

NKT Cells: A Regulator in Both Innate and Acquired Immunity

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Abstract: CD1d-restricted NKT cells are a unique subset of lymphocytes bridging innate and acquired immunity and mediating both effector and regulatory functions in immune responses. NKT cells are essential for the protection against pathogens or tumors, and also play a regulatory role in transplantation tolerance and autoimmune disease development. This review focuses on the various functions of NKT cells and discusses fundamental mechanisms in NKT cell biology.

INTRODUCTION

The immune system provides two arms to elicit effective responses; innate and acquired immunity. The innate immune system is a first line of host defense characterized by rapid response mediated by natural killer (NK) cells, macrophages, dendritic cells (DCs), or granulocytes, etc. In contrast, the acquired immune system is characterized by the clonal expansion of antigen-specific T and B cells and their memory or secondary immune responses. CD1d-restricted NKT cells (or type 1 NKT cells, hereafter referred to as NKT cells) [1-3], a unique lymphocyte subset, have been shown to play an intermediary role bridging the innate and acquired immune system. Therefore, in the absence of NKT cells, both systems fail to accomplish their respective functions.

In this review, we focus mainly on the *in vivo* functions of NKT cells in both innate and acquired immunity, and discuss the cellular and molecular mechanisms of NKT cell-mediated immune responses.

NKT CELLS AS AN AUTO-REACTIVE REPERTOIRE

NKT cells are characterized by an expression of an invariant antigen receptor encoded by V 14-J 281 gene segments in mice, and by V 24-J 18 in humans [4, 5], and recognize a glycolipid antigen, α -galactosylceramide (α -GalCer) presented by MHC class I-like CD1d molecules [6-9]. α -GalCer is a synthetic glycolipid composed of a hydrophilic carbohydrate moiety with an α -linkage to the hydrophobic ceramide portion, whose analogs were initially detected in marine sponge [10]. As glycosphingolipids with β -conformation are not detected in mammals (thus β -GalCer seems to be an exogenous ligand for NKT cells), α -GalCer has been very useful and extensively used to investigate NKT cell functions. The recognition of α -GalCer by NKT cells is in sharp contrast to the recognition of peptidic antigens presented by MHC class II and class I molecules by conventional CD4⁺ and CD8⁺ T cells, which strongly indicates the particular role of NKT cells in the immune system. Furthermore, a gene-targeting approach to

delete the J 281 gene segment resulted in complete loss of V 14 NKT cells whereas other lymphoid cells including T, B and NK cells were left intact, indicating that NKT cells are a unique subset of lymphocytes distinct from conventional lymphocytes [11].

Although the endogenous self ligand for NKT cells has not been identified, NKT cells do respond to CD1d-expressing antigen-presenting cells in the absence of exogenous antigens [6], suggesting that they belong to an autoreactive repertoire [12]. This is in line with the fact that freshly isolated NKT cells express activated or memory phenotypes (CD44⁺CD62L^{low}CD69⁺). Recently, Brenner and his colleagues [13] suggested a model for the role of autoreactivity of NKT cells in protective immunity against microbes. Under physiological condition, NKT cells constitutively recognize self antigens presented by CD1d, resulting in sub-optimal activation without any functional activity (i.e. little cytokine production or proliferation). Once NKT cells receive signals of IL-12 derived from DCs following Toll-like receptor activation by infectious agents, the signals mediated by the sub-optimal activation is amplified, and NKT cells in turn become activated to produce cytokines, such as IFN- γ . Subsequently, IFN- γ produced by NKT cells activates both innate and adaptive immune cells. Therefore, pathogens or their products may not activate NKT cells directly. These findings partly explain the mechanism of rapid production of cytokines upon activation of NKT cells in a variety of microbial infections without specific foreign antigen recognition.

UNUSUAL CYTOKINE PRODUCTION BY NKT CELLS

Upon activation, NKT cells rapidly and abundantly produce both Th1 and Th2 cytokines, including IFN- γ , IL-4, IL-2, IL-10, TNF- α and IL-13 [14, 15]. They can also produce cytotoxic molecules, such as perforin and granzymes, CD95L, TNF-related apoptosis-inducing ligand (TRAIL), and granulysin [16-18], and chemokines [19, 20], indicating their pleomorphic functions different from conventional T cells.

In accordance with their immediate cytokine responses, NKT cells are unique among lymphocytes as concerns the state of cytokine gene transcription [21, 22]. In naïve

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conventional T cells, the cytokine genes remain quiet. However, when they encounter antigens for the first time in the periphery, they begin a process of differentiation that includes commitment to a specific pattern of cytokine production (Th1- or Th2-type) according to their environments. This process is associated with expressions of specific transcription factors, such as T-bet or GATA3, and chromatin remodeling at the relevant cytokine loci [23, 24]. On the other hand, freshly isolated NKT cells contain chromatin modifications at both Th1 and Th2 cytokine genes by histone acetylation, which facilitate access by transcription factors [21]. In fact, naïve NKT cells obtained from the thymus or the periphery already activate both IFN- and IL-4 transcription [21]. This constitutive expression of IFN- and IL-4 mRNA may allow the rapid secretion of the cytokines. Especially in the thymus, NKT cells first produce only Th2 and then Th1 cytokines and then, in association with NK receptor expression, become producers of either cytokine lineage [25]. Thus, the transcriptional activity of Th1 and Th2 cytokine genes in NKT cells seems to be acquired during their development in the thymus.

DYNAMISM OF THE PHENOTYPE AND FUNCTION FOLLOWING ACTIVATION

NKT cells exhibit a dynamic change in their surface phenotypes and functions after activation. Quickly after activation by antigen receptor engagement (by α -GalCer or anti-CD3 cross-linking) or IL-12, NKT cells become undetectable when assessed by flow cytometry with α -GalCer-loaded CD1d tetramers [26-28]. This disappearance of NKT cells is due to transient down-regulation of their antigen receptors, although a contribution of activation-induced cell death had also been suggested [29, 30]. During the down-regulation phase, NKT cells show relative resistance to apoptosis and, after a few days, dramatic expansion while preserving their ability to produce cytokines [26-28]. The anti-apoptotic nature of NKT cells seems to correlate with the up-regulation of several anti-apoptotic genes, such as the NAIP and MyD118 [28]. The meaning of this feature has not been fully understood. However, considering that CD25⁺CD4⁺ regulatory T cells, another type of regulatory cells, have also been reported to exhibit autoreactivity and relatively resistant to activation-induced apoptosis with up-regulation of anti-apoptotic factors [31], their anti-apoptotic nature may help NKT and CD25⁺CD4⁺ regulatory T cells exhibiting their regulatory functions [32].

The mode of α -GalCer-stimulation greatly influences the fate of NKT cells. Notably, when mice were repeatedly administered with α -GalCer, a Th2-type polarization of cytokine production was observed [33, 34]. Furthermore, it was recently reported that, after chronic α -GalCer-stimulation (~8 weeks), NKT cells expressing inhibitory Ly-49 molecules were repopulated in a thymus-dependent fashion [35]. The repopulated NKT cells showed impaired cytokine production and had lost the capacity to reject tumor cells upon α -GalCer administration in vivo [35]. Therefore, upon repeated or chronic stimulation, NKT cells alter their cytokine production pattern, which may lead to distinct biological outcome. Single activation of NKT cells causes a cytokine burst which contributes to the protective immunity, whereas repeated or chronic stimulation of NKT cells

appears to induce suppressive responses ([35] and Fig. 1). The suppressive effect mediated by NKT cells seems at least partly accompanied by a functional change in DCs, which is described below (in the section “NKT cell-mediated immune regulation”).

CELLULAR AND MOLECULAR MECHANISMS BASED ON NKT CELL-MEDIATED PROTECTIVE FUNCTIONS

NKT cells contribute the protection of the hosts from infections or neoplasms. In this section, we summarize the cellular and molecular events in protective immune responses involving NKT cells.

There is a cognate interaction between NKT cells and DCs (Fig. 1). Like conventional T cells, NKT cells express costimulatory or adhesion molecules including CD28 or LFA-1, and their stimulation by cognate partners, such as CD80/86 or ICAM-1, expressed on antigen-presenting cells is important to induce NKT cell activation [10]. Conversely, following TCR-mediated activation with α -GalCer, NKT cells up-regulate the expression of CD40L as well as IFN- secretion, resulting in the activation of DCs to secrete IL-12 [36, 37]. Furthermore, activation of NKT cells with α -GalCer induces the rapid differentiation and maturation of DCs in vivo, followed by augmentation of both CD4 and CD8 immune responses to coadministered antigens [38-40]. This enhancement of acquired immunity seems to be an adjuvant-like effect mediated by NKT cells with the potential to elicit effective immunity to microbes or tumors.

After the initial activation, NKT cells exhibit certain cytotoxicity and produce cytokines including IFN- and IL-2 which lead to the activation and increased IFN- secretion and cytotoxicity of NK cells and macrophages [41, 42]. The IFN- produced by both NKT and NK cells induces Th1-type responses and macrophage activation [20, 43, 44]. These mechanisms are also operated in the elimination of infectious pathogens (including bacteria, fungi, and plasmodia) [19, 20, 45-48] or tumor rejection [10, 16, 43, 49-53]. Especially in the case of bacterial infection, chemokines secreted by NKT cells also recruit other innate immune cells including granulocytes and macrophages [19, 20].

As for the NKT cell-mediated anti-tumor responses, a phase I clinical trial for intravenous injection of α -GalCer in patients with progressing solid tumors such as lung cancer, esophageal cancer, breast cancer, or melanoma has been performed ([54] and our unpublished data). In these trials, α -GalCer was well tolerated in cancer patients over a wide range of doses. Biological effects were observed in patients with relatively high pretreatment NKT cell numbers.

NKT CELL-MEDIATED PATHOGENIC RESPONSES

In some cases, the activation of the CD1d/NKT cell system also induces allergic and/or inflammatory responses. Development of allergen-induced airway hyper-reactivity was diminished in CD1d KO and NKT KO mice, indicating the importance of Th2-type cytokines such as IL-4 and IL-13 produced by NKT in disease development [55, 56]. In another experimental setting, however, IFN- produced by NKT cells has been shown to inhibit Th2-type responses

leading to the suppression of antigen-specific IgE production [57]. In contact hypersensitivity, which is known to require IgM production by B-1 B cells to initiate T cell recruitment, the importance of NKT cells was also demonstrated; very early after skin immunization, NKT cells are stimulated to produce IL-4, which activates B-1 cells to produce antigen-specific IgM. These responses subsequently induce the recruitment of effector T cells to the site of contact sensitivity [58].

In hepatic viral infection, NKT cells can inhibit the virus replication through IFN- γ and IFN- α/β production [59]. However, the activation of intrahepatic NKT cells is also involved in liver injury. In concanavalin A-induced hepatitis, NKT cells appeared to be essential through their perforin and FasL production [60, 61]. This is similar to the cytotoxic mechanism of HBV-specific CTL, in which both signaling pathways must be activated simultaneously in order to kill the hepatocytes *in vivo* [62].

Recent reports have indicated an interesting role of NKT cells in the formation and development of atherosclerosis [63, 64]. In model mice in which atherosclerotic lesions develop (such as low-density lipoprotein receptor deficient mice and apolipoprotein E deficient mice), the absence of NKT cells results in a significant decrease in lesion size. Administration of the synthetic glycolipids that activate NKT cells via CD1d induced a great increase in lesion size in model mice, accompanied by an early burst of cytokines (IFN- γ , MCP-1, TNF- α , IL-2, IL-4, IL-5, and IL-6). The development of atherosclerosis in model mice was associated with the presence of V 14J 18 transcripts in the atherosclerotic arterial walls, indicating that NKT cells were recruited to these lesions. Therefore, NKT cells seem to be pro-atherogenic in mice [63, 64].

Oxazolone colitis is an experimental colitis that has a histologic resemblance to human ulcerative colitis. It has been indicated that IL-13 produced by NKT cells is a significant pathologic factor in this model, since neutralization of IL-13 prevented the colitis and disease could not be induced in NKT cell-deficient mice [65].

These findings suggest that the inhibition of excessive NKT cell activation may be beneficial to preventing certain allergic or inflammatory diseases.

NKT CELL-MEDIATED IMMUNE REGULATION

It has been indicated that NKT cells are also involved in immune regulation. In human and mouse autoimmune diseases, various numerical and/or functional changes in NKT cells have been indicated [66-73]. Studies using type 1 diabetes in NOD mice have shown that the disease could be ameliorated by the augmentation of NKT cells [74-76], while crossing with CD1d-deficient mice caused earlier onset and increased disease frequency [77, 78], thus further confirming the correlation between type 1 diabetes and CD1d/NKT cells. The involvement of NKT cells in the immune regulation has also been shown in transplant tolerance [79-81], anterior chamber-associated immune deviation (ACAID) [82], and tumor recurrence [83, 84].

Potent production of cytokines such as IL-4 and/or IL-10 by NKT cells appears to be an important factor in the

regulatory role of these cells, especially in type 1 diabetes [68, 76, 85] and experimental allergic encephalomyelitis (EAE) [86-88]. Moreover, in ACAID and in a corneal graft model, IL-10 production by NKT cells, which led to an emergence of allospecific T regulatory cells (probably CD8⁺ cells, ref. [89]), is believed to be involved in tolerance induction as a consequence of immune privilege in the eye [81, 82, 90]. A recent study demonstrated paradoxical but interesting results, which may open up new insights for understanding the immune-regulatory property of NKT cells: the α -GalCer-induced protection from EAE was conversely mediated by IFN- γ , but not by IL-4, and in turn, suppressed Th1-cytokine production and fostered secretion of IL-10 from myelin oligodendrocyte glycoprotein-specific T cells [91]. This study also showed that the protective effect of α -GalCer was observed only when injected subcutaneously but not intraperitoneally, suggesting that the route of administration of α -GalCer is critical for eliciting regulatory function.

In addition to the cytokine-mediated control, recent data have suggested that NKT cells also contribute to the generation of regulatory DCs [92] (Fig. 1). Naumov *et al.* have reported that treatment of NOD mice with repeated injection of α -GalCer induced an accumulation of NKT cells and CD8⁺ DCs in pancreatic lymph nodes and inhibited disease development [93]. These results suggest that the interaction between NKT cells and DCs is operated in the regulatory responses mediated by NKT cells. In fact, a single injection of α -GalCer into NOD mice induced rapid maturation of DCs, manifested by up-regulation of co-stimulatory molecules and proinflammatory cytokine production. By contrast, DCs derived from mice after several injections of α -GalCer showed a non-matured phenotype and up-regulation of IL-10 production—an important regulatory cytokine (Seino *et al.*, unpublished data). These data suggest a novel mechanism by which NKT cells regulate the immune system, namely through manipulating the maturation and function of DCs. Similarly, we recently found that NKT cells are accumulated in accepted cardiac grafts in a CXCL16/CXCR6-dependent fashion (manuscript in preparation), suggesting that NKT cells play a protective role in the prevention of graft rejection. This is somewhat similar to the observation by Naumov *et al.* that inhibition of type 1 diabetes is associated with NKT cell accumulation in the pancreases [93]. Together, these findings indicate that the coexistence of regulatory NKT cells and DCs with regulatory functions may help maintaining their respective functional roles.

In another aspect, Beaudoin *et al.* have recently reported that NKT cells could induce anergy in pathogenic effector T cells, using a model of NKT cell transfer into T cell-deficient (C α -deficient) NOD mice with injection of islet-specific BDC2.5 T cells [94]. The transferred V 14 NKT cells prevented late expansion and proliferation of BDC2.5 T cells, leading to anergy. Therefore, in addition to the Th2 deviation, NKT cells seem to induce a kind of tolerogenic DCs and/or induce anergy in effector T cells, when exhibiting their regulatory function.

In a 15-12RM tumor recurrence model, CTL-mediated tumor immunosurveillance was suppressed by IL-13

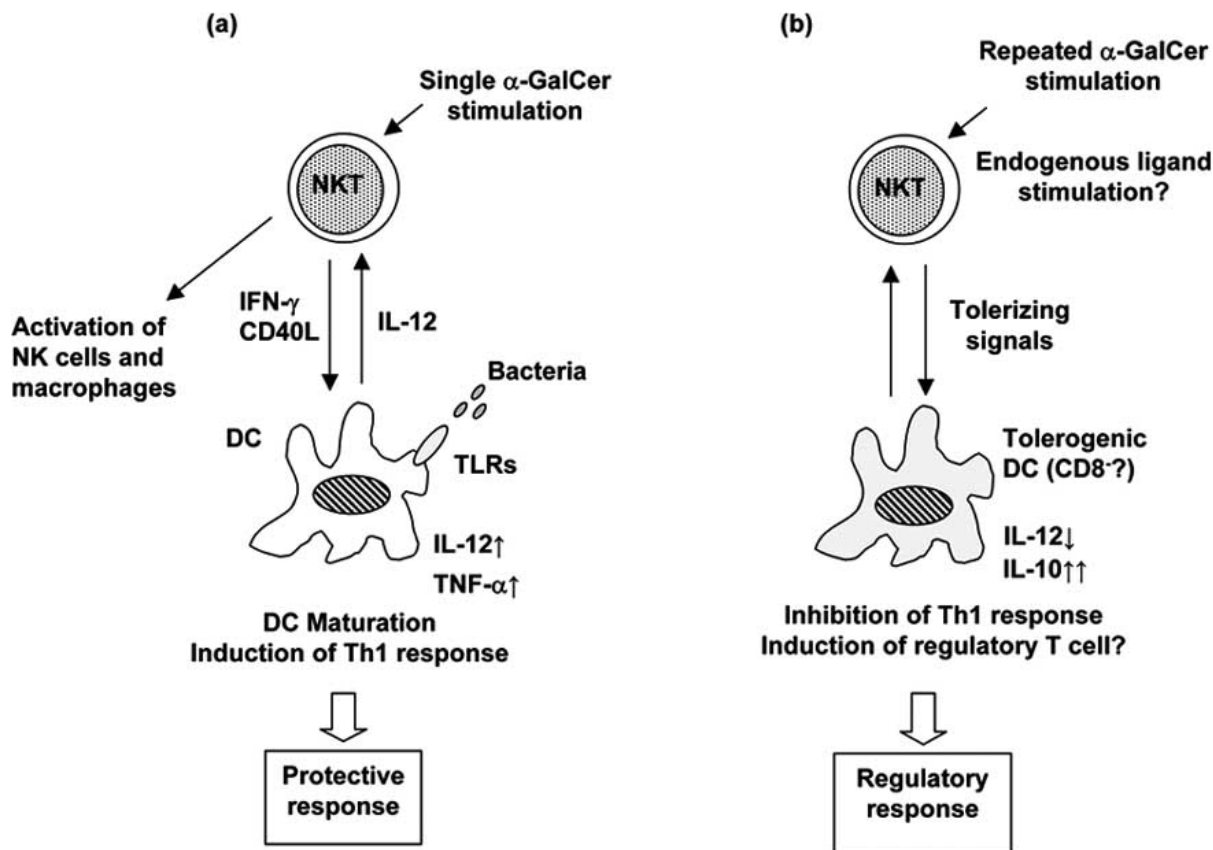


Fig. (1). Possible interactions between NKT cells and DCs. (a) Protective interaction. When NKT cells are activated by a single α -GalCer stimulation, or by a microbial infection through TLRs on DCs, factors such as IFN- γ or CD40L derived from NKT cells and IL-12 from DCs positively act on this interaction to activate these cells and rapidly induce DC maturation. Mature DCs then activate CD4 and CD8 T cells in a Th1-biased fashion, while activated NKT cells also induce the activation of NK cells and macrophages. These interactions between innate and acquired immunity contribute to the induction of protective responses (ref. [36-40]). (b) Regulatory interaction. Activation of NKT cells through repeated stimulation can induce a tolerizing signals, possibly through the secretion of TGF- β , IL-10, or IL-13, that results in the generation of tolerogenic DCs (in a CD8 $^-$ population, [93]) which have the potential to secrete less IL-12 and more IL-10. These regulatory DCs lost the capacity to induce Th1 responses and may be able to induce certain types of regulatory T cells, thus contributing to the prevention of tissue damage (ref. [91-93] and our unpublished data).

produced by NKT cells [84]. IL-13 seems to act on CD11b $^+$ Gr-1 $^+$ cells to induce TGF- β production, which finally suppresses the induction of anti-tumor CTL, while blocking TGF- β or depleting Gr-1 $^+$ cells *in vivo* prevented tumor recurrence [83]. These data indicate that there is an immunoregulatory circuit repressing tumor immunosurveillance in which NKT cells are involved. Taken collectively, it is possible that there are several different mechanisms of NKT cell-mediated regulatory pathways operated in different pathogenic situations.

CONCLUSIONS

Innate immune cells that constitute the first line of host defense, including NK cells, macrophages, neutrophils, and DCs upon activation can produce proinflammatory cytokines such as IL-12. Such proinflammatory cytokines may primarily activate NKT cells. Activated NKT cells, in turn, rapidly activate innate immune cells through their production of various bioreactive molecules and notably IFN- γ and IL-4. It thus seems that, in addition to their effector function, NKT

cells are at the center of a positive feedback machinery that enhances innate immune responses. Furthermore, NKT cells can also augment acquired immune responses directed against specific antigens. Therefore, it appears that NKT cells play an intermediate role between innate and acquired immune responses.

In addition to their important role in innate immunity, NKT cells also serve as regulatory cells that control anti-self and anti-non-self responses in acquired immunity. Recent data have suggested that NKT cells regulate acquired immunity not only via Th1/Th2 modulation but also through regulating DC functions. Several recent studies have suggested the regulatory DCs induced by NKT cell activation can subsequently generate IL-10-producing regulatory T cells, which in turn contribute to the regulating of adaptive immune responses. However, the correlation between NKT and regulatory T cells (including FoxP3 $^+$ regulatory T cells [95]) has not been well clarified and is an issue left for future examination.

Although NKT cells appear to recognize self antigens, the endogenous ligands for NKT cells remain unknown. The identification of endogenous ligands should lead to a better understanding of the physiological roles of NKT cells in either homeostatic processes or in pathological situations. Further detailed investigations about NKT cells will contribute to a more precise understanding of the mechanisms by which these cells govern immune responses. This, in turn, will also contribute to develop a new therapeutic approaches using NKT cells to fight various human diseases, including cancer, infections, autoimmunity, transplant rejection, and allergic disorders.

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