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Natural killer T cells in health and disease

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TABLE OF CONTENTS

1. Abstract
2. Introduction
3. Development and maintenance of NKT cells
4. NKT cell activation
5. NKT cell effector functions
6. NKT cell functions
7. NKT cell-based vaccine adjuvants
8. NKT cell-based immunotherapies
9. Summary and perspective
10. Acknowledgements
11. References

1. ABSTRACT

Natural killer T (NKT) cells are a subset of T lymphocytes that share surface markers and functional characteristics with both conventional T lymphocytes and natural killer cells. Most NKT cells express a semi-invariant T cell receptor that reacts with glycolipid antigens presented by the major histocompatibility complex class I-related protein CD1d on the surface of antigen-presenting cells. NKT cells become activated during a variety of infections and inflammatory conditions, rapidly producing large amounts of immunomodulatory cytokines. NKT cells can influence the activation state and functional properties of multiple other cell types in the immune system and, thus, modulate immune responses against infectious agents, autoantigens, tumors, tissue grafts and allergens. One attractive aspect of NKT cells is that their immunomodulatory activities can be readily harnessed with cognate glycolipid antigens, such as the marine sponge-derived glycosphingolipid alpha-galactosylceramide. These properties of NKT cells are being exploited for therapeutic intervention to prevent or treat cancer, infections, and autoimmune and inflammatory diseases.

2. INTRODUCTION

Cells of the immune system are typically divided into cells that belong to the innate or the adaptive arms of the immune system. Cells of the innate immune system express receptors that recognize molecular patterns shared among many different microbes whereas cells of the adaptive immune system express receptors with exquisite specificity for foreign antigens. Another distinction between cells of the innate and adaptive immune systems is that the former respond in a similar manner to each repeated exposure to the same microbe, whereas the latter respond more vigorously and faster to repeated exposure to the same antigen. Natural killer T (NKT) cells are a subset of immune cells that share characteristics of both the innate and adaptive arms of the immune system (for general reviews on NKT cells see (1-5)). Like conventional T lymphocytes, NKT cells express a T cell receptor (TCR), which is generated by somatic DNA rearrangement. However, whereas the TCR repertoire of conventional T cells is highly diverse, most NKT cells, commonly referred to as invariant or type I NKT cells, express a semi-invariant TCR (6). Murine invariant NKT cells express Valpha14-

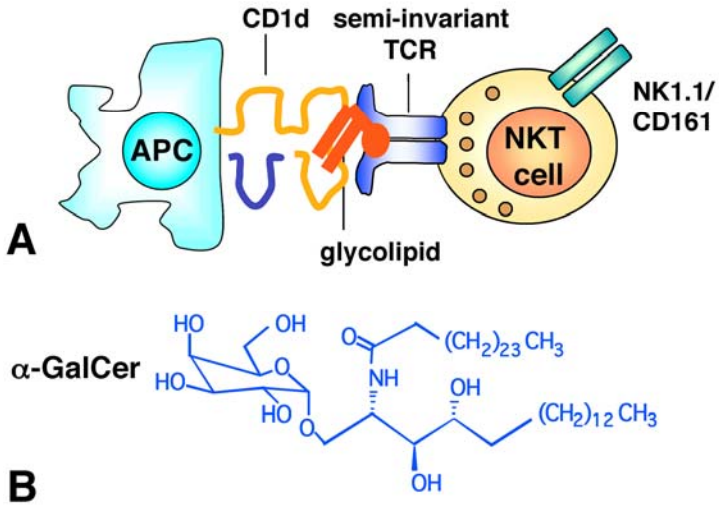

Figure 1. Phenotype and specificity of NKT cells. (A) NKT cells react with CD1d-bound glycolipid antigens. NKT cells express the NK cell marker NK1.1 (or CD161 in humans) together with a semi-invariant T cell receptor (TCR), which is specific for glycolipid antigens bound by CD1d on antigen-presenting cells (APC). (B) Structure of the prototypical NKT cell antigen alpha-galactosylceramide (alpha-GalCer; also called KRN7000).

Jalpha18 chains paired with Vbeta8.2, -7, or -2, and human invariant NKT cells express homologous Valpha24-Jalpha18 chains paired with Vbeta11. This semi-invariant TCR reacts with glycolipid antigens presented by the major histocompatibility complex (MHC) class I-related protein CD1d (see Figure 1A). A substantial fraction of NKT cells in mice and humans expresses the co-receptor CD4, most of the remaining NKT cells lack CD4 and CD8 expression, and a small subset of NKT cells in humans but not mice express CD8α. NKT cells also constitutively express a variety of markers, including CD25, CD44, CD69 and CD122 that are typical of T cells with an activated or memory phenotype. In addition to these T cell markers, NKT cells express receptors such as NK1.1 in mice or CD161 in humans that are characteristic of the natural killer (NK) cell lineage, which belongs to the innate arm of the immune system. Consistent with their activated phenotype, TCR engagement of NKT cells results in rapid elicitation of effector functions, including cytokine secretion and cytotoxicity. A hallmark of these cells is their capacity to rapidly produce a mixture of cytokines, including interleukin (IL)-4 and interferon (IFN)-gamma, which are the signature cytokines produced by T helper type 1 (Th1) cells and Th2 cells, respectively. NKT cells are found in substantial numbers in thymus, spleen, liver and bone marrow, but only low numbers are present in peripheral blood and lymph nodes. The size of the NKT cell population in humans is much lower than that in mice and shows high variability among different individuals.

In addition to type I NKT cells, a subset of NKT cells lacks expression of the invariant TCRalpha chain, and instead expresses a more diverse set of TCRs (6). These cells are referred to as type II NKT cells or variant NKT cells. Like type I NKT cells, type II NKT cells recognize glycolipid antigens presented by CD1d molecules and have potent immunomodulatory activities (7). Additional T cell subsets with surface receptors and functions that are similar to NKT cells have been identified, and these cells are usually referred to as NKT-like cells (6). This review will focus on type I NKT cells, which we will simply call NKT cells.

3. DEVELOPMENT AND MAINTENANCE OF NKT CELLS

Like conventional T cells, NKT cells develop in the thymus (8). Generation of the invariant TCR by VDJ recombination is a stochastic event. Acquisition of the NKT cell phenotype appears to be driven by interaction of the invariant TCR with CD1d. Mice that are deficient in CD1d expression have a complete block in NKT cell development (9-11). Positive selection of NKT cells requires interaction of the invariant TCR on immature NKT cells expressing both CD4 and CD8 with CD1d expressed by cortical thymocytes themselves (12-14). This step in NKT cell development depends on co-stimulatory interactions between immature NKT cells and CD1d-expressing cortical thymocytes mediated by signaling lymphocyte activation molecules (SLAM) (15-17). SLAM molecules engage in homotypic interactions resulting in activation of the SLAM adaptor protein (SAP), which signals through the Src tyrosine kinase Fyn. Consequently, SAP-deficient mice, and patients with X-linked lymphoproliferative disease due to SAP-deficiency, lack NKT cells but contain normal numbers of conventional T cells (18-20). This step in NKT cell development is controlled by the transcription factor c-Myb, which regulates CD1d expression, the half-life of double-positive thymocytes, and the expression of SLAM and SAP (21). The interaction between the invariant TCR of NKT cells likely involves endogenous glycolipid antigens that drive positive selection of NKT cells. It has been suggested that isoglobotrihexosylceramide (iGb3), a lysosomal

glycosphingolipid that weakly activates mature NKT cells, can perform this function (22). However, not all evidence supports this possibility and it is likely that multiple glycolipids are involved.

There is also evidence that NKT cells undergo negative selection in order to avoid overt activation in the periphery (8). During early stages of their development NKT cells express the transcription factor Nur77 (23), which is associated with negative selection in conventional T cells. Treatment of fetal thymus organ cultures with the NKT cell agonist alpha-galactosylceramide (alpha-GalCer; see Figure 1B for the structure of alpha-GalCer) resulted in the loss of NKT cells, suggesting negative selection (24, 25). Further, overexpression of CD1d on dendritic cells, which play a critical role in intrathymic negative selection, resulted in reduced numbers of NKT cells and reduced responsiveness of these cells to TCR engagement (24). Conversely, selective expression of CD1d on thymocytes resulted in NKT cells that were hyperresponsive to stimulation with alpha-GalCer (26). However, in another transgenic mouse where CD1d was selectively expressed on thymocytes and peripheral T cells, the selected NKT cells were hyporesponsive to alpha-GalCer stimulation but developed liver pathology in the absence of exogenous manipulation (27).

Following positive selection, NKT cells undergo a series of differentiation steps that result in the mature NKT cell pool. Our understanding of this process is largely based on studies in mice (8). Early in their maturation, positively selected NKT cells downregulate CD8, and a subset of these cells also downregulate CD4. Silencing of CD8 in mouse NKT cells appears to be driven by the transcription factor Th-POK (Th, poxviruses and Zinc finger, and Kruppel family) (28). These immature NKT cells undergo extensive proliferation and are potent IL-4 producers, but make little IFN-gamma. Most immature NKT cells leave the thymus at this stage, in a process that is controlled by the lymphotoxin-beta receptor and the sphingosine 1-phosphate receptor. Next, immature NKT cells, both in the thymus and the periphery, acquire NK cell receptors such as NK1.1 (CD161) and increase their capacity to produce IFN-gamma. In mice a substantial fraction of mature CD4-negative and CD8-negative NKT cells remain in the thymus as long-term resident cells.

The maturation of NKT cells is guided by a variety of cytokines, cell surface molecules, signal transducers, transcription factors and other regulatory factors, which have been reviewed elsewhere (8). A key step in the maturation of NKT cells is their acquisition of innate effector functions. The transcription factor promyelocytic leukemia zinc finger (PLZF) has been implicated in this process (29). PLZF is expressed by NKT cells and a few other T cell subsets with innate effector functions but not by conventional T lymphocytes. PLZF-deficient mice had sharply reduced numbers of NKT cells and the remaining cells had lost their characteristic memory phenotype and accumulated in lymph nodes (30, 31). Conversely, transgenic expression of PLZF in thymocytes resulted in the development of MHC class II-restricted, CD4-expressing T cells with rapid effector functions (31). Another key step in the maturation of NKT cells is the acquisition of cytokine secretion capability. These cells constitutively express cytokine gene transcripts but require intrathymic signaling mediated by granulocyte monocyte colony-stimulating factor (GM-CSF) in order to become competent for cytokine secretion (32).

The homeostasis and maintenance of NKT cells in the periphery is regulated by the cytokine IL-15 and to a lesser extent IL-7 (33, 34), which is similar to NK and memory CD8 T cells. In contrast to conventional T cells, which require interaction with their MHC ligands for maintenance, NKT cell homeostasis does not require CD1d expression (26, 33, 34). Recruitment and maintenance of NKT cells in the liver further involves the chemokine receptor CXCR6 (35, 36), expression of integrin LFA-1 (37, 38), and the transcription factor Id2 (39). Finally, a recent study further showed that the composition of commensal microbiota in the gut can impact the abundance of NKT cells in mice (40). Specifically, germfree mice had a moderate reduction of these cells as compared with mice maintained under specific pathogen-free conditions, and the precise composition of commensal microorganisms impacted NKT cell abundance as well.

4. NKT CELL ACTIVATION

Several endogenous and exogenous glycolipids that can activate NKT cells have been identified (41). The self-glycolipid iGb3 can activate NKT cells but its role in NKT cell development and functions is unclear (22). Some NKT cell hybridomas reacted with phospholipids, including phosphatidylinositol and phosphatidylethanolamine (42). Further, a substantial portion of human NKT cell clones was shown to react with lyso-phospholipids (43), which serve as lipid messengers during normal physiological responses. A subset of NKT cells can also respond to ganglioside GD3 (44), which is highly expressed in tumors of neuroectodermal origin. NKT cells also react with multiple glycolipids of microbial origin (41). A small fraction of NKT cells reacts with a tetramannosylated form of phosphatidylinositol called PIM_4, derived from *Mycobacterium bovis* BCG (45). Similarly, a substantial portion of mouse and human NKT cells was able to react with diacylglycerol-based glycolipids derived from *Borrelia burgdorferi* (46), the etiological agent of Lyme disease. It has also been reported that a subset of NKT cells can recognize lipophosphoglycan from the protozoan parasite *Leishmania donovani* (47) and it has been suggested that a lipopeptidophosphoglycan in the membrane of *Entamoeba histolytica* (48) can activate NKT cells as well. Alpha-glycuronosylceramides, which are found in bacteria of the *Novosphingobium* (formerly called *Sphingomonas*) and *Ehrlichia* genera, can potently activate both mouse and human NKT cells (49-51). This included alpha-galacturonosylceramide and alpha-glucuronosylceramide from *Novosphingobium* species. These reagents bear profound structural similarity with alpha-GalCer, which had been previously identified as a potent NKT cell agonist present in extracts derived from the marine sponge *Agelas*

mauritianus (52). It is now believed that alpha-GalCer is actually derived from *Novosphingobium* bacteria that colonized the marine sponge. An optimized synthetic version of alpha-GalCer, called KRN7000, has been employed by many laboratories to study the functions and therapeutic properties of NKT cells (53). Many synthetic variants of KRN7000 and other NKT cell ligands have also been studied. Collectively, these studies have revealed a remarkable capacity of NKT cells to react with a wide range of lipid structures.

The structural basis for the capacity of the invariant TCR of NKT cells to react with a wide array of glycolipid and lipid antigens has been investigated (54, 55). Structural studies of human and mouse CD1d have revealed that CD1d contains a hydrophobic antigen-binding groove, with two pockets that can accommodate lipid tails (56). Structures of CD1d with alpha-GalCer confirmed the binding of lipid tails in the CD1d pockets, with the polar head group protruding out of the CD1d binding groove (57). Structures of CD1d with a version of alpha-GalCer with a shortened acyl chain (58), a *Novosphingobium*-derived glycolipid (59) and the putative endogenous antigen iGb3 (60) revealed the presence of a spacer lipid, providing part of the explanation for the capacity of CD1d to bind a wide variety of glycolipid and lipid antigens. The crystal structures of the canonical human and mouse NKT cell receptor bound with CD1d and alpha-GalCer revealed that the NKT cell receptor binds parallel to the long axis of CD1d, rather than the diagonal type of binding that has been observed for TCRs of conventional T cells (61, 62). Interactions of the NKT cell receptor with CD1d and glycolipid were dominated by the TCRalpha chain, which is surprising because the alpha chain is invariant. These observations have been confirmed and extended by extensive mutational analysis of the NKT cell receptor (61, 63-66). These mutational analyses have further indicated that the TCRbeta chain can influence the fine-specificity of the NKT cell receptor (66).

The mechanisms by which glycolipid antigens are processed and presented to NKT cells have also been investigated (67). Following its synthesis in the endoplasmic reticulum (ER), CD1d molecules are loaded with ER-resident phospholipids that largely function as chaperones to stabilize CD1d complexes and facilitate their transport to the cell surface. Loading of CD1d with these phospholipids is assisted by the lipid transport protein microsomal triglyceride transfer protein (MTP) (68). Surface displayed CD1d is subsequently internalized and routed to endosomal-lysosomal compartments where the phospholipids are released from CD1d. Lipids might be delivered to these compartments following engagement of pattern recognition receptors on antigen-presenting cells with microbial molecular patterns (67), or following internalization of apolipoprotein E-containing lipid complexes via lipoprotein receptors (69). A recent study further showed that loading of alpha-GalCer on CD1d is regulated by the enzyme fatty acid amide hydrolase (70). After delivery to endosomal-lysosomal compartments some complex lipids might require further processing by resident lipases and glycosidases (71). Loading of glycolipids on CD1d is assisted by lipid transfer proteins such as the saposins, the GM2-activator protein and the Niemann-Pick C2 protein (72-74). Several pathogens, including Kaposi sarcoma-associated herpes virus, HIV-1, herpes simplex virus-1, vesicular stomatitis virus, vaccinia virus and *Chlamydia trachomatis* have evolved mechanisms to interfere with the CD1d antigen processing pathway and, thus, impair glycolipid antigen presentation to NKT cells (75, 76).

In addition to the direct activation of NKT cells by cognate glycolipid antigens, NKT cells can become activated indirectly (77) (see Figure 2). This has been studied most extensively for Gram-negative bacteria that contain lipopolysaccharide (LPS) in their cell wall. NKT cells became activated when dendritic cells were cultured with *Salmonella* bacteria and this could be blocked by antibodies against IL-12 or CD1d (78). This activation also required signaling through toll-like receptors (TLR). Consistent with this finding, LPS was able to substitute for the bacteria in activating NKT cells. Similar findings have been observed for other bacteria and other types of microorganisms (79). In some cases, activation of antigen-presenting cells by TLR ligands was shown to modify lipid biosynthetic pathways, the composition of CD1d-bound lipids or possibly expression of NKT cell ligands (80-82). In the case of the Gram-positive bacterium *Listeria monocytogenes*, it was shown that production of IFN-beta induced upregulation of CD1d in infected antigen-presenting cells (83). Collectively, these studies have led to the general concept that activation of antigen-presenting cells via TLR signaling results in the production of cytokines (i.e., IL-12, IL-18 and/or type I interferons) by the antigen-presenting cells that can synergize with interactions of the invariant TCR with CD1d-lipid complexes for NKT cell activation (79). Of note, CD1d expression on the antigen-presenting cells was not always required for NKT cell activation (84). This pathway of NKT cell activation might be sufficiently conserved to permit activation of these cells during other situations, such as during the inflammatory responses that are associated with many pathological conditions.

5. NKT CELL EFFECTOR FUNCTIONS

The effector functions of NKT cells have been explored most extensively when these cells were stimulated with alpha-GalCer (53). A hallmark of NKT cells is their capacity to rapidly produce a variety of cytokines following TCR stimulation. This includes typical Th1 cytokines such as IFN-gamma and tumor necrosis factor (TNF) and typical Th2 cytokines such as IL-4, IL-10 and IL-13, but also IL-2, transforming growth factor (TGF)-beta and several chemokines. Further, a subset of NKT cells produces the Th17 cytokine IL-17 (85). NKT cells produce cytokines early after their activation, after which cytokine production ceases. In mice, IL-4 production peaks around 6 hrs and IFN-gamma production peaks around 24 hrs after in vivo alpha-GalCer treatment. NKT cells activated in this manner are also capable of substantial expansion in multiple organs, after which NKT cell numbers gradually decline to reach pre-injection levels around 2 weeks after

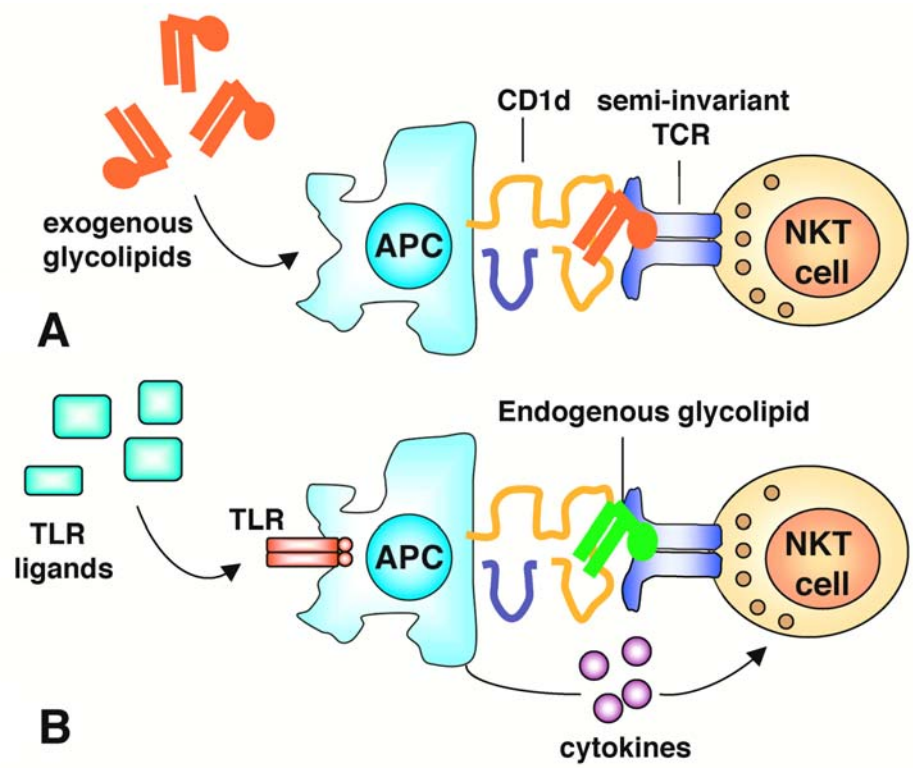

Figure 2. Activation of NKT cells. There are two general mechanisms that can lead to the activation of NKT cells. In the direct pathway of NKT cell activation (A), microbial or other lipid antigens are taken up by antigen-presenting cells (APC) and, after processing, these antigens are delivered to CD1d molecules for presentation to NKT cells. In the indirect pathway of NKT cell activation (B), agonists of toll-like receptors (TLR) interact with TLR on APC, resulting in cytokine production and activation of NKT cells. In many cases the latter pathway requires interaction of the semi-invariant T cell receptor (TCR) on NKT cells with endogenous glycolipid antigens presented by CD1d.

the initial alpha-GalCer treatment (86, 87). These alpha-GalCer-experienced NKT cells are then resistant to restimulation with alpha-GalCer, as they have adopted an anergic phenotype, which lasts for up to 2 months (88, 89).

The level of activation and the profile of cytokines produced by NKT cells can be profoundly modulated by the context in which these cells become activated (53, 90-92). For example, NKT cell activation can be modulated by engagement of co-stimulatory molecules on these cells such as CD28, CD40L, OX40, and programmed death (PD)-1. Likewise, distinct NKT cell agonists can skew the cytokine profile of NKT cells. While KRN7000, the prototypical alpha-GalCer, induces a mixed Th1/Th2 cytokine profile in NKT cells, some structural variants such as OCH (93) and C20:2 (94) promote a Th2-biased cytokine profile, whereas some other structural variants such as alpha-C-GalCer (95) and alpha-carba-GalCer (96) promote a Th1-biased cytokine production profile. These properties of NKT cells have been exploited in the development of vaccine adjuvants and immunotherapies (see below).

NKT cells are capable of substantial crosstalk with many other cell types from the innate and adaptive immune system (91, 92). In particular, activated NKT cells are potent stimulators of dendritic cell functions, a property that has received substantial interest for the development of NKT cell-based adjuvants (97). Activated NKT cells also promote the activation of natural killer (NK) cells, macrophages, neutrophils, and conventional B and T cells. In many cases, NKT cells also influence the quality of an immune response by regulating the differentiation of naïve CD4 T cells into Th cell effectors. These complex interactions of NKT cells with other cell types in the immune system are influenced by the precise nature of the stimulus and form the basis for the capacity of these cells to modulate a variety of immune responses.

6. NKT CELL FUNCTIONS

NKT cells can contribute to protective immune responses against a wide variety of pathogens, including bacteria, viruses, fungi, protozoa and parasites (79, 98). Some of these microorganisms, including *Borrelia burgdorferi* (99) and species of the *Novosphingobium* and *Ehrlichia* genera (50), contain glycolipid or lipid antigens that can directly activate NKT cells. However, most microorganisms likely activate NKT cells in an indirect manner, using the pathway described above and depicted in Figure 2. NKT cells can also contribute to disease pathogenesis during infection. For example, although these

cells contribute to protective immunity against infection with a low dose of *Novosphingobium capsulata*, at high dose inoculation, they can induce a potent cytokine storm that leads to a lethal sepsis (50). These cells also play a pathogenic role following intranasal infection with *Chlamydia muridarum*, but surprisingly, were protective for infection by the related organism *Chlamydia pneumoniae* (100). Some studies further showed that the outcome of infection can be influenced by the genetic background of the animals employed. For example, NKT cells protected BALB/c mice against disease induced by respiratory syncytial virus infection, but exacerbated disease in mice of the C57BL/6 background (101). Similarly, NKT cells protected BALB/c mice against cerebral malaria but exacerbated disease in C57BL/6 mice (102). Interestingly, a recent study showed that NKT cells can regulate colonization of the intestine of mice with commensal and pathogenic bacteria (103).

NKT cells have been implicated in natural immunity against tumors (104). This has been demonstrated in experimental models involving transplantable tumors, tumors induced by the chemical methylcholanthrene, and tumors that develop in mice heterozygous for the p53 tumor suppressor gene. This natural immunity against tumors appears to be due to production of IFN-gamma to activate NK cells and CD8 T cells, and by inducing IL-12 production in dendritic cells. Consistent with this protective role of NKT cells against tumors in mice, infiltration of tumors with NKT cells appears to be positively correlated with survival of cancer patients.

NKT cells contribute to a variety of autoimmune and inflammatory conditions (105, 106). In most mouse models of type 1 diabetes, multiple sclerosis and systemic lupus erythematosus, NKT cells play a protective role. Although NKT cell-deficient animals do not develop any obvious disease pathologies, aged Jalpha18-deficient mice developed signs of kidney disease and antibodies directed against double-stranded DNA (107), which are characteristic of lupus-like disease. A recent study (108) has provided important insight into the potential mechanisms by which NKT cells can modulate lupus development. These investigators showed that apoptotic cells, which are often increased in lupus, can activate NKT cells, resulting in suppression of autoreactive, CD1d-expressing B cells and lupus development in mice (108). NKT cells can also promote autoimmunity in some cases. In models of collagen-induced and antibody-mediated arthritis NKT cells played a pathogenic role (109). Similarly, these cells were important for the development of primary biliary cirrhosis in transgenic mice overexpressing a dominant-negative form of the TGF-beta receptor, and in mice infected with *Novosphingobium aromaticivorans* (110-112). NKT cells also play a pathogenic role in mouse models for multiple inflammatory diseases and hypersensitivities, including allergic airway hyperresponsiveness and asthma (113), atherosclerosis (114-116), contact hypersensitivity (117), rejection of tissue transplants (118), colitis induced by the hapten oxazolone (119), liver disease induced by the T cell mitogen concanavalin A (120), ischemia-reperfusion injury in multiple organs (121) and during sickle cell disease (122), and sepsis syndrome induced by LPS (84). Consistent with the proposed role of NKT cells in autoimmunity and inflammation, mice or patients with autoimmune or inflammatory diseases often have alterations in the abundance and cytokine production profile of NKT cells. However, it is unclear whether these alterations in NKT cells contribute to disease development or if they are secondary to the disease process.

The hypothesis that NKT cells play an important role in immune tolerance is supported by studies with experimental models of tolerance induction. This was first demonstrated in a model of tolerance in the eye, termed anterior chamber-induced immune deviation (123). Tolerance induced by injection of protein antigens in the eye was dependent on NKT cells in the spleen (124). A similar tolerogenic role of NKT cells has been demonstrated in transplant tolerance induced by blocking antibodies directed against CD4 or co-stimulatory molecules (125), immune suppression induced by ultraviolet exposure (126) or following burn wounds (127), and tolerance to the fetus (128). NKT cells in the host also play a protective role in protecting the host from graft-vs-host disease (GVHD) following bone marrow transplantation (129). Importantly, the latter activities of NKT cells did not impair immune responses against tumors in the host (i.e., graft-vs-leukemia responses).

7. NKT CELL-BASED VACCINE ADJUVANTS

The immune potentiating properties of NKT cells have been exploited for the development of vaccine adjuvants (130, 131). These properties of NKT cells are based on their capacity to stimulate the maturation of dendritic cells, which, in turn, promote potent cell-mediated immune responses. In vivo injection of alpha-GalCer into mice led to phenotypic and functional maturation of dendritic cells, resulting in the priming of CD4 and CD8 effector T cells. Dendritic cells potently upregulated a variety of co-stimulatory molecules, including CD80, CD86 and CD70, and secreted copious amounts of cytokines such as IL-12 and TNF-alpha. These activities of NKT cells required CD40-CD40L interactions between the dendritic cells and NKT cells. Additional studies further showed that NKT cells can also promote antibody responses. Mechanistic studies suggested that activation of NKT cells by alpha-GalCer enhances antibody responses to co-administered T cell-dependent antigens by inducing persistent plasma cell responses (132). Because CD1d expression on the B cells was required, it is likely that direct interactions between B cells and NKT cells were critical for these activities of alpha-GalCer on antibody responses. Thus, alpha-GalCer has strong adjuvant activities that promote both cell-mediated and humoral immune responses.

The adjuvant activities of NKT cells have been tested in vaccines against several pathogens and tumors (130, 131, 133, 134). This was first demonstrated for malaria vaccines, using irradiated sporozoites or

recombinant viruses expressing a malaria antigen (135). These adjuvant properties of alpha-GalCer required IFN-gamma production by NKT cells. Alpha-GalCer was also effective as a mucosal adjuvant in vaccine formulations directed against influenza virus, in which case mucosal IgA antibodies were produced. Similarly, alpha-GalCer promoted the efficacy of an HIV-1 DNA vaccine, promoting a 10-fold increase in humoral immune responses. Multiple strategies have been employed to develop tumor vaccines. Some investigators have employed tumors expressing neoantigens such as ovalbumin, in which case mice were immunized with ovalbumin antigens and treated with alpha-GalCer and then challenged with the ovalbumin-expressing tumor cells. Another strategy employed alpha-GalCer treatment at the time of immunization with clinically relevant peptide epitopes, such as the HLA-A2-restricted peptide derived from the NY-ESO-1 antigen. In another approach alpha-GalCer was co-administered with irradiated tumor cells, in which case NKT cell activation promoted the maturation of dendritic cells that captured and presented tumor antigens to CD4 and CD8 T cells. In a variation of this approach, CD1d-expressing tumor cells were pulsed with alpha-GalCer and then used to immunize mice. In the latter case, immunity to the tumors was long-lived, persisting for 6-12 months (136).

8. NKT CELL-BASED IMMUNOTHERAPIES

Alpha-GalCer was originally discovered during a search for reagents derived from a marine sponge with potent anti-metastatic activities in mice (137). These original findings paved the way for numerous studies investigating the anti-tumor activities of alpha-GalCer and its structural variants (104, 138). Tumor models employed included transplantable tumors, tumors induced by the chemical carcinogen methylcholanthrene and spontaneous tumors in Her2/neu transgenic mice and in p53-deficient mice. Mechanistic studies revealed that CD1d expression on the tumors was not required for the anti-metastatic activities of alpha-GalCer, and provided evidence for a critical role of IFN-gamma, produced by NKT cells themselves, NK cells and cytotoxic T lymphocytes. Consistent with the critical role of IFN-gamma, the alpha-GalCer analogue alpha-C-GalCer demonstrated superior anti-metastatic activities (95). Similarly, delivery of the glycolipid by dendritic cells provided improved therapeutic activities (139). The anti-metastatic activities of alpha-GalCer have also been tested in cancer patients (133). Treatment modalities employed included repeated injection with free alpha-GalCer or alpha-GalCer-loaded dendritic cells, and treatment with NKT cells expanded in vitro with alpha-GalCer. These clinical trials revealed that it is very challenging to induce potent NKT cell responses in humans, in part because humans have low numbers of NKT cells as compared with mice. Another challenge is that NKT cells in cancer patients are often dysfunctional. One trial, which involved repeated treatment of patients with non-small lung cancer using alpha-GalCer-stimulated peripheral blood mononuclear cells, has shown some promising clinical benefit to patients (140).

The capacity of alpha-GalCer to impact infection has been investigated for a wide variety of microorganisms (79, 98). In most cases, treatment was associated with pathogen clearance, in a manner that depended on the capacity of alpha-GalCer to activate dendritic cells and other innate immune cells. Consistent with this notion, alpha-C-GalCer was more effective that alpha-GalCer in clearing malaria parasites from mice infected with sporozoites (95). Because alpha-GalCer treatment was typically only effective during a narrow time window of a few days before and after infection, this strategy will likely have limited benefit for treatment of human infectious diseases. Clinical trials with patients chronically infected with hepatitis B virus (141) or hepatitis C virus (142) have found no clinical benefit of alpha-GalCer treatment.

Alpha-GalCer and its structural analogues have been tested in a variety of autoimmune diseases, usually employing repeated injections (105, 143). Alpha-GalCer has proven efficacious for prevention of diabetes in non-obese diabetic mice (144-147), multiple sclerosis-like disease in multiple mouse models (93, 148-150), some induced and genetic models of systemic lupus erythematosus (151, 152), collagen-induced rheumatoid arthritis (153, 154), and experimental models of autoimmune myasthenia gravis (155), autoimmune thyroiditis (156), and ocular autoimmunity (157). However, in some cases, NKT cell activation exacerbated disease, which was observed in an adoptive transfer model for type 1 diabetes in C57BL/6 mice (158), some studies with multiple sclerosis (159) or lupus models (152, 160), and antibody-mediated arthritis (161). The outcome of NKT cell activation on the disease process was often influenced by the glycolipid dose, timing of treatment, genetic background of the animals and precise experimental model tested. Generally, disease prevention was associated with a shift in the immune response towards Th2 immunity and/or suppression of Th1 and/or Th17 cell responses. In many cases cytokines such as IL-4 and IL-10 appeared to be critical for disease protection, but in some models IFN-gamma appeared to play a protective role as well. Consistent with the idea that immune deviation plays a role, the alpha-GalCer analogues OCH and C20:2 demonstrated superior disease protection in some models. In addition to general deviation of the immune response, multiple other mechanisms have been implicated in the capacity of activated NKT cells to suppress autoimmunity, including the induction of tolerogenic dendritic cells and regulatory T cells, induction of anergy in pathogenic T cells, direct effects on B cells, or general immune suppression. It is likely that NKT cell activation by distinct NKT cell agonists impacts distinct autoimmune diseases in a different manner and that mechanisms involved are diverse. For example, a single injection of either alpha-GalCer or alpha-C-GalCer was able to protect DBA/1 mice against collagen-induced arthritis (153). In the case of alpha-GalCer the cytokine IL-10 appeared to play a critical role, whereas the effects of alpha-C-GalCer on disease amelioration were associated with a general immune suppression. Similarly, alpha-GalCer, OCH and alpha-C-GalCer all protected mice against ocular autoimmunity, but alpha-C-GalCer was most effective, in a manner that was

associated with suppression of both Th1 and Th17 cell responses (157). The diverse and often unpredictable outcomes of NKT cell activation on autoimmunity in mouse models will complicate translation of these preclinical studies to the clinical setting.

A few studies have investigated the capacity of NKT cell activation to modulate GVHD responses. In a model of allogeneic bone marrow transplantation where recipients were nonlethally irradiated, alpha-GalCer treatment of the recipient mice reduced GVHD by stimulating host NKT cells and inducing Th2 deviation in donor T cells (162, 163). However, in a model of allogeneic stem cell transplantation where recipient animals received a fully myeloablative dose of irradiation and using delayed glycolipid administration, alpha-GalCer exacerbated GVHD, in a manner involving donor NK and T cell activation (164). Nevertheless, using the latter model, the Th2-deviating alpha-GalCer analogue C20:2 potently inhibited GVHD.

In a model of inflammatory bowel disease induced by dextran sodium sulphate, which elicits inflammation by permitting access of intestinal microorganisms to the subepithelial space in the gut, alpha-GalCer and OCH were able to confer disease protection (165, 166). In the case of OCH, even a single treatment was effective, in a manner that correlated with a reduction in IFN-γ expression and an increase in IL-4 expression in the mucosal tissue (165).

The effects of NKT cell activation in models of atherosclerosis have also been investigated (167). Alpha-GalCer exacerbated spontaneous atherosclerosis in apolipoprotein (apo) E-deficient mice (114-116). However, in a model where atherosclerosis was induced by Western-type diet feeding and collar placement around the carotid arteries, alpha-GalCer treatment had no effect on atherosclerosis in apoE-deficient mice, but led to a reduction in plaque size in low density lipoprotein receptor-deficient mice (168). The investigators of the latter studies proposed that these differences between the two types of knockout animals might be due to the critical role of apoE in delivering glycolipids to NKT cells.

In allergic reactions and asthma, NKT cell activation during the sensitization phase exacerbated disease (169). Conversely, a CD1d-dependent antagonist of NKT cells, di-palmitoyl-phosphatidyl ethanolamine polyethylene glycol, prevented development of allergen-induced airway hyperreactivity in mice (170).

9. SUMMARY AND PERSPECTIVE

NKT cells are regulatory T cells that bridge the innate and adaptive immune system. NKT cells can directly respond to glycolipid antigens that bind with CD1d molecules, and these cells can also become activated indirectly in response to TLR agonists and other inflammatory mediators such as pro-inflammatory cytokines. Activated NKT cells are capable of rapidly producing copious amounts of cytokines that can activate cells of the innate and adaptive immune systems and influence the character of the ensuing immune response. Consequently, NKT cells have been implicated in a variety of immune responses and diseases, including infections, cancer, tissue graft rejection, allergies, autoimmunity and inflammatory diseases. Numerous glycolipid antigens that can differentially induce distinct effector functions of NKT cells have been identified, providing potent tools for the development of vaccine adjuvants and immunotherapies for a variety of diseases. Nevertheless, a number of challenges for applying these tools in the clinical setting remain. Thus far, NKT cell activation during therapy of cancer or infectious disease in humans has had limited success. More will need to be learned about the behavior of NKT cells and how their therapeutic properties can be best exploited. With the proper tools it might be possible to target specific subsets of NKT cells with unique and distinct effector functions. In addition, because humans have lower numbers of NKT cells than mice, it will be critical to develop potent techniques to elicit the biological functions of these cells in patients. In this context, methods to introduce the invariant TCR of human NKT cells into stem cells followed by programming towards the T cell lineage has been considered (171). Finally, combination therapies are being tested in animal models. Collectively, these studies hold much promise for the clinical application of NKT cell-based immunotherapies.

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