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Therapeutic Targeting the Diverse Immunologic Functions Expressed by Hepatic NKT cells

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Abstract

Introduction—NKT cells comprise approximately 30% of the hepatic lymphoid population in mice (~50% in humans). Most mouse hepatic NKT cells [invariant (i)NKT cells] express T cell receptors, composed of invariant Va14Ja18 chains. Unlike conventional T cells, iNKT cells recognize glycolipid molecules presented in association with MHC class Ib (CD1d) molecules. Purportedly, iNKT cells serve a key function in a wide range of immunological events; the precise nature of this function is often unclear. Indeed, the consequences of hepatic iNKT cell activation can be beneficial or detrimental. α -Galactosylceramide, the prototypic glycolipid recognized by the iNKT cell receptor, stimulates the rapid production of both interferon- γ and interleukin-4. The reciprocal suppression exhibited by these cytokines limits the potential therapeutic value of α -galactosylceramide. An extensive research effort is ongoing to develop α -galactosylceramide analogs that modulate iNKT cell activity and selectively promote interferon- γ or interleukin-4.

Areas covered—This review provides a broad overview of hepatic iNKT cells and their purported role in liver disease. Efforts to develop therapeutic agents that promote their beneficial contributions are detailed.

Expert Opinion—While a growing body of literature documents the differential effects of α -GalCer analogs on IFN- γ and IL-4 production, the effects of these analogs on other iNKT cell activities, e.g., cytotoxicity and the production of other cytokines, remain to be determined. Similarly, an exhaustive examination of the effects of these analogs on inflammation and liver injury in animal models remains prior to considering their utility in clinical trials.

1. INTRODUCTION

NK1.1⁺TcR $\alpha\beta$ ^{int} CD1-restricted T (NKT) cells constitute a unique subset of T lymphocytes thought to play an immuno-regulatory role in a wide range of diseases [1]. Indeed, it is widely postulated that NKT cells link innate and adaptive immune responses by responding rapidly and subsequently activating other cell types, e.g., dendritic and NK cells [2]. NKT cells comprise approximately 30% of the hepatic lymphoid population in mice (up to 50% in humans) where they reside within the vascular sinusoids, adherent to endothelial cells, crawling rapidly along the blood vessel walls [3,4]. A much smaller percentage is found in other organs, i.e., thymus, spleen, lymph nodes and peripheral blood, suggesting hepatic NKT cells might serve some unique function [4,5].

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Declaration of interest

The authors declare no conflict of interest.

Most (80–85%) mouse hepatic NKT cells [Type 1, classical or invariant (i)NKT cells] express T cell receptors (TcRs) composed of an invariant V α 14J α 18 chain and a β -chain heavily biased toward V β 8.2, V β 2 and V β 7 [6]. Similarly, a significant portion (5–6%) of the NKT cells in human livers expresses the V α 14J α 18 homologue, V α 24J α 18 [6,7]. In contrast to conventional T cells, these iNKT cells recognize antigenic glycolipids (rather than peptides) derived from self or non-self and presented in association with non-classical MHC class Ib (CD1d) molecules [8]. Notably, not all iNKT cells (e.g., most immature iNKT cells recently emigrated from the thymus) exhibit cell-surface NK1.1 [9]. As such, iNKT cells are defined by the expression of the invariant TcR α -chain [5,10]. The remaining CD1d-restricted NKT cells (Type 2, non-classical or variant NKT cells) express a diverse TcR repertoire; relatively little is known regarding their antigenic specificity or biological function [9]. Clearly, however, the continued presence of Type 2 NKT cells in J α 18-deficient, but not CD1d-deficient, mice can contribute to the disparate responses of these two strains in a number of experimental models.

CD1 molecules are cell surface glycoproteins consisting of 43- to 49-kDa heavy chains noncovalently associated with β_2 -microglobulin [11]. While mice express only CD1d, most mammalian species including human express multiple CD1 isoforms (i.e., CD1a–d and e, which is localized intracellularly within Golgi compartments, endosomes, and lysosomes) [8,12]. Thus, in addition to CD1d-restricted Type 1 and Type 2 NKT cells, human livers contain a heterologous population of T cells [hepatic natural T (NT) cells] that express various combinations of TcR and NK receptors, but are best defined by co-expression of CD3 and CD56 [7]. Activated NT cells exhibit cytolytic activity and the ability to produce a number of cytokines *in vitro* [7].

A variety of cell types express CD1d including B cells, dendritic cells and mononuclear phagocytes, as well as epithelial cells, parenchymal cells and vascular smooth muscle cells comprising non-lymphoid tissues such as the liver [13]. Analysis of the crystal structure of CD1 reveals a narrow, deep binding groove with 2 extremely hydrophobic pockets that accommodate aliphatic hydrocarbon chains [14]. Cell-surface CD1d molecules are internalized and delivered to late endosomes and lysosomes then recycle back to the plasma membrane, thus allowing their access intracellular compartments that contain both exogenous and endogenous glycolipid antigens [8]. The origin and identity of these self-antigens remain to be fully elucidated.

Ostensibly, mouse V α 14J α 18 iNKT cells and their human V α 24J α 18 iNKT cell homologue serve key functions in a wide range of immunological events that occur in the liver and outlined below; the precise nature of these functions, however, is often controversial or unclear [8]. Indeed, while strong evidence supports the role of NKT cells in animal models, clinical studies implicating human NT or NKT cells in the pathogenesis of liver disease are largely corollary demonstrating changes in the size and/or phenotype of the hepatic (or peripheral blood) population while providing little insight into the actual contribution of that population to liver disease [15]. Consequently, “a consensus view has not emerged in support of the true physiologic role of NKT cells [16].”

2. BIOLOGIC FUNCTIONS OF HEPATIC iNKT CELLS

2.1 Metastasis, immune surveillance, tumor rejection

iNKT cells have been implicated in immunity to hepatic tumors in humans, as well as rodent models. The presence of fewer iNKT cells in liver biopsies obtained from patients with metastatic disease than from healthy donor organs suggests, for example, that a diminished iNKT cell population may contribute to metastatic liver disease [17]. In an animal model, the number of metastatic liver tumors increased significantly in NKT cell-depleted mice

inoculated with EL4 tumor cells [18]. Moreover, iNKT cell-deficient mice failed to reject FBL-3 erythroleukemia or B16 melanoma cells inoculated intra-splenically and metastasizing preferentially to the liver [19].

2.2 Antimicrobial activity

Purportedly, NKT cells also play a role in host defenses to infection. Indeed, it has been suggested that iNKT cells evolved primarily to respond to a diverse array of microbial pathogens [20]. Both the direct response of iNKT cells to glycolipids associated with a select few microorganisms (e.g., glycosphingolipids derived from *Sphingomonas sp.*, such as *S. wittichii* and *S. capulata*), and the indirect response to endogenous self-antigens produced by infected host cells have been reported [2,21–23]. The increased replication of a limited number of parasites, bacteria and viruses in the organs of CD1d- or $\alpha 18^{-/-}$ mice outlined below supports the role of iNKT cells in host defenses to certain microbial infections. In many instances, however, iNKT cells are not protective and, at times, are detrimental.

Bacteria—Most mouse and human iNKT cells recognize glycosphingolipids derived from *Sphingomonas capsulata*, *Ehrlichia muris* and *Novosphingobium aromaticivorans*, a member of the Sphingomonadaceae family implicated in the etiology of primary biliary cirrhosis (see discussion below). iNKT cells were activated in mice infected with *Sphingomonas sp.* or exposed to glycosphingolipids; CD1d $^{-/-}$ and $\alpha 18^{-/-}$ mice infected i.v. with a sublethal dose of *S. capsulata* exhibited a marked defect in bacterial clearance from the liver and lungs [22,23]. Similarly, CD1d-deficient mice exhibited a reduced capacity to clear *E. muris* from their spleens [22].

In contrast to a diminished resistance to *S. capsulata* or *E. muris* infections, no significant differences were found in the survival of CD1d $^{-/-}$ mice, relative to wild-type mice, infected with *M. tuberculosis*; CD1d-deficient animals were fully protected against *M. tuberculosis* infection [24]. Likewise, *Mycobacterium bovis* BCG replicated to a similar extent in the spleens, livers and lungs of wild-type and $\alpha 18^{-/-}$ mice [25].

Additionally, iNKT cells played an insignificant role in clearance of *Salmonella choleraesuis* and *Escherichia coli*; the bacterial burden of the livers and/or serum cytokine levels of infected control and NKT cell-deficient mice were equivalent [26,27]. Moreover, serum alanine aminotransferase (ALT, an indicator of liver injury) levels, Fas/Fas ligand-dependent liver injury, and pathological lesions in the liver were decreased significantly in $\alpha 18^{-/-}$ mice suggesting the response of iNKT cells was largely detrimental in this model.

Hepatic iNKT cells also exerted a detrimental effect on host resistance in cecal ligation and puncture (CLP) model of sepsis. Survival was increased in $\alpha 18^{-/-}$ mice and mice administered anti-CD1d monoclonal antibody prior to CLP [28,29]. In contrast to these findings, Etogo and colleagues reported comparable rates of survival among wild-type and CD1d-deficient mice subjected to CLP [30]. While the results of these studies are seemingly at odds, they agree that hepatic iNKT cells fail to exert a beneficial effect on sepsis following CLP.

Listeria monocytogenes is an intracellular bacterial pathogen that, within the liver, replicates primarily within hepatocytes [31]. Studies undertaken to determine the role of iNKT cells in host defenses to *Listeria* yielded disparate results dependent upon the route of infection. Mice lacking CD1d-restricted NKT cells exhibited enhanced resistance to *Listeria* inoculated i.v. Relative to wild-type animals, the listerial burdens of the livers and spleens were reduced substantially [32]. Conversely, survival was increased in mice administered anti-CD1d monoclonal antibody prior to intravenous infection [33]. In contrast, resistance to

Listeria was diminished in both $\text{J}\alpha 18^{-/-}$ and $\text{CD1d}^{-/-}$ mice infected by gavage; significantly more listeriae were recovered in the livers of $\text{CD1d}^{-/-}$ mice compared to WT, mice [34]. Liver sections derived from $\text{CD1d}^{-/-}$ mice showed severe hepatic necrosis associated with the accumulation of neutrophils and lymphocytes within hepatic sinusoids and periportal regions correlating directly with a higher bacterial count [34]. The disparate responses of $\text{CD1d}^{-/-}$ mice to *Listeria* dependent upon route of administration suggest distinctly different contributions of hepatic iNKT cells and iNKT cells associated with the intestinal mucosa. Indeed, other investigators reported stark differences in the composition of the intestinal microbiota of wild-type and $\text{CD1d}^{-/-}$ mice that could exert a significant influence on subsequent infections by the oral route [35].

Viruses—Lymphocytic choriomeningitis virus (LCMV)-induced NK cell activity, virus-specific cytolytic T lymphocyte activity, and viral clearance in mice were independent of CD1d expression [36]. Similarly, $\text{CD1d}^{-/-}$ and wild-type C57BL/6 mice had equivalent titers of murine cytomegalovirus (MCMV) in their spleens and livers following infection with a moderate (5×10^4 PFUs) dose [37]. However, $\text{CD1d}^{-/-}$ mice infected with a high dose exhibited an increased rate of mortality indicating the potential role of Type 2, variant NKT cells. NKT cells were not critical for recovery from Ectromelia (mousepox) virus infection; virus titers in the livers of $\text{CD1d}^{-/-}$ mice were equivalent or less than those found in wild-type animals [38]. Adenovirus infection, on the other hand, induced significant liver pathology not found in iNKT cell-deficient mice [39].

A large population of $\text{CD161}^+\text{CD56}^+$ NKT cells resides in healthy human livers despite low-level CD1d expression. CD1d expression is elevated significantly upon hepatitis C virus (HCV) infection [40]. The resultant response of hepatic NKT cells is a matter of controversy due in large part to the disparate criteria and methods used to quantify the cell population. Durante-Mangoni and colleagues reported, on one hand, that non-classical proinflammatory (IFN- γ -producing) hepatic CD1d -reactive CD161^+ T cells composed a significant proportion of the hepatic cells recovered from HCV-infected patients, and concluded that the response of NKT cells may be beneficial in acute viral clearance [40]. Significant reductions in numbers of non-classical ($\text{CD56}^+\alpha\beta\text{-TcR}^+$), as well as classical ($\text{V}\alpha 24^+$), hepatic NKT cells in cirrhotic HCV-infected livers support this suggestion [41]. In contrast, a positive correlation between the elevated presence of $\text{V}\alpha 24^+$ and $\text{V}\alpha 24^+\text{V}\beta 11^+$ NKT cells infiltrating the liver of chronic HCV patients and the extent of hepatocellular damage lead investigators to suggest, instead, that these cells play a pathologic role [42,43]. Notably, the proportions and absolute numbers of activated $\text{CD56}^+\text{CD3}^+$ and $\text{CD161}^+\text{CD3}^+$ NKT cells in livers were significantly increased in chronic HCV-infected patients exhibiting a sustained response to chemotherapy (pegylated IFN and ribavirin), demonstrating a direct correlation between the presence of NKT cells and treatment efficacy [44].

Protozoa—The surface glycoconjugate lipophosphoglycan, as well as related glycoinositol phospholipids, derived from *Leishmania donovani* induced CD1d -dependent IFN- γ production by naive intrahepatic lymphocytes [45]. Furthermore, CD1d -deficient BALB/c mice exhibited defective granulomas formation and increased susceptibility to infection [45]. Parasitic burden was increased in the livers of $\text{J}\alpha 18^{-/-}$ mice infected i.v. with *L. major* [46]. In sharp contrast to these findings, Stanley and coworkers reported iNKT cells played a negligible role in host resistance to *L. donovani* infections in C57BL/6 mice. The parasitic burden of the livers of wild-type and iNKT cell-deficient were nearly comparable during an eight week period following infection [47].

NKT cells play a relatively minor role in controlling the development of *Plasmodium in vivo* [48]. Comparable numbers of organisms were found in the livers of wild-type, $\text{J}\alpha 18^{-/-}$ and

CD1d^{-/-} mice infected with *P. yoelii* sporozoites [49]. Similarly, CD1-restricted NKT cells were dispensable for protective immunity to liver-stage *P. berghei* infections [50].

Trypanosoma cruzi is the causative agent of Chagas' disease, a chronic inflammatory disease in humans. J α 18^{-/-} mice inoculated i.p. with trypomastigotes exhibited increased liver inflammation and death from infection [51].

2.3 Autoimmune hepatitis

V α 24 transcripts and V α 24⁺ cells were increased in the livers, but decreased in peripheral blood, of children with type I autoimmune hepatitis [52]. The expression of IFN- γ , IL-4, IL-12p40, IL-12R β , and IL-18 mRNA transcripts were detected in liver biopsies obtained from these children, but not from controls. These correlations lead the authors to conclude that NKT cells play a substantial role in the pathogenesis of type I autoimmune hepatitis in children.

Animal models support the role of NKT cells in the pathogenesis of autoimmune hepatitis. In concanavalin A-induced hepatitis in mice, for example, IL-12 promoted the recruitment of iNKT cells to the liver and the subsequent production of IL-4 necessary for the onset of injury [53]. NKT cell depletion abrogated Fas ligand-dependent liver damage [54].

2.4 Primary biliary cirrhosis and cholestatic liver injury

NKT cells have been implicated in the pathogenesis of primary biliary cirrhosis (PBC) and cholestatic liver injury. CD1d expression and numbers of iNKT cells in the livers of patients with PBC are increased [55]. Although the functional characteristics of NKT cells and their precise role have not been delineated, it has been suggested that these cells are a factor in the development of cirrhosis [55].

A strong correlation exists between PBC and infection by *Novosphingobium aromaticivorans*, a member of the Sphingomonadaceae family of gram-negative α -Proteobacteria discussed above [56]. Infection in mice triggered a chronic T cell response against small bile ducts, and the development of lymphoepithelioid granulomas and lesions similar to those observed in PBC [57]. A CD1d-dependent iNKT cell response to α -glycuronosylceramides derived from the cell wall of *N. aromaticivorans* was required for liver injury. Notably, liver disease could be adoptively transferred from infected to non-infected animals by conventional T cells (i.e., in the absence of iNKT cells) demonstrating the importance of the early microbial activation of iNKT cells in initiating autonomous, organ-specific autoimmunity [57].

Common bile duct ligation (BDL) in mice offers an alternative model in which to study the role of NKT cells and the factors that affect biliary obstruction and cholestatic liver injury. A marked increase in activated iNKT cells was found in the livers of BDL animals [58]. Comparing wild-type and iNKT cell-deficient (V α 14J α 18^{-/-}) mice, elevated serum ALT activity evidencing greater liver injury was determined in all bile duct ligated, relative to sham-operated, animals on day 3 post-BDL. Among the ligated groups, however, serum ALT levels were significantly higher in iNKT cell-deficient mice. Histologic analysis confirmed significantly greater liver damage in BDL, iNKT cell-deficient mice. Additional experiments demonstrated the ability of iNKT cells to suppress the proinflammatory response of neutrophils and neutrophil-dependent liver injury.

2.5 Toxin-induced liver injury

The liver is a primary target for carbon tetrachloride (CCl₄) toxicity. J α 18^{-/-} mice are more susceptible than wild-type animals to CCl₄-induced acute liver injury, inflammation and

fibrosis suggesting the beneficial role of iNKT cells [59]. In contrast, chronic CCl₄ administration induced a comparable degree of injury in wild-type and iNKT cell-deficient animals albeit the number of iNKT cells was notably reduced in the former, conceivably accounting for their negligible effect.

2.6 Partial hepatectomy and liver regeneration

Partial hepatectomy (PHx) induced a significant, β -adrenergic-dependent expansion of the hepatic NKT cell population [60]. Treatment with α - or β -adrenergic antagonist suppressed both the increase in NKT cells and liver regeneration suggesting that NKT cells contributed to liver restoration. This suggestion is supported by a significant decrease in hepatocyte mitosis in CD1d^{-/-} mice following PHx [61]. Contradictory finds were reported, however, in which liver injury following PHx was diminished in J α 18^{-/-} mice suggesting the detrimental contribution of iNKT cells to liver regeneration [62].

2.7 Ischemia and reperfusion

Reperfusion of the liver resulted in a marked accumulation of iNKT cells in a mouse model of ischemia and reperfusion injury [63]. Liver injury (neutrophil accumulation, serum ALT levels, and tissue necrosis) was attenuated in CD1d^{-/-} mice or wild-type mice pretreated with anti-CD1d antibody [63]. Reduced reperfusion injury observed in T cell-deficient (RAG-1 knockout) mice was restored to normal levels by adoptive transfer of NKT cells purified from wild-type, but not IFN- γ -deficient mice demonstrating the roles of NKT cells and IFN- γ [64].

2.8 Alcohol-induced liver injury

Hepatic NKT cell numbers and serum ALT values increased concomitantly over 1-4 week period in mice fed alcohol via an intragastric tube; liver injury was delayed significantly in J α 18^{-/-} mice demonstrating the deleterious role of iNKT cells [65]. Alcohol consumption in this model sensitized hepatocytes to iNKT cell-mediated lysis dependent upon TNF- α and Fas ligand expression.

2.9 Nonalcoholic fatty liver disease

Both CD1d expression and the number of CD3⁺CD56⁺ (V α 24⁺) NKT cells increased as nonalcoholic fatty liver disease (NAFLD) activity progressed in humans [66]. Flow cytometric analysis demonstrated these NKT cells produced more IL-4 and IFN- γ as the NAFLD activity score (NAS) increased suggesting that NKT cells and production of cytokines contributed to NAFLD.

3. THERAPEUTIC INTERVENTION

3.1 α -Galactosylceramide

Background— α -Galactosylceramide (α -GalCer) is the general name given to glycosphingolipids composed of a ceramide lipid that has fatty acyl and phytosphingosine chains of variable lengths attached to galactose via an α -linkage. The naturally occurring glycolipid produced by *Agelas mauritanus* (a marine sponge) was discovered in 1993 while screening for novel cancer therapeutic agents [67]. Subsequently, it was synthesized by the Kirin Brewery Company and called KRN7000 [68]. KRN7000 [2S, 3S, 4R-1-O(α -galactopyranosyl)-2(N-hexacosanoylamino)-1,3,4-octadecanetriol], the synthetic form used most often experimentally, is composed of a C18 phytosphingosine base and a saturated C26:0 N-linked acyl chain (Figure 1A) [68]. Notably, references to α -GalCer and the manufactured equivalent (KRN7000) are used interchangeably in the literature. α -GalCer is

the prototypic ligand recognized by the TcR expressed by mouse and human iNKT cell [69,70].

α -GalCer inoculated i.p. into mice stimulates the translation of pre-existing mRNA transcripts and the production of immuno-regulatory T_H1-type (e.g., IFN- γ , IL-2, IL-12 and TNF- α) and T_H2-type (e.g., IL-4, IL-5, IL-10 and IL-13) cytokines [71]. Increases in serum IL-4 are rapid and transient while elevations in serum IFN- γ are delayed, but persistent [72]. Notably, the production of IFN- γ and IL-2 by α -GalCer-stimulated NKT cells in culture requires de novo protein synthesis, IL-4 synthesis does not [73]. Moreover, a thorough examination of the secreting cells indicates that NK (rather than NKT) cells are the principal source of IFN- γ produced *in vivo* subsequent to α -GalCer administration; iNKT cells and CD1 expression are required, however, for NK cell activation [74]. Notably, β -anomeric GalCer retains the ability to stimulate iNKT cell proliferation and cytokine production, but is less potent and requiring higher doses than the α -anomeric form [75]. In this regard, it is also notable that β -anomeric, but not α -anomeric, glycosphingolipids are present in mammalian tissues raising the possibility that β -glycosphingolipids comprise endogenous ligands recognized by iNKT cells [76].

Optimal α -GalCer presentation requires receptor-mediated endocytosis and CD1d loading within the acidic endosomal compartment [77]. The crystalline structure of the complex formed by α -GalCer and CD1d expressed by humans and mice has been elucidated, providing insight into the factors that affect glycolipid presentation by antigen presenting cells and the subsequent response of iNKT cells [14,70,78]. Like classical MHC class I molecules, CD1d molecules bind and present antigens in a groove formed by two anti-parallel α -helices positioned on top of an anti-parallel β -sheet platform. In contrast to classical MHC class I molecules, the CD1 antigen-binding groove is deep, narrow, hydrophobic and, consequently, well suited for binding alkyl chains. The binding groove consists of two pockets: A', which binds the fatty acyl chain, and F', which binds the phytosphingosine chain; the carbohydrate head group remains exposed at the binding groove surface permitting iNKTcR recognition (Figure 2).

Therapeutic potential in treating liver diseases—The results of a number of studies document the beneficial effects of α -GalCer treatment on tumor growth and metastases in animal models. Hepatic tumor bearing mice treated with α -GalCer, for example, exhibited tumor-specific IFN- γ production, and significant NK cell-dependent reductions in liver weight and tumor growth relative to vehicle treated animals [79]. Comparable results were obtained when hepatic tumor bearing mice were administered α -GalCer-pulsed dendritic cells, i.e., marked increases in serum IFN- γ levels, complete NK cell-dependent tumor rejection, and 100% survival [80]. Other groups of investigators reported similar findings: a significant rise in cytokine (IL-2, IL-4, IL-12 and IFN- γ) production; a marked increase in NK cell activity, and an inhibition in hepatic tumor growth or liver metastases in mice administered α -GalCer [81-83]. Notably, IFN- γ (but not IL-4) was absolutely required for the anti-metastatic activity of α -GalCer observed in these studies.

The therapeutic potential of α -GalCer in treating malignancies has also been examined in a number of clinical trials. In a phase I study, for example, 12 patients with metastatic malignancy often involving the liver received α -GalCer-pulsed monocyte-derived dendritic cells [84]. Treatment resulted in the broad activation of NK, NKT, T and B cells, and increased serum IL-12 and IFN- γ levels. While there were no severe adverse effects, no significant benefit of treatment was reported.

α -GalCer-activated iNKT cells also inhibited the replication of virus in a HBV transgenic mouse model [85]. Moreover, co-administration of α -GalCer with hepatitis surface antigen

(HBsAg) induced the proliferation and activity of HBV-specific cytotoxic T lymphocytes in these same mice thought to be tolerant to HBV-encoded antigens; neither α -GalCer nor HBsAg administered alone had an effect [86]. The results of these studies suggest the potential therapeutic value of α -GalCer and the activation of NKT cells in the treatment of patients with chronic HBV infections. Indeed, these findings correlate with results demonstrating the increased expression of activation markers CD69 and CD25, and production of IL-2 and IFN- γ by NKT cells derived from vaccinated versus non-vaccinated individuals ($p < 0.05$) following incubation with HbsAg, supporting the role of NKT cells in the development of immunity following vaccination against HBV [87].

Several clinical trials also tested the therapeutic value of α -GalCer in treating patients infected with hepatitis B (HBV) and/or hepatitis C (HCV) virus [88]. As in the cancer trials, α -GalCer treatment of HCV-infected patients in a phase I/II study was well tolerated, but ineffective. α -GalCer treatment failed to result in significant decreases in either serum ALT levels or viral load [89]. Likewise, α -GalCer treatment of 27 HBV infected patients resulted in a transient reduction of HBV DNA in 3 patients, but a sustained reduction in only one [90].

α -GalCer treatment inhibited the development of intrahepatocytic parasites in mice infected with *P. yoelii* and *P. berghei* [49]. Similarly, murine cytomegalovirus replication was reduced in the spleens and livers of mice treated with α -GalCer, dependent upon NK cells and the production of perforin and IFN- γ [91]. α -GalCer also promoted host defenses to *L. monocytogenes* replicating in the liver dependent upon the enhanced immigration of neutrophils [92].

In additional experimental models, mice administered α -GalCer at 36 hours post-surgery exhibited marked increases in TNF- α - and Fas-ligand-dependent hepatocyte mitosis and liver regeneration following partial hepatectomy [61]. Cao and co-workers reported that α -GalCer pretreatment protected the liver from ischemia-reperfusion injury via a CD-1d-, IL-13- and adenosine A2A receptor-dependent mechanism suggesting the utility of such an approach in alleviating liver injury in a clinical setting [93].

In contrast to the beneficial effects observed in a number of animal models, α -GalCer administered during the course of *L. donovani* infection exacerbated liver disease in mice [47]. In addition, it accelerated carbon tetrachloride-induced acute liver injury and fibrosis, and suppressed liver regeneration following partial hepatectomy when inoculated 5 days prior to surgery (in contrast to inoculation post-surgery described above) [94,95]. α -GalCer inoculated i.p. also resulted in sharp increases in serum ALT levels and death of alcohol-fed mice by Fas- and TNF-receptor-1-dependent mechanisms [65].

In general, the production of T_H1-type cytokines (e.g., IFN- γ) supports cell-mediated immunity and correlates with the adjuvant, anti-tumor, anti-bacterial and anti-viral effects of α -GalCer; T_H2- type (IL-4) cytokine production, on the other hand, correlates with a diminution in certain autoimmune diseases such as type 1 diabetes [96]. The reciprocal suppression exhibited by T_H1- and T_H2-type cytokines and potential side effects that include Fas ligand- and IL-4-mediated, NKT cell-dependent hepatotoxicity demonstrated in animal models limit the efficacy and potential therapeutic uses of α -GalCer [53,97]. Moreover, α -GalCer is hepatotoxic in mice, a serious side effect of any beneficial activity expressed. Inoculation i.p. results in uncontrolled iNKT cells activation, cytokine production, Fas ligand expression, and severe liver damage [97].

As such, an intensive research effort is currently underway to develop α -GalCer analogs that selectively promote either a T_H1- or a T_H2-type cytokine response. Structure analysis of α -GalCer provides considerable insight into the factors that affect cytokine production by

iNKT cells. Indeed, recent studies demonstrate that the response of iNKT cells to α -GalCer can be skewed toward the production of T_H1 - or T_H2 -type cytokines by altering: the length and/or composition of the fatty acyl and sphingosine tails; the galactose-ceramide (α -glycosidic) linkage; and the polar, carbohydrate head group. For the most part, the therapeutic effects of these α -GalCer analogs on the treatment of liver diseases in humans or animal models have not been tested; instances when such studies have been conducted are discussed below.

3.2 α -GalCer Analogs

Glycosidic bond derivatives—A broad correlation exists between modifications in α -GalCer that promote stability of the CD1d-glycolipid complex, and a T_H1 -type iNKT cell response [77,98,99]. Increased stability of the complex extends the period of invariant TcR recognition favoring the production of IFN- γ relative to IL-4. α -C-GalCer is a potent α -GalCer analog created by replacing the glycosidic oxygen atom at the galactose-ceramide linkage with a CH_2 group rendering the bond resistant to α -galactosidase, and allowing freer rotation between the galactose and ceramide moieties [100,101] (Figure 1B). α -C-GalCer inoculated i.p. into mice stimulated a T_H1 -type response and marked increases in the serum levels of IL-12 and IFN- γ produced over an extended period of time relative to mice inoculated with α -GalCer [100,101]. Conversely, serum IL-4 levels were reduced 7- to 10-fold in α -C-GalCer-treated animals. α -C-GalCer also induced the prolonged activation of CD8 α^+ dendritic cells secondarily to iNKT cell activation and was far more potent than α -GalCer in stimulating: the anti-metastatic activity of NK and NKT cells in a mouse model; and IFN- γ -mediated, iNKT cell-dependent protection against infection by the rodent malaria parasite, *Plasmodium yoelii* [100,101].

Sphingosine derivatives—The length and composition of the sphingosine tail that composes glycolipid analogs also influence the response of iNKT cells and the production of T_H1 - versus T_H2 -type cytokines. Specifically, truncation of the 23-carbon sphingosine chain of α -GalCer shortens the duration of iNKT cell stimulation by the CD1d-glycolipid complex, and sharply reduces cell proliferation and production of IFN- γ [69,72,73,102]. OCH, which consists of a 9-carbon sphingosine chain and a 24-carbon fatty acyl chain (Figure 1C), preferentially induces IL-4 production while analogs composed of a longer sphingosine chain induce greater T_H1 -type cytokine secretion [72,73]. OCH treatment suppressed the development of collagen-induced arthritis and experimental autoimmune encephalomyelitis in wild-type C57BL/6 mice, and the progression of diabetes and insulinitis in NOD mice [72,103,104]. In each case, OCH was significantly more effective than α -GalCer in preventing disease. Further truncation of the sphingosine chain increased the ratio of IL-4 to IFN- γ released by mouse iNKT cells and by a human NKT cell line following glycolipid recognition [102]. Notably, the addition of a phenyl group to the end of a truncated sphingosine chain reversed the bias toward a T_H2 -type response and increased the ratio of IFN- γ to IL-4 produced (Figure 1D) [105].

Acyl chain derivatives—Like truncated sphingosine analogs, α -GalCer analogs composed of shorter acyl chains exhibit: a diminished capacity to stimulate cell proliferation; an increased T_H2 -type response; and greater production of IL-4 relative to IFN- γ by mouse splenocytes and a human NKT cell line [69,102]. The introduction of double bonds into the acyl chain, e.g., 11,14-cis-diunsaturated C20 (α -GalCer-C20:2; Figure 1E), also increases the polarity of the ligand, reduces iNKT cell expansion and skews the response toward T_H2 -type cytokine production [106]. The avidity of the ligand for Cd1d is not a significant factor affecting the response of iNKT cells to ligands such as OCH and α -GalCer-C20:2. In contrast to α -GalCer, these ligands are rapidly bound and presented by cell-surface CD1d molecules (Figure 3) [77]. Subsequently, the CD1d-ligand complexes are

internalized, dissociated within the lysosomal compartment, and the ligand replaced by endogenous glycolipids [99]. As such, complexes composed of CD1d and glycolipids such as α -GalCer-C20:2 are excluded from cell surface membrane lipid rafts required for efficient T cell stimulation [77,99,107].

Unlike α -GalCer analogs composed of truncated or unsaturated fatty acyl chains, glycolipids consisting of an aromatic group at the end of the acyl chain promoted a T_H1 -type response in mice, dependent upon the number of alkyl groups intervening between the amide bond and terminal aromatic (phenyl) group [105]. Analogs comprised of ten or more intervening $-CH_2$ groups (Figure 1F) elicited the highest T_H1 -biased response by iNKT cells by extending the phenyl group into the A' pocket of the CD1d molecule, and, thus, enabling additional interactions with aromatic residues within the pocket, enhancing stability of the glycolipid-CD1d complex and prolonging the period of invariant TcR recognition [98,105]. In addition to increasing the ratio of IFN- γ /IL-4 produced, these analogs induced a significant expansion of the NK, NKT, B, CD4, CD8 and dendritic cell populations, and were more potent than α -GalCer in suppressing tumor growth and prolonging survival in an animal model of metastatic lung cancer [105]. Notably, analogs composed of shorter acyl chains terminated by a phenyl group were much less effective in promoting T_H1 -type cytokine production [105].

Carbohydrate head group derivatives—CD1d-dependent activation of iNKT cells is induced by invariant TcR recognition of the carbohydrate head group exposed at the surface of the glycolipid-CD1d complex [108]. While α -GalCer-pulsed dendritic cells readily stimulate the proliferation of iNKT cells, for example, α -mannosylceramide or ceramide alone has no stimulatory activity [69]. In addition, flexibility in positioning of the head group within the CD1d binding groove exerts significant influence on iNKT cell recognition and subsequent response [109]. In this regard, the galactose moiety of α -GalCer is bound tightly at the surface of binding groove enabling multiple contact points between the invariant TcR and the CD1d molecule. In the case of α -C-GalCer, on the other hand, it has been suggested that replacement of the glycosidic oxygen atom with a non-polar CH_2 group changes position of the galactose head group in the groove affecting the affinity of the invariant TcR and altering the response of iNKT cells [100].

Modifications in the galactose moiety itself also influence the response of iNKT cells to the ligands presented by CD1d. The 6'-OH group is only free sugar alcohol comprising the galactose ring that does not form hydrogen bonds with amino acid residues of the invariant TcR α -chain. Mice inoculated with analogs that feature a substituted benzamide group attached at the galactose 6' position exhibit strong T_H1 polarization (Figure 1G) [110]. Similarly, mice inoculated with α -Carba-GalCer comprised of an α -linked carba-galactosyl moiety in which a methylene group replaces the 5a' oxygen atom of galactose exhibit a high ratio of IFN- γ to IL-4 produced [111].

Lastly, β -glycolipids [e.g., β -glucosylceramide; β -GluCer (Figure 1H)] are naturally occurring, metabolic intermediate in anabolic and catabolic pathways of complex glycosphingolipid. As such, they represent potential endogenous iNKT cell ligands [76]. α -GluCer-pulsed dendritic cells stimulated the proliferation of iNKT cells in culture, but (in contrast to α -GalCer) failed to stimulate significant IFN- γ or IL-4 production [69,112]. β -GluCer exerts beneficial effects in a number of mouse models of liver inflammation: ConA-induced hepatitis, CCl₄-induced acute liver injury and fibrosis, non-alcoholic fatty liver disease, autoimmune cholangitis, tumor growth in hepatocellular carcinoma (Hep3B)-bearing mice [76]. The mechanism(s) that underlie these effects remains to be determined. It has been suggested, however, that β -GluCer treatment modulates iNKT cell activity by: interfering with glycolipid metabolism, displacing the activating ligand from the CD1d

molecule, and/or altering the composition and structure of (and thereby influencing the signaling of CD1d-glycolipid complexes localized within) membrane lipid rafts [76,113].

CONCLUDING REMARKS

NKT cells, which comprise an inordinately large percentage of the hepatic lymphoid population in mice and men, have been implicated in a wide range of immunological events that occur within the liver. Unlike conventional T cells, NKT cells recognize and respond to antigenic glycolipids presented in association with CD1 molecules. To date, α -GalCer, the prototypic ligand recognized by iNKT cells, has been used clinically with little success in efforts to treat a variety of liver diseases including chronic HBV and HCV infections. The therapeutic potential of α -GalCer is limited by its ability to stimulate both T_H1- and T_H2-type responses by iNKT cells, and by the reciprocal suppression exhibited by the cytokines produced. As such, an intensive research effort is ongoing to develop α -GalCer analogs for clinical use that favor either T_H1- or T_H2-type cytokine production by iNKT cells.

EXPERT OPINION

The liver constitutes a unique immunological and anatomical site through which 30% of the total blood volume flows each minute; 80% derives from the splanchnic organs via the hepatic portal vein [114]. Blood transported from the gut contains a variety of substances including environmental toxins, bacterial products and food antigens that are capable of eliciting immune responses. The failure of these substances to provoke such responses has lead investigators to describe the liver as immunologically privileged, tolerant to antigenic stimulation; the underlying mechanisms remain to be resolved [115]. Studies to date have focused in part on the contribution of NKT cells. Indeed, the activated phenotype and preponderance of iNKT cells in the liver relative to lymphoid tissues suggest that hepatic iNKT cells serve a unique function in addition or unrelated to innate host defenses to microbial pathogens widely proposed by other investigators [20].

Studies outlined in this review described both the positive and negative effects of hepatic iNKT cells on liver disease. Fewer iNKT cells in the livers of patients with chronic hepatitis C virus infections suggests, for example, the beneficial role of iNKT cells in resistance to viral infection and/or disease progression [116]. Increased iNKT cell numbers in the livers of patients with primary biliary cirrhosis, on the other hand, lead investigators to surmise the negative impact of iNKT cells on liver injury [117,118]. In the latter case, however, one might envisage that an increased iNKT cell number represents an anti-inflammatory response and a mechanism dedicated to minimizing tissue injury. In this regard, iNKT cells inhibited the influx of proinflammatory neutrophils and acute liver injury in an experimental model of cholestasis [58]. Consequently, it was proposed that a primary function of hepatic iNKT cells is to suppress the proinflammatory response of other cell types (e.g., neutrophils) to endogenous glycolipids expressed in the liver consequent to the continuous accumulation of toxic products that would otherwise provoke tissue damage [58].

The role of iNKT cells in suppressing liver injury is supported by experiments demonstrating the rapid expansion of the hepatic iNKT cell population following partial hepatectomy in mice and the purported role of such cells in immune surveillance and hepatocyte regeneration [60]. Further support is provided by results demonstrating significantly more granulomas, large cellular infiltrates, and multinucleated macrophages in the livers of iNKT-deficient, relative to wild-type, mice infected with *Mycobacterium bovis* BCG despite the presence of a comparable numbers of organisms [25]. More recently, Wondimu and co-workers reported that α -GalCer-activated hepatic iNKT cells produced IL-17, which suppressed the influx of proinflammatory neutrophils and mononuclear phagocytes and the severity of the liver injury in a mouse model of hepatitis [119]. Given

the capacity of α -GalCer-activated iNKT cells to induce hepatitis in mice, these latter findings stress the complex nature of the response of hepatic iNKT to α -GalCer, and the critical need to develop analogs that promote the beneficial, while avoiding the detrimental, effects of α -GalCer treatment.

IL-4 plays either a protective or a detrimental role in animal models of liver injury dependent upon the specific model being studied. For example, IL-4 reduces liver damage that results from ischemia and reperfusion in mice [120], but plays an essential role in concanavalin A-induced, iNKT cell-dependent hepatitis [53]. Relevant to the latter, IL-4 exerts a direct effect on hepatocytes inducing apoptosis *in vivo* and *in vitro*, i.e., independent of immune cell activity [121]. In contrast to these effects of IL-4, IFN- γ plays a protective role, reducing the extent of liver injury in mice inoculated with α -GalCer [97]. Similarly, the extent of liver injury in an experimental model of biliary obstruction was diminished in mice treated with anti-IL-4 neutralizing antibody, but increased in mice administered anti-IFN- γ (unpublished observation). Results demonstrating the direct, negative effect of IL-4 on hepatocytes suggest that α -GalCer analogs that promote a T_H1-type response and increased IFN- γ relative to IL-4 production would prove more efficacious in treating liver disease. While a growing body of literature documents the differential effects of these α -GalCer analogs on IFN- γ and IL-4 production, the effects of these analogs on other iNKT cell activities, e.g., cytotoxicity and the production of other cytokines, remain to be determined. Similarly, an exhaustive examination of the effects of these analogs on inflammation and liver injury in animal models remains prior to considering their utility in clinical trials.

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Figure 1.
Schematic representation of α -galactosylceramide (KRN7000) and its derivatives.

Figure 2. Structure of the CD1d- α -GalCer complex in which the fatty acyl and sphingosine chains are bound within the A' and F' pockets, respectively. APC = antigen presenting cell.

Figure 3.

CD1d loading, trafficking and glycolipid presentation. Left Panel. Truncated and polyunsaturated α -GalCer analogs are bound by cell-surface CD1d molecules expressed by antigen presenting cells (APC), rapidly internalized, dissociated within the lysosomal compartment, and exchanged with endogenous self glycolipids; CD1d-self glycolipid complexes cycle back to the plasma membrane. The transient expression of cell-surface CD1d-analog complexes favors a T_H2 -type response and IL-4 production by iNKT cells. Right Panel. α -GalCer undergoes receptor-mediated endocytosis, intracellular CD1d loading, and transport to the cell membrane where the CD1d- α -GalCer complexes localize within lipid rafts required for optimal iNKT cells stimulation, IFN- γ production and a T_H1 -type response.