$LyzMCre^{+} mice have decreased IL-4 production as compared to wild-type (Figure 23A). These data show that the absence of LRP biases the iNKT cells toward a Th1 response. To further

investigate the differences in cytokine production, we examined trans-activation of other spleen cell populations by flow cytometry 24 hours post α-GalCer injection. Our results show that $LRP^{fl/fl}LyzMCre^{+}$ mice have decreased B and T cell activation compared to wild-type (Figure 22B). We also observed a decrease in IgE antibody production in the serum of $LRP^{fl/fl}LyzMCre^{+}$ mice as determined by ELISA (Figure 22C). Given that IL-4 is a major switch factor for IgE production in mice (129), the decrease observed is consistent with decreased IL-4 production.

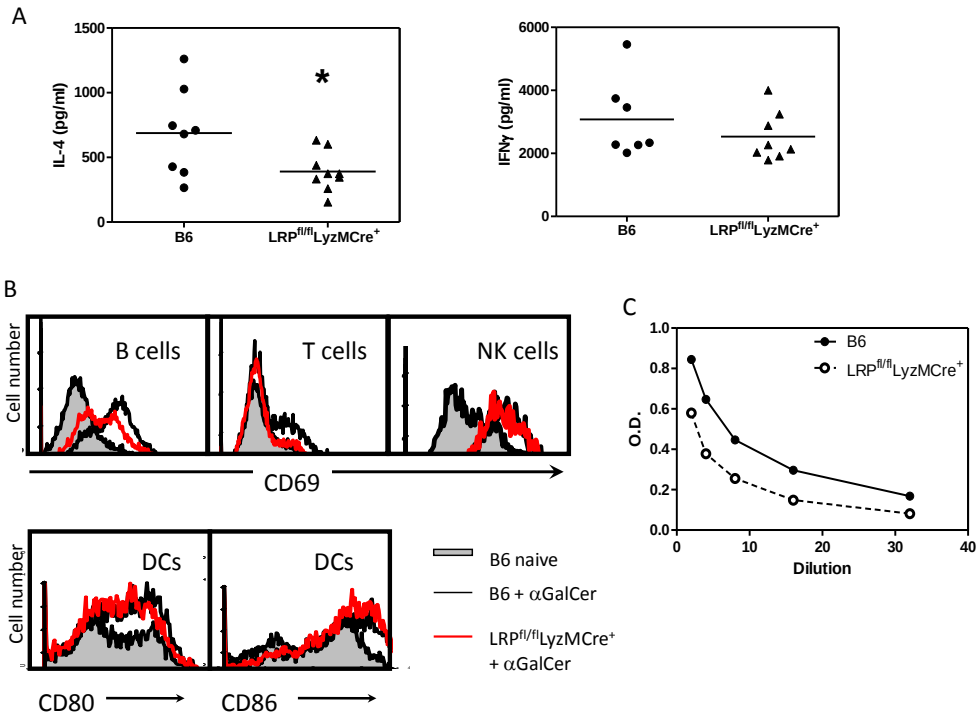

Figure 23.LRP$^{fl/fl}$LyzMCre^{+} mice have altered IL-4 production. A, B6 and LRP mice were injected with 4 µg α-GalCer/mouse and serum IL-4 (left panel) and IFN-γ (right panel) were measured at 2 hours and 24 hours post injection, respectively. **B,** B6 and LRP mice were injected with 4 µg α-GalCer/mouse and splenocytes were analyzed for activation by flow cytometry 24 hours post injection. **C,** Serum IgE titers were measured by ELISA 9 days post injection. *p<0.05 as determined by Student's *t* test.

Finally, to indirectly examine a possible role for the apoE-LRP axis in iNKT cell activation, we used transgenic mice expressing human apoE isoforms from monocytes (130). Human apoE exists as three isoforms (apoE2, apoE3, and apoE4). ApoE3 and apoE4 are recognized by the LDLr while apoE2 preferentially binds to LRP but not the LDLr. ApoE3, E4 and E2 transgenic mice (on the apoE$^{-/-}$ background), apoE$^{-/-}$ and B6 mice, were injected i.p. with 4µg α-GalCer, and serum levels of IL-4 and IFN-γ were measured by ELISA at 2 hours and 24 hours

respectively post injection. Compared to apoE3, the apoE2-expressing mice have a significant decrease in serum IFN-γ with no difference in serum IL-4 (Figure 24), further supporting our data suggesting that not only does the LRP play a role in glycolipid uptake and iNKT cell activation, but that this receptor also modulates the quality of the iNKT cell response.

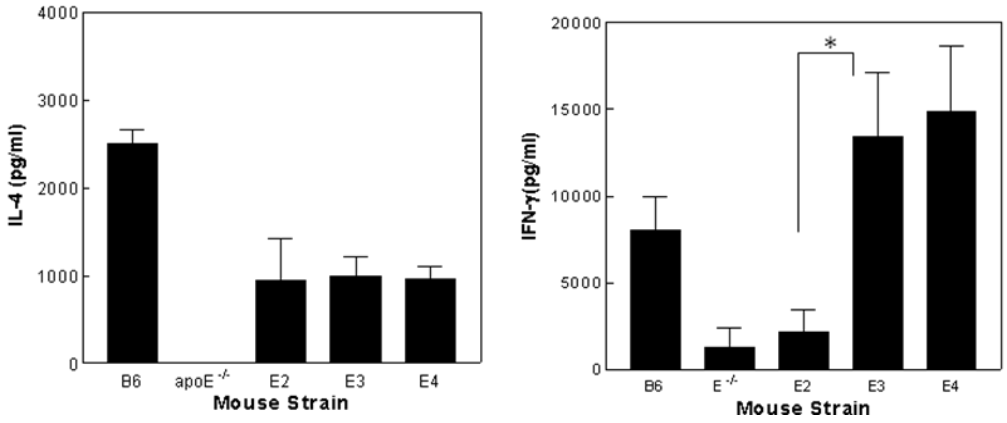

Figure 24. ApoE2 biases iNKT cells toward IL-4 production *in vivo*. B6 mice, apoE-/- mice, and mice transgenic for human apoE isoforms (apoE2, apoE3, and apoE4) were injected with 4ug a-GalCer/mouse. Serum IL-4 (left panel) was measured 2 hours post injection and IFN-γ (right panel) was measured 24 hours post injection. *p<0.05 as determined by Student's t test.

Discussion

In this study, we have shown that LRP deficient BM-DCs have decreased NKT hybridoma activation *in vitro,* and that $LRP^{fl/fl}LyzMCre^{+}$ mice have

decreased IL-4 and IgE production, as well as decreased B and T cell activation, after NKT cell stimulation *in vivo*. These data suggest that LRP plays a role in NKT cell activation and modulation.

ApoE can be taken up by both LDLr and LRP, both of which are expressed on DCs. Recent evidence has implicated both apoE and the LDLr in the activation of iNKT cells via enhancing uptake of glycolipid antigen by DCs (83); however, the authors dismissed the ability of LRP to mediate lipid antigen uptake in their model. In that study, apoE2 (which binds to LRP exclusively) had a diminished ability to enhance CD1d-dependent responses compared to apoE3 (which can bind to both LRP and LDLr); however these data were not shown. Additionally, their data show that exogenous addition of apoB (which binds to LDLr but not LRP) decreased antigen presentation compared to controls. However, in all of these studies, antigen presentation in $LDLr^{-/-}$ mice was shown to be reduced but not completely lost, suggesting that LRP could possibly still be involved in the uptake of apoE-antigen complexes. Another caveat is that IFN-γ production was the primary readout for iNKT activation. According to our data using $LRP^{fl/fl}LyzMCre^{+}$ mice, iNKT cell-associated IFN-γ production remained largely unchanged compared to B6 mice, however we observed a significant reduction in IL-4 production indicating a potential role for LRP in altering the cytokine response in iNKT cells.

We have shown that LRP is involved in iNKT cell activation, indicating that the LDLr may not be the sole route of uptake of apoE-antigen complexes by APCs. Importantly, our results show that activation of iNKT cells through the LRP

pathway can skew their cytokine response. Given that targeted therapies involving iNKT cells are currently being considered in several human pathologies, further investigation to define a mechanism by which LRP modulates the quality of the iNKT cell response is warranted.

CHAPTER V

DISCUSSION

Summary

In summary, the data presented here support the hypothesis that chronic dyslipidemia results in decreased iNKT responsiveness through direct effects on iNKT cells. In apoE$^{-/-}$ mice, a model for spontaneous hyperlipidemia, we have shown that iNKT cell numbers and functions are significantly decreased compared to normolipidemic B6 mice. Importantly, these changes are not observed in young mice, before the onset of hyperlipidemia, suggesting that increased accumulation of circulating lipids over time negatively affects iNKT cell function. Additionally, our studies reveal that iNKT cells from hyperlipidemic apoE$^{-/-}$ mice exhibit an anergic phenotype, as determined by increased TCRβ internalization, as well as decreased proliferation and cytokine secretion in response to exogenous glycolipid stimulation. We have also shown that APCs from apoE$^{-/-}$ mice retain the ability to activate iNKT cells, therefore the defects we observed are iNKT cell intrinsic.

Furthermore, our data suggest that the increase in circulating lipids in apoE$^{-/-}$ mice results in decreased in iNKT cell function through direct effects on the iNKT cells. As determined by flow cytometry, liver iNKT cells from apoE$^{-/-}$ mice have an apparent increase in membrane free cholesterol accumulation as compared to those from B6 mice. Additionally, our *in vitro* studies indicate that

activation of the LXR pathway results in decreased activation of a classical iNKT cell hybridoma. Adoptive transfer of iNKT cells from hyperlipidemic apoE$^{-/-}$ mice into normolipidemic B6 mice does not increase the ability of these cells to proliferate, suggesting that the direct of effects of lipids on iNKT cells may be irreversible once established in our model.

Overall Discussion

We have shown that iNKTs are directly influenced by their environment, and increased circulating lipids render them dysfunctional. In our studies, we have used a mouse model of spontaneous hyperlipidemia which leads to atherosclerosis. Consistent with this, Nakai *et al*. demonstrated that B6 mice fed a high fat diet also have decreased iNKT cell numbers and functions (8). Taken together, these studies demonstrate that iNKT cells are extremely sensitive to their lipid environment.

Interestingly, we have consistently observed decreased iNKT cell responses in apoE$^{-/-}$ mice as these mice age and dyslipidemia continues. These data suggest that chronic hyperlipidemia, as observed in older (>12 weeks of age) apoE$^{-/-}$ mice, directly affects the ability of iNKT cells to respond to exogenous glycolipid antigen stimulation, given that iNKT cells from younger (<6 weeks of age) apoE$^{-/-}$ mice display normal cytokine secretion in response to α-GalCer. These findings represent a novel role for the direct effects of increased

circulating lipids in iNKT cell activation. Although the mechanism by which increased cholesterol directly modulates iNKT cells has yet to be determined, studies utilizing human conventional T cells have suggested a possible link to the effect of cholesterol on TCR signaling via membrane stiffness (131). Previous studies have determined that compared to young subjects, T cell membranes are more rigid in elderly subjects, and that this increased stiffness is due to increased T cell free cholesterol content in the plasma membrane (132). Furthermore, when cholesterol content of the T cell membranes from young subjects was increased to the level observed in elderly subjects, there was a decrease in T cell proliferation and IL-2 secretion similar to that observed in immunosenescence (133). Additionally, Seres *et al* (134) have shown that in patients with hypercholesterolemia, peripheral blood lymphocytes display increased cellular cholesterol content within the plasma membrane. Although these studies were conducted using human subjects rather than mice, the results provide evidence that cholesterol can exert direct effects on the T lymphocyte population. Therefore, it is possible that increased free cholesterol within iNKT cell membranes represents a potential mechanism for the decreased iNKT cell responses observed in our model.

The effects of increased lipids on iNKT cells have not been extensively characterized and represent an important area of research which needs further study. Our data show decreased iNKT cell responsiveness to exogenous stimulation with glycolipid antigen within a hyperlipidemic environment. A recent study from Miyazaki *et al* investigated the ability of dietary lipids to modulate

iNKT cell responses (85). Using wild-type mice fed a high fat diet (HFD), they found decreased numbers of spleen and liver iNKT cells, and activation with α-GalCer results in suppression of IFN-γ but not IL-4 production. Although the mechanism for this skewed cytokine production was not determined in this study, the authors hypothesize that the decreased IFN-γ could be a result of altered antigen presentation, because they observed decreased CD1d expression on DCs from mice fed HFD. In our studies using apoE$^{-/-}$ mice fed a standard chow diet, we have shown a reduction in both IFN-γ and IL-4 production by iNKT cells, and that CD1d expression on DCs is not different compared to B6 mice, therefore this hypothesis may not pertain to our model. Discrepancies between these studies may be explained by the use of increased dietary lipids to induce hyperlipidemia as compared to spontaneously increased lipids as seen in our model. Because iNKT cells have been shown to play diverse roles in various disease models, these studies suggest that dyslipidemia may have grave impact on disease states. Taken together, these data provide evidence that a hyperlipidemic environment has a profound effect on iNKT cell activation and that molecules classically associated with lipid metabolism may regulate normal immune function.

Role of iNKT cells in atherosclerosis

Our laboratory (7) and others (8, 9) have previously shown that iNKT cells are proatherogenic. Specifically, both apoE$^{-/-}$ and LDLr$^{-/-}$ mice lacking iNKT cells, as well as C57BL/6 mice fed a high-fat diet, have a significant reduction in

atherosclerotic lesion formation. In a study to determine the mechanism by which iNKT cells promote atherosclerosis, Aslanian *et al* (80) demonstrated that the role of iNKT cells in this disease is limited to early fatty streak formation. Additionally, activation of iNKT cells by α-GalCer *in vivo* has been shown exacerbate atherosclerosis (7–9); however, a recent study by van Puijvelde *et al* (82) has shown that α-GalCer injections had no effect on lesion size in a model of carotid atherosclerosis using aged apoE$^{-/-}$ mice. Although their data were obtained using a model for carotid atherosclerosis, the results from van Puijvelde *et al* (82) could be explained by our data demonstrating that iNKT cells from aged apoE$^{-/-}$ mice display an anergic phenotype and are hyporesponsive to exogenous stimulation with α-GalCer. Given that, by the time carotid atherosclerosis is initiated in their model, the iNKT cells in older apoE$^{-/-}$ mice are most likely rendered unresponsive to further activation by exogenous stimuli, we would hypothesize that α-GalCer administration would have no effect on iNKT cell activation.

Provided that our goal was to determine the effect of the hyperlipidemic environment on iNKT cell function, and that our lab and others (7-9) have already established a proatherogenic role for iNKT cells, atherosclerosis data was not determined in the current study. However, because apoE$^{-/-}$ mice spontaneously develop atherosclerosis, we have proposed a model for the contribution of dyslipidemia to iNKT cell activation within the context of atherosclerosis (Figure 25). iNKT cells are activated in apoE$^{-/-}$ mice at a young age when circulating lipids begin to spontaneously increase. This activation leads to the production of

proatherogenic cytokines such as IFN-γ and IL-4, therefore the normal iNKT responses we observe in young apoE$^{-/-}$ mice are potentially contributing to fatty streak formation in our model. Supporting this hypothesis, results from Aslanian *et al* (80) demonstrate that the role iNKT cells play in atherogenesis is limited to early fatty streak formation. As atherosclerosis-associated hyperlipidemia continues, we hypothesize that iNKT cells are repeatedly activated, therefore leading to an anergic phenotype. As atherosclerosis progresses in these mice, these cells are rendered dysfunctional possibly by a combination of repeated activation and the direct effects of increased cholesterol, and are no longer contributing to atherosclerotic lesion progression.

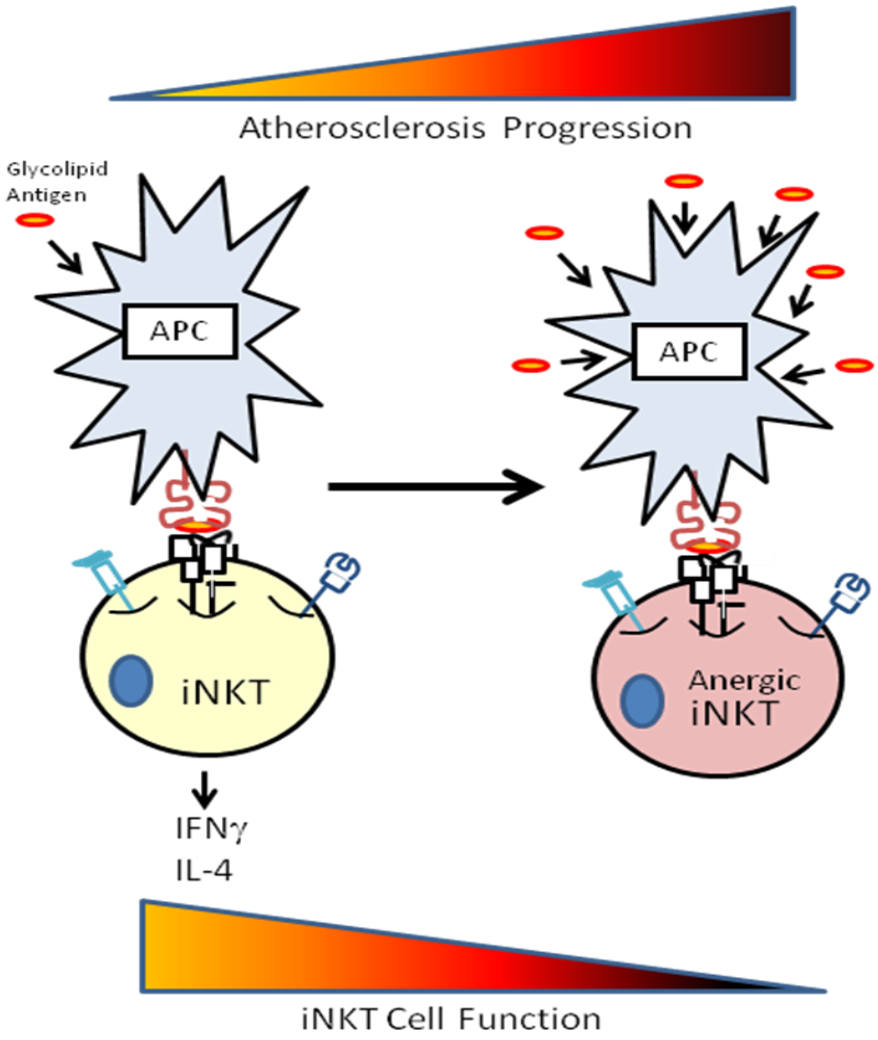

Figure 25. Model for iNKT cell function during spontaneous atherosclerosis in apoE$^{-/-}$ mice. Spontaneous activation of iNKT cells occurs early in apoE$^{-/-}$ mice as circulating lipids begin to increase (≥8 weeks of age). This activation results in the secretion of proatherogenic cytokines (IFN-γ and IL-4) by iNKT cells, therefore contributing to the development of early fatty streaks in these mice. As the atherosclerosis-associated dyslipidemia progresses (<12 weeks of age), iNKT cells become dysfunctional, presumably by repeated activation combined with direct effects of increased cholesterol on these cells. The resulting anergic iNKT cells no longer contribute to atherosclerotic lesion progression in these mice.

iNKT cells as therapeutic targets in human disease

Previous studies have shown that iNKT cells are important in anti-tumor immunity, and current human clinical trials utilizing iNKT cells are being developed for treatment of tumors. Specifically, activating iNKT cells using α-GalCer therapy has been shown to protect against some tumors (135, 136), and phase I/IIa clinical trials are being conducted on patients with advanced lung cancer (137). Because iNKT cells play an important role in the early development of atherosclerosis, these cells also potentially represent a target for the prevention of atherosclerosis. However, given our data shown here, in addition to the previously published data on the regulatory functions of iNKT cells on other cells of the immune system (138), we hypothesize that either targeting iNKT cells in the prevention of atherosclerosis or utilizing iNKT cells as a treatment for patients with this disease would not represent feasible or ideal options, given that the risks involved would outweigh the potential benefits. iNKT cells protect against microbial infections such as *Listeria monocytogenes*, *Streptococcus pneumoniae*, *Leishmania major*, *Plasmodium berghei* (139), as well as provide host protection against viral infections such as herpes simplex virus 1 (HSV-1) (140) and human immunodeficiency virus (HIV) (106). Additionally, iNKT cells prevent the development of several autoimmune diseases and maintain tolerance (141). The activation of iNKT cells with repeated α-GalCer injections has been shown to protect against type 1 diabetes, experimental autoimmune encephalomyelitis (EAE), and RA in mice, but exacerbates lupus, atherosclerosis, and allergic airway inflammation (142). Because of their ability to

rapidly produce large amounts of cytokines and their involvement in several autoimmune diseases, iNKT cells are currently being considered as therapeutic targets in human disease. However, given the importance of iNKT cells in host protection against pathogens, maintenance of immune tolerance, and suppression of allergic airway inflammation, we hypothesize that manipulating these cells to prevent atherosclerosis development could potentially lead to increased susceptibility to infection, development of autoimmune disease, or increased allergic reactions.

Provided that therapies manipulating iNKT cells are being considered in human disease, understanding how iNKT cells are influenced by their environment is imperative. Therefore, we sought to determine the effects of atherosclerosis-associated dyslipidemia on iNKT cell function. Importantly, our data show that iNKT cells are hyporesponsive to exogenous stimulation by α-GalCer within the dyslipidemic environment of apoE$^{-/-}$ mice. These results contribute to our understanding of how iNKT cells function during conditions of increased circulating lipids, and suggest that the ability of dyslipidemia to render iNKT cells anergic should be taken into consideration when designing therapies using iNKT cells in diseases associated with an increased risk of cardiovascular disease such as type 1 diabetes (130), RA (131), and HIV infection (143). Additionally, our preliminary data suggesting that increased circulating cholesterol potentially exerts direct consequences on iNKT cell function represents an exciting, novel finding within the iNKT cell field.

Future Directions

Future studies will be designed to determine the mechanism by which increased circulating lipids mediate iNKT cell dysfunction. One possible mechanism which we have yet to rule out involves the alternative or indirect pathway of iNKT cell activation (Figure 2). In this pathway, TLR-mediated activation of APCs leads to pro-inflammatory IL-12 cytokine production which, along with weak interaction of iNKT cells with endogenous antigens, activates iNKT cells. Although iNKT cell activation by this alternative pathway represents a potential mechanism in our model, we do not anticipate this to be the case; however, future experiments utilizing TLR4$^{-/-}$apoE$^{-/-}$ mice and IL-12$^{-/-}$apoE$^{-/-}$ mice will elucidate the contribution of the key molecules involved in this pathway.

Another mechanism by which a hyperlipidemic environment exerts direct effects on iNKT cell function, which is supported by data from our laboratory, is through intracellular lipid accumulation. Our data, although preliminary, provide novel insight into the direct effect of lipid accumulation on these immune cells. We have shown that iNKT cells from apoE$^{-/-}$ mice are capable of accumulating free cholesterol within their membranes, and that activation of the LXR pathway appears to be occurring in these cells *in vivo*, therefore, we anticipate this mechanism will be confirmed in our model. The next step in determining the importance of the mechanism by which increased cholesterol directly affects iNKT cells in our model will focus on the impact of increased membrane free cholesterol accumulation within iNKT cells on cytokine secretion by these cells.

Lastly, another mechanism by which increased circulating lipids could influence iNKT cell activation may include the interaction of apoE and LRP. Although apoE has been shown to enhance lipid antigen uptake by APCs (83), our data suggest that the lack of apoE alone is not responsible for decreased iNKT cell responsiveness in our apoE$^{-/-}$ mouse model. However, the interplay between apoE and LRP may prove to be an interesting avenue to pursue in determining how iNKT cells are directly influenced by their lipid environment. Further investigations into this potential mechanism are currently being conducted in our laboratory.

In summary, we have shown the effects of dyslipidemia upon iNKT cell activation, and these results indicate that iNKT cells are directly influenced by their lipid environment. Given that iNKT cells are currently being considered as potential therapeutic targets in human pathologies, and that hyperlipidemia may have varying impacts within these different disease states, further investigations into the precise mechanism by which dyslipidemia affects iNKT cell functions are needed.

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