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TOWARD AN UNDERSTANDING OF NKT CELL BIOLOGY: Progress and Paradoxes

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■ **Abstract** Natural killer T (NKT) cells constitute a conserved T cell sublineage with unique properties, including reactivity for a synthetic glycolipid presented by CD1d, expression of an invariant T cell antigen receptor (TCR) α chain, and unusual requirements for thymic selection. They rapidly produce many cytokines after stimulation and thus influence diverse immune responses and pathogenic processes. Because of intensive research effort, we have learned much about factors promoting the development and survival of NKT cells, regulation of their cytokine production, and the means by which they influence dendritic cells and other cell types. Despite this progress, knowledge of the natural antigen(s) they recognize and their physiologic role remain incomplete. The activation of NKT cells paradoxically can lead either to suppression or stimulation of immune responses, and we cannot predict which will occur. Despite this uncertainty, many investigators are hopeful that immune therapies can be developed based on NKT cell stimulation.

INTRODUCTION

There are several remarkable features of natural killer T (NKT) cells that have emerged from studies carried out during the previous decade. First, although they constitute less than 1% of mouse T lymphocytes, they exert a critical influence on a variety of immune responses and pathologic conditions (1–6). Second, to a surprising extent they function differently from conventional T cells. A salient feature that distinguishes NKT cells is their ability to rapidly secrete a variety of cytokines within a few hours after activation (7, 7a). Therefore, not surprisingly, this small lymphocyte subpopulation has captured the attention of many immunologists. Many excellent reviews pertain to NKT cells, including several previous ones in this series (1–6, 8). In this article, I give a brief overview of the known properties of mouse and human NKT cells, while highlighting recent findings and the important unresolved questions concerning the development, specificity, and function of these unique T lymphocytes.

WHAT IS AN NKT CELL?

It is important to define unambiguously what we mean by the term "NKT cell." Operationally, these lymphocytes were originally characterized in mice as cells that express both a T cell antigen receptor (TCR) and NK1.1 (NKR-P1 or CD161c), a C-lectin type NK receptor (8). This definition is not entirely satisfactory, however, and more recently NKT cells have been defined as cells that nearly always have an invariant Vα14-Jα18 rearrangement and reactivity to the glycosphingolipid α-galactosylceramide (αGalCer) (Figure 1) when presented by the class I–like molecule CD1d (9). Defined operationally in this way, NKT cells are the group of lymphocytes that can be detected by flow cytometry using tetramers of CD1d

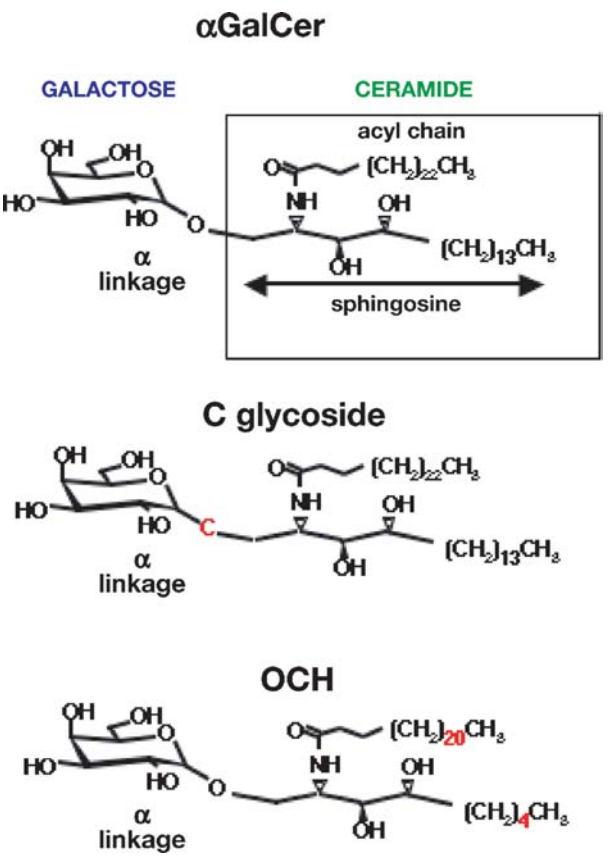

Figure 1 Structure of αGalCer and related compounds. The ceramide lipid portion of the molecule is boxed, and the aliphatic acyl chain (*upper*) and sphingosine base (*lower*) of the ceramide are indicated. How the C-glycoside and OCH differ from αGalCer is indicated in red.

loaded with αGalCer (9). They are known by several other names, including type I NKT cells, invariant (*i*) NKT cells, and Vα14*i* NKT cells (9); the latter term is used here. In many cases, Vα14*i* NKT cells do not express NK1.1 (9), and other T cells, including conventional, virus-specific $CD8^+$ T cells, can induce NK1.1 expression (10), hence making the earlier definition based on NK1.1 and TCR coexpression too imprecise.

Humans have a homologous population of T cells with an invariant Vα24 rearrangement (Vα24*i*) (11, 12) and reactivity to αGalCer when presented by human CD1d (13, 14). In humans, however, the dichotomy between CD161 expression and reactivity with CD1d tetramers is very pronounced. Most $NK1.1^+$ $TCR\alpha\beta^+$ mouse T cells are CD1d tetramer reactive (7, 15). However, although 5%–10% of human peripheral blood T cells express CD161a, only approximately 0.1% are reactive with CD1d tetramers (16–19).

DEVELOPMENT OF Vα14*i* NKT CELLS

Vα14*i* NKT Cells Are a Sublineage with Unique Requirements for their Selection

Using CD1d tetramers permits investigators to detect developing Vα14*i* NKT cells in a normal physiologic setting, without the potentially distorting effects on the repertoire of TCR transgenes. Investigators now agree that Vα14*i* NKT cells arise in the thymus in the perinatal period and do not reach significant levels until at least three weeks after birth (20–22). Their positive selection is mediated by CD1d-expressing bone marrow–derived cells rather than by cortical epithelial cells (8), a finding that helped investigators establish the distinctive nature of this T lymphocyte subset. Among the bone marrow–derived cells, $CD1d^+$ double-positive thymocytes are almost certainly the critical cell type for positive selection (23, 24). In addition to this requirement by Vα14*i* NKT cells for an unusual positively selecting cell type, Vα14*i* NKT cell development and maturation are differentially effected by a number of mutations that have relatively little effect on conventional cells (25). Several recent studies have focused on members of the NF-κB family. Vα14*i* NKT cell development requires the expression of NF-κB1 (p50) in a cell-autonomous manner (26, 27). NKT cell development also requires the expression of the gene for the inhibitor of κB kinase, *Ikk2* (28). Moreover, RelB (p65) expression in an irradiation-resistant cell is required in NKT cell development (26, 29). Recently, it has been shown that, for their differentiation, Vα14*i* NKT cells require the transcription factor T-bet (T-box expressed in T cells) (30), a factor originally identified as important for the induction of IFN-γ synthesis and Th1 immunity in several cell types.

Why are the genetic requirements for Vα14*i* NKT cell development unique and complex? A few of the mutations that specifically diminish the Vα14*i* NKT cell population, such as those affecting AP-3 subunits and prosaposin, probably act by affecting the pathway required for the loading of endogenous glycolipids into the

CD1d groove. The adaptor protein AP-3 binds to the CD1d cytoplasmic tail and is required for CD1d trafficking to lysosomes (31, 32), where endogenous glycolipids may be processed and loaded into CD1d. Sphingolipid activator proteins, four of which are encoded by the prosaposin precursor, are lysosomal proteins that interact with CD1d molecules. They make lipids available for CD1 loading, and they apparently perform an editing or quality control function for the lipids bound to CD1d (33, 34).

In contrast to conventional thymocytes, most of the Vα14*i* NKT cells in the thymus are part of a mature, immune-competent population, capable of producing IL-4 and IFN-γ immediately after TCR stimulation (21, 22, 35). Therefore, the set of genes required for Vα14*i* NKT cell differentiation may reflect not only those required for their early maturation but also those that are required for lymphocyte expansion and differentiation to effector cells. This could explain, for example, the requirement for expression of NF-κB transcription factors in developing Vα14*i* NKT cells, as these transcription factors seem to affect the survival of the Vα14*i* NKT cells after they have expressed the TCR, undergone some expansion, and reached an intermediate stage of their differentiation (27).

Precommitment or Instruction?

The use of αGalCer-loaded CD1d tetramers has permitted several research groups to analyze the phenotype of Vα14*i* NKT cells during these later stages of their differentiation, after they have acquired a TCR. Expression of several molecules, including the IL-7 receptor, CD24, DX5, NK1.1, and Ly49 family NK receptors, occurs during later maturation steps (21, 22, 27, 36). Induction of NK1.1 expression probably can occur in the thymus, although most of the recent thymus emigrants are NK1.1 negative (21, 22), suggesting that the final maturation stages for Vα14*i* NKT cells also occur in the periphery. An unresolved issue concerns the elements that direct developing thymocytes into this sublineage. Two alternative models are illustrated in Figure 2. Investigators have presented evidence suggesting that Vα14*i* NKT cells have a double-positive precursor (20). The existence of a double-positive precursor is consistent with an instructional model in which the expression of the Vα14*i* TCR and recognition of endogenous ligands presented by CD1d commit the developing precursor to become a Vα14*i* NKT cell. A relatively high-affinity recognition of endogenous ligands presented by CD1d and/or the consequences of selection by other double-positive thymocytes may be responsible for this commitment. Other researchers have suggested that a subset of thymocytes is precommitted to become Vα14*i* NKT cells before antigen receptor rearrangement. For example, the requirement for the SRC family kinase Fyn in NKT cell development is cell autonomous (37, 38). In contrast, conventional T lymphocytes undergo relatively normal thymus differentiation in the absence of Fyn. This special requirement exhibited by of Vα14*i* NKT cells can be overcome by expression of a Vα14*i* transgene (39). In mixed bone marrow chimera experiments, in which *fyn*$^{+}$ and *fyn*$^{-/-}$ Vα14*i* transgenic precursors were cotransferred

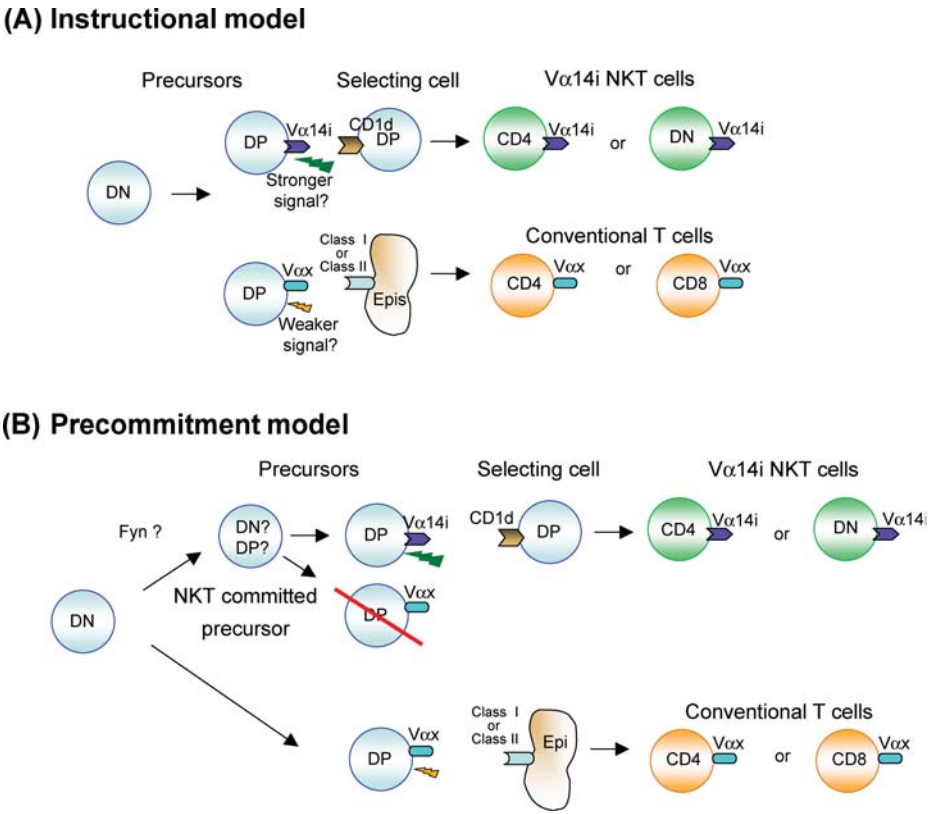

Figure 2 Models for the development of Vα14*i* NKT cells. (*A*) In the instructional model, expression of the Vα14*i* TCR instructs thymocytes to become Vα14*i* NKT cells, perhaps because of a high-affinity interaction with CD1d-presented ligands or interaction with double-positive thymocytes. Expression of other TCRs (Vαx) leads to the positive selection of conventional T cells by MHC class I– and class II–expressing epithelial cells (Epis). (*B*) According to the precommitment model, a precursor decides to become a Vα14*i* NKT cell before TCR rearrangement, as depicted.

to irradiated recipients, the *fyn*$^{-/-}$ precursors contributed to the Vα14*i* NKT cell compartment as effectively as those that express Fyn (39). On the basis of these results, investigators have suggested that Vα14*i* NKT cell deficiency in *fyn*$^{-/-}$ mice is not simply due to a failure to properly signal through the Vα14*i* TCR or to expand Vα14*i* NKT cell precursors. They suggest instead that *fyn* acts upstream of TCR expression, and that it in some way aids in the TCR-independent commitment of thymocytes to the Vα14*i* NKT cell sublineage, with expression of the transgene acting downstream to overcome this defect. Vα14*i* NKT cells do not have a preferential rearrangement of Vα14*i* on the unexpressed allele (40), and therefore even if

a precommitment occurred, the cells precommitted to this sublineage would then also have to undergo selection on the basis of TCR specificity.

Agonist Selection of Vα14*i* NKT Cells?

A growing body of evidence favors the concept that the thymus does not discard all self-reactive T cells and that some potentially self-reactive T lymphocytes are preserved to carry out specialized or regulatory functions (41). Consistent with this theory, evidence suggests that TCR$\alpha\beta^+$ CD8$\alpha\alpha$ intraepithelial lymphocytes (42, 43) and $CD4^+$ $CD25^+$ regulatory T cells are inherently self-reactive (44). The CD1d autoreactivity of Vα14*i* NKT cells has led to the suggestion that these T lymphocytes also are positively selected by self-agonist ligands (8).

The forced expression of CD8α and CD8β transgenes in T cells leads to a decrease in Vα14*i* NKT cells (45), suggesting that CD8-mediated enhancement of the intrinsically high affinity of the Vα14*i* TCR for self-antigens presented by CD1d pushes the developing Vα14*i* NKT cell over the threshold of negative selection. Direct evidence is lacking, however, indicating that CD8 can serve as a coreceptor for CD1d-mediated antigen recognition, and the CD8 transgenic mice have other abnormalities, including a decrease in total thymocyte numbers (45). An alternative model for the lack of CD8 expression by Vα14*i* NKT cells is the finding that increased or prolonged Lck signaling preferentially directs thymocytes to be CD4 positive (46, 47). Therefore, although the reason for the lack of CD8 expression by Vα14*i* NKT cells remains to be determined, the concept that they are positively selected by self-agonists or relatively high-avidity interactions remains an appealing one. Unlike in mice, in humans a minority of Vα24*i* NKT cells expresses CD8α, and some express CD8β as well (17, 19, 48, 49).

Negative Selection of Vα14*i* NKT Cells

Although Vα14*i* NKT cells may require self-agonist for their development, there must be an upper limit to the avidity window that permits their positive selection, as the results from several experiments support the concept that these cells can be negatively selected in the thymus. Addition of αGalCer to fetal thymic organ cultures causes Vα14*i* NKT cell negative selection (50, 51), and increased expression of CD1d in transgenic mice causes a decrease in the number of Vα14*i* NKT cells (50). Moreover, the remaining Vα14*i* NKT cells in these CD1d transgenic mice were hyporesponsive. Mouse Vα14*i* NKT cells tend to coexpress Vβ8.2, constituting more than 50% of the total, with Vβ7 and Vβ2 also highly represented (45). Evidence suggests that the Vβ8.2-containing Vα14*i* TCRs tend to have the highest affinity for αGalCer presented by CD1d (52), and in the CD1d transgenic mice, Vβ8.2 Vα14*i* NKT cells were underrepresented, which is consistent with the elimination of cells expressing the highest affinity TCRs (50). Chronic exposure of young adult mice to αGalCer also led to Vα14*i* NKT cell deletion (53). When treatment was halted, newly exported cells from the thymus reconstituted the Vα14*i* NKT cell population. The recovered cells were hyporesponsive,

however, and they had increased expression of inhibitory NK receptors of the Ly49 family (53). This is consistent with other data indicating that the balance of signals between activating and inhibitory NK receptors, as well as TCR avidity, sets the affinity threshold and regulates the development of Vα14*i* NKT cells (54).

HOMING AND HOMEOSTASIS

Vα14*i* NKT cells are most prevalent in the thymus, spleen, liver, and bone marrow, with at least 5×10^5 cells generally found in each site (7, 15). They are much less abundant in lymph nodes and are rarely found in other tertiary sites such as the intestinal mucosa. They express a set of chemokine receptors consistent with tissue-seeking effector cells, with relatively little expression of CCR7 (55–57). In humans, there are differences in the chemokine receptors expressed by the $CD4^+$ and $CD4^-$ Vα24*i* subsets (55), as well as functional differences in the cytokines they produce (49, 58). There is much less evidence for the existence of functional subsets or of a regional specialization of mouse Vα14*i* NKT cells. Consistent with their chemokine receptor expression, mouse Vα14*i* NKT cells can migrate to sites of inflammation in the lung (59–61), liver, and spleen (62).

IL-15 plays a dominant role in governing the homeostasis of Vα14*i* NKT cells, including survival, turnover, and lymphopenia-induced or homeostatic proliferation (63, 64), which is the proliferation induced upon transfer to irradiated or $RAG^{-/-}$ recipients. This lymphopenia-induced proliferation does not require expression of CD1d. With respect to Vα14*i* NKT cells' IL-15 requirement, and their independence from TCR signals, Vα14*i* NKT cells therefore resemble conventional, $CD8^+$ memory T cells. Vα14*i* NKT cells have the surface phenotype of activated cells, including expression of CD69 and high levels of CD44, even in germ-free mice (65), and they have an activated phenotype in humans when isolated from cord blood (66). This raises the question as to how Vα14*i* NKT cells deal with self-antigens presented in the context of CD1d. One answer is that the self-antigens are not always expressed at levels sufficient to drive the full activation of these cells, but that their expression or presentation by CD1d may be induced upon infection, cell stress, or even apoptosis. Additionally, runaway activation of self-reactive Vα14*i* NKT cells may be prevented by the expression of inhibitory NK receptors.

Vα14*i* NKT cell clonal diversity can be assessed by analyzing the diversity of TCRβ complementarity-determining region (CDR) 3. Even though Vα14*i* NKT cells express a limited subset of Vβ segments, the CDR3β diversity for the major Vβ segments, Vβ8.2, Vβ7, and Vβ2, is enormous (67–69), and the likely average clone size, or number of cells with the same rearrangement, is quite small ($<$10 cells). Several conclusions can be drawn from these data. First, the natural antigen(s) driving the expansion of Vα14*i* NKT cells likely does not contact the CDR3 region of TCRβ. Furthermore, the continual turnover of these cells, measured by BrdU incorporation (23, 63), must be balanced by cell death and the

export of new clones form the thymus. A puzzling finding is that the repertoire of Vβ segments in Vα14*i* NKT cells in different organs is distinct (69). Perhaps Vα14*i* NKT cells do not rapidly circulate from one organ to another, compared with the rate at which they are replenished from the thymus. Although this is a cogent explanation, analysis of adult thymectomized mice indicates that peripheral replenishment of the Vα14*i* NKT cell pool is possible (53). In contrast, in human adults Vα24*i* NKT cells have a limited Vβ diversity, although the diversity is much greater in cord blood (66). Therefore, the Vβ diversity in mouse Vα14*i* NKT cells may contract as a result of infection, repeated antigenic stimulation, or aging.

Following stimulation in vivo with αGalCer, anti-CD3, or IL-12, Vα14*i* NKT cells disappear within a few hours (7, 70). This was originally attributed to activation-induced cell death (70–74). It is now evident, however, that after αGalCer treatment many of the Vα14*i* NKT cells do not die, but they downregulate their TCR and NK1.1 (7, 75–77), making them undetectable. Vα14*i* NKT cells reexpress their TCR and expand dramatically by two days after αGalCer exposure, although many of them are NK1.1 negative (75–77). By approximately nine days after αGalCer, the number of Vα14*i* NKT cells returns to the level found before antigen exposure (75–77). What happens upon reexposure to αGalCer? The question of Vα14*i* NKT cell memory is a difficult one because Vα14*i* NKT cells exhibit characteristics of memory cells even before αGalCer exposure. It remains possible, however, that the behavior of Vα14*i* NKT cells changes after antigenic exposure. It has been reported that repeated exposure to αGalCer causes Th2 cytokine skewing of both the Vα14*i* NKT cell (78) and concomitant conventional T cell responses (79), but this has not been found in every study (80).

SPECIFICITY

In this review, I have treated the expression of the Vα14*i* TCR and reactivity to αGalCer presented by CD1d as synonymous. However, relatively rare Vα14*i* TCR^+ cells have been found that respond preferentially to phosphatidylinositol and phosphoethanolamine presented by CD1d (81, 82), and in humans αGalCer plus CD1d-reactive T cells have been identified that do not have a canonical Vα24*i* TCR (83). Nonetheless, equivalence of Vαi TCR expression and reactivity to αGalCer presented by CD1d remain a reasonably accurate generalization. This conclusion is supported by the results from a variety of studies, including one in which 71/73 hybridomas with a Vα14*i* TCR responded to αGalCer presented by CD1d (84).

Within the population of cells with a Vα14*i* TCR or a Vα24*i* TCR, subpopulations can be found that respond to other glycolipids. Given the expression of an invariant TCRα chain, these additional specificities must be based on the Vβ segment expressed or on features of the CDR3β region. For example, T cells reactive to ganglioside GD_3 presented by CD1d can be detected after immunization with GD_3 (85). The GD_3-reactive T cells can be depleted with αGalCer/CD1d tetramers, and they constitute a subset of the αGalCer plus CD1d-reactive cells.

Investigators generally accept that Vα14*i* NKT cells are reactive to self-ligands or antigens presented by CD1d, and reactivity of these cells to microbial glycolipids has been difficult to prove unequivocally. Recently, however, Fischer et al. (86) reported that a subset of mouse Vα14*i* and human Vα24*i* T cells react to material purified from bacterial cell walls enriched for phosphatidylinositol tetramannoside (PIM_4). The reactive cells were a minority, consisting in some of the experiments of approximately 1% of the αGalCer/CD1d tetramer–reactive cells (86). Interestingly, PIM_2, a similar compound but with only two as opposed to four mannose sugars, was less able to stimulate NKT cells, although PIM_2 was reported earlier to have TCR-independent effects on the recruitment of Vα14*i* NKT cells (62).

On the basis of the crystal structures of glycolipids with human CD1b and CD1a molecules (87–89), investigators have concluded that the aliphatic chains of αGalCer likely fill the CD1d binding groove, with the more hydrophilic portions exposed for TCR recognition. Extensive structure-function studies with compounds related to αGalCer have mapped the sites likely to be important for the TCR interaction (90–92). The most critical features are in the area around the sugar-lipid linkage, including the 2′ OH in the equatorial position in the sugar, the α linkage of the sugar to the 1 carbon of the ceramide, and the hydroxyl on the 3 carbon of the sphingosine (Figure 1). The results from several studies indicate that the Vα14*i* TCR binds to complexes of αGalCer plus CD1d with a relatively high affinity and a particularly long half-life ($t_{1/2}$), on the order of minutes rather than the seconds characterizing conventional peptide plus MHC class I– or class II–reactive TCRs (93–96). Many Vα14*i* NKT cells are CD4, CD8 double negative, and this strong interaction may be a requirement for activating the TCR in the absence of cooperation from CD4 or CD8 coreceptors. It remains possible, however, that synthetic αGalCer is an agonist with an exceptionally strong potency. More recently, investigators have measured the TCR affinity for a series of six αGalCer analogs, and this measurement was correlated with their antigenic potency (96). In this study, a TCR $t_{1/2}$ of at least one minute was required for effective agonist activity. Vα14*i* NKT cells can also respond to signals from cytokines such as IL-12 in the absence of TCR cross-linking (97, 98), and, as suggested by one recent study (99), in the context of an inflammatory response including IL-12, the Vα14*i* TCR may be activated by much weaker agonists.

Evidence strongly suggests, however, that Vα14*i* NKT cells are reactive to a self-antigen bound to CD1d. Vα14*i* NKT cells have an activated phenotype in cord blood (66) and in germ-free mice (65), and CD1d autoreactivity can be measured in vitro under some conditions, such as (*a*) when CD1d is overexpressed; (*b*) when the inhibitory NK receptors expressed by Vα14*i* NKT cells are not expressed, as in hybridomas (100); or (*c*) when they cannot interact with their ligands on D^b-deficient antigen-presenting cells (APCs) (101). The self-ligand is likely to be a glycolipid, perhaps even a glycosphingolipid similar to αGalCer, although other types of molecules, such as lipopeptides, have been shown to be presented by different CD1 molecules (102).

CYTOKINE PRODUCTION

Activated Vα14*i* and Vα24*i* NKT cells have both perforin-dependent and FasL-dependent cytotoxic function, which is dependent upon TCR recognition of cognate antigen, as opposed to the killing of classic NK targets (103). The hallmark of the Vα14*i* NKT cell response, however, is the rapid and copious production of cytokines. Secretion of the prototypical Th1 and Th2 cytokines, IFN-γ and IL-4, respectively, has been most thoroughly documented, but TCR-activated NKT cells produce many other cytokines, including IL-2, tumor necrosis factor (TNF), IL-5, IL-13, and GM-CSF. The question remains as to how this mélange leads to a regulated immune response, but experimental evidence favors several possible explanations, which are not mutually exclusive.

One possibility is that there are subsets of Vα14*i* and Vα24*i* NKT cells, based on anatomic location and/or cell surface phenotype, and that the activation of one subset or the other could have a selective influence. In humans, there is evidence that the Vα24*i* NKT cells produce more IL-4 (49, 58), but strong evidence for functional subsets of mouse Vα14*i* NKT cells is lacking.

A second possibility is that the quality of the TCR signal influences the cytokine profile produced, by analogy with the effects of altered peptide ligands on conventional $CD4^+$ T cells. Consistent with this, OCH, an αGalCer analog with a shortened sphingosine (Figure 1), reportedly stimulates a higher ratio of IL-4 to IFN-γ secretion when added to total spleen cell cultures (104, 105). Promotion of Th2 cytokine secretion correlates with the reduced aliphatic chain length of OCH and related compounds (106), and evidence indicates that poor induction of the c-Rel transcription factor may be in part responsible for the reduced IFN-γ gene transcription (106). By contrast, a C-glycoside analog of αGalCer (Figure 1) has been reported to stimulate a higher ratio of IFN-γ to IL-4 (107). So far, there is no structural basis for predicting how a compound will affect the cytokines produced by activated Vα14*i* NKT cells. Moreover, there is recent evidence indicating that Vα14*i* NKT cells contain at least some mRNA for both IL-4 and IFN-γ, even before TCR activation (35, 108), although it remains to be demonstrated that these amounts are physiologically significant. However, Vα14*i* NKT cell cytokine production resulting from TCR stimulation is not influenced by IL-4, IL-12, or other factors that influence the cytokine production pattern of conventional T cells (108). Collectively, these data suggest that Vα14*i* NKT cells are poised to produce immediately both Th1 and Th2 cytokines following a TCR signal. It is possible that this immediate response, which occurs by two hours, is not sustained under some conditions, thereby allowing for Th1 or Th2 polarized cytokine production by Vα14*i* NKT cells (76). However, activation of Vα14*i* NKT cells also leads to the immediate activation of other cells types. For example, within hours of αGalCer stimulation of Vα14*i* NKT cells, NK cells are stimulated to secrete IFN-γ (108–110). In fact, the bulk of the systemic IFN-γ detected after αGalCer administration in vivo is due to the activity of NK cells (108). Therefore, although a direct effect of altered lipid ligands on the Vα14*i* NKT cell needs to

be considered, the Th1 polarizing effects of a particular glycolipid on the immune response may depend more on how much it stimulates communication between Vα14*i* NKT cells and NK cells or other cell types. This cell-cell communication could be a function of several factors, such as CD40L induction by the Vα14*i* NKT cells, rather than alterations in the cytokine profile produced by the activated Vα14*i* NKT cells themselves.

A third possibility is that cytokine production by Vα14*i* NKT cells can be determined by the integration of signals from different types of receptors, and that this can influence the pattern of cytokines produced. For example, although IL-12 signals are not required for Vα14*i* NKT cells to produce IFN-γ after αGalCer stimulation, IL-12 can selectively stimulate IFN-γ production by Vα14*i* NKT cells in the absence of αGalCer (97, 98), and NK1.1 cross-linking has been reported to do the same (111). In a recent series of experiments, IL-12, produced by APCs stimulated with lipopolysaccharide, promotes IFN-γ but not IL-4 synthesis by Vα14*i* and Vα24*i* NKT cells (99). Such IFN-γ synthesis may also require a TCR signal delivered by weak agonists, however, because anti-CD1d antibodies could block the Vα14*i* NKT cell response to IL-12 (99). Therefore, according to this view, integration of weak TCR signals and IL-12R-mediated signals may favor a Th1 polarized pattern of cytokine production by Vα14*i* NKT cells, although stronger TCR signals favor a Th0 pattern.

A fourth possibility is that the context in which αGalCer is presented has an influence on the pattern of cytokines produced. When bone marrow–derived dendritic cells (DCs) pulsed with αGalCer were administered to mice, the response was different from the one engendered by injection of the compound itself (112). The glycolipid-pulsed DCs caused an increased and prolonged release of IFN-γ compared with the free compound. This could be due to the DC-influenced increased activation of NK cells and/or the integration of signals from other receptors on the Vα14*i* NKT cells, as outlined above. Another important factor is the previous exposure to αGalCer, which, as outlined above, seems to favor Th2 responses (78, 79). In conclusion, cytokine production induced in other cells by activated Vα14*i* NKT cells, and the cytokines they produce themselves, can be modulated, but they are not influenced by the same factors that determine the cytokine response of conventional $CD4^+$ T cells. In general, the pattern of cytokines produced immediately following TCR stimulation of Vα14*i* NKT cells is relatively difficult to alter.

Communication with other Cell Types

Activation of Vα14*i* and Vα24*i* NKT cells has an effect on nearly every hematopoietic cell type, including DCs, NK cells, and B and T lymphocytes. This has usually been studied in the context of stimulation with αGalCer, however, which is a highly potent stimulus that might not reflect the outcome when these NKT cells are activated in a more physiologic way. Nevertheless, under conditions of stimulation with this synthetic antigen, DC maturation is enhanced (113–116). NK cells proliferate and secrete IFN-γ, and their cytotoxic activity is increased (103, 108–110).

B cells express activation markers, total serum Ig increases, and Vα24*i* NKT cells help B cells secrete Ig (78, 117, 118). Vα14*i* NKT cells can affect the cytokine profile elicited by $CD4^+$ T cells (79). They also provide effective help for CD8 T cells (119). Moreover, αGalCer acts as an adjuvant that can increase the magnitude of the CD8 T cell response to protein antigens (120, 121). Although it is possible that Vα14*i* and Vα24*i* NKT cells provide cognate help for CD1d-expressing B cells, many of the diverse effects of activated NKT cells may be mediated by their communication with $CD1d^+$ DC. Activated Vα14*i* NKT cells induce CD40L, which can interact with CD40 to stimulate release of IL-12 by DCs (122, 123).

It is appealing to speculate that physiologic activation of Vα14*i* NKT cells could under some circumstances be an important source of the CD4 help required during the priming of CD8 T cells, as the frequency of this population is 1% or greater than the total T cells in spleen, bone marrow, and elsewhere. During the primary response, the frequency of antigen-specific, conventional $CD4^+$ T cells might not be high enough to provide effective help, but the frequency of Vα14*i* NKT cells is lower in the lymph nodes, where T cell priming occurs and T cell help is needed, than in the spleen and liver.

Vα14*i* NKT cells are sometimes considered to be regulatory T cells. Regulation is often thought of as suppression, but in many cases Vα14*i* NKT cells tend to have an activating effect on immune responses, although this may be in a Th1 or a Th2 direction. In this regard, they are unlike $CD4^+$ $CD25^+$ regulatory T cells, the best-characterized regulatory population, which almost uniformly inhibit different types of immune responses. Surprisingly, there is little information as to how these two populations influence one another, but recent studies reported that activation of self-reactive $CD4^+$ $CD25^+$ regulatory T cells could inhibit the antitumor response of Vα14*i* NKT cells (124, 125).

Vα14*i* NKT Cells in the Systemic Immune Response

Vα14*i* NKT cells play a pivotal role in influencing a very diverse group of immune responses (1). There are controversies regarding some of these reports, which may reflect technical differences in the way the studies were carried out or differences in genetic background of the mice. For example, in a cerebral malaria model, NKT cells promoted pathogenesis in C57BL/6 mice, but they inhibited it in BALB/c mice (126). Because of these controversies, investigators should consider the experimental means by which a pivotal role for Vα14*i* NKT cells has been assigned. The involvement of Vα14*i* NKT cells has been tested in different experimental contexts by analyzing the effects of stimulation with αGalCer (1) or with related compounds such as OCH (104). As noted above, the limitation of αGalCer is that the response to this strong TCR agonist may not reflect the physiologic activation of Vα14*i* NKT cells. This limitation is highlighted by the results from studies of the immune response to cancers. There is a potent role for αGalCer in preventing tumor metastases (2), but in the absence of αGalCer treatment, tumor surveillance is mainly unaffected when Vα14*i* NKT cells are absent, although the response

to methylcholanthrene-induced sarcomas (127) and GM-CSF-transfected tumors (128) constitute exceptions in which Vα14*i* NKT cells play an important role even in the absence of αGalCer. A second method is to analyze mice deficient for Vα14*i* NKT cells or their activity. Acute depletion Vα14*i* NKT cells can be achieved by in vivo treatment with anti-NK1.1 antibodies, but this also depletes NK cells. In vivo treatment with anti-CD1d antibodies has been used successfully to block Vα14*i* NKT cell activity (59). Typically, however, analysis of immune responses in the absence of Vα14*i* NKT cells is carried out using either CD1d$^{-/-}$ or Jα18$^{-/-}$ mice. Deletion of CD1d can have several effects, including the loss of those CD1d-reactive T cells with more diverse TCRs that are not αGalCer reactive. In the response to several viruses and in ulcerative colitis patients (129), these CD1d-reactive T cells with more diverse TCRs may be important for influencing the magnitude of the immune response (130–132).

As reviewed previously (1), the beneficial immune responses in which Vα14*i* NKT cells have been reported to participate include (*a*) the response to tumors; (*b*) host protection from a variety of infectious agents, including bacteria, parasites, and viruses; (*c*) the prevention of autoimmune diseases; and (*d*) the maintenance of self-tolerance. Surprisingly, however, there is no single mechanism through which they exert their influence. For example, antitumor responses (127) and the response to infectious agents (4) depend on the stimulation of IFN-γ secretion by Vα14*i* NKT cells, whereas the prevention of diabetes may depend on IL-4 and IL-10 secretion (133). The prevention of experimental autoimmune encephalomyelitis may depend on IL-4 secretion (104, 134). The role of Vα14*i* NKT cells in anterior chamber immune deviation depends on IL-10 (135). Even when there is relative agreement among researchers that Vα14*i* NKT cells are important, some controversy remains regarding mechanism. For example, although many studies implicate IL-4 in the protective effect of Vα14*i* NKT cells in diabetes (3), others do not (136). Additionally, the sources of these beneficial cytokines remain incompletely defined. Because the activation of Vα14*i* NKT cells results in a network of cellular activation events, it need not be the case that the Vα14*i* NKT cells are the sole or even the major source of the relevant cytokine, although they may be critical for the initial induction of its synthesis. Antitumor responses stimulated by αGalCer provide a model for understanding these types of interactions, as the Vα14*i* NKT cell–mediated stimulation of NK cells to release IFN-γ may be critical (137).

In many cases, Vα14*i* NKT cells are required for or participate in the development of detrimental immune responses, including the induction of airway hypersensitivity, which requires IL-4 and IL-13 (60, 61), the development of oxazalone-induced colitis, which requires IL-13 (138), and the abrogation of maternal tolerance of the fetus, dependent on TNF and IFN-γ (139). More recently, Vα14*i* NKT cells have been shown to play a harmful role in the atherosclerosis that develops in apolipoprotein E–deficient mice fed a high-fat diet (140, 141). This disease-promoting role for Vα14*i* NKT cells is consistent with the emerging view of atherosclerotic plaque as an inflammatory lesion.

Despite many studies, a consensus view has not emerged in support of the true physiologic role of Vα14*i* NKT cells. This difficulty provides a stark contrast with the CD4$^+$ CD25$^+$ regulatory T cell population, which, despite some uncertainty regarding mechanism, clearly functions as a suppressive population. Vα14*i* NKT cells may either suppress or activate immune responses. They may act as regulators that direct Th2 responses but may, in some cases, promote Th1 responses. Moreover, they may act as effectors as well as regulators by accumulating in sites of inflammation and by exhibiting cytotoxic activity. Furthermore, rules have not yet emerged that would allow investigators to predict the function of these cells in a particular context. One appealing speculation that can account for the myriad effects of Vα14*i* NKT cell stimulation is that the main function of Vα14*i* NKT cells is to condition or educate various types of DCs so that they might in turn program appropriate adaptive immune responses. Chronic stimulation of Vα14*i* NKT cells might result from CD1d-mediated presentation by DCs of endogenous ligands resulting from cellular stress or danger. The DCs may take up these ligands by endocytosis, which is an effective pathway for CD1d-mediated antigen presentation. The "interpretation" of the resulting Vα14*i* NKT cell response may depend on a variety factors, including the types of DCs affected, as well as the strength or quality of the TCR signal and the cytokine milieu.

Clinical Relevance of Vα24*i* NKT Cells

Although basic scientists have intensely studied the unique features of NKT cell responses to lipid antigens, these cells also have fascinated more clinically oriented scientists, and clinical trials of αGalCer therapy in cancer are already underway. A number of investigators have reported decreased Vα24*i* NKT cells in a variety of autoimmune diseases (142). A striking example is type I diabetes, in which the frequency of invariant Vα24-Jα18 sequences among Vα24$^+$ double-negative T cells was reported to be lower in diabetics than in their disease-free identical twins (143). The ability of Vα24*i* NKT cell clones derived from diabetics to produce IL-4 was also impaired. These findings were especially compelling, given their similarity to those from NOD mice, but they could only be corroborated by some subsequent studies (144) and not by others (18). In addition to the generally lower frequency of Vα24*i* NKT cells in humans compared with their counterparts in mice, another problem in their investigation is that study of peripheral blood NKT cells, which often is the only site accessible in human studies, may not adequately reflect the frequency and function of NKT cells in other sites. In NOD mice, the decreased numbers of Vα14*i* NKT cells in organs such as the spleen are not reflected by a similar decrease in the blood (145). Moreover, there is a great range in the frequency of Vα24*i* NKT cells in the peripheral blood of normal individuals, making the detection of differences between diseased and normal populations challenging.

In addition to differences in cell number, correlations between disease status and Vα24*i* NKT cell function have been reported in several cases. In advanced prostate cancer patients, for example, expanded lines of Vα24*i* NKT cells had a reduced capacity to produce IFN-γ upon stimulation (146). The expanded Vα24*i* NKT

cells from multiple sclerosis patients in remission showed a Th2 bias compared with Vα24*i* NKT cells obtained from healthy individuals or individuals who had undergone a relapse (147), suggesting that this Th2 bias is protective. Additionally, there is a report of an 11-year-old girl with disseminated varicella infection after vaccination who had a deficiency of NKT cells and no other detectable immune defect (148).

Despite uncertainties about Vα24*i* NKT cells' mode of action, modulation of Vα24*i* NKT cell responses remains an attractive potential target for immune therapy. Positive features of this strategy include the presence of at least some Vα24*i* NKT cells in all individuals, the ability to specifically target these cells by αGalCer, the relatively low toxicity of this compound (149), and the ability to expand Vα24*i* NKT cells in vitro in the presence of cytokines and αGalCer (13). Adoptive transfer of activated Vα24*i* NKT cells may be cumbersome and expensive. Thus, a more attractive strategy is direct activation of these cells in vivo. Although intravenous administration of αGalCer had only limited effects in cancer patients (150), injection of DCs pulsed with αGalCer did activate innate and acquired immunity (149), including increases in serum IFN-γ. Therefore, as evidenced in mice, transferring DCs pulsed with αGalCer could lead to a much more potent Th1 response than injecting the free compound.

CONCLUSIONS

Vα14*i* and Vα24*i* NKT cells are surprisingly different from conventional T cells in several ways, including their TCR diversity, coreceptor expression, specificity, development, homing, and cytokine production. However, they share some properties with other populations of what may be considered natural memory lymphocytes, including $\gamma\delta$ T cells, intraepithelial lymphocytes, and B-1 B cells, in that they are capable of rapid effector functions without need for priming and clonal expansion. Despite significant progress in understanding the biology of these NKT cells, their specificity and the means by which they influence immune responses remain to be defined better. Nevertheless, the manipulation of Vα24*i* NKT cell responses retains some promise as the possible basis for immune therapy.

NOTE ADDED IN PROOF

Recently it was shown that isoglobotrihexosylceramide, a glycosphingolipid, is recognized by both mouse and human NKT cells as an autologous antigen (151).

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