CD1d-Restricted Natural Killer T Cells: Roles in Tumor Immunosurveillance and Tolerance

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Key Words

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Summary

Natural killer T (NKT) cells are a heterogeneous group of T cells that share properties characteristic of both T cells and NK cells and possess a variety of unusual properties with regard to antigen recognition and function. Many of these cells recognize the non-polymorphic CD1d molecule, an antigen-presenting molecule that binds self- and foreign lipids. The best known subset of CD1d-dependent NKT cells expresses an invariant T cell receptor α (TCR- α) chain. These are referred to as type I or invariant NKT cells (iNKT cells). These cells, which are the main focus of the current review, are conserved between humans and mice. Detailed work in mouse models has implicated iNKT cells in many immunological processes, and related studies in humans suggest important roles in health and disease. By virtue of their ability to produce a variety of immunoregulatory cytokines and to acquire a broad spectrum of effector activities, iNKT cells may both induce or suppress immune reactions in healthy and pathologic settings. We review the role of iNKT cells in the induction of tolerance to solid organ and hematologic transplants and malignancies, as well as their importance in maintaining normal self-tolerance and involvement in autoimmune diseases.

Schlüsselwörter

NKT-Zellen · CD1d · Lipidantigene · Immuntoleranz · Neoplasie

Zusammenfassung

Natürliche-Killer-T-Zellen (NKT-Zellen) sind eine heterogene Gruppe von T-Zellen, die Charakteristika von sowohl T-Zellen als auch NK-Zellen aufweisen und eine Vielzahl ungewöhnlicher Eigenschaften hinsichtlich Antigenerkennung und Funktion besitzen. Viele dieser Zellen erkennen das nichtpolymorphe Molekül CD1d, ein Antigen präsentierendes Molekül, dass autolog und allogene exprimierten Lipide bindet. Die am besten untersuchte Teilgruppe CD1d-abhängiger NKT-Zellen exprimiert eine invariante T-Zell-Rezeptor- α (TCR- α)-Kette. Solche Zellen werden als Typ-I- oder invariante NKT-Zellen (iNKT-Zellen) bezeichnet. Sie kommen gleichermaßen im Human- und im Maussystem vor und sind der Schwerpunkt der vorliegenden Übersichtsarbeit. Ausführliche experimentelle Untersuchungen im Maussystem zeigen, dass iNKT-Zellen in viele immunologisch relevante Prozesse involviert sind, und Studien im Humansystem legen eine wichtige Rolle dieses Zelltyps unter physiologischen und pathologischen Bedingungen nahe. Aufgrund ihrer Fähigkeit, eine Vielzahl immunregulatorischer Zytokine zu produzieren und ein breites Spektrum an Effektorfunktionen zu realisieren, können iNKT-Zellen Immunreaktionen unter physiologischen und pathologischen Bedingungen sowohl induzieren als auch unterdrücken. Die vorliegende Übersichtsarbeit konzentriert sich auf die Rolle der iNKT-Zellen bei der Induktion von Immuntoleranz im Rahmen der Transplantation solider Organe und hämatopoetischer Stammzellen bei Tumorerkrankungen sowie auf die Bedeutung der iNKT-Zellen für die Aufrechterhaltung von Immuntoleranz unter physiologischen Bedingungen und im Rahmen von Autoimmunerkrankungen.

Introduction

Natural killer T (NKT) cells express a T cell receptor (TCR) and mature in the thymus, but they also frequently express a variety of molecular markers and perform functions that are typically associated with NK cells. Unlike most conventional T cells, many NKT cells do not recognize peptide antigens bound to MHC class I or MHC class II molecules. Instead, these cells are now known to respond to lipid antigens, which they recognize with their TCRs, as ligands bound to MHC-like CD1d molecules. NKT cells rapidly produce large quantities of cytokines and other effector molecules upon stimulation and, as a result, have the capacity to modulate and regulate a variety of immune reactions [1]. To date, NKT cells have been shown to influence the outcome of host immune reactions during microbial challenge, autoimmune disease, malignancy and transplantation. The role of NKT cells and CD1d in the response to infection and host defense has been the subject of several recent reviews [2, 3]. In this article, we focus on the role of NKT cells in promoting and preventing tolerance to self, altered self, and non-self tissues. Endogenous lipid ligands produced in health and disease may be the key to understanding the role of NKT cells in these processes.

Defining NKT Cells

In the mid to late 1980s and early 1990s, an unusual population of thymic and peripheral CD3+ cells was identified that expressed a TCR- $\alpha\beta$ and a C-type lectin receptor, NK1.1 or CD161, which is typically expressed on NK cells [4]. These T cells were found to share other features with NK cells as well, such as CD16 and CD56 expression and granzyme production [5, 6]. Although NKT cells continue to be defined in this way by some, it has become apparent that defining NKT cells as T cells with the NK1.1 marker is neither sensitive nor specific enough to label a single group of cells. For example, many NKT cells that are destined to express NK1.1 initially lack this marker during development in the thymus and soon after export [7, 8]. Furthermore, mature NKT cells may transiently down-modulate NK1.1 from their surfaces following activation in peripheral tissues [9]. Conversely, a substantial fraction of conventional T cells may also express the NK1.1 marker, particularly following activation [10, 11].

Major progress towards understanding and characterizing NKT cells was made when it was discovered that many T cells co-expressing TCR- $\alpha\beta$ and NK1.1 recognize the MHC-class-I-like CD1d molecule and are dependent upon interactions with this molecule for their thymic selection and subsequent development [12]. In humans and mice, CD1d-dependent T cells include NK1.1+ and NK1.1- cells as well as CD4+, CD4- CD8- and CD8+ T cells (the latter found thus far only in humans). Among the CD1d-dependent NKT cells, the best studied and probably most abundant subset is characterized by the expres-

Fig. 1. Overlapping subsets of comprising NKT cells (not to scale). CD1d-restricted T cells, also called NKT cells, are a small subset of the total T cell population. iNKT cells are a subset of CD1d-restricted (NKT) cells. These cells typically comprise less than 1% of circulating T cells, although their numbers are higher in some organs. NK1.1/CD161+ cells are an overlapping subset of cells which may comprise up to 25% of circulating T cells.

sion of an invariant TCR- α chain (V α 14 J α 18 in mice and V α 24 J α 18 in humans), which is identical in all cells of this subset and formed by the precise joining of 2 germline gene segments without template-independent nucleotide additions [13–16]. The invariant TCR- α of these cells is paired with a limited number of TCR- β chains containing a limited diversity of V β segments (V β 8.2, V β 7, V β 2 in mice and V β 11 in humans). Cells expressing this canonical TCR have been called iNKT, V α 14i NKT, classical NKT and type I NKT cells (fig. 1, table 1) [4].

A second important observation that revolutionized the field of NKT cell research was the finding that virtually all type I NKT cells recognized a specific type of ceramide glycolipid structure when bound to CD1d [17]. The α -galactosylceramide agelasphin-9b was originally identified as a component of extracts of a marine sponge during a screen for compounds that could inhibit tumor growth in mice [18, 19]. A synthetic analogue called KRN7000 (often referred to simply as α -GalCer in the literature (fig. 2)) was then produced and selected for its ability to delay tumor growth and trigger both murine and human lymphocyte proliferation [19]. The lymphocytes responding to KRN7000 were subsequently identified as the V α 14i NKT cells (i.e., type I or iNKT cells) which required CD1d presentation of the α -GalCer to be activated in a TCR-dependent manner [17]. These findings provided an enormously valuable tool for characterizing and manipulating iNKT cells both in vitro and in vivo. For example, recombinant CD1d loaded with α -GalCer is now a staining agent used to identify and separate iNKT cells from cell suspensions [20, 21]. This methodology is now widely used in laboratories studying this T cell subset.

Following the more precise characterization of the iNKT cell subset, a second population of CD1d-dependent T cells was

Table 1. Definitions and characteristics of NKT and NKT-like cells. Adapted from [4]

	Type I NKT	Type II NKT	NKT-like
Other names	classical NKT invariant NKT (iNKT) V α 14i NKT (mouse) V α 24i NKT (human)	non-classical NKT diverse NKT	NK1.1+ T cells CD3+ CD56+ cells
Restriction	CD1d	CD1d	MHC, other?
α -GalCer reactivity	+	–	–
TCR repertoire	V α 14-J α 18: V β 8.2, 7, 2 (mouse) V α 24-J α 18: V β 11 (human)	diverse	diverse

recognized to exist which does not express the invariant TCR- α chain and has a broad range of TCRs [22, 23]. This population has been less well studied, in part because it is difficult to identify and isolate these cells with current methodologies. It has been proposed that these CD1d-dependent T cells with diverse TCRs should be designated as type II or non-classical NKT cells [4]. Finally, there are T cells that express NK1.1 (CD161) and are not CD1d-dependent. For example, while CD1d-dependent NKT cells are less than 1% of circulating lymphocytes in a healthy donor, up to 25% of circulating T cells from healthy donors co-express CD161 [24]. Most of these CD161+ T cells are MHC class I or class II restricted $\alpha\beta$ or MHC unrestricted $\gamma\delta$ T cells and are thus distinct in their development and function from the CD1d-dependent population [10, 11]. A recently proposed classification scheme summarized in table 1 suggests that CD1d-independent T cells expressing CD161 be referred to as ‘NKT-like cells’ [4]. Based on information from a variety of studies, a more detailed understanding of the relative frequencies of the different NKT cell subsets and their relationships to each other is now beginning to develop, as summarized in simplified graphical form in figure 1. This article will focus mainly on type I CD1d-dependent NKT cells, referred to henceforth as iNKT cells. Although iNKT cells are the majority of NKT cells in normal mice, in absolute numbers they are a relatively small population, typically comprising 1% or less of total T cells in the mouse spleen or lymph nodes [25]. They are substantially more prominent, however, among the lymphoid populations of some other tissues, such as in the mouse liver, where they represent up to 50% of all T cells [21, 26]. Similarly, among human circulating lymphocytes, iNKT represent a relatively minor percentage of total T cells (around 0.5% or less) [27, 28]. As a result of rapidly advancing techniques to study and manipulate iNKT cells, it is now clear that these unique T cells are important effectors and regulators of a wide variety of immune responses. In some disease processes, circulating iNKT numbers are reduced. Most iNKT cells express inflammatory lymphocyte chemokine receptors, and migration from the periphery to become distributed at sites of inflammation may explain this phenomenon [29].

The CD1 Family of Antigen-Presenting Molecules

CD1d, which is critical for the development and function of iNKT cells and also type II NKT cells, is a member of the CD1 family of MHC-like antigen-presenting molecules. All CD1 molecules studied to date are β 2-microglobulin-associated surface glycoproteins that bear significant structural similarity to MHC class I molecules [30]. In humans, the CD1 family consists of 5 members, designated CD1a, CD1b, CD1c, CD1d and CD1e. Based on homology relationships and the details of their expression and function, CD1a/b/c/e are classified as group 1 CD1 molecules. CD1d comprises a separate group and is the only member of the group 2 CD1 molecules [31]. Both group 1 and group 2 CD1 molecules are lipid-binding proteins that can present foreign and self-lipids and glycolipids to T cells. Group 1 CD1 molecules have been characterized most extensively in humans and have been demonstrated to present microbial lipid antigens and certain self-lipids to a diverse group of T cells [32]. Group 2 CD1 molecules (i.e., CD1d) have been extensively studied in mice as well as humans. In fact, CD1d is the only member of the CD1 family that is conserved in rodents, and this has greatly facilitated the study of its role in a wide variety of mouse models. In marked contrast to the highly polymorphic MHC-encoded protein antigen-presenting molecules, CD1 proteins appear to have limited polymorphism between individuals of a given species [33]. CD1d is also highly conserved across species, as murine CD1d can interact with human iNKT cells and vice versa [34].

Cell Types and Tissues Expressing CD1d

In contrast to group 1 CD1 molecules, which are expressed by relatively few cell types in humans, CD1d is expressed on cells of numerous hematopoietic lineages and also by a number of non-hematopoietic cell types [35]. Low levels of CD1d are present on many cells of myeloid lineage, including most monocytes, monocyte-derived dendritic cells (DCs) and macrophages [36]. The expression of CD1d on these cells is constitutive, unlike group 1 CD1 molecules that show inducible expression on monocytes in response to GM-CSF and other cytokines [35]. Despite low levels of CD1d expression,

these cells are capable of potently activating CD1d-dependent NKT cells [35, 36].

High levels of CD1d expression are found on cortical thymocytes, and low levels are found on mature activated T cells, whereas medullary thymocytes and resting peripheral blood T cells are CD1d⁻ [37]. All B cells in peripheral blood have been found to be CD1d⁺. B cells in the mantle zones of peripheral lymphoid organs and marginal zones of the spleen show particularly high expression of CD1d, whereas expression is low or absent on germinal center B cells [36, 38]. Early studies on CD1d expression found that it could be detected in significant amounts on human intestinal epithelial cells [39]. Subsequently, CD1d has been found on other human non-hematopoietic tissues, including keratinocytes and epithelial cells in the biliary tract [40, 41].

Antigens Presented by CD1d to iNKT Cells

The main function attributed thus far to CD1d is its role in antigen presentation to iNKT and type II NKT cells. It is known that iNKT cells arise from CD4⁺ CD8⁺ T cell precursors in the thymus and require CD1d expression for their selection and development in mice, and this appears likely to be the case in humans as well [42, 43]. In the peripheral tissues, CD1d molecules provide at least one of the signals that can activate mature iNKT cells and trigger the expression of a wide range of T cell effector functions. This interaction is analogous to the classical CD8⁺ and CD4⁺ T cell interactions with antigen presented by MHC class I and II. However, instead of presenting peptides like classical MHC-encoded antigen-presenting molecules, CD1d is dedicated mainly or solely to the presentation of lipid ligands [44]. The full range of natural antigens to which iNKT cells respond remains unknown, but studies of the synthetic glycolipid antigen α -GalCer have provided enormous insight into CD1d-mediated lipid presentation and recognition by iNKT cells. The synthetic α -GalCer KRN7000 (fig. 2) binds to CD1d to generate a CD1d/glycolipid complex that is strongly recognized by all iNKT cells. The crystal structures of human and murine CD1d with bound α -GalCer have been recently solved, providing a high level of precise molecular information about the mechanism underlying the formation of this complex [45, 46]. These crystal structures reveal that CD1d contains 2 deep hydrophobic pockets that accommodate the 2 hydrophobic tails of α -GalCer and presumably the tails of other lipid ligands that are presented by CD1d as well. The lipid tails of KRN7000, consisting of a 26 carbon acyl and 18 carbon phytosphingosine chain, fit within the pockets of the antigen-binding site in a way that is fully space occupying, which may in part explain why it activates iNKT cells so strongly.

KRN7000 is currently the only form of α -GalCer that has been tested in human subjects, and is also the best studied activator of iNKT cells in mouse models of disease. Several laboratories have recently created synthetic analogues of α -GalCer with a variety of structural modifications, and have tested

Fig. 2. Synthetic analogues of α -GalCer. KRN7000 has been most extensively studied. It is most closely related to the original marine sponge-derived α -GalCer and activates NKT cells to produce Th1 (e.g. IFN- γ) and Th2 (e.g. IL-4) cytokines. The second and third compounds, C20:2 and OCH, induce a strong early IL-4 and IFN- γ production, but do not stimulate the delayed IFN- γ burst as much as KRN7000. They incorporate several of the changes typical of Th2-biasing compounds, such as tail length shortening and the introduction of double bonds. The C-glycoside of KRN7000, in which a carbon rather than an oxygen atom links the carbohydrate head group, has been shown in one study to increase sustained IFN- γ production beyond levels seen with KRN7000.

these for their ability to activate iNKT cells (fig. 2) [47–51]. Some of these analogues possess novel properties in vivo and in vitro. Modified forms of α -GalCer with altered lipid tails have been shown to preserve the stimulation of IL-4 secretion by iNKT cells, while greatly diminishing production of IFN- γ . The reduction in IFN- γ secretion is particularly notable during a late sustained phase of its production, which typically follows an initial transient IL-4 and IFN- γ burst and likely reflects downstream iNKT activation of NK cells [46]. Another synthetic compound, with a carbon atom linking the galactose sugar of KRN7000 to the ceramide backbone rather than an oxygen atom, has been shown to shift cytokine production in the opposite manner, causing substantially higher levels and more sustained production of IFN- γ (fig. 2) [52, 53].

The mechanism by which these analogues of α -GalCer lead to altered cytokine responses are not yet known, although initial studies suggest that it may be related to their association with different subsets of CD1d-expressing antigen-presenting cells [47]. Alternatively, there is also evidence favoring the hypothesis that this phenomenon may be attributable to the stability of the CD1d/glycolipid complex or the affinity of the interaction with the iNKT TCR [54, 55]. As a third possibility, the location where different analogues are loaded onto CD1d, which can potentially include the cell surface, the endoplasmic reticulum or endosomes, may also contribute to the functional outcomes of activation. Compounds with longer tails seem to require endosomal loading onto CD1d, and molecules transported to the cell surface from endosomes may be preferentially sorted into lipid rafts [56]. Sphingolipids, of which α -

GalCer and other iNKT agonists are a part, are preferentially sorted into lipid rafts [55]. Two studies found that CD1d presentation in lipid rafts was necessary for efficient NKT activation [57, 58]. Another possible explanation is that, in some cases, the altered glycolipid antigens may preferentially activate only certain subsets of iNKT cells that are biased in their cytokine secretion patterns or in other aspects of their function. Many questions remain to be resolved, and this is an active area for current and future research.

While KRN7000 and other related forms of α -GalCer are by far the most well-known and studied CD1d-presented ligands, data on other potentially relevant ligands have gradually emerged in recent years. One early study demonstrated the capacity for CD1d to bind hydrophobic peptides with a specific sequence motif [59], although subsequent studies have identified only lipid and glycolipid molecules as physiologically relevant ligands. Recently, the catalogue of CD1d-presented agonists was expanded to include several naturally occurring bacterial glycosphingolipids from organisms in the bacterial genera *Sphingomonas* and *Ehrlichia*. These are Gram-negative bacteria that lack lipopolysaccharide (LPS) in their cell walls and instead express abundant α -glycosylsphingolipids. Several of these compounds have been shown to be CD1d-presented antigens that are strong activators of iNKT cells [50–62]. The relevant *Sphingomonas* antigens were structurally characterized as α -glucuronylceramides which are closely related in their structure to the synthetic α -GalCer compounds that have been widely studied. A glycolipid in mycobacteria, phosphatidylinositol tetramannoside (PIM₄), has been identified as another bacterially derived CD1d-presented activator of iNKT cells [63]. A similar role has been attributed to lipophosphoglycans of *Leishmania donovani* [64]. It has been speculated that the presence of iNKT cell activators in the cell walls of microbes, such as *Sphingomonas* species, *Leishmania* or mycobacteria, which lack LPS and thus are not able to activate innate immune responses through Toll-like receptor 4, may provide an alternative innate immune recognition for these organisms via their stimulation of iNKT cells.

It is widely believed that endogenous lipids and glycolipids also exist that bind to CD1d and may be involved in the development and activation of iNKT cells under certain conditions. Self-lipid ligands of CD1d are generally thought to be involved in the positive and negative selection of iNKT cells in the thymus, and they may also contribute to stimulation of mature iNKT cells in peripheral tissues. Studies of human or murine cells have shown that CD1d molecules are associated with bound cellular lipids, which in some instances have been identified as phosphatidylinositols (PI) [65, 66]. It has been suggested that PI may play a chaperone-like role in CD1d assembly, possibly to preserve the integrity of the antigen-binding groove until other lipids are bound at the cell surface or in the endocytic pathway. In addition, an endogenous glycolipid ligand has recently been described that has been proposed to be required for thymic development of iNKT cells. This is a

trisaccharide-containing glycosphingolipid called isogloboside-3 (iGb3) [67]. Data in mice that are deficient in iGb3 processing suggest that this endogenous molecule may be required for thymic selection of iNKT cells, and it has been shown to have significant although relatively weak activating properties for mature peripheral murine and human iNKT cells in vitro.

Effector Functions of iNKT Cells

Upon iNKT stimulation, numerous cytokines are produced. The best characterized response is an early phase of IL-4 and IFN- γ production followed by a larger and more sustained IFN- γ release. A 3-step model has been proposed for the activation of iNKT cells [68]. In the first step, interaction between the iNKT cell TCR and CD1d, and between CD28 constitutively expressed on iNKT cells and B7 (CD80/86) expressed on DCs, triggers IL-4 and IFN- γ production by iNKT cells and up-regulates iNKT expression of CD40 ligand (CD40L). Second, IFN- γ and CD40L induce DCs to produce IL-12. In the third step, IL-12 further activates iNKT cells, inducing a second wave of IFN- γ production. Although the steps of this model have not yet been fully validated, it provides a useful framework for describing the complex temporal series of events that follows iNKT activation (fig. 3).

Resting and activated iNKT cells produce mediators of cytotoxicity as well. Target cell killing by iNKT cells has been proposed to be mediated through several pathways. Apoptosis of CD1d-expressing target cells may directly follow specific recognition by iNKT cells [69]. Transcripts for proteins associated with cytotoxicity, such as perforin and granzymes, are constitutively expressed at high levels by iNKT cells at baseline. Also, upon stimulation by α -GalCer, expression of these transcripts increases several-fold from baseline and expression of FAS ligand, TNF and TNF-related apoptosis-inducing ligand (TRAIL) is observed [69]. Non-specific interactions are a second pathway by which iNKT cells may mediate cytotoxicity. Following activation by CD1d-expressing antigen-presenting cells, iNKT cells have been found to lyse non-CD1d expressing tumor cells [70]. Finally, activation of the cytolytic properties of NK cells may amplify the ability of iNKT cells to induce target cell apoptosis [71]. There is evidence to suggest that the physiologic role of iNKT cells is more congruent with activation of other cytolytic cells, such as NK cells, rather than with direct killing [72, 73].

In addition to phenotypic changes, murine iNKT cells expand to at least several times their original numbers in peripheral tissues in vivo 2–3 days after α -GalCer stimulation [74]. This is coupled with an initial down-regulation of surface receptors, such as the TCR and NK1.1. However, these markers recover, and following the initial expansion the activated iNKT cells shift from an initial mixed IL-4 and IFN- γ production to predominant IFN- γ expression [9, 74]. In a recent experiment, a

Fig. 3. Development and differentiation of iNKT cells. **A** iNKT cells are positively selected in the thymus by interaction with cortical thymocytes through specific CD1d-TCR interactions and apparently through SLAM-SLAM interaction.

B Positive selection leads to clonal expansion of double-positive NK1.1-negative thymic iNKT with subsequent loss of CD8 or both CD8 and CD4, and migration of these cells into the periphery. Most iNKT cells express the NK1.1 marker upon maturation in the thymus or shortly after. **C** In the periphery, iNKT cells

interact with CD1d-presenting cells, and most strongly with DCs. Activation of iNKT cells leads to IL-4 and IFN- γ production and CD40L upregulation on the iNKT cell surface. This leads to DC IL-12 production which induces further iNKT activation. The DN iNKT cells may produce a more Th1-biased cytokine profile and have more cytotoxic mediators, such as perforin and granzymes. **D** The activated cells then go on to perform multiple functions which continue to be elucidated, but include cytokine production, cytotoxicity and activation of other lymphatic cells, such as NK and T cells.

state of hyporesponsiveness and a shift to predominant IL-4 production, which was described as a state of anergy, was induced for up to 6 months following a single injection of α -Gal-Cer [75]. These cells were capable of ameliorating clinical symptoms in experimental autoimmune encephalomyelitis (EAE), a model of Th1-type inflammatory autoimmune disease, but were not active in a model of tumor metastasis prevention [75].

In some ways, iNKT cells appear to be a part of the acquired immune system, particularly in their surface expression of a TCR- $\alpha\beta$ and in their activation through specific interaction with CD1d. However, in several other ways, such as their ability to respond very rapidly to various antigens to which they have not been previously exposed and their limited T cell receptor diversity, iNKT cells also resemble cell types that comprise the more primitive innate immune system, such as NK cells and the B1 and marginal zone B cell subsets [2, 3, 76]. Adaptive immunity appears to have first developed approximately 500 million years ago within the early vertebrates that immediately preceded the emergence of cartilaginous fish. At present, CD1 is known to exist in a variety of mammals, and 2 recent reports show that it is also found in birds, suggesting that CD1 molecules were present at least 300 million years ago [77, 78]. It remains to be seen whether CD1 extends all

the way back in evolutionary time to the initial foundations of the adaptive immune system.

The iNKT cell subset is similarly known to be conserved between several mammalian species (mice, rats, humans, non-human primates), but may be absent in others (guinea pigs, cows) and has not yet been identified in non-mammalian species other than birds [79, 80]. Thus, while the evolutionary origins remain incompletely discovered, it is interesting to speculate that iNKT cells might represent an archaic form of T cell that arose before the MHC-restricted subsets and mediated a combination of innate-like and adaptive responses to infection. This may explain why iNKT cells appear to have such a wide range of effector and regulatory activities and seem to play several distinct roles that have been relegated to separate cells in more evolutionarily advanced branches of the adaptive immune system.

Phenotypic and Functional Subsets of iNKT Cells

Studies correlating the various surface markers expressed by iNKT cells with developmental and functional properties have led to the conclusion that there are several distinct subsets of these cells. In this regard, CD4 has been a particularly infor-

mative marker. For example, it has been shown that both human and mouse iNKT cells differ considerably in their function depending on whether they express CD4 or not [81–83]. In mice, the majority of iNKT cells in spleen, liver and peripheral blood have usually been found to be CD4+, while the remainder are cells known as double-negative (DN) cells that lack expression of both CD4 and CD8 [84, 85]. The predominance of CD4+ iNKT cells is not as clear in humans, although both CD4+ and DN subsets are present in addition to a CD8+ population (predominantly CD8 α , with very little or no expression of the CD8 β subunit) [85, 86]. In both species, clear differences in the patterns of cytokines secreted following activation have been documented for CD4+ versus DN iNKT cells. Cells with the CD4 marker have a Th0 cytokine response upon activation, producing a mixture of both Th2-associated cytokines, such as IL-4 and IL-13, and Th1-associated cytokines, such as IFN- γ and TNF. Human CD4- iNKT cells (i.e., DN or CD8 α +) have been found to selectively produce Th1 cytokines at levels similar to those expressed by CD4+ iNKT cells but without concomitant levels of IL-4 production [81, 82]. It has also been shown that CD4- iNKT cells also preferentially express mediators of cytotoxicity, such as perforin and the C-type lectin receptor NKG2D [81]. This suggests that the CD4- iNKT cell subset may be predominantly responsible for the anti-tumor activities that have been attributed to CD1d-dependent T cells and for the therapeutic benefits of α -GalCer in tumor-bearing mice.

In mouse studies, the NK1.1 marker has also been shown to be relevant for distinguishing functionally distinct subsets of iNKT cells. The development of iNKT cells proceeds from a CD4+ CD8+ NK1.1- precursor in the thymus to mature cells which become either CD4 or DN and gain the NK1.1 marker (fig. 3) [8, 87]. A difference in cytokine production was found between the early NK1.1- and the more mature NK1.1+ populations. The NK1.1- iNKT cells preferentially produced more of the Th2 cytokine IL-4 than IFN- γ , in a ratio varying from 300:1 to 15:1 depending on the presence of other developmental markers. In contrast, NK1.1+ iNKT cells produced much higher levels of Th1 cytokines, with a 1:4 ratio of IL-4 to IFN- γ [8]. The NK1.1 marker is also lost during the incompletely characterized state of anergy that iNKT cells have been shown to enter in vivo following a single α -GalCer injection [9, 75]. In this state, IL-4 production also predominates over IFN- γ .

NKT Cells and CD1d Expression on Hematologic Malignancies

Several studies have implicated CD1d expression in hematologic malignancies. In some cases, CD1d is expressed on the malignant cells, and these cells can be induced to undergo apoptosis by α -GalCer-activated iNKT cells. In other studies, CD1d has been reported to be down-regulated on malignant cells, suggesting the possibility of an immune-editing process

by which loss of CD1d expression could help the tumor escape detection and elimination [88]. The expression of CD1d on malignant cells may provide a target for iNKT-associated therapy. Decreased expression of CD1d on some hematologic malignancies suggests either that some neoplastic cells are derived from lineages without CD1d expression or that NKT cells have exerted a selective force that leads to expansion of malignant cells that have lost CD1d expression.

Chronic and Acute Lymphocytic Leukemias

A study of malignant cells from patients with B cell chronic lymphocytic leukemia (B-CLL) compared the expression profile of these cells with normal B cells and found significant down-regulation of CD1d and CD1c transcripts in all 8 patients studied [89]. The gene microarray that was used for this study also identified the up-regulation of IL-4-responsive gene transcripts and down-regulation of IFN- γ -responsive transcripts. These findings suggest that iNKT cells and other CD1-dependent T cells may exert a negative selective pressure on B-CLL cells through recognition of CD1d and by IFN- γ production. The malignant cells may up-regulate IL-4-responsive genes in part to take advantage of the pro-survival effects of the IL-4 produced by iNKT and other cells.

Another study of B-CLL examined surface expression of the CD1d protein on malignant cells and found CD1d to be expressed on all 38 B-CLL cases analyzed [90]. CD1d expression at similar levels was found as well on peripheral-blood B cells of all normal subjects tested, although the malignant cells exhibited more inter-patient variability in CD1d expression levels than did B cells from healthy donors. An iNKT cell line derived from a healthy donor induced apoptosis in B-CLL cells, but only after the malignant cells were pre-incubated with α -GalCer. The observed increase in variability of CD1d expression among malignant cell lines is consistent with immune editing leading to loss of expression of this molecule from cancer cells. CD1d-restricted T cells might exert a negative selective pressure in some patients but not others, depending on the duration of disease and individual variation in host response.

A correlation of CD1d expression on B cell acute lymphoblastic leukemia (B-ALL) with decreased survival was recently published [91]. This is the first such paper to demonstrate a relationship between CD1d expression and prognosis. CD1d expression was inversely correlated with survival as well as positively correlated with pro-B immunophenotype, MLL rearrangement and infant leukemia. CD1d was expressed on blast cells from only 15% of patients. In 9 cases, CD1d was found on all blasts, while in 3 other cases it was detected on a subset of cells. The overall 5-year survival rate was 44.4% for CD1d+ ALL and 86.8% for CD1d- disease ($p = 0.00001$). Although it could not be stated whether CD1d was an independent prognostic marker or if its expression overlapped completely with other subgroups with poor prognosis, the results of this study suggest that CD1d expression might be useful as

a marker of disease severity as well as a target for therapy. The investigators showed that a cytotoxic iNKT cell line termed NKTA co-incubated with these cells and α -GalCer induced in vitro apoptosis of CD1d+ ALL cells in all but 1 case. The authors suggest using iNKT cells engineered with drug-inducible suicide genes [92], co-administered with α -GalCer to treat these CD1d+ refractory cases of ALL [91].

A study of T cell ALL (T-ALL) found that CD1d was expressed on a number of T-ALL cell lines examined as well as on 5/8 primary cell lines derived from patients with T-ALL [93]. Similar to other studies, it was also demonstrated that human iNKT cells could directly kill these cells in vitro and that killing was enhanced by α -GalCer stimulation.

Acute and Chronic Myeloid Leukemia

Acute myeloid leukemia (AML) cells with a more differentiated phenotype (M4, M5) expressed CD1d on their surface, while the molecule was almost completely absent from malignant cells with less mature blasts (M1-M3) and chronic myeloid leukemia (CML) cells with non-monocytic differentiation [69]. M4 and M5 cells were found to be targets for CD1d-dependent iNKT cell-mediated in vitro killing. Culturing with α -GalCer enhanced the in vitro cytotoxicity of iNKT cells against these cells. In a separate study, an iNKT cell line was derived from a patient with AML M4 and was shown to be phenotypically similar to iNKT cells from a healthy donor [94]. These cells were then shown to induce apoptosis of AML M4 leukemic cells, but not M0 or M1 cells, through the TRAIL pathway after iNKT incubation with α -GalCer-pulsed DCs. Findings reported by 2 other groups have also shown that human iNKT cells that are activated by α -GalCer or α -GalCer-pulsed DCs mediate cytotoxic anti-tumor activity against a number of cell lines derived from hematologic malignancies of myeloid, B and T cell lineages [95, 96]. The physiologic significance of iNKT direct cytotoxicity and its effect in vivo are currently unknown.

Multiple Myeloma and Myelodysplastic Syndrome

Multiple myeloma cells also express CD1d and are effectively killed by autologous iNKT cells. In 1 study, all primary myeloma cells had the CD1d marker, although it had been lost on several multiple myeloma cell lines [97]. Patients with pre-malignant gammopathy, non-progressive myeloma or progressive multiple myeloma were compared for the ability of their peripheral and tumor-localizing iNKT cells to produce cytokines. It was found that patients with progressive disease had a marked deficiency in iNKT IFN- γ production, which was not observed in patients with stable non-progressive disease [97]. IFN- γ production was recovered after incubating these iNKT cells with α -GalCer-pulsed DCs. Patients with myelodysplastic syndromes have also been reported to have iNKT cells deficient in IFN- γ [98].

Alteration of iNKT cytokine production raises the possibility that a tumor-derived glycolipid may be influencing iNKT ac-

tivity. The glycosphingolipid globotriaosylceramide (Gb3) has been found to be shed into the culture medium of a murine T cell lymphoma line [99]. It is interesting to note that Gb3 has a similar structure to iGb3, the endogenous ligand of iNKT cells that was recently characterized [67]. The 2 molecules are derived from the same immediate precursor molecule, lactosylceramide, but Gb3 does not appear to be an activator of iNKT cells. Conversely, it was shown that shed Gb3 could inhibit iNKT recognition of the CD1d+ murine T cell lymphoma and other CD1d expressing cells. Inhibition of glycolipid shedding rescued the iNKT recognition of these cells [99]. Ganglioside shedding in humans has been reported for solid tumors in vivo as well, and has been described as a mechanism of inducing immunosuppression by tumor cells [100, 101]. One rodent tumor cell line was characterized as over-expressing iGb4, a member of the isogloboside family of glycosphingolipids of which iGb3 is also a member [102]. Interestingly, almost all multi-drug resistant cancer cell lines that have been examined have increased levels of secreted β -glucosylceramide [103, 104]. It was found that this glycolipid can prevent ConA-induced hepatitis, a murine model of hepatitis mediated by iNKT cells, suggesting that the glycolipid may somehow inhibit iNKT recognition of CD1d [105, 106]. The significance of endogenous lipid antigen presentation for hematologic and solid tumors has not been extensively characterized, and is an area that is well deserving of further study. In addition to the functional abnormalities observed in multiple myeloma and myelodysplastic syndrome patients, circulating iNKT cells have been found to be depleted in a number of other hematologic malignancies [107]. In 70 patients with a variety of disorders including CML, AML, malignant lymphoma and myelodysplastic syndrome, cells co-expressing V α 24 and CD161, which is a reasonably sensitive and specific combination of markers for identification of iNKT cells in humans, were reduced in peripheral blood compared to healthy controls. In particular, the DN iNKT cell subset was found to be most depleted, and the absolute number of these cells in peripheral blood was correlated positively with survival [107] (table 2).

iNKT Cells in Solid Tumor Immunosurveillance

Activities of α -GalCer or Exogenous IL-12-Activated Mouse iNKT Cells

iNKT cells are considered a promising target for immunotherapy targeted to solid tumors. Several approaches toward this translational goal have been developed in mouse models. Hepatic murine iNKT cells display particularly potent anti-metastatic effects in vivo upon activation with synthetic α -GalCer administered by a variety of different protocols [108]. Early studies documenting the effects of iNKT cells in controlling solid tumors in mice showed that the beneficial

Table 2. CD1d expression and iNKT cells in hematologic malignancies

	CD1d expression on tumor cells	NKT-induced apoptosis*	NKT phenotypic alterations	NKT numbers in peripheral blood
B-CLL	↓ transcription [89] ↑ variability of expression [90]	inducible with α GalCer [90]	N/A	N/A
B-ALL	positively correlated with survival [91]	inducible with α GalCer [91]	N/A	N/A
T-ALL	expressed on some cell lines and primary tumor cells [93]	enhanced with α GalCer [93]	N/A	N/A
AML	present on differentiated phenotypes (M4, M5); absent on M1-M3 [69, 94]	enhanced with α GalCer [69, 94]	N/A	decreased [107]
CML	absent on cells with non-monocytic differentiation [69]	N/A	N/A	decreased [107]
Multiple myeloma	present on all primary cells studied to date, absent on some cell lines [97]	Inducible with α GalCer [97]	Decreased IFN- γ by iNKT cells of patients with malignant type [97]	N/A
Myelodysplastic syndrome	N/A	N/A	Decreased IFN- γ by iNKT cells [98]	decreased [107]
Lymphoma	N/A	N/A	N/A	decreased in malignant lymphoma [107]

N/A = not assessed.

*Only *in vitro* data available. In all cases, NKT-mediated apoptosis was only seen with CD1d+ malignant cells.

effect of exogenous IL-12 on B16 melanoma metastasis to the liver was primarily mediated by iNKT cells, as anti-tumor effect was lost in iNKT cell-deficient mice ($J\alpha 18^{-/-}$ knockout mice) [109]. Many subsequent studies concerning the anti-tumor activity of α -GalCer and IL-12 have been performed, expanding the spectrum of responsive tumor types and furthering the understanding of the mechanisms involved in the anti-tumor effects of iNKT cells. Significant efficacy of α -GalCer in the inhibition of tumor development has been demonstrated for a wide variety of injected tumors including C26 colon carcinoma [71], EL-4 lymphoma [110], 3LL lung/RM-1 prostate/DA3 mammary carcinoma [111] and BNL hepatoma [112]. In addition to a response to injected tumors, efficacy has been shown against spontaneously arising tumors in specific mouse models, such as mammary carcinomas in $Her2/Neu^{-/-}$ mice and sarcomas in $p53^{-/-}$ mice [113].

There are multiple mechanisms that are likely to be involved in the therapeutic effects of activated iNKT cells against solid tumors in mice. As mentioned earlier, there is a sequential cross talk between iNKT cells and DCs. After activation by α -GalCer stimulation, iNKT cells induce and respond to IL-12 production by DCs, which can stimulate their cytolytic T lymphocyte (CTL) activity [114]. Activated iNKT cells produce IFN- γ , which in turn activate NK cytotoxic activity and IFN- γ release, resulting in a rapid wave of IFN- γ production derived from iNKT cells and a second amplified wave generated by

NK cells together with cytolytic activity from both cell types [115]. Aside from its inflammatory properties, IFN- γ also inhibits tumor angiogenesis [111]. The activation of iNKT cells by α -GalCer may also have an impact on other leukocytes, such as the activation of bystander CD8+ T cells or the recruitment of DCs and neutrophils [116–119].

Spontaneous Anti-Tumor Activities of Mouse iNKT Cells

A more physiological role of iNKT cells in immunity versus solid tumors not requiring administration of exogenous IL-12 or α -GalCer can be observed in the prevention of fibrosarcoma development induced in mice by the chemical carcinogen methylcholanthrene (MCA). It was initially found that the presence of iNKT cells and endogenous IL-12 production were critical for the immunosurveillance of spontaneous tumors in this model, as demonstrated by the increased appearance of tumors in $J\alpha 18^{-/-}$ and IL-12p40 $^{-/-}$ mice [120]. *In vitro* studies showed that iNKT cells exerted direct lysis of fibrosarcoma cells. NK cells were also found to contribute to the tumor rejection in this model since treatment of mice with an antibody that selectively depletes NK cells but not iNKT cells also allowed increased tumor growth [121]. Notably, experiments have shown that the spontaneous activity of iNKT cells in controlling MCA-induced sarcomas does not require CD1d expression by the tumor cells [122]. This suggests that the actual killing of tumor cells in this model most likely involves di-

rect recognition by either NK cells or classical CTL, and that the role of iNKT cells is likely to be in facilitating the expansion and activation of these other effector cell populations. In an analogous study in humans, the ability of α -GalCer to enhance the cytotoxicity in vitro of hepatic lymphocytes obtained from 4 patients bearing intrahepatic tumors (of various histological origins) indicated that the cytolytic activity observed was mediated by the activation of CD3⁺ CD56⁺ NK cells. These were probably activated as a result of IL-2 production by iNKT cells in a comparable manner to bystander activation that occurs in mice [123, 124].

Involvement of Mouse CD1d-Dependent NKT Cells in Tumor Recurrence

In a fascinating model of spontaneous tumor regression followed by recurrence in mice employing inoculation with 15–12RM fibroblast-derived tumor cells, it has been found that the recurrence of the tumor is dependent on a population of CD4⁺ and CD1d-restricted NK1.1⁺ T cells [125]. This population of CD1d-restricted T cells mediates tumor recurrence through IL-13 production that leads to TGF- β secretion from myeloid suppressor cells [126]. The production of TGF- β then inhibits the priming or activation of a tumor-specific classical CD8⁺ CTL response which is required to maintain remission. The involvement of a similar or identical population of CD1d-restricted T cells in the inhibition of anti-tumor responses has been recently extended to C26 colon carcinoma metastases in lung by the same group [127]. Other studies that may be detecting a similar phenomenon have reported that CD1d-restricted T cells expressing CD4 and DX5 can suppress immune responses against skin cancer [128]. In all of these studies, the CD1d-restricted T cells responsible for the suppression of anti-tumor CTL have been shown to be absent from CD1d-deficient knockout mice, consistent with a role for either type I or II NKT cells or both. However, none of the published studies have investigated whether the phenomenon is retained or absent in iNKT cell-deficient J α 18^{-/-} mice.

iNKT Cells in Patients with Solid Tumors

iNKT cells have been investigated in patients with a variety of solid tumors. In general, the status of iNKT cells in cancer patients has been documented in peripheral blood lymphocytes (PBL), but rarely in the tumor or in the corresponding healthy tissue. This has led to the observation of numerical and/or functional deficiencies in most cases. As mentioned above, decreased numbers of peripheral iNKT cells may indicate homing to target tissues. In some cases, the iNKT cells from PBL of patients with cancer have also shown impaired proliferation upon α -GalCer stimulation, although their lytic activity could be restored in vitro [129]. In a study of patients with cutaneous melanoma, a decreased number of iNKT cells in peripheral blood was observed [130]. This was confirmed in another study that also reported a significant loss of iNKT cells in the circulation of patients with breast cancer but not with

colon carcinoma [131]. Numbers of iNKT were decreased in samples from 10 liver samples bearing colon carcinoma metastases (0.098% of CD3⁺ T cells as compared to 0.48% in normal liver), and these cells retained the ability to produce IFN- γ upon stimulation ex vivo with α -GalCer [132]. A decreased peripheral iNKT cell frequency has been reported in patients with prostate cancer. In addition, these cells were deficient in IFN- γ release [133]. Decreased circulating iNKT cell numbers have also been reported in lung carcinoma patients, but they retained detectable expression of IFN- γ mRNA ex vivo. In tumoral lung, iNKT cells were increased to over twice the number found in non-tumoral lung, as assessed by semi-quantitative PCR (polymerase chain reaction) specific for the invariant V α 24-J α 18 rearrangement [134]. Another study reported a weak peripheral decrease of circulating iNKT cells in patients with stages I–IV lung carcinoma, and significant reductions in iNKT cell numbers and decreased in vitro proliferation in response to α -GalCer were found in patients with tumor recurrence [135].

iNKT cells have also been studied in brain malignancies. In the circulation of patients with glioma, iNKT cells displayed similar frequency, distribution of the CD4⁺/DN/CD8⁺ subsets and reactivity to α -GalCer when compared to healthy donors [136]. No differences in peripheral iNKT cell frequencies were identified in neuroblastoma patients when compared to healthy donors [136]. iNKT cells were readily detectable by RT-PCR (reverse transcription PCR) of V α 24-J α 18 transcripts in 54% of neuroblastoma tissues, and the recruitment was suggested to be dependent upon CCL2/CCR2 chemokine interaction. In addition, the specific detection of the invariant TCR- α chain mRNA in neuroblastomas was correlated with a better prognosis [137].

Clinical Trials of α -GalCer in Cancer Patients

Given the often dramatic effects of the α -GalCer iNKT activator KRN7000 on inducing regression of murine cancers, there has been a strong impetus to translate these findings into clinical practice. To date, 4 phase I clinical trials using KRN7000 have been reported in patients with tumors of various histological origins. In all trials, treatment was well tolerated, demonstrating the lack of serious acute toxicity of α -GalCer. In the first phase I clinical trial, patients with advanced cancers were given intravenous injections of α -GalCer [138]. 24 patients with diverse solid tumors were given 3 consecutive weekly doses of KRN7000 ranging from 50 μ g/m² per week to 4,800 μ g/m². No serious drug-related adverse events occurred during the study. Even at the lowest dose tested, evidence of an iNKT cell response in vivo was observed, as measured by cytokine production and reduction of detectable circulating iNKT cells indicating receptor down-modulation following activation. The responses seen were not dose-dependent but did appear to correlate with the patient's initial iNKT cell count. Biological effects could be observed in 10 out of 24 patients, representing the subgroup that had high pretreat-

ment levels of iNKT cells. All of these patients showed a loss of detectable iNKT cells after injection. Among them, only 5 displayed cytokine induction in serum samples, with detectable levels of INF- γ , IL-12, GM-CSF, TNF or various combinations of these cytokines. A transient decrease of blood NK cell numbers and cytotoxicity was also observed in 7 patients. This trial was not designed to assess clinical outcomes as an endpoint. Although no statistically significant clinical responses were observed, several patients in the treatment arm had relatively long-lasting stable disease.

Another trial using adoptive transfer of α -GalCer-pulsed immature DC administered by 2 intravenous infusions separated by a 2 week interval was performed in 12 patients with diverse cancers [139]. A transient decrease in detectable iNKT, NK, T and B cells was observed. Levels of IFN- γ and IL-12 were detectable in serum samples from 6 out of 9 patients assessed after the second infusion, together with T, B and NK cell activation shown by expression of the CD69 activation marker. An increase in NK cell cytotoxicity was observed with 5 out of 11 patients. Again, no clinical response was observed. Interestingly, ^{111}In -labelled α -GalCer-pulsed DCs were found to mainly home to the liver after 24 h, suggesting that this tropism may be exploited for the treatment of malignancies resident in the liver.

A third trial included 9 patients with advanced and recurrent lung carcinoma who received 1 intravenous injection of control immature DCs, followed by 4 intravenous injections of α -GalCer-pulsed immature DCs at 1–5 week intervals [140]. This study divided the patients into 3 groups of 3 patients each, who received different numbers of DCs. In only the three patients who received the highest dose (10^9 cells/m 2) was there an observed biological effect. This consisted of an increase in iNKT cell frequency 2 weeks after the first α -GalCer loaded DC injection and an iNKT cell-derived IFN- γ mRNA increase 1 week after the second injection. Again, no objective clinical response was observed.

The fourth clinical trial, with 1 injection of control mature DCs, followed by 2 injections of α -GalCer-loaded mature DCs, revealed the strongest biological effects to date [141]. Of 5 patients who displayed weakly detectable iNKT cell numbers prior to therapy, all showed an increase in iNKT cell numbers, especially after the second injection of α -GalCer-pulsed mature DCs. Up to a 100-fold increase was observed, and this was associated with IL-12 and IFN- γ -inducible protein-10 release in serum. Investigation of the CD4 $^+$ /DN/CD8 $^+$ iNKT cell subsets from these patients revealed that the CD4 $^+$ iNKT cell subset predominated after the first α -GalCer/DC injection, but that the DN and CD8 $^+$ subsets became dominant following the second injection. Freshly isolated iNKT cells displayed IFN- γ cytokine production after the second injection in 1 patient. In addition, the 3 HLA-A2 patients who could be assessed for virus-specific CTL showed an increase in influenza- or CMV-specific CD8 $^+$ T cells following treatment, and these cells displayed higher in vitro proliferation in re-

sponse to specific peptide stimulation. This suggests that iNKT cell recovery in patients treated with α -GalCer-pulsed mature DCs favors the development of MHC class I restricted conventional CD8 $^+$ T cell immunity. This may be exploited during vaccination with tumor antigen-specific peptides as has been suggested elsewhere [142].

Although these 4 phase I clinical trials have not yet demonstrated that this approach can lead to significant clinical benefit, it is encouraging that the therapy has been well tolerated and that biologic effects on iNKT cells can be observed in some cases. The potential anti-tumor activity of α -GalCer-activated iNKT cells as a single agent therapy may have been overestimated because of the dramatic effects in mice, which are attributable in part to the high frequency of both iNKT and NK cells in the murine liver and the extensive usage of models with tumor metastases to that organ. Such activity may not be expected from human intrahepatic lymphocytes, as the numbers of iNKT cells are normally much lower, more similar to what is found in the blood [26]. In addition, murine tumor models are usually not representative of the long window of silent tumor development and associated changes to the immune system that often occurs during the progression to malignancy before the tumor becomes clinically detectable. Immunotherapy of cancers with iNKT cell activators may be a safe and effective addition to currently used regimens for treating cancers. Once its safety has been sufficiently assessed, it may be expected that studies with more statistical power and a primary outcome of prolonged disease-free survival will be designed.

iNKT Cells in Prevention of GVHD

Host iNKT cells are important in preventing graft versus host disease (GVHD) in mouse models. In an MHC-mismatched bone marrow (BM) transplantation model, GVHD is accelerated in the absence of iNKT cells [143]. The same study found that iNKT cells caused delayed engraftment and in some cases rejection of the transplanted cells. Adoptive transfer of iNKT cells together with the transplanted BM cells attenuated GVHD, but the presence of host-residual iNKT cells was needed for this protective effect. The protection offered by transferred iNKT cells was lost in a $\text{J}\alpha 18^{-/-}$ iNKT cell-deficient mouse. These results suggest a beneficial effect of host-specific iNKT cells that can be enhanced but not replaced by adoptive transfer of iNKT cells.

A role for iNKT cells in hematopoietic stem cell transplantation and GVHD in humans has also been proposed [144]. In patients receiving peripheral blood stem cell transplant, iNKT cells were reconstituted within 1 month, while for BM transplant recipients, the numbers of iNKT cells remained low 1 year after engraftment. iNKT cell counts were then compared to clinical outcomes of transplantation. iNKT cell numbers were reduced in the peripheral blood of patients with

acute, extensive and chronic GVHD, with the lowest numbers found in patients with chronic extensive GVHD, thus suggesting a role for iNKT cells in the prevention of GVHD in humans as well as in mice.

Type II NKT cells may also play a role in preventing GVHD. In mice it has been found that peripheral blood T cells are 30 times more potent at inducing lethal GVHD than BM T cells. NK1.1+ T cells are less than 1% of the population of murine peripheral blood T cells, but they are over 30% of BM T cells. Sorted NK1.1- BM T cells had increased potency in the induction of GVHD, but adding back NK1.1+ cells abrogated this effect [145]. Analysis of BM-derived NK1.1+ cells showed that the large majority are CD1d-dependent with over-representation of type II NKT cells [146]. While not definitive proof, this is suggestive that type II NKT cells play a role in suppressing GVHD in addition to iNKT cells. Human BM has also been found to be enriched in CD161+ cells, over half of which are CD1d-restricted. Of these, most express a diverse TCR and are thus type II NKT cells [147]. This study also found that human BM-derived NKT cells were Th2-biased and could suppress a mixed lymphocyte response.

A selected non-myeloablative conditioning regimen that preserves cells expressing DX5 (an NK marker that is also expressed on a fraction of iNKT cells) or NK1.1+ cells in the host prior to transplantation has been shown to attenuate the acute GVHD reaction in mice [148, 149]. CD1d expression by the mouse was required for the protective effect of this regimen. Further phenotyping of the iNKT cells from these mice showed that they were biased towards a Th2 response. The adoptive transfer of DX5+ cells decreased GVHD in a murine semi-allogeneic BM transplant GVHD model. Transfer of these cells led to 85% survival of the host on day 28 post-transplant, while depletion of these cells in the host mouse led to 100% mortality [150]. Peripheral blood cytokine profiling again demonstrated a Th2 bias, with decreased IL-12 and IFN- γ production relative to IL-10 and IL-4 production. Activation of host iNKT cells by α -GalCer injection was also sufficient to prevent GVHD in mice. Treatment prevented the development of GVHD in almost all mice receiving haploidentical MHC-mismatched splenocytes. Nearly all α -GalCer-treated mice remained healthy for more than 235 days, while vehicle-treated mice died after an average of 3 weeks [151]. The beneficial effect of α -GalCer is dependent on IL-4 production, since protection from GVHD by α -GalCer injection was lost in an IL-4-/- knockout mouse [152].

iNKT Cells in Promotion of Tolerance to Solid Organ Transplants

iNKT cells are important, and in some cases required, for solid tissue graft survival. This is in agreement with their putative role in delaying hematologic cell transplantation. The same role that iNKT cells play in suppressing transplanted

hematologic cells may also suppress the body's immune cells from responding to a solid tissue transplant. In both cases, iNKT cells may serve as immunosuppressive regulatory cells. Several studies have shown that iNKT cells are required for successful transplant of heart allografts in a mouse model. Rejection of MHC-mismatched allografts was not different between wild type (WT) and iNKT cell-deficient mice, but in models in which tolerance was induced against the graft by blockade of lymphocyte function-associated antigen-1/intercellular adhesion molecule-1 or CD28/B7 interactions, long-term acceptance of the grafts was observed only in WT but not in iNKT cell-deficient mice [153]. Parallel experiments using IFN- γ - and IL-4-deficient mice suggest that IFN- γ is required for allograft acceptance in vivo, apparently opposite to the IL-4/Th2 biasing of iNKT cells seen in suppression of GVHD [153].

In a similar study, post-transplant conditioning with donor BM cells to develop immune cell chimerism and promote tolerance of an allogeneic heart transplant was performed with CD1d-/- host and donor mice [154]. Host mice without type I and II NKT cells (CD1d-/-) or specifically lacking type I NKT cells (J α 18-/- knockout mice) failed to achieve tolerance to the graft despite the development of mixed chimerism. Tolerance could be restored in these mice by injection of enriched BM T cells from WT mice but not from CD1d-/- mice that lack CD1d-restricted NKT cells. Tolerance could not be induced in this model in either IL-4-/- or IL-10-/- hosts, suggesting that induction of a Th2 response by NKT cells might be critical for allograft tolerance.

In a study that might be relevant to the future treatment of diabetes by pancreatic islet transplantation, the role of iNKT cells was examined when rat pancreatic islet cells were transplanted intrahepatically into a mouse host. In this study, it was found that an anti-CD4 monoclonal antibody (mAb) was sufficient to allow acceptance of the transplant without immunosuppressive drugs [155]. However, rat islet xenografts were rejected, despite the anti-CD4 mAb treatment, in iNKT cell-deficient mice, and adoptive transfer of iNKT cells into these mice restored the acceptance of the islet xenografts. The conclusion was drawn that iNKT cells were essential for acceptance of pancreatic islet xenografts in this model. The choice of intrahepatic transplantation may have been a fortuitous experimental design, since iNKT cells are a remarkably large percentage of hepatic lymphoid cells in the mouse [26]. In contrast to most experimental settings where iNKT cells are found to enhance survival of transplants, one study using a porcine neuron xenotransplant mouse model found that survival of the neurons was enhanced in a CD1d-/- mouse [156].

Pregnancy can be considered a special model of rejection-free introduction of allogeneic tissue. A study of the maternal-fetal interface has shown that CD1d is expressed on fetal trophoblastic cells in the decidua, and maternal iNKT cells are present at the decidua maternal-fetal interface at levels 10 times seen in maternal peripheral blood [157]. The role of

iNKT cells at the maternal-fetal interface may be to induce tolerance to a foreign tissue, which may be relevant to understanding iNKT cells in transplant tolerance.

Information from human studies on the possible roles of iNKT cells in transplant tolerance are lacking at present. A study of human kidney transplant recipients compared iNKT cells of patients with 10 years of long-term rejection-free (LTRF) cadaver kidney allograft survival to those with acute rejection and to patients with acute tubular necrosis [158]. Total numbers of circulating iNKT cells did not differ significantly between the groups, although the frequency was lowest in the LTRF group. However, CD4⁺ iNKT cells were more frequent in acute rejection at levels 3–7 times higher than seen in the other groups.

iNKT Cells and Autoimmunity

Many experimental models of autoimmune diseases are improved or worsened by the presence and activation of iNKT cells. Autoimmune type 1 diabetes has been most extensively studied, since the discovery that non-obese diabetic (NOD) mice are deficient in iNKT cells [159]. It has been shown that manipulations that increase the numbers of iNKT cells or activation of the residual iNKT cells with α -GalCer in these mice prevent disease progression [160, 161]. Some studies have linked the protective effect of iNKT cells to the production of Th2 cytokines such as IL-4 and IL-10 [162]. Consistent with this view, the Th2-biasing compound OCH, which abrogates IFN- γ production by iNKT cells but induces strong IL-4 production, prevented diabetes progression as well or better than the KRN7000 form of α -GalCer which elicits a more mixed cytokine response that includes high levels of both IL-4 and IFN- γ [163]. However, other studies have found that KRN 7000 treatment elicits equivalent levels of protection against diabetes in standard NOD mice and in NOD mice genetically deficient in IL-4 and/or IL-10 [164], and suggest that the protective effects of KRN7000 may be due to the recruitment of mature tolerogenic myeloid DCs into peripheral lymph nodes [165]. An iNKT deficiency was also reported in the genetically similar non-obese resistant (NOR) mice that do not develop diabetes, proving that a deficiency of iNKT cells is not sufficient to cause diabetes in mice [166]. However, iNKT adoptive transfer or activation can prevent disease in NOD mice. Some studies of human subjects have confirmed that there are differences in iNKT phenotype, numbers and function between healthy and diabetic subjects, although at least one study refutes this, and the point remains controversial [167–169].

The Th2 cytokine production by iNKT cells that is believed to have a protective effect against diabetes is thought to prevent several other autoimmune diseases as well. iNKT cells are also implicated in multiple sclerosis (MS) and the animal model of this CNS demyelinating syndrome, experimental autoimmune encephalitis (EAE). iNKT cells are reduced in the peripheral

blood of MS patients [169]. iNKT cell lines derived from patients in remission have a Th2 bias compared to cell lines from patients with active disease [170]. However, the protective effect of α -GalCer is less clearly defined for EAE than for diabetes. Studies have found iNKT activation to alternatively protect from EAE disease progression [171, 172] to have no effect [49, 173] or to accelerate disease [171]. The Th2-biasing α -GalCer analogue OCH has also been shown to protect against EAE [49]. Timing of α -GalCer administration may determine the role of iNKT cells in this model, as simultaneous injection of α -GalCer and EAE-inducing myelin-reactive T cells accelerated disease, while prior activation of iNKT cells was protective [171].

Systemic lupus erythematosus (SLE) is another disease that has received attention for the role of iNKT cells in its pathogenesis. In (NZB $\times$ NZW)F1 lupus-prone mice, iNKT cells expand dramatically prior to and after onset of clinical disease and appear to exert an aggressive pro-inflammatory influence [174]. Recent studies in a model of murine lupus induced by injection of the hydrocarbon oil pristane showed that iNKT cell activation with KRN7000 can either suppress or promote pristane-induced lupus-like autoimmunity, depending on the strain of mice used [175]. In patients with clinically active SLE, circulating iNKT cells are reduced compared to healthy controls [176].

Autoimmune diseases such as rheumatoid arthritis, Sjögren's syndrome, dermatomyositis, systemic sclerosis and others are also reported to have reduced numbers of peripheral blood iNKT cells [177, 178]. It is unclear whether the aberrant features of iNKT cells in various autoimmune diseases is related to recognition of specific auto-antigens, or whether they are being influenced by changes in the cytokine milieu or other non-specific mechanisms. Decreased numbers of iNKT cells in autoimmune diseases may be causing a loss of tolerance to self-antigens, or they may be leaving the periphery to actively participate in tolerance induction. Peripheral blood is more readily available than biopsy samples and is often the only available tissue to analyze. However, patients with primary biliary cirrhosis (PBC) were found to have similarly reduced peripheral blood iNKT cells, but liver biopsies found these cells to be substantially elevated [179]. This finding illustrates the difficulty in interpreting results from studies using only peripheral blood, as decreased circulating iNKT cells may either indicate an absolute deficiency of these cells in the body, or conversely could indicate that they are actually normal or expanded but have left the blood to home to an affected organ.

NKT Cells and Foreign Antigen Tolerance Induction

Systemic tolerance can be elicited by introduction of a foreign antigen into an immune-privileged site, such as the eye in which it is known as associated immune deviation (ACAID). It can also be produced by intravenous or oral introduction of

the antigen. Tolerance to a foreign antigen abrogates the delayed-type hypersensitivity that is normally elicited when the same antigen is reintroduced. Several studies now implicate iNKT cells in ACAID [180]. iNKT cells appear to be essential for this mechanism of tolerance induction, as CD1d^{-/-} mice were unable to develop ACAID unless reconstituted with iNKT cells and CD1d⁺ antigen-presenting cells [181]. Furthermore, either antibody depletion of NK1.1⁺ iNKT cells or administration of anti-CD1d antibody which blocks the interaction between CD1d and the iNKT cell TCR, were sufficient to prevent tolerance to an antigen introduced in the eye. Further investigation into the role of iNKT cells in the ACAID model has elucidated part of the mechanism of this phenomenon, showing that iNKT cells cause the differentiation and activation of T regulatory (Tr) cells in the spleen through an interaction that requires iNKT cell production of IL-10 [182]. It has also been shown that CD1d expression is necessary on antigen-presenting cells similar to those which travel from the eye to the spleen in ACAID and on splenic marginal zone B cells for the generation of Tr cells by iNKT cells [183]. Other studies have documented similar phenomena involving tolerance to antigens administered via the oral or portal venous route [184].

X-Linked Lymphoproliferative Disorder and iNKT Cells

X-linked lymphoproliferative disorder (XLP) is a disorder of immunity manifested by Epstein Barr Virus (EBV) sensitivity leading to fulminant infectious mononucleosis, impaired anti-tumor immunity, lymphoma and hypogammaglobulinemia. A surprising story with NKT cells as a protagonist is unfolding for this rare but uniformly fatal inherited disease. A defect in the signal transduction gene SH2D1A and its protein product, SAP, is responsible for the XLP phenotype, and NKT cells have been shown to be markedly reduced in patients with the disease and in a SAP knockout mouse [185–187].

NKT cells development has been shown to be uniquely dependent on the signal transduction protein FynT, which is not required for NK or conventional T cell development [188, 189]. FynT was found to be activated by interaction with 2 other signaling proteins, signaling lymphocytic activation molecule (SLAM) and SLAM-associated protein (SAP) [190]. Because the SAP protein is responsible for XLP, iNKT cells were examined from patients with the disease. In one study, 17 patients with germline mutations of SAP were deficient in iNKT cells [191]. A female carrier of the XLP mutation showed completely skewed X chromosome inactivation in iNKT cells but not in T or B cells, illustrating that the mutation is specific for iNKT development. NKT cell deficiency may be in part responsible for the phenotype of XLP, although SAP is also expressed in T and NK cells where it regulates cytokine production and cytotoxicity. At this time, the extent to which the deficiency of NKT cells is responsible for the clinical immunodeficiency of XLP is unclear.

NKT Leukemia/Lymphoma

The syndrome that is often designated NK-like T cell leukemia/lymphoma in the literature is an aggressive malignancy with a clinical course that is similar to blastic NK cell leukemia/lymphoma [192]. The tumor is associated with EBV infection, is found most commonly in Asia and South America and is often intranasal. Tumors within this classification can express a TCR on their surface, although most cases do not and are believed to be derived from an NK cell precursor. Two studies of 42 intranasal and 47 extranasal cases found that 27 and 15% of tumors, respectively, expressed a monoclonal TCR gene rearrangement indicating a T cell derivation [193, 194]. The clinical course was not correlated with the immune cell of origin. These cells are likely NKT-like cells, and there is no current evidence to suggest that these tumors are derived from CD1d-restricted NKT cells. Testing of these cells for CD1d restriction may be useful for further characterization. It is interesting to note that both XLP and NK-like T cell lymphoma/leukemia are associated with EBV infection, suggesting a possible connection despite different causality.

Conclusions

Markers of NK cells are now appreciated to be fairly widely expressed on human T cells, particularly with activation. On the other hand, CD1d-restricted T cells, which frequently express NK markers, are a small population that is increasingly recognized as being important for fully understanding many disease processes. NKT cells, as defined here, are activated by interaction with CD1d-presented lipid agonists which in many cases have yet to be defined. The subset known as iNKT cells is unique in the limited diversity of its TCRs and, as a result, the ease with which it can be studied. However, even within this subset, substantial functional and phenotypic diversity seems to exist. The iNKT cell subset may turn out to be one group of cells that can switch phenotypes, or it may be that there are several distinct lineages of these cells. In addition to the cytokines and mediators of cytotoxicity that have been described for activated iNKT cells, these cells also coordinate the activation of downstream effector cells such as NK cells, Tr cells, DCs and B cells. There is evidence to suggest that the most important function of NKT cells is to regulate the activation of other immune cells, fine-tuning the immune system based on subtle changes in the lipid pool. How NKT cells respond differently to various stimuli is currently an area of active investigation and will be important for designing therapies that are safe and effective.

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