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Fine tuning a well-oiled machine: Influence of NK1.1 and NKG2D on NKT cell development and function

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Abstract

Natural Killer T cells (NKT) represent a group of CD1d-restricted T-lineage cells that that provide a functional interface between innate and adaptive immune responses in infectious disease, cancer, allergy and autoimmunity. There have been remarkable advances in understanding the molecular events that underpin NKT development in the thymus and in the complex array of functions in the periphery. Most functional studies have focused on activation of T cell antigen receptors expressed by NKT cells and their responses to CD1d presentation of glycolipid and related antigens. Receiving less attention has been several molecules that are hallmarks of Natural Killer (NK) cells, but nonetheless expressed by NKT cells. These include several activating and inhibitory receptors that may fine-tune NKT development and survival, as well as activation via antigen receptors. Herein, we review the possible roles of the NK1.1 and NKG2D receptors in regulating development and function of NKT cells in health and disease. We suggest that pharmacological alteration of NKT activity should consider the potential complexities commensurate with NK1.1 and NKG2D expression.

Keywords

NKT cells; NK1.1; NKG2D; adaptive immunity; pathogenesis

INTRODUCTION

Natural Killer T cells

Natural Killer T (NKT) cells represent a group of T lineage cells of thymic origin with several features that distinguish them from ‘conventional T cells’ [1]. Mature NKT cells are restricted by the MHC class I-related molecule and express a T cell antigen receptor (TCR)

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capable of recognizing lipid-containing antigens (Ags) bound to CD1d. Since the discovery of the marine sponge *Agelas mauritianus*-derived alpha-galactosylceramide CD1d-binding glycolipid (α-GC) [2], numerous synthetic glycolipids have been devised and natural self and foreign CD1d ligands have been discovered [3–7]. Several outstanding structural studies have elucidated the manner in which CD1d binds to various ligands and in some cases how the CD1d ligand complex interacts with the NKT TCR and are described elsewhere [8–12].

NKT cells are broadly grouped into two categories, namely Type I and Type II NKT cells. Murine Type I NKT cells express an invariant Vα14/Jα18 TCRα chain (Vα24/Jα18 in humans) paired with a restricted Vβ2, Vβ7 or Vβ8 repertoire (Vβ11 in humans) and are thus termed 'semi-invariant' NKT cells [1,6,7] These are by far the most well studied subset of NKT cells and are reactive to the α-GC ligand. Recently a Type I-like subset was discovered by the Godfrey laboratory which expresses an invariant Vα10 TCRα chain and is reactive to a glucosylceramide ligand [13].

Type II NKT cells represent a group of CD1d-restricted T lineage cells with variable, likely oligoclonal TCR usage [14–17]. Type II NKT cell subsets have proven quite elusive due to their variable TCR usage meaning that a given CD1d ligand such as myelin-derived sulfatide may only detect a fraction of the Type II NKT population [18,19]. Nonetheless, interest in this subset is growing rapidly and investigators are deepening their understanding of Type II NKT functions in health and disease [16]. The information presented in this review focuses on Type I NKT cells.

The classical model for Type I NKT activation is based upon observations that $CD1d^+$ professional APCs present CD1d/foreign ligand complexes to NKT cells which are subsequently activated via engagement and cross-linking of the TCR [1,2,20,21]. Several co-receptors such as ICOS, CD40L and CD28 also regulate TCR signaling in NKT cells [22]. More recently, it was reported that Toll-like receptor (TLR)-stimulated IL-12 production by professional APCs in concert with presentation of self CD1d ligand can potently activate NKT cells [23,24]. Clearly, there are at least two major modes of Type I NKT activation and the relative extent to which they occur in vivo could well be influenced by several factors.

Type I NKT cells regulate adaptive immune responses to pathogens, allergens, self-antigens and tumors. Type I NKT cells can boost cytotoxic T cell responses to tumors [25–27] as well as Ag-specific Ab responses to foreign Ags (including allergens) and associated pathogens [28–35]. Type I NKT cells also appear to regulate autoimmune reactions [36]. In a recent study auto-reactive B cell homeostasis in SLE patients was linked to abnormal NKT function and subject to CD1d-dependent control [37]. Consequently, there is much interest in understanding the molecular events that govern the interaction of Type I NKT cells with other immune cell types [28,38,39].

Natural Killer Cells

Natural Killer (NK) cells should not be confused with NKT cells. NK cells develop in the bone marrow, are of the myeloid lineage, and do not express T cell antigen receptors. NK cells are particularly responsive to virus-infected cells and cancer cells, responding often to target cells that have down-regulated expression of the classical MHC. This is achieved via multiple receptors that govern activation. NK cells exert effector function on target cells, by production of IFNγ and release of perforin from intracellular granules, leading to target cell killing. For a review on NK cells and their receptors, the reader is directed to the outstanding review by Dr. Lanier [40]. The biological functions of NK cells and NKT cells therefore differ but overlap. As we will discuss, the surface receptors that constitute the major modes

of activation of NK cells, may play a supporting role by influencing activation of NKT cells via the TCR.

Natural Killer Cell Receptors Expressed by NKT and NK Cells

The natural killer gene complex (NKC) encodes multiple C-type lectin-like receptors which are responsible for the regulation of development and functions of NK and NKT cells depending on the cellular environment. These include NK1.1, the Ly49 family of proteins, and NKG2D. The balance of expression and engagement of these receptors fine tunes NK activation [40] and it is expected that many of these properties also apply to NKT cells.

NK1.1 (also known as KLRB1C and NKRP1C), is a type II integral membrane glycoprotein with a C-type lectin domain and is a member of the NKR-P1 family of cell surface receptors [41]. This activating receptor associates with the ITAM-bearing γ-chain adaptor molecule normally associated with Fc receptors. NK1.1 is expressed as a disulfide-linked homodimer on all NK and NKT cells in some strains of mice and is a particularly good marker of these cell types in the C57BL/6 genetic background. NKRP1 receptors family members (to which NK1.1 belongs) do not engage ligands with an MHC class-I-like fold, but rather interact with C-type lectin-like CLEC2 glycoproteins [41,42]. Although crosslinking of the NK1.1 receptor using an NK1.1-specific mAb (clone PK136) induces NK cell-mediated cytotoxicity and effector cytokine secretion, the *in vivo* functions of NK1.1 and the identity of its ligand(s) remain incompletely understood [43].

NKG2D is encoded by the *KLRK1* gene (killer cell lectin-like receptor subfamily K, member 1) on chromosomes 6 and 12 mouse and human respectively. NKG2D is a C-type lectin-like type-2 trans-membrane glycoprotein expressed as a disulfide linked homo-dimer on the surface of natural killer (NK) cells, NKT cells, $CD8^+$ cytotoxic T cells, γδ T cells, and under certain conditions $CD4^+$ T cells [44–47].

NKG2D molecules bind to cell surface glycoproteins of the major histocompatibility complex (MHC) class I family and thereby facilitate detection of stressed cells or cells exhibiting aberrant MHC class I expression. In human, the ligands of NKG2D receptor are MHC class I–related protein A (MICA), MICB, UL-16 binding proteins (ULBP) 1 to 3, and lymphocyte effector cell toxicity-activating ligand (Letal/ULBP4/Raet1E) while in mice NKG2D ligands include retinoic acid early transcript-1 proteins (Rae1α-ε), a minor histocompatibility antigen (H-60), and mouse ULBP-like transcript 1 (Mult-1) [48]. As NKG2D ligands are typically expressed by distressed cells, NKG2D ligands can potentially be used as tumor-specific targets with minimal cross-reaction with normal tissues. Indeed, it has been demonstrated that engineered expression of NKG2D (as TCR complex-associated fusion proteins) can lead to enhanced tumor killing by tumor-specific $CD8^+$ T cells [49,50].

We will now discuss how NK1.1 and NKG2D may contribute to the development and functions of Type I NKT cells. Understanding the relationship between these molecules and NKT activation will be valuable is designing therapies that pharmacologically alter the activation of NKT cells.

NK1.1 and NKG2D IN NKT DEVELOPMENT

Impressive progress has been made in understanding the mechanisms regulating NKT cell development and is reviewed elsewhere [51,52]. In brief NKT development is highly dependent on homotypic interactions between CD1d-expressing thymocytes [53,54]. The correct TCR gene arrangement is also required, since deletion of the Jα18 gene segment from the TCR locus blocks development [55]. Other studies have reported that self CD1d binding ligands such as the lysosomal glycosphingolipid isoglobotrihexosylceramide (iGb3)

is required for NKT development, but debate exists regarding how many self-ligands could influence development [56]. In more recent studies, co-receptor molecules such as the SLAM (signaling lymphocytic activation molecule) family members (Slamf1 and Slamf6) have been identified as critical for NKT development [53]. Signaling mediators including MAP Kinases, and Erk target protein Egr2 and transcription factors that include PLZF (promyelocytic leukemia zinc finger), E protein transcription factor HEB, c-Myb, and Hobit (Homologue of Blimp-1 in T cells) regulate NKT development [57–61].

Thymic precursor cells receiving the correct balance of signals differentiate through discreet checkpoints, whereby CD4/CD8 double-positive thymocytes are selected based on CD1d expression [53,54]. NKT precursors (in mouse) then differentiate into CD4 single-positive and CD4/CD8 double-negative cells. CD8 single-positive NKT cells are also found in humans [62]. Expression of NK molecules is acquired relatively late during thymic development with NK1.1 being examined closely than other molecules [63,64]. Nonetheless, up-regulation of other NK markers appears commensurate with NK1.1 expression and thus potential contributions of NKG2D might be inferred from such studies. In elegant studies by the Bendelac and Stein groups respectively, several important findings regarding NK receptors were reported [63,64]. NK1.1 expression was increased after commitment to the CD4 or CD8 subsets and recent thymic emigrants were NK1.1− or had low expression which increased considerably in the periphery over the few days following export. $NK1.1^+$ NKT cells were shown to have considerably lower cell division than their $NK1.1^-$ counterparts, suggesting that they represent a more terminally differentiated subset. $NK1.1^+$ cells were also detected in a population of mature NKT cells retained in the thymus [65]. NK1.1 expression is regulated by the Tec family kinase Itk and $Itk^{-/-}$ mice were reported to progressively lose peripheral NKT cells with aging [64]. These findings demonstrate an important contribution of NK molecules in the maintenance of NKT cells in the periphery. A flow cytometric analysis of thymic and peripheral NKT cells from $NKG2D^{-/-}$ mice (kind gift from David H. Raulet, University of California, Berkeley) did not reveal any differences in frequency, number or phenotype as compared to cells derived from C57Bl/6 controls (*Lang and Joshi, unpublished observation*). These findings are consistent with the notion that NK receptors including NK1.1 and NKG2D have little if any role to play in early stages of NKT development. However, more detailed studies on the impact of NKT NK1.1 and NKG2D expression in the periphery with regard to differentiation into distinct functional subsets is warranted. Given the diversity of tissue-expressed NK1.1 and NKG2D ligands in the periphery (Table I), it is possible that select ligand/NK1.1 and ligand/NKG2D pairings can deliver a form of ‘tonic’ but non-mitotic signaling to NKT cells that could promote their survival in the periphery (Figure 1a).

NK1.1 AND NKG2D IN NKT FUNCTION

NK1.1 and NKG2D delineate functional NKT subsets

NKT cells express numerous cell surface receptors that were first identified as markers of Natural Killer (NK) cells. These include NK1.1, NKG2D, and the Ly49 family of proteins [66], the balance of expression and engagement of which may fine tune NKT activation. To assist the reader, Table II shows NKT subsets in mouse and human according to CD4, CD8 and NK1.1 expression. While Table II indicates that NKT function with regard to Th1-, Th2- or Th17-skewed responses can be ascribed to different subsets, it is also worth noting that anatomical location and microenvironment can affect NKT function [67]. Arguably, exposure to diverse NK1.1 and NKG2D ligands in different microenvironments could influence these events.

As discussed earlier, NKT cells express NK receptors including NK1.1 during the later stages of thymic development and expression increases further in the periphery. Increased

expression of these receptors is concomitant with a change in the expression of cytokines following stimulation of the TCR with CD1d/α-GC. Several studies have now confirmed that $NK1.1^+$ NKT cells are skewed away from Th2 responses and IL-4 production towards Th1 responses and IFNγ production [52,63,64]. Indeed, NKG2D activation on NKT cells can act synergistically with IL-12 to promote a Th1-skewed response [68]. A subset of $CD4^-NK1.1^-$ NKT cells has also been defined which produces very high concentrations of the pro-inflammatory cytokine IL-17 within a few hours of stimulation [69–72]. IL-17-secreting NKT cells, designated as NKT-17 cells, represent a small subset of the NKT cell populations in the thymus, spleen, liver and lung but is enriched in the peripheral lymph nodes [71]. In contrast, a largely $CD4^+NKG2D^-$ NKT cell subset has been reported to express the IL-25 receptor (IL-25R/IL-17RB) and play a major role in Th2-mediated allergy and airway hyper-reactivity by producing large amounts of IL-4 and IL-13 [73]. These findings collectively reinforce the notion that NK1.1 and NKG2D expression by NKT cells are associated with Th1 rather than Th2 or Th17 responses.

A recent study by Kuylenstierna and co-workers provided the most important demonstration to date that NKG2D is important for NKT cell function [74]. Using primary human NKT cells, the authors established NKG2D expression primarily in the $CD4^-$ subset, consistent with findings in mice. $NKG2D^+$ NKT cells expressed perforin which localized to the contact site between these cells and NKG2D-ligand-expressing target cells. Furthermore, NKG2D engagement led to degranulation and target cell killing. In further experiments, they demonstrated that NKG2D engagement enhanced TCR-mediated NKT activation (represented in model in Figure 1b). Collectively, this study showed that NKG2D plays two important roles in NKT cells. Firstly, NKG2D directly stimulates NKT effector functions and secondly, it acts as a TCR co-receptor to influence cell activation. Understanding how different NKG2D ligands differentially stimulate these diverse biological outcomes is of considerable interest.

Clinically-relevant observations on NKT-expressed NK receptors

Some clinically-oriented studies have highlighted linkages between NKG2D and NKT function in disease. Consistent with the Kuylenstierna study, Wang and colleagues showed that the NKG2D ligand class MHC class I chain-related molecules (MICs) derived from patients tumors could down-regulate NKT cell NKG2D expression and tumor cell killing in vitro [75]. This perhaps provides one explanation of why NKT cells are functionally compromised in cancer patients [75]. In another study, patients with Type II diabetes were shown to have an increased frequency of $NKG2D^+$ NKT cells in the peripheral blood as compared to healthy controls [76]. Early onset SLE was associated with changes in the ratio of activating NKG2D/inhibitory NKG2A receptors in multiple cell types including NKT cells [77]. Th1-skewed NKT cells were also elevated in pre-eclamptic women as compared to those undergoing a healthy pregnancy [78]. Interestingly, in pre-eclamptic patients, expression of NKG2A was decreased on $NKG2D^+$ NKT cells, suggesting the balance of critical activating and inhibitory signals was altered [78]. Together, these studies suggest an important role for NKG2D in human NKT cells regulating immunity during a variety of disease conditions.

Interestingly, NKT cell NKG2D, but not NK1.1 may represent a target for bacterial pathogenesis. We reported that anthrax toxin-treated murine NKT cells were functionally anergic, responding poorly to TCR stimulation [79]. In that study NKG2D expression by NKT cells but not NK cells was down-regulated following anthrax toxin treatment. NK1.1 expression was not down-regulated indicating a degree of selectivity of this effect. While the anthrax toxin study did not establish causality, the association between NKG2D expression and TCR function was clear and warrants further investigation. Whether bacterial toxins

impact the immune system in part through effects on intrinsic NKT function or extrinsic function is also of considerable interest.

Effect of NKT NK1.1 and NKG2D on transactivation of other immune cells

As described, NKT cells influence a range of adaptive immune responses to tumors, self-antigens, allergens and pathogens. In order to do this, NKT cells appear to be able to carry out some functions directly (intrinsic function) or to influence the behavior of other immune cell types (extrinsic function). There are several reports in the literature which to describe in their entirety would constitute a separate review article. However, the selected examples described in Table III illustrate some likely extrinsic functions of NKT cells.

Hepatic injury following hepatitis B virus infection of mice was reported to be mediated by the immune response to viral antigens. Disease was mediated at least in part by CD1d-restricted but α-GC non-reactive NKT cells which in turn activated NK cells [80]. Vilarinho and colleagues demonstrated that mAb blockade of NKG2D interaction with its ligands prevented the acute immune response and liver damage in a transgenic mouse expressing HBV envelope proteins on a $RAG^{-/-}$ background [81]. Adoptive transfer of NKG2D-depleted donor splenocytes induced less liver damage than transferred NK-depleted splenocytes or whole mixed splenocytes. This work therefore implicated $NKG2D^{+}$ in the ability of NKT cells (possibly Type II NKT cells) to influence the immunological milieu and thus the complex immune response to HBV.

As referenced in Table III, the Steinman laboratory demonstrated that NKT activation with α-GC was able to boost DC maturation and elicit improved tumor-specific CTL responses [26,82,83]. We therefore generated chimeric mice in which NKT cells were $NKG2D^{+}$ or NKG2D-null. Re-constituted mice were immunized with OVA or OVA plus α-GC before adoptive transfer of CFSE-labeled OVA SIINFEKL peptide-specific OT-1 cells. Subsequent flow cytometry analysis revealed no difference in expansion of the OT-1 cells in the presence of $NKG2D^{+}$ or NKG2D-null NKT cells (Figure 2). This observation was surprising in light of the known Th1-skewing effect of $NKG2D^{+}$ NKT cells, but suggested that NKG2D was dispensable for NKT-enhanced CTL expansion. This result would also suggest that NKT cell NKG2D is not required for NKT-enhanced DC maturation and experiments to test this hypothesis are warranted.

The growing number of available examples therefore illustrate an important point, namely that NK1.1 and NKG2D may influence the ability of NKT cells to alter the activity and behaviors of other immune cell types. More experimentation is required to delineate the circumstances under which this occurs and those in which it does not.

CONCLUSIONS AND REMAINING QUESTIONS

There is a paucity of information on the exact contribution of NK1.1 and NKG2D to NKT cell development, homeostasis and activation. However, the information available indicates that NK1.1 and NKG2D could be of importance in the life of an NKT cell and that these molecules represent potential targets for immune system avoidance by pathogens and for immunotherapy. We therefore feel that investigators should consider the following questions in future studies:

- Does NKG2D expression contribute to homeostasis and maintenance in the periphery?
- Which ligands engage NK1.1 and NKG2D expressed by NKT cells?
- To what extent do NK1.1 and NKG2D signaling interact with TCR signaling and affect intrinsic and extrinsic NKT function?

- Do human and murine NKT cells differentially depend on NK1.1 and NKG2D for function?
- How do NK1.1 and NKG2D work in concert with other NK receptors to control cellular activation?
- How does pharmacological manipulation of NK1.1 and NKG2D activity affect NKT function?

NK1.1 and NKG2D are molecules of pivotal importance in the immune system. It will be of significant interest to determine the contribution of NKT cell NK1.1 and NKG2D to the immune response to pathogens, allergens, tumors and auto-antigens.

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HIGHLIGHTS

- CD1d-restricted Natural Killer T (NKT) cells are appreciated as an important bridge between innate and adaptive immune responses.
- There is less awareness of how molecules normally expressed by NK cells, but also expressed by NKT cells, regulates NKT development and function.
- The NK1.1 and NKG2D receptors may influence later stages of NKT development, survival in the periphery, intrinsic function, and extrinsic function.

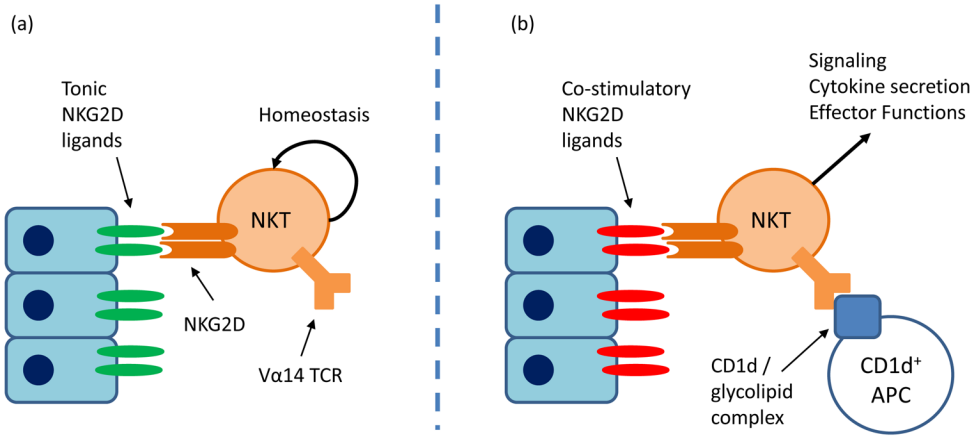

Figure 1. Possible effects of NKG2D engagement on peripheral NKT cells

(a) In this model, tonic signaling provided by tissue-expressed NKG2D ligands could help maintain NKT cells in the periphery (b) NKG2D signaling provided by tissue-expressed NKG2D ligands could potentiate the TCR-driven effector functions of NKT cells.

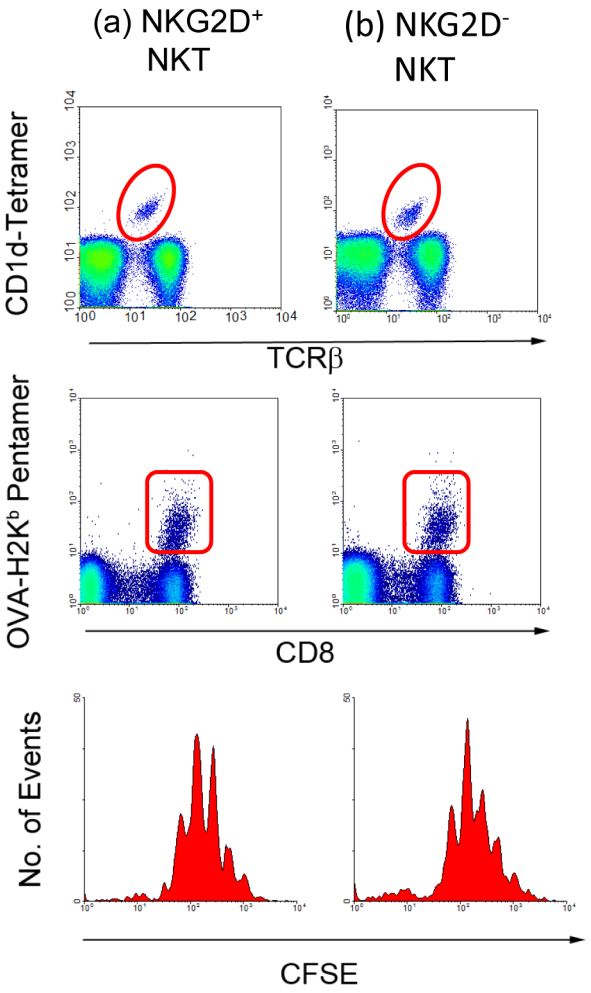

Figure 2. Potentiation of CD8^{+} T cell expansion by NKG2D-null NKT cells

Mixed bone marrow chimeric mice were generated by engrafting irradiated CD45.1 congenic recipients with donor bone marrow mixtures (a) Jα18$^{-/-}$ plus C57Bl/6 or (b) Jα18$^{-/-}$ plus NKG2D$^{-/-}$. Mice were immunized with OVA plus α-GC before adoptive transfer of CFSE-labeled MHC I-restricted (OVA SIINFEKL-specific) OT-1 cells. After 96 hours splenocytes were harvested and analyzed by flow cytometry. Dot plots on top row show re-constituted NKT cells in the mixed chimeras. Dot plots on center row show OT-1 cells detected by SIINFEKL-loaded MHC 1 pentamers. Histograms on lower row represent dilution of CFSE in the OT-1 cells. Immunization with OVA alone or a heterologous Ag did not lead to OT-1 expansion (*not depicted*). Jα18$^{-/-}$ refers to mice with a TCR Jα18 gene segment deletion that lack Type I NKT cells [55]. Data are representative of three mice per group.

Table I

NKG2D ligands

Shows known ligands that could potentially interact with NKG2D expressed on human and murine NKT cells. MIC (MHC-class I polypeptide-related sequence, ULBP (cytomegalovirus UL-16-binding protein), RAE 1 (Retinoic acid early protein 1), RAET 1 (Retinoic acid early transcript 1), MULT1 (murine UL-16 binding protein-like transcript 1), H60 (minor histocompatibility antigen) [47,48,84]. Definitive ligands have not been identified for NK1.1

	NKG2D Ligands
Mouse	RAE-1α
	RAE-1β
	RAE-1γ
	RAE-1δ
	RAE-1ε
	H60
	H60a
	H60b
	MULT-1
Human	MICA
	MICB
	ULBP-1/RAET1I
	ULBP-2/RAET1H
	ULBP-3/RAET1N
	ULBP-4/RAET1E
	ULBP-6/RAET1L
	RAET1G

Table II

NKT subsets

Shows major subsets of NKT cells grouped according to CD4, CD8 and NK1.1 expression in humans and mice.

	Type-1 NKT Subtypes	Cytokine Profile	Ref.
Mouse	$CD4^+$	T_H1 (IFNγ/TNFα); T_H2 (IL-4/IL-10/IL-13)	[52,89–91]
	$CD4^+NK1.1^-$	$IFN\gamma^{LO}/IL\text{-}4^{HI}$	
	$CD4^-NK1.1^+$	$IFN\gamma^{HI}/IL\text{-}4^{LO}$	
	$CD4^+NK1.1^+$	$IFN\gamma^{LO}/IL\text{-}4^{HI}/IL\text{-}13^{HI}$	
	$CD8^+$	IFNγ/TNFα	
	$CD4^-CD8^-$ (DN)	T_H1 (IFNγ/TNFα); T_H2 (IL-4/IL-10/IL-13)	
	$CD4^-NK1.1^-$ (NKT-17)	IL-17/IL-21/IL-22	[69,72,73,92,93]
Human	$CD4^+CD8^-$	T_H1 (IFNγ/TNFα)^{LO}; T_H2 (IL-4/IL-10/IL-13)^{HI}	[62,94]
	$CD4^-CD8^+$	T_H1 (IFNγ/TNFα)^{HI}; T_H2 (IL-4/IL-10/IL-13)^{LO}	
	$CD4^-CD8^-$ (DN)	T_H1 (IFNγ/TNFα); T_H2 (IL-4/IL-10/IL-13)	

Table III

NKT extrinsic functions

Shows selected examples of major immune cell types affected by NKT activation. Specific references to original work are cited in the table. The topics are broadly reviewed in [26,85–88]

Cell Type	Effector Function	Ref.
DC	DC Maturation/IL-12 secretion	[26]
CTL	Augment B16 melanoma killing	[83,95]
NK	Immuno-surveillance against sarcoma	[96,97]
B	Enhanced specific Ab responses	[30–32,35,98,99]
$CD4^+$ T	Enhancement of T cell responses	[95]