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REVIEW ARTICLE

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Potential of targeting natural killer T cells for the treatment of autoimmune diseases

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Abstract Natural killer (NK) T cells emerge as unique lymphocytes subsets implicated in the regulation of autoimmunity. Abnormalities in the numbers and functions of NKT cells have been observed in patients with diverse autoimmune diseases as well as in a variety of mouse strains that are genetically predisposed for the development of autoimmune diseases. Unlike conventional T cells that recognize peptides in association with major histocompatibility complex (MHC), NKT cells recognize glycolipid antigens presented by the nonpolymorphic MHC class I-like protein, CD1d. Recently, vigorous activation of NKT cells by synthetic glycolipids such as α-galactosylceramide (α-GC) or its sphingosine truncated derivative OCH have been shown to suppress autoimmune diseases such as experimental autoimmune encephalomyelitis (EAE), diabetes in nonobese diabetic (NOD) mice, and collagen-induced arthritis (CIA) by inducing T helper (Th) 2 bias of autoimmune T cells. In this review, we examine the potential roles of NKT cells in the pathogenesis of autoimmune disease regulation, and the recent advances in glycolipid therapy for autoimmune disease models. In addition, we summarize studies suggesting a role for NKT cells in human autoimmune disease, and discuss the potential of targeting NKT cells for the treatment of autoimmunity.

Key words α-Galactosylceramide (α-GC) · Autoimmune disease · Natural killer (NK) T cells · OCH · Th1/Th2

Introduction

Natural killer (NK) T cells are usually defined as cells coexpressing the natural killer receptors such as NK1.1 or NKR-P1A (CD161) and an αβT cell receptor (TCR). Although $NK1.1^+$ TCR^+ lymphocytes are heterogeneous, the majority of NKT cells have a restricted TCR diversity, with an invariant TCRα chain, composed of Vα14-Jα281 segments in mice and Vα24-JαQ segments in humans, which is associated with TCR β chains using a restricted set of Vβ genes. These Vα14 invariant NKT cells recognize glycolipid antigens such as α-GC presented by a nonpolymorphic MHC class I-like molecule, CD1d[1–5] (Fig. 1). As little is known about CD1d nonrestricted NKT cells or α-GC-independent CD1d restricted NKT cells, in this review we focus on the α-GC responsive NKT cells, and the term "NKT cells" will be used for α-GC responsive NKT cells.

Subsets of mouse and human NKT cells have a similar memory phenotype, and recognize α-GC in association with CD1d molecules highly conserved through mammalian evolution. Whereas human and mouse NKT cells share many characteristics, the frequency is much lower in humans.[2–4] Consistent with their preactivation status, NKT cells release large amounts of cytokines, including IL-4 and IFN-γ promptly upon antigen stimulation, and these affect the functions of neighboring cell populations such as T cells, B cells, NK cells, and dendritic cells.[2,5] NKT cells are composed of two subsets: $CD4^+$ or $CD4^-CD8^-$ (double negative DN). $CD4^+$ and DN NKT cells appear to be different in terms of cytokine production in humans but not in mice.[2,6] The $CD4^+$ subset of human NKT cells produces both Th1 and Th2 cytokines upon antigen stimulation, whereas the DN subset produces Th1 cytokines and upregulates the production of perforin after exposure to cytokines.[6]

Natural antigens for NKT cells have not yet been identified. However, it is speculated that self glycolipid antigens probably function as activating ligands for NKT cells owing to the self-reactivity of NKT cells and the activated memory phenotype of NKT cells isolated from human umbilical-cord blood[7,8] and germ-fee mice.[9] α-GC is a synthetic glycolipid originally isolated from marine sponges *Agelas mauritanius*, and later, a synthetic analogue of this compound was developed for experimental studies and clinical trials (Fig. 1).[10] α-GC has been shown to be a potent stimulator of both murine and human NKT cells.[10–12] NKT cells

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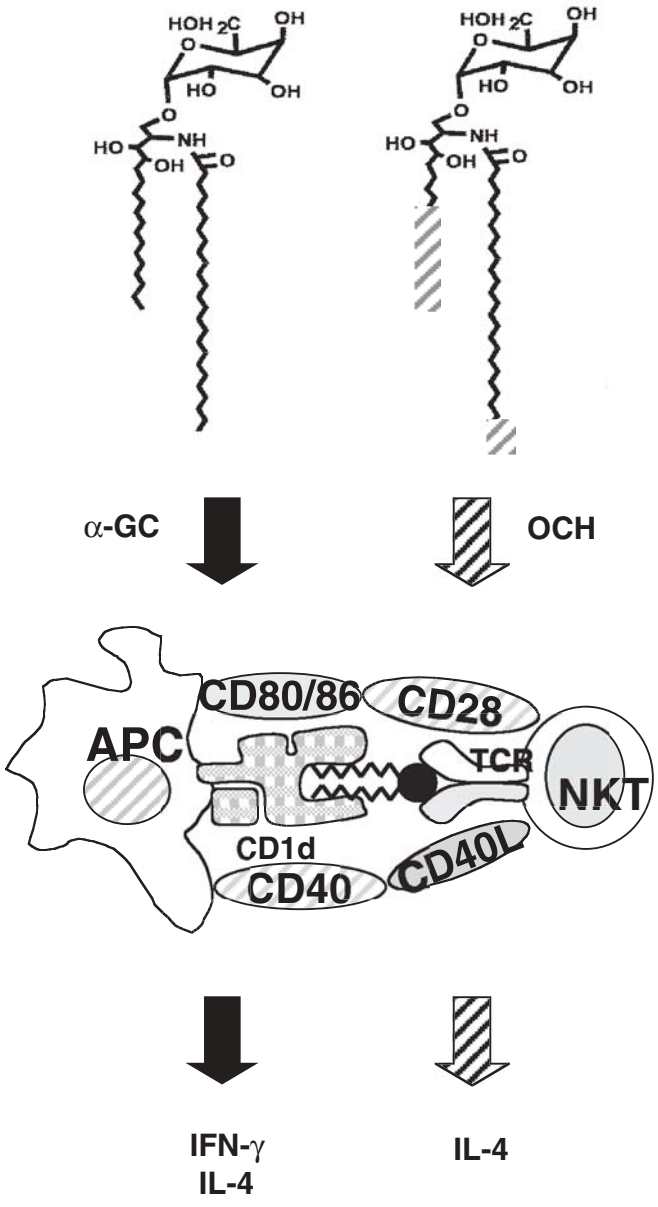

Fig. 1. Structure and function of α-galactosylceramide (α-*GC*) and an altered ligand *OCH*. *NKT* cells recognize glycolipid ligand presented by *CD1d* molecules. The α-anomeric conformation of sugar moiety, the configuration of the 2-hydroxyl group on the sugar moiety, and 3,4-hydroxyl groups of the phytosphingosine are important for NKT cell recognition of α-GC. The OCH analogue has a shorter sphingosine chain. Upon stimulation, NKT cells produce a variety of cytokines and exert effector functions. α-GC stimulates NKT cells to produce both anti-inflammatory (e.g., *IL-4* and IL-10) and pro-inflammatory (e.g., *IFN*-γ) factors. This response can be modified by stimulation with an altered ligand such as OCH, or stimulation in the absence of *CD28*/B7.2 co-stimulation. These modifications are a potentially important therapeutic approach to suppressing Th1-mediated autoimmune diseases. *APC*, antigen presenting cell; *TCR*, T cell receptor

respond to sphingolipids substituted with an α-linked galactose or glucose, but not α-linked mannose and sphingolipids containing β-linked galactose or glucose.[10] Sphingolipids containing β-linked sugars resemble common mammalian lipids, whereas α-glycosyl sphingolipids have not been found in normal mammalian tissues. Recently, we have demonstrated that a sphingosine truncated analogue of α-GC, OCH, is a unique ligand for NKT cells.[13]

OCH stimulates NKT cells to preferentially produce IL-4, in contrast to α-GC which induces a variety of cytokines including IFN-γ, IL-2, tumor necrotic factor-α, IL-4, and IL-13 from NKT cells (Fig. 1).

NKT cells in rheumatoid arthritis and collagen-induced arthritis (CIA)

Abnormalities of NKT cells in autoimmune diseases were first reported in scleroderma patients. In such patients, αβ+ DN T cells were increased and there was an oligoclonal expansion of $V\alpha24^+TCR^+$ cells among them.[14] However, the invariant Vα24JαQ T cells were reduced in scleroderma patients, although the invariant Vα24JαQ T cells were dominant among these cells from healthy donors. Van der Vliet et al.,[15] Kojo et al.,[16] and other groups investigated the number of NKT cells by using Vα24 and Vβ11 mAb to detect NKT cells in patients with several different autoimmune diseases, including rheumatoid arthritis (RA). They found lower numbers of $V\alpha24^+V\beta11^+$ NKT cells in the peripheral blood of patients than in that of controls. In this study, Kojo et al.[16] showed that half of the patients with autoimmune disease responded to α-GC in culture. In addition, Maeda et al.[17] reported the expansion of noninvariant Vα24 TCR^+ cells but not Vα24JαQ T cells in the synovium of RA patients.

CIA is a murine experimental model of RA induced by immunization with type II collagen. The activation of NKT cells by glycolipid ligands was examined by Chiba et al.[18] The effect of α-GC on CIA was marginal. In contrast, Th2 skewing ligand OCH inhibited the clinical course of CIA. Histological analysis revealed that OCH treatment protected against the infiltration of inflammatory cells and the destruction of cartilage and bone. The suppressive effect of OCH was not observed for CIA induced either in CD1d knockout mice or in Jα281 knockout mice deficient in NKT cells, suggesting that OCH-mediated suppression requires NKT cells. Moreover, the injection of OCH strongly suppressed CIA in SJL mice even though these mice have defects in the numbers and functions of NKT cells, and even after the arthritis had already developed (Fig. 2). In contrast, the administration of α-GC did not suppress arthritis in SJL mice. The suppression of arthritis was associated with an elevation of the IgG1:IgG2a ratio, indicating the Th2 bias of type II collagen-reactive T cells. The injection of neutralizing antibody to either IL-10 or IL-4 reversed the beneficial effect of OCH treatment. These results imply that IL-10 and IL-4 are critical in the OCH-mediated suppression of CIA, and are consistent with the idea that OCH modulated CIA by stimulating the production of Th2 cytokines from NKT cells, although the source of IL-10 remains to be elucidated.

The role of NKT cells in CIA is still not clear. The clinical score of CIA induced in NKT cell-deficient mice

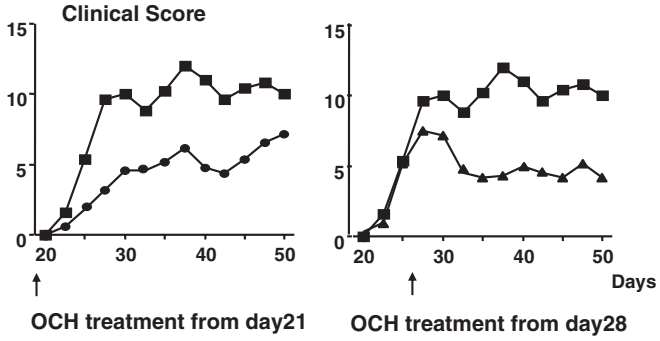

Fig. 2. The suppressive effect of *OCH* on collagen-induced arthritis (CIA) in SJL mice. Clinical score of CIA in SJL mice treated with 500 μg/kg of vehicle (*squares*) or OCH (*circles*) twice per week starting from day 21 or day 28

appeared lower than that in wild-type mice. The finding of a natural antigen for NKT cells would give us further insight into the precise role of NKT cells in the pathogenesis of autoimmune diseases such as arthritis.

NKT cells in systemic lupus erythematosus and lupus murine models

Sumida and co-workers[19,20] observed the expansion of noninvariant Vα24 TCR^+ clones in patients with active systemic lupus erythematosus (SLE). These authors and other groups found lower numbers of $V\alpha24^+V\beta11^+$ NKT cells in the peripheral blood of patients with SLE than in that of controls.[15,16]

In lupus murine models such as MLR lpr/lpr mice, it has been reported that a selective reduction in $NK1.1^+$ T cells precedes the development of disease. Mieza et al.[21] also found a decrease in the expression of invariant Vα14 TCR mRNA of NKT cells before the onset of lymphocyte accumulation and autoimmune disease in MRL lpr/lpr mice, C3H gld/gld mice, and NZB/W F1 mice when compared with control mice. Morshed et al.[22] reported that the number of NKT cells increased after the onset of disease, and then the transfer of NK1.1+ T cells from diseased mice to young F1 mice (before the onset of renal failure) induced proteinuria and swelling of the glomeruli. Moreover, Zeng et al.[23] demonstrated that treatment of NZB/W F1 mice with anti-CD1d monoclonal antibody augmented Th2-type responses, increased serum levels of IgE, decreased levels of IgG2a and IgG2a antidouble-stranded DNA (dsDNA) antibodies, and ameliorated lupus. They also showed that multiple injections of α-GC treatment induced an enhanced Th1-type response and exacerbated lupus associated with decreased serum levels of IgE and increased levels of IgG2a and IgG2a anti-ds DNA antibodies. This exacerbation of disease was associated with reduced IL-4 and tumor necrotic factor-α production and an expansion of marginal zone B cells. These results suggested that the activation of NKT cells by α-GC augmented Th1-type responses and autoantibody production that contribute to lupus development in NZB/W F1 mice. In contrast, CD1d deficiency did not lead to disease acceleration in MRL lpr/lpr mice,[24] and pristine-induced lupus nephritis was accelerated when induced in CD1d-deficient mice.[25] They also demonstrated that repeated injections of α-GC resulted in the expansion of NKT cells and ameliorated dermatitis in MRL lpr/lpr mice.[26] Therefore, they postulated that NKT cells may play a protective role in lupus models. Since lupus models are not simply explained by only Th1-mediated or Th2-mediated pathology, the complexity of these models may explain the differences in results in these studies.

NKT cells in multiple sclerosis and experimental autoimmune encephalomyelitis (EAE)

Multiple sclerosis (MS) is an autoimmune demyelinating disease of the central nervous system (CNS). A reduction in VαJαQ cells among $V\alpha24^+$ cells from the peripheral blood of patients with MS compared with healthy subjects has been found by using a single-strand conformation polymorphism method to detect TCR gene rearrangements.[27] In support of this finding, Van der Vliet et al.[15] showed a decrease in the number of NKT cells by screening $V\alpha24^+V\beta11^+$ cells in the blood using monoclonal antibodies specific for $V\alpha24^+$ and $V\beta11^+$TCR. Araki et al.[28] demonstrated that DN NKT cells in the periphery were greatly reduced in remission, whereas the reduction of $CD4^+$ NKT cells was marginal. Furthermore, $CD4^+$ NKT line cells expanded from MS in remission produced a larger amount of IL-4 than those from healthy subjects or from MS in relapse, suggesting that the Th2 bias of $CD4^+$ NKT cells may play a role in the regulation of Th1-type autoantigen-reactive T cells. Conversely, Gausling et al.[29] did not find a significant difference in the number of DN $V\alpha24^+$ NKT cells in peripheral blood lymphocytes between MS patients and healthy controls. Considering that the proportion of VαJαQ T cells in normal individuals varies among studies, it may not be easy to compare these studies, and the basis for the discrepancy between the numbers of NKT cells is not clear.

EAE is a Th1-mediated autoimmune inflammatory disease affecting the CNS that serves as a model for MS. EAE can be induced in susceptible mouse strains by immunization with CNS proteins or peptides in adjuvant, or by the passive transfer of T cells reactive against such CNS antigens. The presence of a Vα14 transgene reduced myelin oligodendrocyte glycoprotein (MOG)-induced EAE in nonobese diabetic (NOD) mice.[30] The disease severity in CD1d deficient mice has been reported as being reduced, unaltered, or enhanced.[30–34] Even though the basis for these inconsistencies is not clear, breeding genetic alterations onto C57BL/6(B6) background, the variability in the B6 strains used, and the marked impact of colony health on the disease severity of EAE could explain some differences. Although the role of NKT cells in the course of EAE is not yet clear, the stimulation of NKT cells to produce Th2

cytokines would be a powerful strategy to deliver protective cytokines to autoimmune-mediated inflammatory lesions, since NKT cells are known to invade rapidly and to accumulate in inflammatory lesions in a manner similar to inflammatory cells and produce cytokines.

The results obtained from α-GC treatment of EAE generated conflicting results. The administration of α-GC was found either to prevent disease, to have no effect, or to accelerate disease.[13,31–35] These differences could be due to the differences in the protocols of the α-GC treatment and the differences in strains and antigens used for the induction of EAE. The timing and the route of administration appear to be critical to modulation of the disease. Since NKT cells produce both IFN-γ and IL-4 upon stimulation with α-GC, α-GC may have different effects on EAE depending on the stage of the disease and the strains used. NKT cell-derived IFN-γ would mask the protective effect of the IL-4 simultaneously produced by the NKT cells, and sometimes even worsen the disease. We have shown several lines of evidence supporting this idea.[35] First, α-GC treatment inhibited EAE induced in IFN-γ-deficient mice. Second, α-GC treatment augmented the clinical signs of EAE induced in IL-4-deficient mice. Third, blockade of CD86 polarized NKT cells toward a Th2-like phenotype with a concomitant suppression of EAE, and the activation of APCs by treatment with CD40 biased them toward a Th1-like phenotype and exacerbated the EAE.

Thus, EAE could be prevented if ligand stimulation led to the selective production of Th2 cytokines by NKT cells in vivo. Manipulating NKT cells through adoptive transfer may not be practical in humans, and therefore a more attractive strategy would be the direct activation of these cells in vivo. Therefore we synthesized several analogues of α-GC and found that a sphingosine-truncated analogue, OCH, induced selective IL-4 production by NKT cells (see Fig. 1). As expected, the administration of OCH prevented the development of EAE in both clinical and pathological parameters. The inhibitory effect of OCH was not observed for EAE induced either in NKT cell-deficient or IL-4-deficient mice, confirming that IL-4 produced by NKT cells is critical for OCH-mediated suppression of EAE.[13] In addition to B6 mice, SJL mice are highly susceptible to EAE, and EAE induced by immunization with proteolipid protein (PLP)-derived peptides $PLP_{139–151}$ is used as a remitting–relapsing MS model. SJL mice have been reported to have markedly fewer NKT cells and markedly lower cytokine production upon activation.[36] Singh et al.[32] reported that SJL mice responded poorly to treatment with α-GC. When SJL mice were treated with α-GC, their morbidity and mortality were exacerbated although the onset of disease was delayed. In contrast, multiple injections of OCH protected SJL mice against EAE (S. Miyake and T. Yamamura, unpublished observation). Furthermore, OCH protected SJL mice against a relapse in EAE, suggesting that OCH holds possibilities as a therapeutic agent to prevent relapses in MS. On the whole, treatment with OCH might be preferable to α-GC, as OCH preferentially induces IL-4 production and inhibits disease in several different strains of mice.

NKT cells in type I diabetes and NOD mice

Studies of the frequency of human NKT cells in peripheral blood in patients with type I diabetes have shown conflicting results. In initial studies analyzing identical twin/triplet sets discordant for disease, there was a lower frequency of invariant Vα24Jα18 sequences among DN $V\alpha24^+$ T cells in diabetic siblings than in nondiabetic siblings.[37] In support of these data, Kukreja et al.[38] showed a reduction in the number of NKT cells in newly diagnosed patients using an antibody specific for the conserved-determining region (CDR) 3 of the Vα24-Jα18 rearrangement. However, more recent papers reported unaltered or increased NKT cells in recent-onset patients with type I diabetes.[39,40] Wilson et al.[37] also showed that DN Vα24JαQ T cell clones isolated from diabetics had an impaired ability to produce IL-4. In contrast, Lee et al.[39] reported that IL-4 production by NKT cells was similar among these groups as assessed by intracytoplasmic staining following short-term PMA and ionomycin stimulation. At this stage, it is hard to interpret the discrepancies between these results, since the methods for detecting NKT cells and the functional assays used differ between studies. In addition, the patients analyzed in the different reports were of different ethnic groups and different age groups.

NOD mice develop a spontaneous autoimmune diabetes which is similar to the human disease insulin-dependent type 1 diabetes mellitus. Many studies have indicated that Th1-type $CD4^+$ cells and $CD8^+$ T cells have been implicated in the development of diabetes in NOD mice. In parallel with these effector cells, it has been suggested that the regulatory cells, including NKT cells, inhibit the development of diabetes. Deficiencies in the number and function of NKT cells have been found in NOD mice.[41] Although the correlation between a defect in NKT cells and a susceptibility to diabetes in NOD mice is still being debated,[3–5,42,43] the putative involvement of NKT cells in the control of islet β-cell reactive T cells in NOD mice was suggested by examples of diabetes being prevented following the infusion of NKT cell-enriched thymocyte preparations,[44] and also by the increase in NKT cells in Vα14Jα281 transgenic NOD mice.[45]

Several recent studies have investigated the effect of treating NOD mice with α-GC.[43,46–48] When started at around 3 or 4 weeks of age, repeated injections at least once a week delayed the onset and reduced the incidence of diabetes. After treatment, splenocytes from NOD mice produced a greater amount of IL-4 in response to islet antigens, and the IgG1/IgG2a (Th2/Th1) ratio of anti-GAD antibody increased. It therefore appears that the mechanism of protection is similar to that observed by increasing the numbers of NKT cells in NOD mice and by α-GC treatment in other autoimmune disease models such as EAE and CIA. We also observed the protective effect of OCH treatment in NOD mice as well as that of α-GC treatment. The protective effect of OCH against insulitis was more profound than that of α-GC (M. Mizuno and S. Miyake, unpublished observation).

Prospects for glycolipid therapy for autoimmune diseases

There is still controversy about whether defects in NKT cells cause autoimmune disease or occur as a secondary consequence of the autoimmune process. However, given the efficacy of glycolipid ligands such as OCH and α-GC in mouse models, the stimulation of NKT cells with glycolipid seems to be an attractive strategy for the treatment of autoimmune diseases. Although several studies have shown that the administration of α-GC caused liver damage, the hepatotoxicity was minimal in phase I trials of α-GC for patients with cancer. Considering the low toxicity in humans, it seems reasonable to use glycolipids for the prevention or therapy of selected human autoimmune disorders. α-GC has been shown to exacerbate EAE, depending on the strain of mouse and stage of disease tested, and to have only a marginal effect on CIA. In this situation, treatment with OCH might be preferable to α-GC for Th1-mediated diseases such as MS, type I diabetes, and RA, as OCH elicits a predominantly IL-4 response rather than IFN-γ. Both rodent and human NKT cells have been reported to recognize α-GC in the context of CD1d. OCH also stimulates human NKT cells, particularly $CD4^+$ NKT cells, and induces greater Th2 cytokine production from NKT cells compared with α-GC stimulation (M. Araki and T. Yamamura, unpublished observation). The evolutionary conservation and the homogeneous ligand specificity of NKT cells allow us to apply a glycolipid ligand such as OCH for the treatment of human disease without considering species barriers or the genetic heterogeneity of humans.

Conclusion

Ligand stimulation of NKT cells is an attractive strategy for the prevention or treatment of autoimmune diseases. The mechanisms by which NKT cells exert their immunoregulatory functions are still largely unknown and a number of questions require further investigation, including the mechanism to recruit NKT cells and control their functions at inflammatory sites, and the interactions of other subsets of cells. The identification of the nature of natural ligands for NKT cells is a major question, and the answer would provide us with more information about NKT cells and autoimmunity. It could also provide us with an interesting natural source of useful stimulators for CD1-restricted regulatory cells.

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