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The Role of Invariant Natural Killer T Cells in Lupus and Atherogenesis

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Abstract

Systemic lupus erythematosus (SLE) is increasingly recognized as a risk factor for the development of premature atherosclerosis. The inflammatory process in both of these diseases is controlled by a variety of cell types of the innate and adaptive immune systems. Recent studies from several groups, including ours, have revealed a critical role of a unique subset of lymphocytes, termed invariant natural killer T (iNKT) cells, in the development of lupus-like autoimmunity and atherosclerosis in animal models. iNKT cells appear to play complex and divergent roles in the development of SLE and atherosclerosis. Our findings suggest that alterations in iNKT cell functions during the development of SLE may be related to the increased risk of SLE patients to develop atherosclerosis and coronary heart disease. We found that iNKT cell activation with the sponge-derived glycolipid α -galactosylceramide generally protects against the development of lupus-like autoimmunity in mice, whereas it exacerbates atherosclerosis. Therefore, while our studies have identified iNKT cells as potential therapeutic targets for SLE, further studies are necessary to design drugs that will avoid the underlying harmful effects of iNKT cell activation on the development of atherosclerosis.

Keywords

α -Galactosylceramide; Atherosclerosis; CD1d; Glycolipids; Immunomodulation; Immunotherapy; Invariant natural killer T cells; Systemic lupus erythematosus

Introduction

Inflammation plays a critical role in the development of an effective immune response. However, resolution of inflammation is critically important for maintaining the balance between health and disease. A breakdown in the regulation of inflammatory responses may result in a wide range of chronic diseases, including allergic asthma, type 1 diabetes (T1D), multiple sclerosis (MS) and systemic lupus erythematosus (SLE). In recent years, it has become clear that the development of certain cancers and vascular diseases such as atherosclerosis also involve inflammation. For example, atherosclerosis is characterized by inflammatory responses that develop at sites of lipid accumulation in arteries (1,2). Infection by common

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bacteria and viruses can contribute to the development of atherosclerosis. In addition, chronic autoimmunity such as SLE can accelerate the development of atherosclerosis. With all other risk factors being equal, the risk of coronary events in SLE patients is approximately 8 times greater than in non-autoimmune subjects and approximately 30% of deaths in SLE are related to atherogenesis (3). Therefore, understanding how SLE influences the atherosclerotic condition is essential to optimize risk reduction for cardiovascular disease while treating the SLE-associated inflammation.

Recent studies in immunology have focused on subsets of T cells that regulate immune responses. Of particular interest is a subset of glycolipid-reactive T cells, called invariant natural killer (iNKT) cells. iNKT cells bridge the innate and adaptive immune systems and hold significant promise for development of immunotherapies for a variety of diseases. iNKT cells play a role in the development of both SLE and atherosclerosis and thus represent a potential target for therapeutic intervention. Here, we review our understanding of iNKT cell biology and the roles these cells play in the progression of SLE and atherosclerosis.

iNKT Cell Specificity and Functions

iNKT cells represent a major T cell subset in bone marrow, thymus, and liver, a significant T cell subset in spleen and peripheral blood, but a minor population in lymph nodes and most tissues. The prevalence of iNKT cells in humans is significantly lower than in mice. Unlike conventional T cells, which express diverse T cell receptors (TCR), iNKT cells express a semi-invariant TCR formed by the combination of V α 14-J α 18 and V β 8.2/V β 7/V β 2 chains in mice and homologous V α 24-J α 18 and V β 11 chains in humans (for reviews regarding iNKT cells see refs. 4–10). This invariant TCR recognizes glycolipid antigens presented by the conserved major histocompatibility complex (MHC) class I-like molecule CD1d (Fig. 1A). Thus, the invariant iNKT cell receptor more closely resembles the pattern-recognition receptors expressed by members of the innate immune system than the diverse antigen-specific receptors expressed by cells of the adaptive immune system. Most murine iNKT cells express CD4, and the remaining cells express neither CD4 nor CD8, whereas a small proportion of human iNKT cells express CD8 α . In addition to the invariant TCR, iNKT cells express surface receptors such as CD161 (termed NK1.1 or NKR-P1C in mice) that are characteristic of the natural killer (NK) cell lineage. iNKT cells also constitutively express a variety of markers such as CD69, CD44 and CD122 that are typical of activated or memory T cells. Consistent with this activated phenotype, stimulation of the TCR of iNKT cells results in the rapid and robust production of a variety of cytokines, including interferon (IFN)- γ , interleukin (IL)-4, tumor necrosis factor (TNF)- α , and IL-2, and these cells also rapidly acquire cytolytic activity. Therefore, despite expression of a receptor of the adaptive immune system, iNKT cells behave more like cells of the innate immune system.

Because of their tendency to react with autologous cells, it is generally assumed that iNKT cells can recognize both exogenous and endogenous glycolipids. While the precise glycolipid antigens that control the physiological functions of iNKT cells remain to be fully characterized, several recent studies have provided insight into the antigen-specificity of these cells. First, in 1997, it was reported that all iNKT cells recognize α -galactosylceramide (α -GalCer; see Fig. 1B) (11), a marine sponge-derived glycosphingolipid with a multitude of immunomodulatory activities that are being exploited for therapeutic purposes (see below). However, because α -anomeric glycosphingolipids are rare among microorganisms and absent in mammalian cells, α -GalCer is not a physiological antigen for iNKT cells. Several studies subsequently demonstrated reactivity of iNKT cells with select cellular (12), tumor-derived (13) and microbial glycolipids (14,15), but these reagents are only recognized by small subsets of iNKT cells. Second, several years ago, we identified a cell line, deficient in the enzyme β -glucosylceramide (β -GlcCer) synthase, which was unable to activate iNKT cell hybridomas

(16). Recognition of the cell line was restored by transfection of the β -GlcCer synthase gene, but synthetic β -GlcCer was unable to activate the iNKT cell hybridomas, suggesting that β -GlcCer may be a biosynthetic precursor to an endogenous iNKT cell antigen. Indeed, a recent study has identified isoglobotrihexosylceramide (iGb3; Fig. 1B), which is downstream of β -GlcCer in the biosynthetic pathway of the isoglobos series of glycosphingolipids, as an activating ligand for iNKT cells (17). Finally, it has recently been reported that two glycosylceramides, α -glucuronosylceramide and α -galacturonosylceramide (Fig. 1B), isolated from the cell wall of Gram-negative *Sphingomonas* bacteria, can activate iNKT cells (18–21). Thus, iNKT cells react with both exogenous and endogenous glycolipids. Nevertheless, our current understanding of the immune response of iNKT cells to these antigens remains limited.

The loading of CD1d molecules with glycolipids is controlled by a variety of accessory molecules (Fig. 1C). Several years ago, we discovered that CD1d molecules that are prevented from engaging endosomal compartments are bound with phospholipids (22). We further demonstrated that CD1d molecules are loaded with phospholipids in the endoplasmic reticulum (ER) (23,24). Recent studies have further shown that microsomal triglyceride transfer protein, a lipid transfer protein that also lipidates apolipoprotein B, facilitates the loading of newly synthesized CD1d molecules with phospholipids (25,26). Because recognition of phospholipids by iNKT cells is the exception rather than the rule, it is likely that phospholipids largely play a role in stabilizing CD1d molecules and facilitating their export from the ER (akin to the function of the invariant chain in the classical MHC class II antigen processing pathway). Indeed, current evidence indicates that physiological iNKT cell ligands engage with CD1d molecules in endosomal compartments, after internalization of CD1d molecules from the cell surface. Several lipid transfer proteins, including saposins and the GM2 activator protein, have been implicated in the loading of CD1d molecules with physiological iNKT cell antigens in endosomal compartments (27,28). A recent study further showed that proteins such as apolipoprotein E (apoE) play a role in delivering glycolipid antigens after receptor-mediated uptake to endosomal compartments for binding with CD1d (29). Some of these glycolipids may require processing by carbohydrate hydrolases prior to CD1d binding (30). iNKT cells can interact with multiple cell types of both the innate and acquired immune systems, including antigen-presenting cells (APC), neutrophils, NK cells, B cells, and conventional T cells. As such, iNKT cells have been implicated in a wide variety of immune responses (reviewed in refs. 4–10). iNKT cells play a protective role in host immunity against some infections, provide natural immunity against certain tumors, generally play a suppressive role during autoimmunity, contribute to the development of allergic inflammation, contact hypersensitivity, certain models of inflammatory colitis and hepatitis, rejection of tissue grafts and the induction of tolerance in a variety of models.

In Vivo Response of iNKT Cells to α -GalCer Stimulation

α -GalCer was identified during a screen for natural products with antimetastatic activities in mice (31). More recent studies have shown that α -GalCer and related glycolipids hold significant promise for development of immunotherapies for a variety of diseases (see below). For these reasons, we investigated the in vivo response of iNKT cells to α -GalCer activation.

While a number of studies had shown that α -GalCer rapidly induces IL-4 and IFN- γ production by iNKT cells, the relevant APC responsible for these activities remained unknown. We hypothesized that α -GalCer presentation by distinct CD1d-expressing cell types elicits distinct iNKT cell functions. Thus, we compared the roles of DC, macrophages, and B cells in the in vivo response of iNKT cells to α -GalCer stimulation, using a variety of approaches (32). We transiently depleted DC from transgenic mice that selectively expressed the human receptor for diphtheria toxin on DC. Injection of α -GalCer into these animals resulted in reduced iNKT

cell responses, particularly with regard to IFN- γ production. iNKT cell responses in B cell-deficient μ MT animals that were transiently depleted of DC showed minimal iNKT cell activation upon α -GalCer administration, indicating that macrophages, hepatocytes, and other CD1d⁺ cell types other than DC and B cells, are not critical for stimulating cytokine secretion by iNKT cells in vivo. To further evaluate the role of B cells, we analyzed μ MT mice, which elicited increased cytokine responses by iNKT cells, indicating that B cells actually suppress DC-mediated iNKT cell activation. Finally, to more directly define the role of DC vs B cells, we enriched these cells from the spleens of mice, loaded them with α -GalCer ex vivo, and injected these cells into mice for evaluation of iNKT cell responses. Results demonstrated that α -GalCer-loaded DC elicited robust iNKT cell responses, whereas α -GalCer-loaded B cells elicited reduced iNKT cell responses with an IL-4-biased cytokine production profile. Collectively, these studies, as well as those conducted by other investigators (33), demonstrated that DC are critical APC for glycolipid antigen presentation and for the elicitation of robust iNKT cell responses in vivo.

Until recently, the fate of iNKT cells after in vivo activation was an issue of significant disagreement. Prior studies suggested that, within 24 h after in vivo activation with reagents such as anti-CD3 antibodies, the cytokine IL-12, or α -GalCer, most iNKT cells in peripheral organs succumb to activation-induced cell death (34–36). It was further suggested that the bone marrow provided a source of extensive homeostatic proliferation of iNKT cells important for the repopulation of peripheral organs with iNKT cells (34). This proposed model of in vivo iNKT cell activation did not explain the response of these cells to glycolipids ex vivo, which is characterized by extensive expansion (11). Therefore, we decided to re-evaluate the in vivo response of iNKT cells to α -GalCer. We demonstrated that the rapid disappearance of iNKT cells after α -GalCer administration to mice is due to downregulation of surface receptors (i.e., the iNKT cell receptor and NK1.1) that are utilized to identify these cells (37). The iNKT cell receptor was downregulated within 2 h after α -GalCer injection, became virtually undetectable by 6 h, and returned to pre-injection levels around 18–24 h. NK1.1 downregulation became evident at 8–12 h after α -GalCer injection, was maximal around 3–5 d, and remained substantially suppressed for up to 6 mo. Using CD1d- α -GalCer tetramers to identify iNKT cells, we further showed that these cells became substantially expanded (10- to 15-fold in the spleen and 2- to 3-fold in liver) around 2–4 d after α -GalCer injection (37), an observation that was missed in prior studies, because NK1.1-specific antibodies, which failed to react with the expanded population of iNKT cells due to NK1.1 downregulation, were utilized to identify these cells. Our studies further demonstrated that after their extensive expansion, numbers of iNKT cells in peripheral tissues gradually declined, reaching pre-injection levels around 2 wk after α -GalCer injection. With regard to cytokine expression, we found that α -GalCer-activated iNKT cells produced multiple cytokines for several days, with an early peak (2 h) in IL-4 production and a delayed peak (around 12–18 h) in IFN- γ production, after which cytokine production by these cells declined (38).

The realization that iNKT cells remained viable for an extended time period after α -GalCer injection raised the possibility that these cells may generate modified responses to secondary exposure to the same or different glycolipid antigens. Prior studies showed that iNKT cells are incapable of generating classical memory responses, that is, to generate increased immune responses upon secondary encounter with antigen (4). Instead, a number of groups, including ours, demonstrated that the recall response of mice to α -GalCer was typically blunted (39–41). We further showed that, upon secondary challenge with α -GalCer, iNKT cells failed to substantially downregulate their surface TCR, produce cytokines (with a more profound effect on IFN- γ than IL-4) and expand (32,42). This hyporesponsive phenotype persisted for at least 1 mo. α -GalCer presented to these iNKT cells by DC from naïve animals was unable to overcome the hyporesponsive phenotype, implying an iNKT cell intrinsic mechanism. Therefore, our findings suggested that α -GalCer induced long-term anergy in iNKT cells. Akin

to conventional T cell anergy, the anergic state of iNKT cells could be overcome by culture of these cells in the presence of α -GalCer and IL-2, and by stimuli (i.e., ionomycin plus phorbol myristate acetate) that bypass proximal TCR signaling events.

To determine the mechanism of anergy induction, we compared the capacity of α -GalCer-pulsed DC vs B cells to induce iNKT cell anergy. Consistent with prior studies (40), we found that α -GalCer-loaded DC failed to induce iNKT cell hyporesponsiveness, whereas α -GalCer-loaded B cells induced iNKT cell hyporesponsiveness (32,42). These findings suggested that DC are not only critically important for effective primary activation of iNKT cells in vivo, but also for avoiding anergy induction. Whether the differential capacity of distinct APC to induce iNKT cell anergy is due to differences in the expression of co-stimulatory molecules or other factors will require further investigation.

Collectively, our findings indicate that, after a single injection of free α -GalCer, most iNKT cells quickly downregulate surface TCR and NK1.1 receptors and secrete cytokines, then expand, undergo homeostatic contraction, and acquire an anergic phenotype (Fig. 2). This model of iNKT cell activation has been supported by studies from other laboratories (43–45).

Investigations of the effects of repeated α -GalCer administration on the numbers and functions of iNKT cells have revealed a substantial decline in iNKT cell numbers and functions, which is likely due to increased sensitivity of anergic iNKT cells to activation-induced cell death (39,46–48). A recent study further showed that depletion of iNKT cells after chronic α -GalCer treatment is followed by thymus-dependent repopulation of iNKT cells with an anergic phenotype, due to expression of increased levels of MHC class I-specific inhibitory NK cell receptors (49). Therefore, in contrast with the peripheral anergy induced in iNKT cells following a single injection of α -GalCer, chronic α -GalCer treatment induced the development of anergic iNKT cells in the thymus. Of note, iNKT cell anergy induced after a single injection of α -GalCer was independent of the thymus and did not involve expression of inhibitory MHC class I receptors on these cells (42,45).

Immunomodulatory and Therapeutic Activities of α -GalCer

To investigate the capacity of α -GalCer to modulate the activities of other cell types, we tested the capacity of this reagent to induce the expression of activation markers and co-stimulatory molecules on a variety of cell types (39). We found that within 24 h of α -GalCer administration NK cells, conventional T cells, and B cells expressed the activation marker CD69, and B cells expressed the co-stimulatory molecule CD86. These findings have been confirmed by many laboratories and it is now clear that α -GalCer-activated iNKT cells induce the activation of a variety of cell types of both the innate and adaptive immune systems (50). Because iNKT cells were originally implicated in the capacity of conventional T cells to differentiate into T helper (Th) type 2 cells, we investigated the capacity of α -GalCer to modulate the quality of an adaptive immune response. Evaluation of the effects of α -GalCer on the generation of Th2 immune responses and the production of IgE antibodies demonstrated that, within 6 d of a single α -GalCer injection, serum IgE levels started to rise, whereas all other Ig isotypes and IgG subtypes remained unaltered (39). To confirm and extend these findings, we mixed α -GalCer together with a protein antigen, ovalbumin, suspended this mixture into complete Freund's adjuvant, immunized mice, and measured ovalbumin-specific T cell cytokine and antibody responses. Our results revealed enhanced production of ovalbumin-specific Th2 cytokines and IgE and IgG1 antibodies, suggesting Th2 deviation (39). Although one group of investigators suggested that α -GalCer promoted Th1 rather than Th2 responses (51), most published studies are consistent with the idea that α -GalCer promotes Th2 immune responses in mice of a variety of strains, including C57BL/6, BALB/c, PL/J, and non-obese diabetic (NOD) (reviewed in ref. 50). Polarized immune responses play a critical role in the outcome of

a variety of pathological conditions, including autoimmune, infectious, and allergic diseases. Therefore, we hypothesized that α -GalCer can prevent the onset of autoimmune diseases that are characterized by Th1-mediated pathology. We tested this idea in mouse models of T1D, MS, and myasthenia gravis (MG).

Although NOD mice have low numbers of iNKT cells, we demonstrated that these cells are preferentially activated by α -GalCer presented by B cells rather than DC (32). Hence, α -GalCer was able to elicit IL-4 production by iNKT cells in these animals (32,46). We showed that repeated injection (twice per week, starting from 5 wk of age) of α -GalCer to NOD mice resulted in the CD1d- and dose-dependent protection against diabetes (46). This protection was associated with a Th2-biased cytokine profile of islet antigen-specific T cells and suppressed autoantigen-specific antibodies, suggesting a role of Th2 deviation in the protective effects of α -GalCer. To extend these findings to other models of organ-specific autoimmunity, we evaluated the capacity of α -GalCer to modulate disease in the experimental autoimmune encephalomyelitis (EAE) model of MS (47). Our results showed that α -GalCer protected C57BL/6 and PL/J mice against EAE in a dose- and CD1d-dependent manner. Consistent with our findings in NOD mice, protection from disease was associated with polarization of autoantigen-specific T cell responses toward a Th2 phenotype. Importantly, α -GalCer was unable to protect IL-4- and IL-10-deficient C57BL/6 mice against EAE, indicating a role for both of these Th2 cytokines. To determine whether α -GalCer also provides protection against autoimmunity that is predominantly antibody-mediated, we evaluated its capacity to modulate disease in an experimental model of MG. These studies, which were performed in collaboration with the laboratory of Dr. Fu-Dong Shi (Barrow Neurological Institute, St. Joseph's Hospital and Medical Center, Phoenix, AZ), revealed that α -GalCer treatment prevented the development of experimental autoimmune MG, by a partial IL-4-dependent mechanism (52). Collectively, these studies, which have been confirmed by other investigators (reviewed in ref. 50), revealed that α -GalCer may be useful for therapeutic intervention in autoimmune conditions with a Th1 pathology. The mechanisms responsible for disease protection afforded by α -GalCer remain poorly understood and likely depend on the particular disease model studied (reviewed in refs. 50,53, and 54).

Role of iNKT Cells in the Development of Lupus

In contrast with T1D, MS, and MG, many autoimmune and inflammatory conditions, including SLE and atherosclerosis, cannot be easily classified as Th1- or Th2-mediated.

SLE is a systemic autoimmune disease that is characterized by autoantibodies directed against a variety of self-antigens, including double-stranded DNA, which ultimately leads to the development of multiorgan failure (55,56). Development of SLE is associated with the generation of autoreactive Th cells and a reduction in regulatory T cells. A potential role of iNKT cells in regulating the development of SLE was suggested by the finding that humans and mice with SLE have defects in the numbers and functions of iNKT cells (57–61).

Several hereditary and induced models of SLE in mice are available that resemble many aspects of the pathogenesis of the human disease and exemplify the heterogeneous nature of disease (56). We have evaluated the role of iNKT cells to the development of SLE in two hereditary models, MRL-MpJ mice and (NZB/NZW)F1 mice, and in a hydrocarbon oil-induced model for this disease.

MRL-MpJ mice spontaneously develop inflammatory lesions in the skin, kidneys and blood vessels, which are markedly accelerated in congenic MRL-Fas^{lpr/lpr} mice that are genetically deficient in the death receptor Fas. Based on prior studies, indicating that C57BL/6-Fas^{lpr/lpr} mice have decreased numbers of NK1.1⁺ cells (57), we performed a detailed analysis of iNKT cell numbers and functions during the progression of lupus-like autoimmunity in MRL-MpJ

mice. We found that, prior to the onset of autoimmunity (at 5–6 wk of age), MRL-Fas^{lpr/lpr} mice exhibited a selective reduction in the numbers and functions of iNKT cells, with reduced capacity to produce cytokines (IFN- γ , IL-2, and IL-4) in response to α -GalCer stimulation (62). Interestingly, these defects in iNKT cell functions appeared to correlate with the severity of disease that developed in the animals. Therefore, MRL-Fas^{lpr/lpr} mice that develop severe disease in early life have a more profound reduction in numbers and functions of iNKT cells than MRL-Fas^{+/+} mice that experience a milder disease course. To determine whether iNKT cells promote or protect against disease, we established MRL-Fas^{lpr/lpr} mice carrying a germline deletion in the CD1d gene. Our results indicated that these animals developed more frequent and more severe skin lesions but exhibited no significant difference in the development of kidney disease, as compared with CD1d-expressing littermates (63). These effects of CD1d-deficiency on inflammatory dermatitis in MRL-Fas^{lpr/lpr} mice were associated with decreased T cell production of Th2 cytokines (IL-4 and IL-10) and increased or unchanged production of Th1 cytokines. To determine whether α -GalCer can modulate lupus-like autoimmunity in these animals, we treated young MRL-Fas^{lpr/lpr} mice with a series of α -GalCer injections (twice per week for 5 mo), and monitored them for various disease manifestations. Results showed significant alleviation of inflammatory dermatitis, but no significant effects on kidney disease (62). Although repeated injection of α -GalCer in most strains, including MRL-Fas^{+/+} mice, induces profound iNKT cell depletion (see above), repeated α -GalCer treatment of MRL-Fas^{lpr/lpr} mice resulted in iNKT cell expansion, suggesting that Fas/FasL interactions play a critical role in inducing iNKT cell death in mice with a functional Fas/FasL activation pathway. This expansion of iNKT cells in MRL-Fas^{lpr/lpr} mice correlated with an increase in serum IgE antibodies, suggesting Th2 deviation.

(NZB/NZW)F1 mice spontaneously develop systemic autoimmunity with severe glomerulonephritis. Similar to MRL-Fas^{lpr/lpr} mice, young (NZB/NZW)F1 mice exhibited a 2.5-fold, 4- to 5-fold, and 7-fold reduction in the proportion and total numbers of iNKT cells in the thymus, as compared with normal C57BL/6 (64), BALB/c, and NZW mice, respectively (unpublished data). In the periphery, however, iNKT cell numbers were similar between young (NZB/NZW)F1 and C57BL/6 (64) or BALB/c mice (unpublished data). Furthermore, during the development of disease in (NZB/NZW)F1 mice, iNKT cells became progressively expanded and activated (64). Nevertheless, we showed that CD1d-deficiency in these animals exacerbated disease, whereas repeated α -GalCer treatment of young animals ameliorated disease (unpublished data). In addition, although we did not observe any effects of repeated α -GalCer treatment on disease in older (NZB/NZW)F1 mice (unpublished data), other investigators have reported disease exacerbation by this treatment, which correlated with abnormal development of Th1 immune responses (65). Collectively, these findings suggest a protective effect of iNKT cells against lupus in young (NZB/NZW)F1 mice and a potential pathogenic role of these cells in older animals.

We have also analyzed the role of iNKT cells in lupus-like autoimmunity induced by the hydrocarbon oil pristane, which induces disease characterized by mesangial and focal kidney lesions. We found that, quickly after pristane injection of BALB/c mice, numbers and functions of iNKT cells declined, with a shift in their cytokine production profile from predominantly IL-4 toward predominantly IFN- γ (66). CD1d-deficiency in BALB/c mice resulted in profound exacerbation of pristane-induced lupus nephritis (66). This disease exacerbation was associated with reduced T cell IL-4 production and expansion of CD1d-expressing marginal zone B (MZB) cells. Conversely, repeated α -GalCer injection (twice per week for 1 mo) of pristane-treated BALB/c mice resulted in disease amelioration, in a manner that was dependent on the cytokine IL-4 and correlated with Th2 deviation of immune responses (48). To further evaluate mechanisms of disease protection, we cultured MZB cells with lipopolysaccharide (LPS) and α -GalCer, and found significant suppression of autoantibody production, as compared with stimulation with LPS alone (66). We also evaluated the capacity of α -GalCer to modulate

pristane-induced lupus-like autoimmunity in SJL/J mice, which develop a particularly severe form of SLE in response to pristane injection. Surprisingly, we found that α -GalCer exacerbated disease in these animals, some of which developed serositis (i.e., inflammation and fluid accumulation in serosal cavities such as the peritoneum) (48). Disease exacerbation correlated with a tendency of α -GalCer to promote unusual Th1 responses in these animals, as contrasted with its capacity to promote Th2 immune responses in most other mouse strains. Consistent with this possibility, it has been shown that SJL/J mice have significant defects in iNKT cell numbers and that the remaining cells exhibit a Th1 cytokine production profile (47,48,67).

Our findings, at least in young animals and in most mouse strains, are consistent with a protective role of iNKT cells in the development of lupus-like autoimmunity. While the precise mechanisms of this protection remain unclear, our studies, taken together with those of others, are consistent with the idea that iNKT cells suppress SLE by promoting Th2 cytokine production and inhibiting autoantibody production by MZB cells (Fig. 3). Additional studies will be necessary to elucidate the complex roles that iNKT cells play during different stages of the disease process. Furthermore, the mechanisms that are responsible for iNKT cell activation, degeneration or expansion in different models of this disease also require further investigation.

Role of iNKT cells in the Development of Atherosclerosis

Atherosclerosis is a disease characterized by the development of inflammatory foci, referred to as atherosclerotic plaques, at sites of lipid accumulation in arteries. Rupture of atherosclerotic plaques may result in infarction of the heart and brain. Increasing evidence indicates that atherosclerosis has many features in common with autoimmune disorders (2). Macrophages play a critical role in atherosclerosis, by entering the progressing arterial plaque and taking up lipids, which eventually transforms these cells into lipid-laden foam cells. However, it is now clear that many other cell types, including T cells, B cells, DC, and NK cells, also contribute to the development of atherosclerosis. Atherosclerotic lesions are infiltrated by T lymphocytes, and it has been generally assumed that these cells are specific for peptide antigens (1). However, atherosclerotic plaques contain abundant lipids and glycolipids (68,69), providing a potentially rich source of antigens for iNKT cells. Atherosclerotic plaques in humans and mice express CD1d molecules and contain iNKT cell infiltrates (70–72), suggesting the possibility that this T cell subset can regulate atherosclerosis.

ApoE-deficient mice suffer from hypercholesterolemia and spontaneously develop atherosclerosis (73). We utilized these animals to study the role of iNKT cells in the development of atherosclerosis. To evaluate whether iNKT cells that accumulate in atherosclerotic plaques are capable of cytokine secretion, we injected 16-wk-old apoE^{-/-} mice with α -GalCer, isolated aortas, and measured cytokines by a real-time polymerase chain reaction assay (74). Results demonstrated a profound increase in IFN- γ and IL-10 mRNA production, and a less dramatic increase in IL-4 mRNA at 16 h (but note that the 16-h time point is relatively late for detection of an increase in IL-4 production by iNKT cells). These findings indicated that iNKT cells that are present in established atherosclerotic lesions are capable of producing cytokines in response to glycolipid activation.

To determine whether the atherosclerotic environment impacts iNKT cell numbers and functions outside of atherosclerotic plaques, we evaluated iNKT cells in the spleen and liver of wild-type and apoE^{-/-} mice prior to the development of atherosclerotic lesions (4 wk) and at times of early (8 wk), intermediate (16 wk), and complex (24 wk) atherosclerotic lesions (74). At 4 wk of age, numbers of iNKT cells in the spleen and liver of apoE^{-/-} mice were comparable to those observed in wild-type animals. However, at later time points, numbers of iNKT cells gradually declined, and at 24 wk of age, numbers of iNKT cells were reduced

approximately twofold. We obtained similar results in C57BL/6 mice fed an atherosclerotic diet compared with mice fed a normal chow diet (74), findings that have been confirmed by other investigators (75). ApoE^{-/-} mice also exhibited changes in iNKT cell functions. In young (8-wk-old) apoE^{-/-} mice, spleen cells produced enhanced IFN- γ but normal IL-4 levels in response to α -GalCer stimulation, as compared with wild-type mice. However, in older apoE^{-/-} mice, cell proliferation and cytokine (IFN- γ and IL-4) production were substantially suppressed. Similar increases in IFN- γ production by iNKT cells in young C57BL/6 mice fed an atherosclerotic diet have been observed by other investigators but whether iNKT cell functions changed during atherogenesis was not investigated (75).

Our findings in the apoE^{-/-} model suggested that the atherosclerotic environment in apoE^{-/-} mice leads to dampening of iNKT cell responses, akin to iNKT cells from α -GalCer-treated mice (see above). The most likely explanation for these findings is that iNKT cells become chronically activated during the progression of atherosclerosis. How iNKT cells become activated during this process remains unclear, but we envision several possibilities (Fig. 4). First, it is possible that iNKT cells become activated indirectly by the inflammatory process that accompanies atherogenesis. iNKT cells are capable of responding to a variety of inflammatory stimuli, in a mechanism that involves interaction with APC (76). For example, iNKT cells become activated in response to LPS, a component of the cell wall of Gram-negative bacteria, that promotes atherosclerosis (77) and induces the recruitment of iNKT cells into atherosclerotic plaques (77). LPS and similar pathogen-associated molecular patterns can bind with pattern-recognition receptors on APC (most notably DC) and induce these cells to produce proinflammatory cytokines such as IL-12. Recent studies have provided evidence that proinflammatory cytokines produced by DC synergize with CD1d molecules in activating iNKT cells, in a mechanism that likely involves interaction of the iNKT cell receptor with iGb3 (18). Second, in a similar manner, atherosclerotic lipoproteins such as oxidized low density lipoproteins (oxLDL), via interaction with scavenger receptors, may activate macrophages and/or DC to produce inflammatory cytokines and activate iNKT cells. Interestingly, one group showed that LDL and oxLDL were able to induce CD1d expression on macrophages and induce a modest increase in IFN- γ production by iNKT cells (75). Third, it is possible that the iNKT cells recognize specific glycolipids, perhaps associated within oxLDL. In this context, the finding that lipid-associated proteins such as apoE and lipoprotein receptors such as the LDL receptor (LDLR) have been implicated in providing CD1d with glycolipids is particularly intriguing (29). In this manner, apolipoproteins may deliver glycolipids by receptor-mediated uptake to endosomal compartments for the loading of CD1d molecules. One candidate glycolipid is the disialoganglioside GD3, which is induced in atherosclerotic tissues and in the circulation (68,69). GD3 is recognized by a small subset of iNKT cells (13) and its overexpression during atherosclerosis may therefore activate this subset of iNKT cells. Finally, atherosclerosis may induce production of the endogenous iNKT cell antigen iGb3, leading to enhanced iNKT cell activation. Interestingly, two glycolipid intermediates for the biosynthesis of iGb3, β -GlcCer and lactosylceramide, are overexpressed during atherogenesis (69), suggesting that iGb3 expression may be enhanced as well. Additional studies will be required to investigate the mechanisms responsible for iNKT cell activation during atherosclerosis.

iNKT cells can produce a variety of cytokines, including the pro-atherogenic cytokines IFN- γ and IL-4 and the anti-atherogenic cytokine IL-10, and can induce APC to produce the pro-atherogenic cytokine IL-12 (78). Our finding that iNKT cells from young apoE^{-/-} mice have a Th1-biased cytokine production profile (see above) suggested that iNKT cells may be pro-atherogenic in this model. To test this hypothesis, we bred apoE^{-/-} mice with iNKT cell-deficient CD1d^{-/-} mice and compared these compound knockout animals with apoE^{-/-} single mutant mice for the development of atherosclerosis (74). Our results revealed a significant decrease (68%) in atherosclerosis in CD1d^{-/-}apoE^{-/-} mice compared with apoE^{-/-} mice at 16 wk. No differences in serum cholesterol or triglyceride levels were observed. Other

investigators have reported similar effects of CD1d-deficiency on atherogenesis in apoE^{-/-} mice (75,79). Additionally, it has been reported that CD1d-deficiency in C57BL/6 mice fed an atherogenic diet and in Ldlr^{-/-} mice similarly ameliorates atherosclerosis (75,80), suggesting that iNKT cells generally play a pathogenic role in atherogenesis.

If iNKT cells are pro-atherogenic, α -GalCer would be expected to exacerbate atherosclerosis. Indeed, we found that repeated (twice weekly) α -GalCer administration to apoE^{-/-} mice, starting from 4 wk of age for a period of 10 wk, resulted in a twofold increase in atherosclerosis when mice were analyzed at 16 wk of age (74). An increase in atherosclerosis was also observed at 24 wk, but was less dramatic. Consistent with prior studies (54), we (74) and others (75, 79), found that repeated α -GalCer injection led to a dramatic decrease in iNKT cell numbers and functions. This treatment also resulted in Th2 polarization in the aortas of apoE^{-/-} mice. Th2 polarization is consistent with the pro-atherogenic role of iNKT cells because recent studies have indicated a pathogenic role of IL-4 in atherosclerosis (81,82). Although α -GalCer is not a physiological iNKT cell antigen, glycolipids produced during the atherosclerotic process, or within the cell walls of bacteria, may activate iNKT cells and exacerbate atherogenesis in a similar manner. In addition, the finding that α -GalCer treatment promotes atherogenesis represents a significant impediment for development of α -GalCer as an immunotherapeutic.

Conclusions

iNKT cells are a unique cell type of the immune system in that they express a receptor (i.e., TCR) that is typical of cells of the adaptive immune system, yet the iNKT cell receptor is semi-invariant, functioning more like a pattern-recognition receptor of the innate immune system. Our studies have shown that effective in vivo activation of iNKT cells with glycolipids such as the sponge-derived antigen α -GalCer requires glycolipid antigen presentation by DC. The typical in vivo response of iNKT cells to glycolipids is characterized by rapid cytokine production, receptor downregulation, expansion, homeostatic contraction, and development of an anergic phenotype. As of yet, there is no evidence that iNKT cells can develop classical memory responses. Activation of iNKT cells in this manner also results in transactivation of a variety of cell types of the innate and adaptive immune systems. In most mouse strains, repeated injection of α -GalCer promotes Th2 immune responses, which can prevent the development of Th1-mediated autoimmunity. iNKT cells generally play a protective role in the development of lupus-like autoimmunity in both hereditary and induced mouse models of this disease, in a manner that appeared to be related to Th2 cytokine production. As lupus progresses, however, iNKT cell numbers and functions become either suppressed or enhanced, with typically a shift in the cytokine production profile from IL-4 to IFN- γ . These findings raise the possibility that alterations in iNKT cell function during the progression of SLE may contribute to the increased risk of SLE patients for development of atherosclerosis, a disease where iNKT cells appear to play a pathogenic role. When initiated early in the disease process, α -GalCer treatment generally protects against the development of lupus; however, this treatment exacerbates atherosclerosis. Thus, the predicted pro-atherogenic properties of α -GalCer temper the use of this glycolipid as an immunotherapeutic for SLE in humans. Nevertheless, it may be possible to develop structural variants of α -GalCer with beneficial effects on both lupus and atherosclerosis.

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Fig. 1.

The CD1d-iNKT cell recognition system. (A) iNKT cells recognize glycolipids presented by CD1d molecules. iNKT cells express an invariant T cell receptor (TCR) together with receptors such as NK1.1 that are characteristic of the NK cell lineage. The iNKT cell receptor interacts with glycolipids presented by CD1d molecules on antigen-presenting cells (APC). (B) Structures of iNKT cell antigens. α -Galactosylceramide (α -GalCer) is derived from a marine sponge, α -glucuronosylceramide (α -GlcA-Cer) and the related glycolipid α -galacturonosylceramide (not shown) are derived from *Sphingomonas* bacteria, and isoglobotrihexosylceramide (iGb3) is an endogenous iNKT cell antigen. (C) The major CD1d antigen processing and presentation pathway. Quickly after their biosynthesis in the endoplasmic reticulum (ER), CD1d molecules are loaded with phospholipid chaperones, catalyzed by lipid transfer proteins (LTP) such as microsomal triglyceride transfer protein. CD1d molecules then traffic to the cell surface and are internalized into endosomal

compartments via their cytoplasmic endosomal targeting motif. Phospholipid chaperones are then exchanged for exogenous or endogenous glycolipids, catalyzed by LTP such as saponins or the GM2 activator protein. Exogenous glycolipids may be delivered to endosomal compartments via lipoproteins and their receptors. Some glycolipids may undergo processing by carbohydrate hydrolases (Hy).

Fig. 2.

In vivo response of iNKT cells to α -GalCer. Rapidly after α -GalCer administration to mice, iNKT cells produce immunomodulatory cytokines (with an early peak of IL-4 at 2 h and a later peak of IFN- γ at 12–18 h) that can activate a variety of other cell types and downregulate critical surface receptors, including the iNKT cell receptor and NK1.1, making these cells invisible by flow cytometry at 8–20 h. TCR levels are restored by 24 h whereas NK1.1 levels remain suppressed for up to 6 mo. iNKT cells expand extensively, reaching peak expansion levels around 3 d, after which the iNKT cell population contracts by homeostatic mechanisms, reaching pre-injection levels around 1–2 wks after α -GalCer injection. Activation of iNKT cells in this manner induces long-term anergy in these cells.

Fig. 3.

A simplified model for the protective role of iNKT cells during the development of SLE. iNKT cells, activated repeatedly by endogenous or exogenous glycolipids, predominantly produce T helper (Th) type 2 cytokines that promote Th2 deviation of the immune response and suppress autoantibody secretion by CD1d-expressing marginal zone B (MZB) cells. Note that the protective effect of iNKT cells may not be observed during every lupus model, mouse strain, or stage of the disease.

Fig. 4.

Possible mechanisms of iNKT cell activation during atherogenesis. **(A)** Indirect activation of iNKT cells by inflammatory mediators and endogenous glycolipids. Infection or inflammation such as the inflammatory response induced during SLE may result in the production of inflammatory mediators such as ligands of Toll-like receptors (TLR) that can activate dendritic cells (DC) to produce pro-inflammatory cytokines with pro-atherogenic properties. Oxidized low density lipoprotein (oxLDL) may similarly activate DC to produce inflammatory cytokines and induce CD1d expression. Pro-inflammatory cytokines may then synergize with CD1d in activating iNKT cells to produce pro-atherogenic cytokines. It is likely that endogenous glycolipids such as isoglobotrihexosylceramide (iGb3), which may be induced during this process, play a significant role during this mechanism of iNKT cell activation. **(B)** Direct activation of iNKT cells by glycolipids. Glycolipids such as α -GalCer, the disialoganglioside GD3, which is overproduced during atherogenesis, and bacterial glycolipids may directly activate iNKT cells to produce pro-atherogenic cytokines.