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EDITORIAL

Potential role of NKT regulatory cell ligands for the treatment of immune mediated colitis

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Abstract

Natural killer T lymphocytes (NKT) have been implicated in the regulation of autoimmune processes in both mice and humans. In response to stimuli, this subset of cells rapidly produces large amounts of cytokines thereby provoking immune responses, including protection against autoimmune diseases. NKT cells are present in all lymphoid compartments, but are most abundant in the liver and bone marrow. They are activated by interaction of their T-cell receptor with glycolipids presented by CD1d, a nonpolymorphic, major histocompatibility complex class I-like molecule expressed by antigen presenting cells. Several possible ligands for NKT cells have recently been suggested. β-glucosylceramide, a naturally occurring glycolipid, is a metabolic intermediate in the anabolic and catabolic pathways of complex glycosphingolipids. Like other β-glycolipids, β-glucosylceramide has an immunomodulatory effect in several immune mediated disorders, including immune mediated colitis. Due to the broad impact that NKT cells have on the immune system, there is intense interest in understanding how NKT cells are stimulated and the extent to which NKT cell responses can be controlled. These novel ligands are currently being evaluated in animal models of colitis. Here, we discuss strategies to alter NKT lymphocyte function in various settings and the potential clinical applications of natural glycolipids.

Key words: Natural killer T lymphocyte; Immunomodulatory; Colitis; Inflammatory bowel disease; Ligand

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NKT REGULATORY LYMPHOCYTES

The term 'NKT cells' was first described in 1995[1] and defines a broad subset of mouse T-cells that share some characteristics with natural killer (NK cells), expression of the NK1.1 marker in particular. This is a heterogeneous subset of lymphocytes some of which do not express the NK1.1 marker[2]. NKT cells develop from thymocyte progenitor cells similarly to conventional T-cells. However, unlike conventional T-cells, NKT cells express a T-cell receptor (TCR) that recognizes glycolipids rather than protein antigens[3]. The largest subset of NKT cells expresses a highly restricted TCR comprised of an invariant TCR α chain with a single rearrangement (in mice Vα14-Jα18, and in humans Vα24-Jα18)[4] coupled with TCR β chains with limited heterogeneity due to marked skewing of Vβ gene usage (mostly Vβ8.2 in mice and Vβ11 in humans)[5]. This population, also referred to as invariant NKT cells (iNKT), is highly conserved in most mammals studied to date. iNKT cells are restricted by the major histocompatibility complex (MHC) class I-like molecule CD1d, which is expressed by conventional antigen presenting cells (APCs) including macrophages, dendritic cells, and marginal zone B cells[2].

CD1d-mediated glycolipid presentation to NKT cells is an important aspect of immune regulation. However, as an illustration of NKT complexity, there is a type of NKT-cell that expresses the NK1.1 marker, but is CD1d independent. There are two broad classes of cells that satisfy the criteria of being CD1d dependent NKT cells. For the purposes of this review, we classify these as type I NKT cells, being the Vα14-Jα18 (mouse) or Vα24-Jα18 (human) population, and type II NKT cells, which includes all other CD1d-dependent T cells[6].

The inherent, low-level auto-reactivity of certain specialized immune cell types that have both innate and adaptive characteristics, such as CD1d restricted NKT cells, γδ T cells, and B1 cells, suggests that these cell types may also have the potential to stimulate autoimmunity[2]. Activation of iNKT cells occurs early in a number of microbial infection models in mice, and such activation can lead to reinforcement of the innate immunity and promote subsequent adaptive immunity. Thus, immune responses to certain bacterial, viral, and parasitic infections and tumors can be enhanced whereas autoimmune disease and allograft rejection can be suppressed[5].

THE ROLE OF NKT CELLS IN IMMUNE RESPONSES

NKT cell Th1 and Th2 responses can offset one another;

therefore, polarizing cytokine release toward either one may serve as an important therapeutic tool[7]. These lymphocytes constitutively express cytokine mRNA, and within hours of activation produce large amounts of cytokines such as IFN-γ, TNF, IL-4, and IL-10[5]. NKT cell-mediated regulation of immune responses has been demonstrated to influence a large number of disease states[5]. These cells have received considerable attention in recent years as innate lymphocytes that can modulate T-cell and APC functions in autoimmunity. A potential link between NKT cells and autoimmunity was suggested by the finding that various mouse strains, including non-obese diabetic (NOD) mice that are genetically susceptible to autoimmunity[8,9], have a reduced number and defective function of iNKT cells as compared with non-autoimmune mouse strains[10]. Diminished numbers of NKT cells have been correlated with an increased incidence of autoimmune diseases including systemic lupus erythematosus, scleroderma, type I diabetes, multiple sclerosis, and rheumatoid arthritis[11-16].

The adoptive transfer of NKT cells has ameliorated disease in several immune-mediated animal models, including experimental autoimmune encephalomyelitis[17], immune mediated colitis[18], and graft versus host disease (GVHD)[19]. In addition, NKT lymphocytes play an important role in diverse neoplastic and infectious processes, and as such may serve as a target for potential new immune-therapeutic strategies[20,21]. NKT cells are now known to be a major source of IFN-γ, which is required for early activation of macrophage bactericidal activity[22]. Several studies have demonstrated a role for NKT lymphocytes in anti-tumor immunity[23]. Mouse and human NKT cells were shown to exert cytotoxic activity towards several tumor cell lines[24]. NKT lymphocytes were found to promote tumor rejection in experimental models of tumor immunotherapy by administration of IL-12 or α-GalCer[25]. In a murine hepatocellular carcinoma (HCC) model, NKT cells were shown to have a role in oral immune regulation with HCC lysate and HBV envelope proteins, and in adoptive transfer of dendritic cells pulsed *ex vivo* with the same antigens[20].

LIGANDS FOR NKT REGULATORY CELLS

Through their semi-invariant TCR, NKT cells recognize glycolipids presented in the context of the CD1d molecule[26]. CD1 proteins are a family of molecules that have structural homology to MHC class I molecules, but are unusual in their ability to present glycolipid antigens to T-cells[27]. Because NKT cells can produce cytokines that result in conflicting responses, the possibility exists that the ligand structure can polarize NKT cell responses toward either a Th1 or a Th2 response[28].

Glycosphingolipids, or glycolipids, are a family of both naturally occurring and synthetic molecules composed of a hydrophobic ceramide backbone, N-acylsphingosine, and a hydrophilic head group made of carbohydrates, mono- or oligosaccharides[29]. Enzymatic defects and subsequent accumulation of certain glycolipids can lead to "storage" diseases such as metachromatic leukodystrophy, Gaucher's or Fabry's disease[30]. Patients with Gaucher's disease have altered humoral and cellular immune profiles[31] and increased peripheral blood NKT lymphocytes[32]. In the context of stimulatory glycolipids, an understanding of how glycolipid structure affects cytokine release profiles is essential.

α-galactosylceramide (α-GalCer) was originally discovered during a screen for reagents derived from the marine sponge *Agelas mauritianus* that prevented tumor metastasis in mice[33]. KRN7000, the synthetic α-GalCer analogue, is a high-affinity ligand for the CD1d molecule[34]. *In vivo* administration of α-GalCer to mice or humans results in rapid and robust cytokine secretion by iNKT cells, followed by the activation of a variety of cell types of the innate and adaptive immune systems[35].

OCH is a truncated analogue of α-GalCer in which the sphingosine chain has been shortened from 18 to 9 carbons. Following its administration to mice, the early production of IL-4 by NKT cells remained intact while the bulk of IFN-γ, mostly derived from NK cells, was lost, leading to a Th-2 biased response[36]. The ratio of IL-4 to IFN-γ released by NKT cells is influenced by the length of the lipid chain; shorter chain lengths increase this ratio[3]. Administration of α-C-GalCer leads to a strong Th-1 biased response with sustained IFN-γ levels for several days compared to the 24-h response induced by α-GalCer[37]. Treatment with α-C-GalCer was more potent than α-GalCer in mouse models of malaria and malignant tumors, while treatment with OCH was more efficacious than α-GalCer in the Th-1 mediated autoimmune disease models of encephalomyelitis and colitis[38].

Activation of NKT cells *via* α-GalCer has been shown to affect numerous models of malignancy, infection, and autoimmune disease[3]. In models with strong NKT cell involvement, such as in type I diabetes-prone NOD mice, activation of NKT cells with α-GalCer delayed disease induction and prevented its recurrence[39,40]. On the other hand, treatment with α-GalCer can cause disease exacerbation, an effect noted mainly in models where these molecules play a "pathogenic" role such as in the F1 mouse model of lupus nephritis (NZB × NZW)[41], or the apolipoprotein E knockout mouse model of atherosclerosis[42,43]. Despite their promising effects in diverse disease situations, the clinical use of α-glycolipids has been limited by their side effects, mainly hepatotoxicity[44,45].

NATURAL LIGANDS FOR NKT CELLS

The discovery of the marine sponge-derived glycolipids as ligands for NKT cells led to studies looking for possible natural ligands. These natural antigens can be separated into two groups: (1) antigens that are produced by the host (endogenous antigens), and (2) antigens from foreign pathogens (exogenous antigens). The strongest evidence for the presence of an endogenous antigen is that positive selection of NKT cells in the thymus requires presentation of an antigen recognized by the TCR[3]. The best evidence for the presence of exogenous antigens is that antigen presentation proteins related to CD1d have been characterized as presenters of microbial glycolipids, and it was speculated that NKT cells might survey for the

presence of infectious agents[46-48].

Given the auto-reactivity of the NKT TCR to CD1d and the limited diversity of TCRs that NKT cells express, it is generally accepted that a single, or set of closely related, autologous glycolipid ligands are responsible for the activation of these cells. These endogenous ligands have yet to be identified. Recently, the lysosomal glycolipid, isoglobotrihexosylceramide (iGb3) has been proposed as a natural ligand for NKT cells[49]. This beta structured-glycolipid, in its natural or synthetic forms, has the ability to activate most human or mouse NKT cells *in vitro*. Impaired generation of lysosomal iGb3 in mice lacking β-hexosaminidase *b* resulted in severe NKT cell deficiency, suggesting a role for iGb3 in murine NKT cell development[49]. Recently, some NKT cell activating antigens of microbial origin have been found[50]. NKT cells have been found to play a role in controlling infection by organisms such as *Mycobacterium tuberculosis* where NKT cells predominate in the anti-mycobacterial granulomatous reaction[51,52], *Plasmodium bergehi*, *Listeria monocytogenes*[53], *Ehrlichia muris*, and *Sphingomonas capsulata*[54].

At least two mechanisms have been proposed for NKT cell activation. The first is "enhanced auto reactivity", where APC recognition of microbial antigen results in IL-12 mediated APC-NKT cell activation. The second is a CD1d presented microbial glycolipid that triggers iNKT cells through TCR recognition[2,3]. There has been some success in identifying specific microbial glycolipid ligands of CD1d that can activate NKT cells, most notably, α-glycuronosylceramides (α-galacturonosyl and α-glucuronosylceramide) derived from the lipopolysaccharide-negative *Sphingomonas* bacteria cell wall[55]. These α-glycuronosylceramides are of specific significance because they share structural homology with α-GalCer. Other examples include the CD1-restricted presentation of *Plasmodium berghei* sporozoite-derived GPI anchor that stimulates NKT-cell-mediated B-cell activation and antibody production[56], and the phosphatidylinositol tetramannoside (PIM4) produced by *Mycobacterium bovis*[57]. These activities suggest a role for NKT cells in the innate response against pathogens that do not activate classical pattern-recognition receptors, such as Toll-like receptor 4.

β-GLYCOLIPIDS AS NKT LIGANDS

Recent studies have shown that different glycolipids preferentially target different organelles. Because different isoforms of CD1 localize to different subcellular compartments, they allow APCs to present a variety of glycolipid antigens that enter the cell by different pathways and are targeted to different locations[58]. β-glycolipids are naturally occurring intermediates in the anabolic and catabolic pathways of complex glycosphingolipids and are found in cell membranes[59]. Past studies have suggested that β-glycolipids do not possess stimulatory properties on NKT cells[59]. However, recent data have suggested that these compounds may have an important NKT cell mediated immune modulatory effect. β-glucosylceramide (GC) is a beta glycolipid that is degraded into ceramide by glucocerebrosidase. CD1d-bound GC does not stimulate NKT cells directly[60]. β-glycolipids may inhibit NKT activation and even block the stimulatory effect of α-GalCer on these cells. Glucosylceramide-synthase deficiency leads to defective ligand presentation by CD1d, with secondary inhibition of NKT cell activation[60]. *In vitro*, administration of GC led to a 42% decrease in NKT cell proliferation in the presence of DCs, but not in their absence[61]. Additional naturally occurring β-glycolipids such as β-lactosylceramide (LC) and β-galactosylceramide (GLC) are being tested for their immunomodulatory effects (unpublished data).

Administration of β-glycolipids in several Th1 mediated disease models such as auto-immune hepatitis, metabolic syndrome, and acute GVHD, alleviated the disease while inducing a Th2 cytokine profile[61-63]. In a murine model of concanavalin A-induced hepatitis, administration of GC led to significant amelioration of liver damage[61]. This beneficial effect was associated with a 20% decrease in intrahepatic NKT lymphocytes, a significant lowering of serum IFN-γ levels, and decreased STAT-1 and STAT-6 expression. The administration of GC to leptin-deficient ob/ob mice, an NKT dependent model, significantly improved the metabolic alterations[62]. Liver fat content was reduced significantly in both MRI and histological examinations. In addition, treated mice achieved near-normalization of glucose tolerance and decreased serum triglyceride levels. These effects have been associated with a marked increase of the peripheral/intrahepatic NKT cell ratio. In a semi-allogeneic model of acute GVHD, GC-treated mice manifested a significant decrease in skin, bowel, and liver GVHD manifestations[64]. The beneficial effect of GC was associated with decreased IFN-γ and increased serum IL-4 levels, as well as a significant increase in the intrahepatic to peripheral NKT lymphocyte ratio and in intrahepatic $CD8^+$ lymphocyte trapping[64]. In contrast, in Th2 mediated models of disease, administration of β-glycolipids also led to NKT mediated disease alleviation associated with an opposite Th1 immune shift. In a murine model of hepatocellular carcinoma, GC led to improved survival rates and a decreased tumor volume[63]. These effects have been associated with an 11-fold increase in intrahepatic NKT lymphocyte number. Taken together, these results suggest that certain β-glycolipids may serve as a "fine tuners" for NKT lymphocyte-mediated immune responses and may have a beneficial effect in seemingly opposing disease models.

NKT CELLS IN INFLAMMATORY BOWEL DISEASE

Inflammatory bowel diseases (IBD) are chronic inflammatory disorders of the gastrointestinal tract that are associated with an imbalance between Th1 pro-inflammatory and Th2 anti-inflammatory subtypes of immune responses. The abundance of CD1d-positive cells in the human intestine suggests a role for these cells in chronic inflammatory disorders of the bowel. NKT cells have been proposed to make both protective and pathogenic contributions to IBD[65]. Ulcerative colitis (UC) is a subtype of IBD that is limited to the superficial

layers of the colon and is dominated by the production of Th2 cytokines. Studies have shown that classical (type I) CD1d-restricted NKT cells contribute to a murine model for UC[66,67]. NKT cells exerted protective effects against DSS colitis, a model for intestinal inflammation that primarily targets mucosal macrophages. In this model, administration of α-GalCer and adoptive transfer of NKT cells resulted in reduction of inflammation.

The role of NKT cells in chronic bowel inflammation is complex. They can play either a protective or a pathogenic role in intestinal inflammation, depending on the type of inflammatory process and the antigen presented in the gut. NKT cells support a pro-inflammatory immune response in TNBS-colitis, a Th1 model. Thus, depletion of NKT cells results in alleviation of the disease[68], effects which were mediated by altered intrahepatic $CD8^+$ trapping and that increased INF-γ producing lymphocytes[69]. Feeding colitis-extracted proteins (CEP) to mice with TNBS-induced colitis induces oral tolerance and alleviates TNBS-induced colitis[70]; NKT depletion prevents oral tolerance induction18. Adoptive transfer of *ex vivo* CEP-pulsed NKT cells also alleviated colitis[69]. NKT cells exerted protective effects against DSS colitis, a model for intestinal inflammation (Th2 model) that primarily targets mucosal macrophages. In this model, administration of α-GalCer and adoptive transfer of NKT cells reduced inflammation. In contrast, oxazlone-colitis could not be induced in animals lacking NKT[71]. Several studies proposed a role for NKT cell activation in IBD patients. Expression of CD1d is higher in the epithelia of the affected terminal ilea of CD patients and in the affected cecum of UC patients, which may lead to recruitment of proinflammatory CD1d-reactive cells from the periphery, resulting in mucosal destruction[72]. However, a more recent report suggested that, in contrast to normal colon surface epithelium, epithelial cells derived from UC or CD patients do not express CD1d[73]. The diminished expression of CD1d was suggested as a possible mechanism for impaired regulatory NKT cell function in IBD. Taken together, these data suggest a complex role for NKT cells in chronic inflammatory disorders of the bowel, which may involve various factors in the immune microenvironment.

EFFECT OF NKT LIGANDS IN ANIMAL MODELS OF IMMUNE MEDIATED COLITIS

Experimental colitis induced by intracolonic installation of TNBS, is associated with a Th-1 immune response as evidenced by increased IFN-γ secretion, decreased IL-10 and IL-4 secretion, and reduction in the intrahepatic $CD8^+$ trapping. These effects were hypothesized to be mediated by regulatory NKT cells[69]. Several glycolipid derivatives have been shown to alleviate hapten mediated colitis. OCH, and α-Gal-Cer analogue with truncated sphingosine chain, attenuates colonic inflammation as defined by body weights and histological injury[38]. The protective effects could not be observed in Vα14 NKT cell-deficient mice, further evidence of an NKT role in the pathogenesis of colitis. The immunomodulatory effect of several β-glycolipids, including GC (glucosylceramide), LC (lactosylceramide), GLC (galactosylceramide), and IGL (GC + LC), was shown to be associated with increased survival and significant alleviation of colitis with improvement in the macroscopic and microscopic scores[63]. Administration of GC alleviated immune mediated experimental colitis, improving both the macroscopic and microscopic scores. The beneficial effects of GC were associated with an increased peripheral/intrahepatic CD4/CD8 lymphocyte ratio and a Th2 immune shift.

In summary, NKT cells may make both protective and pathogenic contributions to IBD[65]. Studies show that these cells are involved in the maintenance of mucosal homeostasis. On the other hand, this subset of cells plays a pathogenic role in human ulcerative colitis. Similar contrasting data have been generated in murine models of IBD[65]. Whether the apparent differences in NKT response patterns depends on variations in NKT ligands and/or on the presence of specific subsets of mucosal NKT cells remains to be elucidated. Further studies that determine the subset of NKT cells and the specific ligands involved in these disorders may facilitate the development of novel therapies for IBD.

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