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The Regulation of Vα14 NKT Cells

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Abstract

Natural Killer T (NKT) cells are a distinct subset of T cells with unique properties. Upon activation, NKT cells release large amounts of both Th1 (IFN-γ) and Th2 (IL-4) cytokines. It was found that NKT cells play a very important role in regulation of various immune responses in disease settings, such as autoimmune disorders, anti-infectious and anti-tumor responses. However, the regulatory mechanisms of the activation of NKT cells are not well understood. We found that autoreactivity against dendritic cell-specific endogenous lipid antigens, which has been known to be required for the development of NKT cells, is also crucial for efficient activation of NKT cells in the periphery. In addition to endogenous lipid antigens, we have also found that CD28/B7 costimulatory signals are required for cytokine release, but not for the activation of NKT cells. Moreover, the development of NKT cells is severely impaired in the absence of CD28/B7 costimulatory signals. These results indicate that ontogenesis and functions of NKT cells are regulated by multiple factors through unique pathways that remain to be further characterized.

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List of Symbols, Abbreviations and Nomenclature

Symbol	Definition
αCD3	Anti-CD3 antibody
αCD28	Anti-CD28 antibody
Ag	antigen
APC	allophycocyanin
APCs	antigen presentation cells
αGC/αGalCer	alpha-galactosylceramide
Baf	bafilomycin A1
β2m	beta-2 microglobulin
CD4/CD8	T cell co-receptors
CMA	concanamycin A
CTLA-4	cytotoxic-T lymphocyte-associated 4
DC	dentritical cell
DGJ	deoxygalactonojirimycin
DN	double negative thymocytes
DP	double positive thymocytes
EAE	Experimental Autoimmune Encephalomyelitis
ELISA	enzyme-linked immunoabsorbant assay
FACs	fluorescence-activated cell sorter
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
GD3	disialoganglioside
HexB	ß-hexosaminidase
IB4	isolectin B4
IDDM	insulin-dependent diabetes mellitus
IκB	inhibitor kappa B
iGb3	isoglobotrihexosylceramide
IL-2	interleukin-2
IL-4	interleukin-4
IFN-γ	interferon-γ
IS	immune synapse
LFA-I	Leukocyte function-associated antigen-1
mAb	monoclonal antibody
MCP-1	monocyte chemotactic protein-1
MHC	major histocompatibility complex
MIP-2	macrophage inflammatory protein-2
mRNA	messenger ribonucleic acid
MS	Multiple Sclerosis
NK	natural killer cells
NKT	natural killer T cells
NOD	non-obese diabetic
PE	phycoerythrin
PerCP	peridinin chlorophyll

PBS	phosphate buffer saline
PDMP	threo-1-phenyl-2-decanoylamino-3-morpholine-1-propanol hydrochloride
PI	phosphatidylinositol
PPMP	DL-thero-1-phenyl-2-palmitoylamino-3-morpholine-1-propanol
PM	plasma membrane
RPMI	media developed at Roswell Park Memorial Institute
SCID	severe combined immunodeficiency
S-APC	antigen presentation cells from splenocytes of scid mice
TAP-1	transporter 1
T1D	Type I diabetes
TCR	T cell receptor
TGF-β1	Transforming growth factor-β1
Th1/Th2	T helper cell (suset 1 or 2)
Treg	regulatory T cells
WT	wide type

CHAPTER ONE: INTRODUCTION

1.1 Overview of NKT Cells

As a small subset of T lymphocytes, Natural Killer T (NKT) cells uniquely display phenotypic and functional characteristics of both T and natural killer (NK) cells. They co-express a rearranged T cell receptor (TCR), as well as several NK cell-associated receptors, including CD161 in humans, NK1.1 in mice, NKG2D, members of the Ly49 family and CD94/NKG2 (1-3). NKT cells express TCRs with much less diversity than conventional alpha/beta T lymphocytes. Most NKT cells in the mouse express an invariant TCRα chain (Vα14-Jα18) that possesses a conserved CDR3 region associated with Vβ8.2, Vβ2 or Vβ7 chains (4). Humans have a homologous population of T cells expressing an invariant Vα24 rearrangement (Vα24-Jα18), associated with Vβ11 (4). In mice, most NKT cells are $CD4^+$ or $CD4^-CD8^-$ (DN) cells, whereas in humans, in addition to $CD4^+$ and DN subsets, a small portion of NKT cells express CD8α marker (5, 6). Like conventional T cells, NKT cells express costimulatory molecules, CD28 (8) and CD40L (9), and other surface markers, CD44, CD69 and CD122 that are characteristic of an activation or memory phenotype (7). Importantly, the expression of unique TCR molecules renders NKT cells an ability to recognize various glycolipids in the context of MHC class I like CD1d/β2 microglobulin complex, and NKT cells can be activated by TCR engagement with glycolipid/CD1d complex (8). Although NKT cells constitute only a very small portion of T cells, they play an important role in initiation and modulation of immune responses. Activated NKT cells demonstrate killing ability against tumor and infected cells similar to NK cells (9, 10). Like other populations of T lymphocytes,

activated NKT cells also produce various cytokines. Most strikingly, NKT cells can produce a large amount of both Th1 (IFN-γ) and Th2 (IL-4) cytokines within a few hours after TCR ligation (4). The burst release of large amount of cytokines by the activated NKT cells mobilizes both innate and adaptive immunity by transactivation of several other immune cells, including B cells, NK cells, and dentritic cells (11-13). An accumulation of evidence has demonstrated there are critical effects of the cytokines produced by NKT cells in anti-infection, anti-tumor responses and immune regulation, although mechanisms underlying NKT cell mediated immune responses and modulation remain to be characterized. In particular, the mechanisms that govern the efficient activation and burst release of cytokines of NKT cells are poorly understood. My project is focused on studying the regulatory mechanisms of NKT cell activation, and trying to understand why and how NKT cells respond rapidly to various stimuli.

1.2 CD1, CD1 Ligands and CD1d–glycolipid Tetramers

1.21 CD1

Unlike most alpha/beta T cells that recognize peptide antigens presented by MHC class I or class II molecules, NKT cells recognize glycolipid antigens presented by MHC class I like molecules, CD1. CD1 molecules are encoded by the CD1 locus that is comprised of a group of non-polymorphic genes located on chromosome 1 in humans and chromosome 3 in mice (14-16). There are 4 members of the CD1 family in humans, CD1a, CD1b, CD1c and CD1d. These members can be separated into 2 groups, CD1a, CD1b and CD1c belong to group I, and CD1d belongs to group II. Mice only express two CD1d molecules, CD1d1 and CD1d2; therefore NKT cells in mice are all restricted by CD1d (17). The group I CD1 molecules can present antigens to a wide variety of T cells. However, the

group II CD1 molecules (CD1d molecules) are mostly NKT cell-restricted, although they also present antigens to some non-invariant NKT cells (18, 19).

Similar to MHC class I molecules, CD1d molecules contain three domains (α1, α2, α3) that associate non-covalently with β2 micro-globulin. CD1d molecules are broadly distributed in various tissues and organs, including thymus, spleen, bone marrow and liver. Within these tissues, CD1d molecules are constitutively expressed on a number of hematopoietic-derived antigen-presenting cells (APCs), such as macrophages and dendritic cells, as well as B and T lymphocytes. In addition, cortical thymocytes, hepatocytes, and some intestinal epithelial cells also express CD1d molecules (20-22). Because the expression of mouse CD1d is detected both in early and late endosomes and/or lysosomes by intracellular staining (23), it is likely that CD1d is capable of binding endocytosed antigens. It is clear that development of NKT cells is CD1d-dependent since NKT cell development is severely impaired in CD1d gene deficient animals (24).

1.22 CD1 Ligands

1.221 Synthetic Glycosylceramides

Early efforts of identification and isolation of mammalian lipid antigens recognized by NKT cells were not successful. To date, the best-described CD1d-presented antigens are synthetic glycosylceramides, and in particular alpha-galactosylceramide (αGalCer) that was synthesized by a group led by Masaru Taniguchi in 1995 (25). αGalCer was originally derived from a marine sponge as an anti-tumor agent. In 1997, it was found that αGalCer was a ligand of NKT cells and its anti-tumor effect was mediated by

activated NKT cells (8, 9). As a glycosphingolipid, the structure of αGalCer is distinguished from other natural compounds in this class. It consists of a sugar head and two hydrophobic alkyl tails (Fig.1). The alkyl tails of αGalCer bind to two hydrophobic pockets in the antigen binding groove of CD1d molecules, and the sugar head is presented to the TCR on NKT cells (22, 26). The identification of αGalCer as a ligand for NKT cells significantly facilitated the study of NKT cell biology and their role in immunity. Recently, other synthetic glycolipids, such as α-glucuronosylceramide (PBS 30) and α-galacturonosylceramide (PBS 59) with structures identical to the compounds derived from the Sphingomonas cell wall, are also found to be able to strongly bind to mouse and human CD1d and be recognized by NKT cells (27).

1.222 Natural, Endogenous CD1d Ligands

So far, numerous endogenous CD1d-presented glycolipids, including phosphatidylinositol (PI), isoglobotrihexosylceramide (iGb3) and disialoganglioside GD3, have also been identified (18, 31-34). Among these endogenous glycolipids, iGb3 is the only mammalian glycolipid with clear agonist activity for both mouse and human NKT cells in a CD1d-dependent manner (34). Structurally, iGb3 consists of a ceramide and a sugar head group linked in a β-anomeric conformation (35). In contrast to other glycolipids, such as αGalCer which only has one sugar head (22, 26), the sugar group of iGb3 consists of three sugar heads (Fig.1) (28).

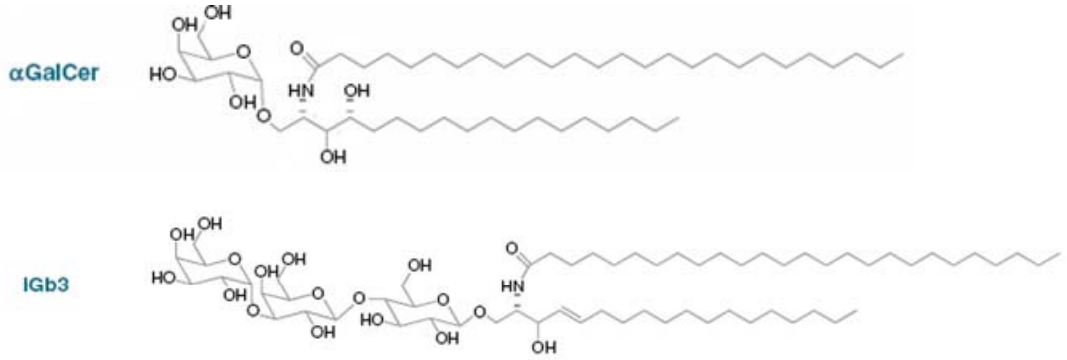

Figure 1 Structure of αGalCer and iGb3. αGalCer (alpha-galactosylceramide) consists of a sugar head and a ceramide as the lipid moiety. iGb3 (isoglobotrihexosylceramide) consists of a ceramide and a sugar group with three heads that are composed of two galactose rings and one glucose.

1.23 CD1d–glycolipid Tetramers

In early studies, CD1d-dependent NKT cells were mostly identified as NK1.1 expressing α/ß TCR^+ cells. However, most mouse strains do not express the NK1.1 marker (20). Although DX5 (CD49b) has been used as a surrogate marker to identify NKT cells in NK1.1 negative mice, there has been debate regarding the specificity of DX5 since other subsets of T cells may also express DX5 in the periphery. In addition, upon activation, NKT cells may downregulate the expression of the NK1.1 marker (29). Moreover, the NK1.1 marker was also found expressed on some CD1d-independent T cells (5). Therefore, it is difficult to identify NKT cells using NK cell markers. The successful development of fluorescent CD1d/αGalCer tetramers has led to a clearer definition of NKT cell subsets and provides us an excellent tool to study NKT cell development and function. The CD1d/αGalCer tetramer is made by loading mouse CD1d tetramers with αGalCer (37), which is able to bind CD1d and efficiently activate NKT cells both in vitro and in vivo (8). These CD1d/αGalCer tetramers bind stably and specifically to mouse NKT cells bearing the invariant Vα14–Jα18 TCR. By loading human CD1d tetramers with αGalCer, tetramers with similar specificity for human NKT cells were also produced (30). CD1d tetramers are a sensitive and highly specific reagent for identifying both mouse Vα14 and human Vα24 NKT cells.

1.3 Tissue Distribution of NKT Cells

As a small population, NKT cells distribute in lymphoid organs in a tissue-specific manner. They are mainly found in the thymus, liver, spleen, and bone marrow, and in blood with reduced frequencies, whereas they are rare in lymph node and gut (31). The

frequency of NKT cells in different tissues varies. For instance, 30-50% of lymphocytes in the liver are NKT cells, whereas NKT cells only consist of about 2-3% of splenic T lymphocytes. In the thymus, where NKT cells develop and mature, less than 1% of total thymocytes are NKT cells (32). However, the absolute number of NKT cells is similar in thymus, spleen and liver (5), in a range of 1 million cells per organ (20). In humans, most studies of NKT cells have been limited to those in peripheral blood. However, a much lower frequency (1 %) of Vα24 NKT cells are present in the human liver compared with mice (33). The distribution of NKT cells in other human tissues remains to be determined. Three subsets ($CD4^+$, $CD8^+$ and DN) of NKT cells are not evenly distributed in different tissues. In thymus, spleen, blood and liver, $CD4^+$ NKT cells are the major NKT cell subset, whereas in other tissues including lymph node and bone marrow, the majority of NKT cells are DN and/or $CD8^+$ (6).

1.4 Development of NKT Cells

1.41 Two Models of NKT Cell Development

In early studies, the original location where NKT cells develop was controversial. It was suggested that NKT cells might have either thymus-dependent or thymus-independent origin (22, 42). However, studies tracking development process with CD1d/αGalCer tetramers (34) have clearly demonstrated that NKT cells developed in the thymus (2, 3, 35, 36). The development progress of NKT cells is highly regulated. NKT cells do not appear in the periphery until day 7 after birth, expand significantly after 3 weeks, and reach a peak plateau at about 6 weeks of age (3). NKT cells experience positive selection during the double positive (DP) stage and branch out from conventional T cells. Two models explain the development of NKT cells in the thymus, the ‘Pre-commitment’

model and the ‘Mainstream’ model (37). In the pre-commitment model (38), Vα14 NKT cells develop from precursor cells that are already distinct from conventional T lymphocytes before TCR rearrangement. This model requires that pre-NKT cells subsequently rearrange the semi-invariant TCR in order to be selected by CD1d for further maturation. According to the mainstream model (20), Vα14 NKT cells originate from a small population of conventional $CD4^+CD8^+$ thymocytes with randomly rearranged Vα14 TCR α and β chains. Their further development depends upon positive selection mediated by TCR engagement with CD1d. Recent evidence supports the mechanism that $CD4^+CD8^+$ thymocytes are diverted to the NKT lineage as a result of CD1d engagement by randomly generated semi-invariant TCRs (37).

1.42 Maturation Progress of NKT Cells

Unlike conventional T lymphocytes that are positively selected by MHC molecules expressed on cortical epithelial cells, CD1d expression in bone marrow derived cortical DP thymocytes is necessary and sufficient for the positive selection of NKT cells (39). Several experiments support that NKT cells also experience negative selection in the thymus. The addition of αGalCer to fetal thymic organ cultures causes Vα14 NKT cell disappearance, and increased expression of CD1d in transgenic mice causes a decrease in the number of Vα14 NKT cells (40). Furthermore, it was found that the negative selection of NKT cells is mediated by CD1d-expressing BM-derived dendritic cells, but not thymic epithelial cells (40).

During the development and maturation, NKT cells express different surface markers. The changes of surface markers reflect a series of activation and differentiation events in

this developmental process. The differentiating thymic NKT cells undergo multiple rounds of cell division leading to a massive lineage expansion. In early stage, NKT cells are $CD44^{low}NK1.1^{-}$ (Stage1); then they acquire $CD44^{high}$ memory phenotype (Stage2) followed by expression of NK cell markers, including NK1.1, CD94/NKG2 and Ly49; and later mature NKT cells are $CD44^{high}NK1.1^{+}$ (Stage3) (Fig.2) (36). Although majority of NKT cells in thymus of adult mice are $NK1.1^{+}$, recent studies have indicated that cells exported from thymus are mostly $NK1.1^{-}$ (2, 3). Thus the peripheral acquisition of NK1.1 expression is possibly stimulated by CD1d expression in the periphery (41). Therefore, it seems that the maturation of NKT cells in thymus and periphery is mediated by separate pathways (Fig.2).

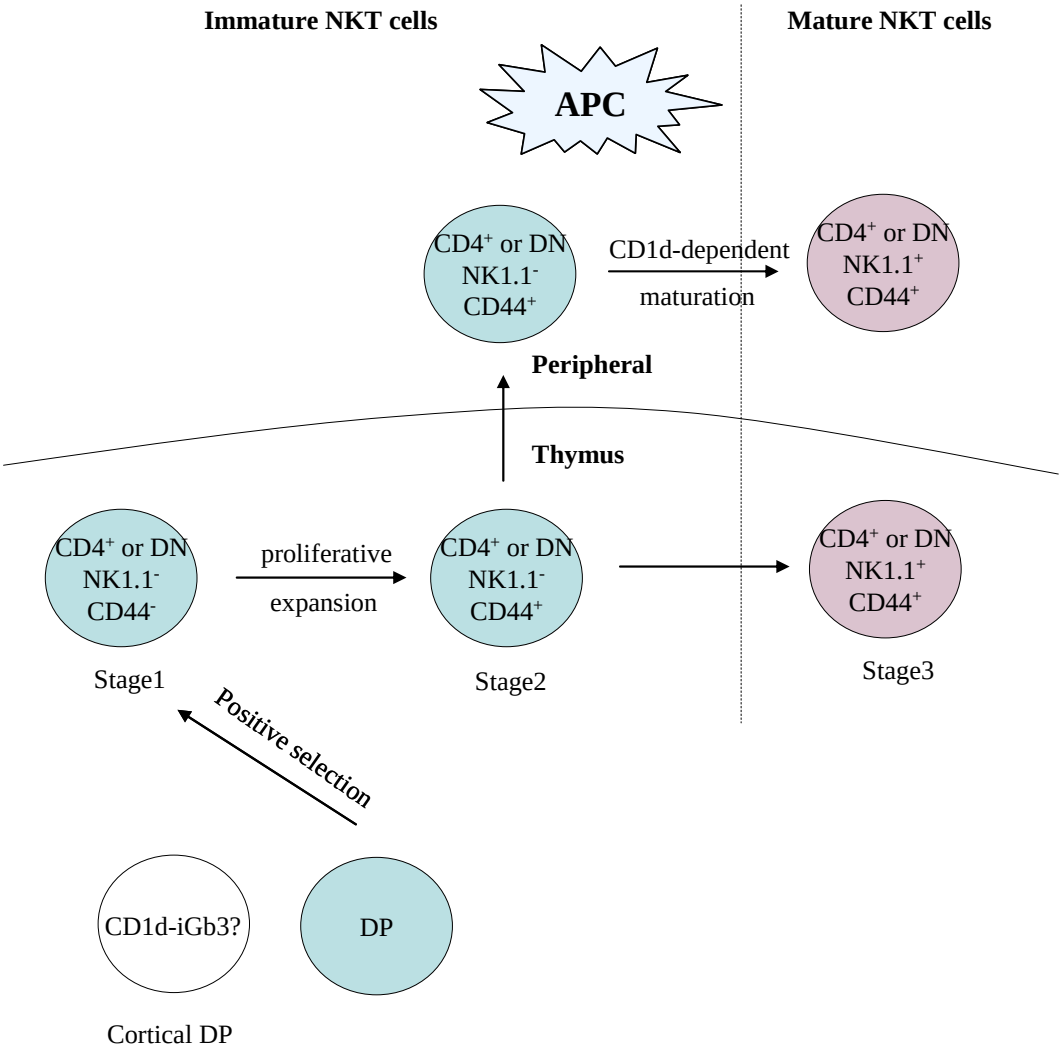

Figure 2 Outline of Vα14 NKT Cell Differentiation. Shown NKT cell development, maturation and phenotypic changes in thymus and spleen. Abbreviations: APC, antigen-presenting cells; DN, double negative ($CD4^-CD8^-$); DP, double positive ($CD4^+CD8^+$).

1.43 Signals required for NKT Cell Development

1.431 CD1d Engagement and molecular Events Downstream of Positive Selection

Although it is clearly demonstrated that CD1d expression is required for the development of NKT cells (24), the molecular events downstream of CD1d selection are poorly understood. Accumulating evidence indicates that signal pathways required for the development of NKT cells are unique. Several transcription factors are involved in NKT cell development, but not in the development of conventional αβ T cells. For instance, Vα14 NKT cell development requires the expression of NF-κB1 (p50), RelB (p65) (42) and κB inhibitor gene, IKK2 (43), whereas development of conventional T cells appears not affected by these factors. In contrast, Ras-MAP kinase signaling pathway is essential for development of conventional T cells, but dispensable for selection of NKT cells (44). The specific signal requirement may reflect unique features of cortical $CD4^+CD8^+$ thymocytes through which NKT cells are selected. Recently, several other transcription factors including T-bet (T-box expressed in T cells) (45), and GATA-3 (46) were identified to play important roles in the NKT cell development. Well established evidence demonstrates that NKT cells fail to develop in mice lacking components of the IL-15 pathway, such as IL-15, IL-15Rα, and IL-2/15Rβ (47). The regulation of NKT cell development by RelB is possibly IL-15 related, since RelB decreases IL-15 production by thymic epithelial cells (42). On the other hand, T-bet deficiency causes a blockage in NKT cell development possibly through a CD122 (IL-2/IL-15 receptor common β subunit)-independent mechanism (45). It is possible that the mechanisms through which other transcription factors control development of NKT cells are also associated with

unique requirements for cytokine signals, such as signals from TGF-β1. It has been shown recently that a defect in TGF-β1 receptor in the T cell lineage resulted in a specific and severe deficiency of NKT cell development (48, 49).

1.432 Ligands required for Positive Selection

NKT cells are positively selected by CD1d-expressing thymocytes derived from the bone marrow (20). It has been shown that lysosomal location of CD1d is necessary for the development of NKT cells, suggesting that selecting ligands may be loaded on CD1d molecules in lysosomes/endosomes (50). Although MHC class II and CD1d molecules are both found in late endosomes/lysosomes, their intracellular trafficking pathways are very different (50). During NKT cell development, the ligand-loaded CD1d molecules mostly have to traffic through endosomal/lysosomal pathway of double positive cells before being displayed on the cell surface to present the ligands to developing NKT cells (Fig.3). Defect of CD1d trafficking results in impaired NKT cell development. For example, APCs from mice that express a germ line deletion of the cytoplasmic tail of murine CD1d (mCD1d) have a defect in glycolipid antigen presentation. These mice also have impaired development of NKT cells despite their greater than normal levels of surface mCD1d expression (51).

One important question for the development of NKT cells is the specific ligand by which NKT cells are positively selected. In 2005, iGb3, a mammalian glycosphingolipid, was suggested to be the ligand responsible for the positive selection of NKT cells (52). This conclusion is based on the finding that mice with a mutant gene (β-hexosaminidase) whose product is a key subunit of enzymes (β-hexosaminidase A and B) necessary for the lysosomal degradation of iGb4 to iGb3 have markedly impaired NKT cell development

(Fig.3), however, iGb3 may not be the only selecting ligand for NKT cells since mutations in several lysosomal proteins with unaffected synthesis of iGb3 can also cause impaired NKT cell development (53). In addition, it was found in a recent study that NKT cell development was nearly normal in mice with deficient iGb3 synthase, further supporting the role of ligands other than iGb3 for NKT cell development (54). Therefore, it is conceivable that multiple glycolipid ligands are involved in the positive selection of NKT cells. It is also possible that different ligands preferentially select subsets of NKT cells expressing different co-receptors or TCR β chains. Although the selecting ligands remain to be clarified, it is clear that normal lipid trafficking and processing in the lysosome is critical for CD1d loading and NKT cell development (55). Taken together, the development of NKT cells depends on processing, presentation and synthesis of endogenous lipid antigens. The involvement of specific transcriptional factors reflects the unique development program of NKT cells. It is possible that the specific transcriptional factors are also required for different membrane signals, in addition to signals from TCR ligation with selecting ligands, for positive and negative selection of NKT cells.

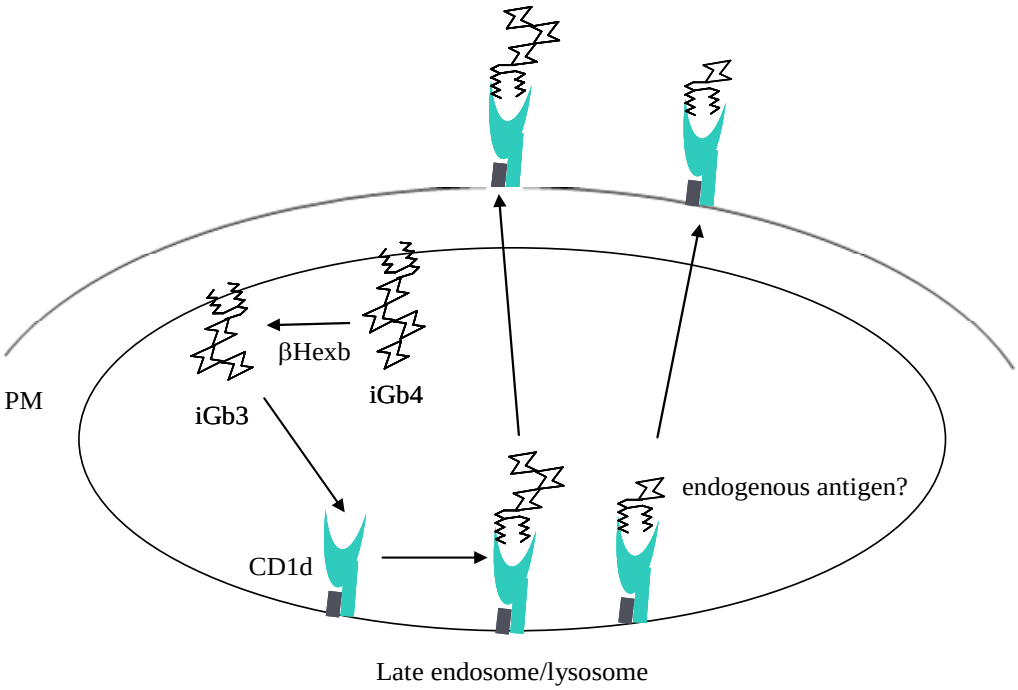

Figure 3 Intracellular trafficking and Lipid loading of CD1d. The endogenous ligand iGb3 is produced through lysosomal degradation of iGb4 by β-hexosaminidase. iGb3 or other endogenous antigens loaded on CD1d traffic through late endosome/lysosome before being displayed on the cell surface. Abbreviations: iGb3, isoglobotrihexosylceramide; βHexb, β-hexosaminidase; PM, plasma membrane

1.5 NKT Cell Homing and Migration

NKT cells develop in the thymus (2, 3, 35, 36), and accumulate selectively at high levels in several peripheral organs including liver, spleen and bone marrow (31). NKT cells have also been found to accumulate in microbial agents-affected tissues (56). However, the homing mechanisms that NKT cells use to regulate their distribution in the periphery, or migrate to inflamed tissues are not well established, although it is clear that similar to conventional T cell migration, chemokines are involved in driving NKT cell trafficking to different organs. Leukocyte function-associated antigen-1 (LFA-I) is proposed to be important for the accumulation of NKT cells in the liver (57). In addition, macrophage inflammatory protein-2 (MIP-2) is able to recruit NKT cells to the spleen during tolerance induction (58). Similarly, monocyte chemotactic protein-1 (MCP-1) plays a critical role in accumulation of NKT cells in the lung after cryptococcal infection (59).

1.6 Activation and Function of NKT Cells

1.61 Glycolipid Antigens of NKT Cells

NKT cells recognize glycolipid antigens in the context of MHC class I like CD1d/β2 microglobulin complex. Among the glycolipids, αGalCer is the most potent ligand for both human and murine CD1d-restricted NKT cells, and Vα14 NKT cells can be strongly activated by αGalCer in the context of CD1d/β2m complex (8, 60). Recently, some foreign microbial glycolipid ligands were also found to be able to activate NKT cells in a CD1d-dependent manner. These glycolipid ligands are isolated from bacteria, protozoa, and viruses (16, 61). These findings provided further supporting evidence for an important role of NKT cells in anti-infection responses. For a long time, NKT cells have

been known for their strong auto-reactivity in a tissue- or cell-specific manner (21, 62, 63), suggesting that endogenous ligands presented by certain types of cells can be recognized by NKT cells (21, 26, 64). However, in spite of extensive searching of lipids presented on cell surface, identification of endogenous ligands functioning as strong stimulators for NKT cells was not successful until the recent finding of iGb3 (isoglobotrihexosylceramide), a lysosomal glycolipid ligand, is likely to be required for NKT cell development, and also has the ability to activate most human or mouse NKT cells in vitro in either natural or synthetic forms (52). However, iGb3 appears to be a weaker agonist to NKT cells than αGalCer, since a much higher concentration of iGb3 is required to reach a similar level of activation induced by a low level of αGalCer (65).

1.62 Activation of NKT Cells and Downstream Events

Since αGalCer is the most potent ligand for NKT cells, it has been widely used to study the activation of NKT cells. In mice, NKT cells can be activated within 2 hours in vivo after αGalCer injection, or they can be activated within 12-24 hours exposure to αGalCer in vitro (8). Activation of NKT cells in vivo and in vitro leads to rapid and robust IL-4 responses (Th2), but also high levels of Th1 cytokine production, especially IFN-γ (20). The rapid production of a large amount of cytokines is the most important characteristic of NKT cells and probably defines the role of NKT cells in immune responses. IFN-γ, one of the major Th1 cytokines, is critical to activate innate and Th1-dependent immune reactions required for anti-tumor and anti-infection activity. In contrast, Th2 cytokine IL-4 downregulates immune reactions required for immune regulatory functions and for prevention of some autoimmune disease (66-68). Thus, the activation of NKT cells

directs immune responses in seemingly opposite directions.

The activation of NKT cells in vivo can also trigger several other downstream events (Fig.4). For example, IL-4 produced by NKT cells triggers upregulation of the activation marker CD69 in most peripheral B cells (13). Upon activation by αGalCer, NKT cells not only upregulate their Th1 and Th2 cytokines, but also increase their CD40L expression. CD40/CD40L interaction between activated NKT cells and Dendritic cells (DCs) induces DC maturation with upregulated expression of costimulatory signals including CD40, CD80 and CD86, as well as MHC class I, MHC class II and IL-12 (69). DCs are professional APCs that play a key role in initiating effector T cell responses in secondary lymphoid organs, and participate in the presentation of self-antigens during the course of central and peripheral T cell tolerance induction (70, 71). Up-regulation of DC costimulatory signals and MHC class I- and class II–mediated antigen presentation is crucial for initiating robust adaptive cellular immune responses mediated by $CD4^+$ and $CD8^+$ T cells (72). Moreover, the cytokine storm released by activated NKT cells can transactivate NK cells to produce IFN-γ 2-24 h post-activation. In fact, NK cells are responsible for the large amounts of "late-phase" IFN-γ observed in serum following in vivo αGalCer stimulation (73). $CD8^+$ T cells are important immune mediators against malignancies and intracellular pathogens such as viruses. NK cells play an important role in viral infections, and can function against intracellular pathogens and tumors. Clearly, the activation of NKT cells mobilizes both innate and adaptive immunity.

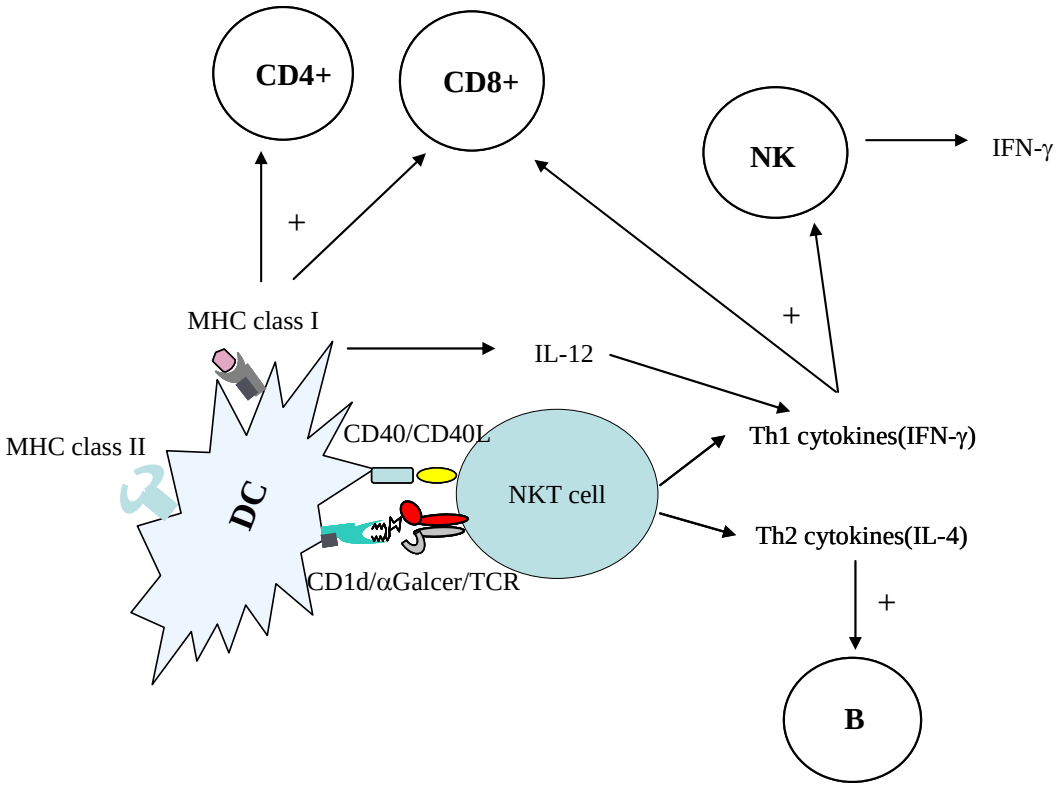

Figure 4 Overview of the Activation of NKT Cells by Glycolipid and Downstream Events. Upon αGalCer stimulation, NKT cells activate and produce large amounts of both Th1 and Th2 cytokines, and upregulate the expression of CD40L on the cell surface. CD40L-CD40 interaction induces DCs maturation by upregulating the expression of MHC class I, MHC class II and costimulatory signals as well as IL-12, which is crucial for initiating robust adaptive cellular immune responses mediated by $CD4^+$ and $CD8^+$ T cells. Furthermore, IL-4 produced by NKT cells can trigger activation of peripheral B cells; whereas IFN-γ production stimulates NK cells and CD8 CTLs mediating anti-tumor cytotoxicity.

1.63 A Critical Role of NKT Cells in Immune Responses

The robust Th1 and Th2 cytokine production together with the bystander activation of other immune cells, including DCs, B, CD8 T and NK cells, by NKT cells indicates an important role of NKT cells in regulating innate and adaptive immunity. Although the mechanisms of NKT cell mediated immune regulation are not well understood, it is clear that NKT cells play an important role in a wide range of immune responses, including anti-tumor and anti-infection responses as well as suppression of autoimmunity.

1.631 NKT Cells and Immune Regulation

From early studies, NKT cells were considered to play a role in immune regulation since they are able to produce large amounts of anti-inflammatory cytokines such as IL-4 and IL-13 (74), and accumulating evidence supports the idea that activation of NKT cells favors Th2 differentiation in subsequent immune responses (75, 76). It has been found that the frequencies and functions of NKT cells in the circulation were abnormal in patients with autoimmune disorders and autoimmune-prone mouse strains, suggesting a strong linkage between immune regulation and NKT cells (76). The regulatory function of NKT cells has been clearly demonstrated in studies in which activated NKT cells inhibit the development of certain autoimmune disorders in animal models. However, whether the regulatory function of NKT cells depends on Th2 cytokine production is still not clear and the detailed mechanisms of immune regulation mediated by NKT cells remain to be identified.

NKT cells and Type I diabetes

Type I diabetes (T1D) also known as insulin-dependent diabetes mellitus (IDDM), is a chronic autoimmune disease where the body’s own immune system turns against its own

pancreatic beta cells (77, 78). Nonobese diabetic (NOD) mice spontaneously develop Type I diabetes, and pathogenic progress in NOD mice has been found to be associated with numerically and functionally deficient NKT cells in these animals (79). Increase in the number of NKT cells in NOD mice by adoptive transfer of thymic NKT cells or transgenic expression of CD1d molecules protected NOD mice from the development of T1D, and the protection has been suggested to be in an IL-4 and/or IL-10-dependent mechanism (68, 80, 81). Targeted mutation of CD1d gene in NOD mice abolished development of NKT cells thus accelerated development of T1D (82). Administration of αGalCer significantly reduced incidence of T1D in NOD mice (75, 81). Deficient frequencies and functions of NKT cells were also reported in T1D patients (83, 84). Although some controversial results remain to be clarified, evidence strongly supports a role of NKT cells in prevention of T1D. However, the mechanisms of NKT cell mediated T1D protection are still not well understood. Numbers of studies suggest that the protection of NOD mice by αGalCer activated NKT cells is associated with the induction of Th2 responses to islet auto-antigens (68, 85).

NKT cells and Multiple Sclerosis (MS)

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system, resulting from T cell mediated immune attack. Experimental Autoimmune Encephalomyelitis (EAE) is an important animal model developed for study of MS by immunizing susceptible rodent strains with CNS antigens. Like NOD mice, administration of αGalCer usually prevented EAE by shifting the balance from a pathogenic Th1 towards a protective Th2 responses to CNS antigens (86). Administration of a novel ligand, which is a structurally modified derivative of αGalCer that

preferentially induced IL-4 production of NKT cells and protected animals from EAE more effectively than αGalCer administration (87).

1.632 NKT Cells and Anti-tumor Responses

NKT cells play a critical role in tumor surveillance and rejection triggered by IL-12. The production of IFN-γ by activated NKT cells is required for generation of anti-tumor Th1 responses. Administration of αGalCer activates NKT cells, leading to the activation of DCs to produce IL-12 (11). IL-12 from DCs enhances NKT cells to produce a large amount of IFN-γ, which in turn stimulates NK cells and CD8 CTLs mediating anti-tumor cytotoxicity (9, 88) (Fig.4). The critical role of IFN-γ production by the activated NKT cells has been demonstrated in IFN-γ or CD1d deficient mice that failed to mount anti-tumor responses triggered by αGalCer (14, 89).

1.633 NKT Cells and Host Defense (anti-infection)

NKT cells have also been shown to participate in protection of mice from a variety of bacterial, protozoan, and viral infections. The participation of NKT cells in anti-microbial responses is thoroughly studied by using NKT cell deficient mice, such as Jα18$^{-/-}$ (9) and CD1d$^{-/-}$ (90) mice. It has been clarified that IFN-γ secretion is an important factor regulating the protective function of NKT cells. At least two mechanisms have been proposed for NKT cells to detect microbial infections through direct and indirect recognition (91). In the direct mechanism, glycolipids from bacteria and parasite are foreign to their mammalian hosts, so they could be recognized as antigens by the invariant TCR of NKT cells (91). On the other hand, some microbes cannot be recognized by NKT cell directly, but they trigger DC maturation and production of

inflammatory signals such as IL-12. NKT cells are activated by IL-12 in the presence of self-glycolipid presented by CD1d on DCs that engaged with invariant TCRs of NKT cells (92).

1.7 Factors Regulating NKT Cell Activation

Although it has been known that NKT cells produce large amounts of both Th1 and Th2 cytokines shortly after activation, and such early cytokine production has significant effects on subsequent immune responses, the exact mechanisms through which NKT cells promote Th1 or Th2 immune responses in different settings remain to be clarified and characterized. In addition, the mechanisms that govern cytokine profiles produced by NKT cells remain to be identified. Clearly, identification of the mechanisms controlling cytokine profile of activated NKT cells will advance our understanding of the regulatory mechanisms mediated by NKT cells. The activation of NKT cells undergoes a series of complex sequential events, and a few factors have been demonstrated to have significant influence on NKT cell activation during the course of activation.

1.71 Subsets of NKT Cells

According to co-receptor expression, NKT cells can be divided into 3 subsets, including $CD4^+$, $CD8^+$ or DN NKT cells (5). These subsets of NKT cells are functionally distinct. The human $CD8^+$ and DN NKT cell subpopulations predominantly produce Th1-like cytokines (93, 94). However, $CD4^+$ NKT cells produce both Th1 and Th2 cytokines, but the level of Th2 cytokines produced by these cells are significantly higher than other NKT subsets (93-96). Since it is difficult to detect $CD8^+$ NKT cells in mouse, it remains unclear whether different cytokine profiles are also associated with subsets of murine NKT cells. CD4 and CD8 coreceptors on the surface of conventional T cells bind to

antigen/MHC class II or class I molecules on the surface of APCs during activation. In contrast, there is no evidence for binding of CD4 or CD8 with CD1d. Therefore, it is unlikely that different coreceptors control cytokine production of NKT cells. In addition to different cytokine profiles, subsets of human NKT cells were also reported to express different chemokine receptors and cytotoxic molecules, further suggesting differentiated functions of subset of NKT cells (93, 96, 97). In mice, although dichotomy of cytokine production has not been observed, functional differentiation between subsets of NKT cells was also reported (98).

1.72 Structure of Lipid Antigens

Most of the glycolipids recognized by NKT cells share similar structure of one or several sugar heads and two hydrophobic alkyl tails (Fig.1) (22, 26, 99, 100), however, subtle structural variations in lipid antigens may modulate cytokine production, since the modifications in either hydrophobic alkyl tails or sugar moiety of αGalCer can preferentially affect cytokine production of responding NKT cells. For example, derivatives of αGalCer with a truncated sphingosine chain, such as OCH and C20:2 predominantly induce IL-4 production by NKT cells. Conversely, an analogue of αGalCer that has modified sugar moiety (α-C-GalCer) biases NKT cells for IFN-γ production (87, 101-104).

1.73 Cytokines

Several cytokines are also found to be able to stimulate and regulate NKT cells. These cytokines include common γ-chain (γ_c) receptor cytokines such as IL-2, IL-7, IL-15, and recently identified IL-21, as well as DC-derived cytokines such as IL-12 and IL-18 (105).

These cytokines function differently on NKT cell regulation. IL-2, IL-7, IL-15 and IL-21 increase the proliferation of NKT cells (105), whereas IL-12 and IL-18 can stimulate NKT cell cytotoxicity against tumors (106). Moreover, cytokines can also enhance NKT cell cytokine production. For example, IL-7 enhances IL-4 production by thymic and splenic NKT cells, whereas IL-12 enhances IFN-γ production by these cells (81, 82).

1.74 Ly49 Inhibitory Receptors on NKT Cells

The expression of NK inhibitory receptors, including Ly49 receptor family and CD94/NKG2 receptors may be another important factor influencing NKT cell activation. Ly49 is a multi-gene family, comprised of at least nine C-type lectin-like receptors. The inhibitory and activating forms of Ly49 family members contain immunoreceptor-tyrosine inhibition motifs (ITIMs) or immunoreceptor-tyrosine activation motifs (ITAMs), respectively (107). It was shown that the Ly49 receptor family plays an important role in NK and CD8 T cell activation. Ly49 receptor family binds classical MHC class I molecules (108). Upon MHC class I binding, most Ly49 receptors on NK or CD8 T cells mediate inhibitory signals that counteract constitutive activating signals through several surface receptors and prevent the NK and CD8 T cells from activation or killing target cells (109, 110). The surface receptors providing positive signals on NK cells are CD69 and CD28 and several other NK cell receptors (111). In addition to Ly49, CD94/NKG2 receptors that recognize the nonclassical MHC class Qa-1b are also known to inhibit NK cell activation (112). However, the role of Ly49 receptor family and CD94/NKG2 receptors on NKT cell activation has not been thoroughly studied. Several studies have demonstrated that engagement of Ly49 receptors can inhibit NKT cell proliferation, cytokine production and cytotoxic activity (113-116). Moreover, CD94/NKG2 receptors

are also proposed to regulate NKT cell activation since blockage of the CD94/NKG2A inhibitory pathway augmented recall and primary responses of NKT cells (117).

1.75 Subsets of APCs

It was also found that subsets of APCs (antigen presenting cells) display different effects on cytokine profile of NKT cells (118). Splenic APCs can enhance IFN-γ production by thymic NKT cells (67, 118). Among subsets of APCs, DCs play a specific role in NKT cell activation, since splenic NKT cell activation is dependent upon DC following in vivo αGalCer stimulation, but not B cells, macrophages or Kupffer cells that appear to be important for liver NKT cell activation (119, 120). In addition, αGalCer-loaded DCs induce a prolonged IFN-γ responses mediated by NKT cells (121). The unique ability of DCs for activation of NKT cells indicates specific but unidentified mechanisms that regulate NKT cells.

1.76 Costimulatory Signals

It has been well characterized that costimulatory signals are essential for full activation of conventional T cells, and TCR ligation in the absence of co-stimulatory signals results in anergic T cells that are hyporesponsive to subsequent antigenic stimulations (122). Therefore, in the early studies, the role of cotstimulatory signals in the activation of NKT cells was carefully investigated. Studies have shown that NKT cells constitutively express CD28 (123) and CD40L (69) on the cell surface. Numerous early studies have reported that costimulatory molecules play a role in activation and cytokine production of NKT cells (8, 123, 124), since the blockage of CD28 signals by antibody reduced cytokine production of NKT cells, whereas blockage of CD40L reduced IFN-γ but not

IL-4 production. In addition, injection of αGalCer into CD28 deficient mice triggered much lower levels of both IFN-γ and IL-4 production by NKT cells, whereas αGalCer injection into CD40 deficient mice resulted in decreased IFN-γ but not IL-4 production (123). Furthermore, the preventive effect of αGalCer injection on EAE was abolished in CD28 deficient mice (125). In contrast to the above reports, a recent study suggested that CD28 and CD40 costimulatory signal pathways were not required for the activation and cytokine production of NKT cells in vivo. This conclusion was based on the observation that αGalCer injection into CTLA4-Ig transgenic mice in which the B7/CD28 signaling pathway was blocked by soluble CTLA4-Ig stimulated a significant portion of NKT cells to produce IFN-γ in the spleen similar to that in WT mice (124). The different methods used in these studies for detection of cytokine production may associate with the controversial results. However, these controversial results raised a possibility that costimulatory signals control cytokine production of NKT cells through mechanisms different than that in conventional T cells. Therefore, the role of costimulatory signals in NKT cell activation needs further investigation, particularly in view of the fact that NKT cells undergo a unique development process compared with conventional T cells. More recently, another costimulartory molecule, GITR (Glucocorticoid-induced TNF receptor), was found constitutively expressed on NKT cells. GITR was proposed to be a potent costimulator of NKT cell activation both in vitro and in vivo (126). This study is very intriguing since GITR is highly expressed on another small subset of T cells, regulatory T cells (Treg).

1.8 General Hypotheses and Research Objectives

1.81 General Hypotheses

Although comprising only a minor subpopulation of T lymphocytes, NKT cells play important roles in both innate and adaptive immune regulation because of rapid IL-4 and IFN-γ secretion. Many factors, including structural variations of ligands, cytokines and NK receptors, have been known to impact the activation and cytokine secretion of NKT cells. However, the mechanisms underlying NKT cell regulation by critical factors remain to be identified. For instance, it is known that NKT cell activation is dependent on subsets of APCs and costimulatory signals as summarized in previous sections, but it is not known why it is the DCs but not other APCs that are required for the presentation of glycolipid ligands to activate NKT cells and how CD28/B7 co-stimulatory signals are required in some studies but not others. These are two basic questions with fundamental importance for us to understand the unique activation profile of NKT cells.

1.82 Specific Hypothesis 1

The **first objective** of my research is to identify the mechanisms involved in regulation of the activation of NKT cells by subsets of APCs. It has been shown that subsets of APCs display different effects on cytokine profile of NKT cells in vivo (118). The different effects of subsets of APCs to initiate NKT cell activation may be due to their different ability for antigen presentation, as well as their different cytokine secretion or co-stimulatory ability. Although it is most likely that combinational effects of multiple factors determine the activation of NKT cells, especially under in vivo conditions, the role of these factors needs to be analyzed and clarified individually. Among these factors, autoreactivity of NKT cells against endogenous lipid antigens has been clarified to be

crucial for the development of NKT cells (50-53, 55), and is also required for activation of NKT cells during infection by microbes that are not recognized by NKT cells (27). However, it is not known whether autoreactivity plays any role when NKT cells encounter exogenous lipid antigens. Our **specific hypothesis 1** is that **subsets of APCs display different patterns or levels of endogenous lipid antigens, and that subsets of APCs can equally present lipid antigens, but their ability to trigger NKT cell activation depends on cell-specific display of endogenous lipid antigens**.

We will test this hypothesis in the following objectives:

Objective 1A. To determine if different APCs have different antigen presentation ability.

Objective 1B. To determine whether cytokine production, co-receptors and costimulatory signals delivered by APCs are critical for triggering the activation of NKT cells.

Objective 1C. To determine whether the different abilities of subsets of APCs for activation of NKT cells are due to cell-specific presentation of endogenous lipid antigens.

1.83 Specific Hypothesis 2

In addition to endogenous lipid antigens, APCs also express many other molecules, including CD80 (B7.1), CD86 (B7.2) and CD40 molecules that are important for the activation of both conventional T and NKT cells. Costimulatory signals are required for full activation of conventional T cells. In the absence of costimulatory signals, T cells are unable to proliferate and produce cytokines (122). However, the role of costimulatory molecules in activation of NKT cells may be different than that of conventional T cells.

There was strong evidence either supporting or against the role of co-stimulatory signals in the activation of NKT cells (123, 124). The discrepancy may be due to technique issues such as different mouse models or detection methods used in different studies. Alternatively, the discrepancy may reveal unique mechanisms by which co-stimulatory molecules define the function of activated NKT cells. Therefore, it is necessary to reevaluate the role of co-stimulatory signals using combined approaches to measure the activation and cytokine production of NKT cells. Based on the results of previous studies, our **specific hypothesis 2** is as follows: **in the absence of costimulatory signals, NKT cells can be activated to synthesize cytokines, but fail to secrete these intracellular cytokines**. **Alternatively, deficient costimulatory pathway may affect development or homeostasis of NKT cells, resulting in reduced cytokine production.** Therefore, the **second objective** of my research project is to define the role of CD28/B7 costimulatory signals in the activation and development of NKT cells. We will test the above hypotheses in objectives proposed below.

Objective 2A. To determine whether CD28/B7 costimulatory signals are required for the activation and cytokine production of NKT cells.

Objective 2B. To determine whether CD28/B7 costimulatory singals are required for NKT cell development.

CHAPTER TWO: MATERIALS AND EXPERIMENTAL PROCEDURES

2.1 Mice, Materials, Chemicals and Reagents

2.11 Mice

Wild type, CD4, CD28, CD40, CD80, CD86, CD80/86, IL-12 and IFN-γ gene-deficient C57BL/6 mice, as well as C57BL/6.scid mice were purchased from Jackson Laboratory (Bar Harbor, ME). C57BL/6$^{Cd1d-/-}$ mice were developed as previously described (127). C57BL/6$^{HexB-/-}$ mice were a kind gift from Dr. Roy Gravel (University of Calgary). 8.3-NOD mice were a kind gift from Dr. Pere Santamaria (University of Calgary). All mice were maintained in a specific pathogen-free facility at the University of Calgary, in accordance with Institutional Animal Care and Use Committee Guidelines.

2.12 Chemicals, Antibodies, CD1d monomers, αGalCer/CD1d Tetramers and Cell Lines

Alpha-galactosylceramide (αGalCer) was provided by Kirlin Brewery Co. (Tokyo, Japan). Isoglobotrihexosylceramide (iGb3) was a kind gift from Dr. Albert Bendelac (University of Chicago). Concanamycin A (CMA), bafilomycin A1 (Baf), DL-thero-1-phenyl-2-palmitoylamino-3-morpholine-1-propanol (PPMP), threo-1-phenyl-2-decanoylamine-3-morpholine-propanol hydrochloride (PDMP), and Deoxygalactonojirimycin (DGJ) were purchased from Sigma (Saint Louis, MO). Isolectin B4 (IB4) was obtained from Vector laboratories (Burlingame, CA). All antibodies were purchased from BD Pharmingen Canada (Mississauga, ON). A20 B lymphoma cell lines, CD1d monomers and αGalCer/CD1d tetramers were provided by Dr. Steven Porcelli (Albert Einstein College of Medicine, New York).

2.2 Experimental Procedures

2.21 Lymophocyte Isolation

Cells including S-APCs, DCs, B cells and NKT cells were isolated from 6 to 8 week-old mice.

2.211 Isolation of S-APCs, DCs, B Cells and T cells from Spleen

Spleens were processed into single cell suspension, and red blood cells lysed using Hemolysis Buffer (containing 155 mM ammonium chloride, 10 mM potassium hydrogen carbonate, and 0.1 mM EDTA-2Na). After hemolysis, cells were washed with complete RPMI medium that contains 10% fetal calf serum (FCS), 5 x 10^{-4} M 2-mercaptoethanol, 2 mM L-glutamine, 100 U/ml penicillin and 2 mM sodium pyruvate. Splenocytes from C57BL/6.scid mice, which do not respond to either anti-CD3 antibody or αGalCer in cultures, were prepared for S-APCs. To purify B cells from C57BL/6 mice, red blood cells were removed by Hemolysis Buffer, then splenocytes were washed and incubated with anti-CD19 antibody conjugated microbeads (40 μl beads per spleen) (Miltenyi Biotec Inc., Auburn, CA) for 15 minutes at 4^{o}C, and then washed with MACs buffer which contains 1% BSA, 2 mM EDTA in PBS, then CD19^{+} B cells were purified via positive selection in the MiniMACS system (Miltenyi Biotec, Auburn, CA). To purify DCs from splenocytes of wild type, CD40, CD80, CD86, CD80/86, IL-12 and IFN-γ gene-deficient C57BL/6 mice, splenocytes were incubated with anti-CD11c antibody conjugated microbeads (12 μl beads per spleen) for 15 minutes at 4^{o}C, and then washed with MACs buffer. Subsequently, CD11c^{+} DCs were purified via positive selection in the MiniMACS system (Miltenyi Biotec, Auburn, CA). To purify T cells from splenocytes of

wild type C57BL/6 mice, splenocytes were incubated with anti-Thy1.2 antibody conjugated microbeads (40 μl beads per spleen) for 15 minutes at 4^{o}C, and then washed with MACs buffer. Following wash, Thy1.2^{+} T cells were purified via positive selection in the MiniMACS system (Miltenyi Biotec, Auburn, CA).

To prepare αGalCer loaded DCs or B cells, isolated DCs (2 x 10^6 cells/mL) or B cells (10 x 10^6 cells/mL) were incubated with αGalCer at various concentrations at room temperature for 4 hours. The cells were then washed with complete RPMI medium.

To fix the cells, DCs (2 x 10^6 cells/mL) or αGalCer loaded DCs (2 x 10^6 cells/mL) were fixed by washing the cells with PBS before the addition of 0.06% glutaraldehyde for 30 seconds, followed by an equal volume of 0.2 M glycine. Cells were then washed again with PBS and resuspended in complete RPMI medium.

To block the processing and presentation of endogenous antigens, DCs (2 x 10^6 cells/mL) were incubated with 0.5 nM concanamycin A (CMA), 10 nM bafilomycin A1 (Baf), 2.5 μM DL-thero-1-phenyl-2-palmitoylamino-3-morpholine-1-propanol (PPMP) plus 10 μM threo-1-phenyl-2-decanoylamine-3-morpholine-1-propanol hydrochloride (PDMP), 1 mM Deoxygalactonojirimycin hydrochloride (DGJ), or incubated with different concentrations of isolectin B4 (IB4) at room temperature for 4 hrs. The cells were then washed with complete RPMI medium.

2.212 Isolation of Thymic NKT Cells

Thymic NKT cells were isolated from WT, CD4 and CD28 gene deficient C57BL/6 mice. In brief, thymi were processed into single cell suspension, washed with complete RPMI medium, and then labeled with phycoerythrin (PE) conjugated anti-NK1.1 antibody (8 μl

antibody per thymus) at 4°C for 15 minutes. After washing twice, cells were incubated with anti-PE antibody conjugated microbeads (10 μl beads per thymus) at 4°C for 15 minutes, and then washed with MACs buffer. Subsequently, $NK1.1^+$ NKT cells were purified via positive selection in the MiniMACS system (Miltenyi Biotec, Auburn, CA). The purity of isolated NKT cells was examined by FACS analysis using anti-TCRβ-FITC, anti-NK1.1-PE and allophycocyanin (APC)-labeled αGalCer/CD1d tetramer staining (Fig.1a).

2.213 Preparation of DN32.D3 and A20 Cell Lines

The DN32.D3 and A20 B lymphoma cell lines were maintained in RPMI medium with 10% fetal calf serum (FCS), 5×10^{-4} M 2-mercaptoethanol, 2 mM L-glutamine, 100 U/ml penicillin and 2 mM sodium pyruvate at 37°C in a humidified 5% CO_2 atmosphere. The construction of the cytoplasmic tail mutant CD1.1 TD and its expression in A20 cells has been described elsewhere (64).

2.22 In vitro αGalCer-induced Activation Assay

2.221 Activation of Thymic NKT Cells by Plate-bound anti-CD3 Antibody and αGalCer/CD1d Monomers

Anti-CD3 antibody was diluted at various concentrations (1 μg/mL, 2 μg/mL, 5 μg/mL) in PBS to coat 96-well plates (Becton Dickinson, NJ. USA) at 37°C for 6 hrs. The plates were then washed with PBS twice before use. Plate-bound αGalCer/CD1d monomers were prepared by incubating various concentrations of αGalCer (10 ng/mL, 100 ng/mL, 500 ng/mL) and CD1d monomers (1 μg/mL, 3 μg/mL) at 4°C overnight, and then coated at 37°C for 6 hrs in 96-well plates. The coated plates were washed twice with PBS before

use. Plate-bound αGalCer/CD1d was fixed by adding 0.06% glutaraldehyde to the plates for 30 seconds followed by an equal volume of 0.2 M glycine. The plates were then washed again using PBS. Thymic NKT cells (2.5 x 10^5 cells/mL) were added to the previously described plates and incubated at 37^{o}C for 48 hrs with 5% CO_2 in the absence or presence of DCs, drug-treated DCs, S-APCs, B cells or A20 cell lines. B cells are purified from WT C57BL/6 mice. DCs are purified from WT, IFN-γ, or IL-12 gene-deficient C57BL/6 mice.

2.222 Activation of Splenic NKT Cells by Plate-bound anti-CD3 Antibody and αGalCer/CD1d Monomers

Plate-bound αGalCer/CD1d monomers were prepared by incubating αGalCer (100 ng/mL) and CD1d monomers (1 μg/mL) at 4^{o}C overnight, and then coated at 37^{o}C for 6 hrs in 96-well plates. The coated plates were washed twice with PBS before use. Splenic T cells (2.5 x 10^6 cells/mL) were added to the previously described plates and incubated at 37^{o}C for 48 hrs with 5% CO_2 in the absence or presence of different numbers of DCs or B cells.

2.223 Activation of Thymic NKT Cells by soluble αGalCer or αGalCer-loaded DCs

Thymic NKT cells (2.5 x 10^5 cells/mL) were cultured with different concentrations of soluble αGalCer (10 ng/mL, 100 ng/mL, 500 ng/mL) together with DCs (1 x 10^5 cells/mL), or cultured with different concentrations of αGalCer (100 ng/mL)-loaded DCs (0.2 x 10^5 cells/mL, 1 x 10^5 cells/mL, 5 x 10^5 cells/mL) in 96-well round bottom tissue culture plates for 48 hrs at 37^{o}C with 5% CO_2 in complete RPMI medium. DCs are purified from WT, CD80, CD86, and CD80/86 gene-deficient C57BL/6 mice.

2.224 Activation of Thymic NKT Cells by αGalCer-loaded B Cells

Thymic NKT cells (2.5 x 10^5 cells/mL) were cultured with different concentrations of αGalCer-loaded B cells (2 x 10^5 cells/mL, 10 x 10^5 cells/mL, 50 x 10^5 cells/mL) in the absence or presence of different concentrations of DCs (0.2 x 10^5 cells/mL, 1 x 10^5 cells/mL, 5 x 10^5 cells/mL) in 96-well round bottom tissue culture plates for 48 hrs at 37^{o}C with 5% CO_2 in the complete RPMI medium. DCs and B cells were purified from WT C57BL/6 mice.

2.23 In vivo Treatment with αGalCer

Wild type, CD28, CD80, CD86 or CD80/86 gene deficient C57BL/6 mice were injected I.P. with 200 μl PBS or 5 μg of αGalCer in 200 μl PBS per mouse. 3 hrs later, mice were euthanized and bled. For ELISA, the blood was allowed to clot, and the serum was separated from the clot by centrifugation. For intracellular cytokine staining, splenocytes were collected from the euthanized mice (see 2.27 for detail).

2.24 Conjugate Formation

Isolated DCs or B cells (10 x10^6 cells/mL) were first stained with biotinylated anti-I-A^b antibody (1 μg/mL), followed by Streptavidin-PerCP. After incubating with thymic NKT cells (NK1.1-PE stained, 10 x10^6 cells/mL) for various durations (0, 20min, 40min) at 37^{o}C, the cells were collected, fixed with 1% paraformaldehyde for 10 min at room temperature, washed and re-suspended for FACS analysis.

2.25 ELISA

Supernatants collected from cultures or serum collected form αGalCer injected mice were tested for IFN-γ and IL-4 production in an ELISA according to standard protocols

used in our laboratory. Capture antibody was coated in the 96-well ELISA plate in room temperature overnight. After blocking with phosphate-buffered saline containing 5% neonatal calf serum, standard antibody and serum collected from cultures will be added on the ELISA plate. 2 hours later, the plates will be incubated with detection antibody for another 2 hours, followed by Streptavidin-HRP for 20 minutes. In the last step, substrate solution will be incubated on the plates for 20 minutes and stop by 2 N H_2SO_4. The result will be analyzed in an ELISA reader at 450 nm and 570 nm.

2.26 Flow Cytometric Analysis

Surface phenotype of the DCs was characterized by one-color flow cytometry. Briefly, 1-3 x 10^6 DCs in 30 µl FACS buffer (containing 1% FCS in PBS) were incubated on ice for 15 min with PE-conjugated anti-mouse CD1 (0.3µl), FITC-conjugated anti-mouse CD11c (0.3µl), PE-conjugated anti-mouse K^b (0.3µl), PE-conjugated anti-mouse I-A^b (0.3µl), FITC-conjugated anti-mouse CD40 (0.3µl), or FITC-conjugated anti-mouse CD80 (0.3µl), separately, followed by two washes in FACS buffer, then the stained cells were resuspended in PBS and analyzed on a FACS Calibur (Becton Dickinson, San Jose, CA).

NKT cell population was characterized by two or three-color flow cytometry. Thymocytes and splenocytes were stained differently. In brief, for thymocytes, 1-3 x 10^6 cells in 30 µl FACS buffer (containing 1% FCS in PBS) were incubated on ice for 15 min with Streptavidin (0.3µl). After washed, cells were incubated in 40 µl FACS buffer at room temperature for 90 min with PE-conjugated αGalCer/CD1d tetramer (2µl), followed by two washes in FACS buffer. Then, cells were incubated in 30 µl FACS buffer on ice for another 15 min with FITC-conjugated anti-mouse TCRβ antibody

(0.3μl). For splenocytes, 1-3 x 10^6 cells in 30 μl FACS buffer were incubated on ice for 15 min with Streptavidin (0.3μl), after washed, cells were incubated in 40 μl FACS buffer at room temperature for 90 min with APC-conjugated αGalCer/CD1d tetramer (1μl), followed by two washes in FACS buffer. Then, cells were incubated in 30 μl FACS buffer on ice for another 15 min with FITC-conjugated anti-mouse TCRβ (0.3μl) and PE-conjugated anti-mouse CD19 antibody (0.3μl), followed by two washes in FACS buffer, then the stained cells were resuspended in PBS and analyzed on a FACS Calibur (Becton Dickinson, San Jose, CA).

Conventional T cell population was characterized by two-color flow cytometry. 1-3 x 10^6 thymocytes in 30 μl FACS buffer were incubated on ice for 15 min with PE-conjugated anti-mouse CD4 (0.3μl) and FITC-conjugated anti-mouse CD8 (0.3μl) antibody, followed by two washes in FACS buffer, then the stained cells were resuspended in PBS and analyzed on a FACS Calibur (Becton Dickinson, San Jose, CA).

Treg cell population in spleen was characterized by two-color flow cytometry. 1-3 x 10^6 splenocytes in 30 ml FACS buffer were incubated on ice for 15 min with PE-conjugated anti-mouse CD4 (0.3μl) and FITC-conjugated anti-mouse CD25 (0.3μl) antibody, followed by two washes in FACS buffer, then the stained cells were resuspended in PBS and analyzed on a FACS Calibur (Becton Dickinson, San Jose, CA).

2.27 Intracellular Cytokine Staining

BD Biosciences intracellular staining kit with GolgiStop was used in this step. 4 hrs after αGalCer injection, splenocytes were collected from the euthanized mice, and then T cells were isolated from splenocytes using anti-thy1.2 antibody conjugated microbeads (40 μl

beads per spleen) (Miltenyi Biotec Inc., Auburn, CA). Thy1.2^{+} T cells were cultured for 2 hours in 2.0 μM monensin (GolgiStop; BD Biosciences) without in vitro stimulation. 2 hrs later, cells were surface labeled first. 1-3 x 10^6 cells in 30 μl FACS buffer (PBS containing 1% FCS) were incubated on ice for 15 min with Streptavidin (0.3μl), after wash, cells were incubated in 40 μl FACS buffer at room temperature for 90 min with APC-conjugated αGalCer/CD1d tetramer (1μl), followed by two washes in FACS buffer. Then, cells were incubated in 30 μl FACS buffer on ice for another 15 minutes with FITC-conjugated anti-mouse TCRβ antibody (0.3 μl). After surface antiboday labeling, lymphocytes were washed once before fixed in 250 μl perm/fix buffer (BD Biosciences) at 4^{o}C in the dark for 20 min. Cells were then washed twice in 1 x perm/wash buffer (BD Biosciences) before incubation with PE-conjugated antibodies to IFN-γ (5 μg/mL) or IL-4 (20 μg/mL). Intracellular staining was performed in 50 μl 1 x perm/wash buffer (BD Biosciences) for 30 min at room temperature in the dark.

2.28 Proliferation Assay

For proliferation assay, NKT cells were activated for 48 hours, and then the cultures were pulsed with 1uCi of H^3-thymidine for an additional 16 hours and then harvested. The incorporation of thymidine was measured by scintillation counting.

2.29 Statistical Analysis

Data were presented as mean ± SD. Statistical analysis was performed with SPSS 11.5 software. For all the variables included in the analysis, we did not find evidence of non-normal distribution (Shapiro-Wilk test). For comparisons between two means an unpaired *t*-test was used. Comparisons among three or more experimental groups were performed

using a two-way analysis of variance (ANOVA) or one-way analysis of variance (ANOVA) followed by either Dunnett's multiple comparison or Student-Newman-Keuls post hoc test. The differences were assumed to be significant with a p value < 0.05.

CHAPTER THREE: RESULTS

3.1 The Regulation of NKT Cells by CD1d-dependent Lipid Antigen Autoreactivity

3.11 NKT Cells are activated differently by different APCs

In order to clarify the role of different APC subsets in the presentation of αGalCer to activate NKT cells in vitro, we purified DCs and B cells from C57BL/6 mice, and S-APCs (splenocytes) from C57BL/6.scid mice. S-APCs are splenocytes isolated from C57BL/6.scid mice, which do not have T and B cells and do not respond to either anti-CD3 antibody or αGalCer in cultures. Since mature NKT cells are retained in thymus and can produce very high levels of cytokines when activated, we also isolated $NK1.1^+$ thymocytes that were mostly $CD4^+$ αGalCer/CD1d $tetramer^+$ Vα14 NKT cells (Fig.5a). DCs, B cells and S-APCs express similar levels of CD1d molecules on the cell surface (Fig.5b). When these DCs, B cells and S-APCs were used to present αGalCer to activate thymic NKT cells, we found that B cells functioned poorly as APCs to present αGalCer to activate NKT cells. αGalCer presented by B cells triggered NKT cells to produce a very low level of IL-4 only if the concentration of B cells was as high as $1x10^5$/mL, however, when presented by B cells, αGalCer hardly induced any IFN-γ production of NKT cells. In contrast, DCs presented αGalCer to activate NKT cells potently even at a concentration as low as $0.1x10^3$/mL, and induced both IFN-γ and IL-4 production by NKT cells (Fig.6a). The ability of S-APCs to present αGalCer to activate NKT cells was intermediate between B cells and DCs (Fig.6a). This experiment demonstrated the different abilities of subsets of APCs to trigger NKT cell activation in the presence of an identical concentration of exogenous lipid antigens. Clearly, as APCs for NKT cells, DCs

are much more efficient. The difference in cytokine production by NKT cells was not due to different abilities of subsets of APCs to present αGalCer, since hybridoma DN32.D3 cells produced similar levels of IL-2 when activated by αGalCer presented by different subsets of APCs (Fig.6b), suggesting that it is APC function rather than αGalCer presentation that determine the activation of NKT cells.

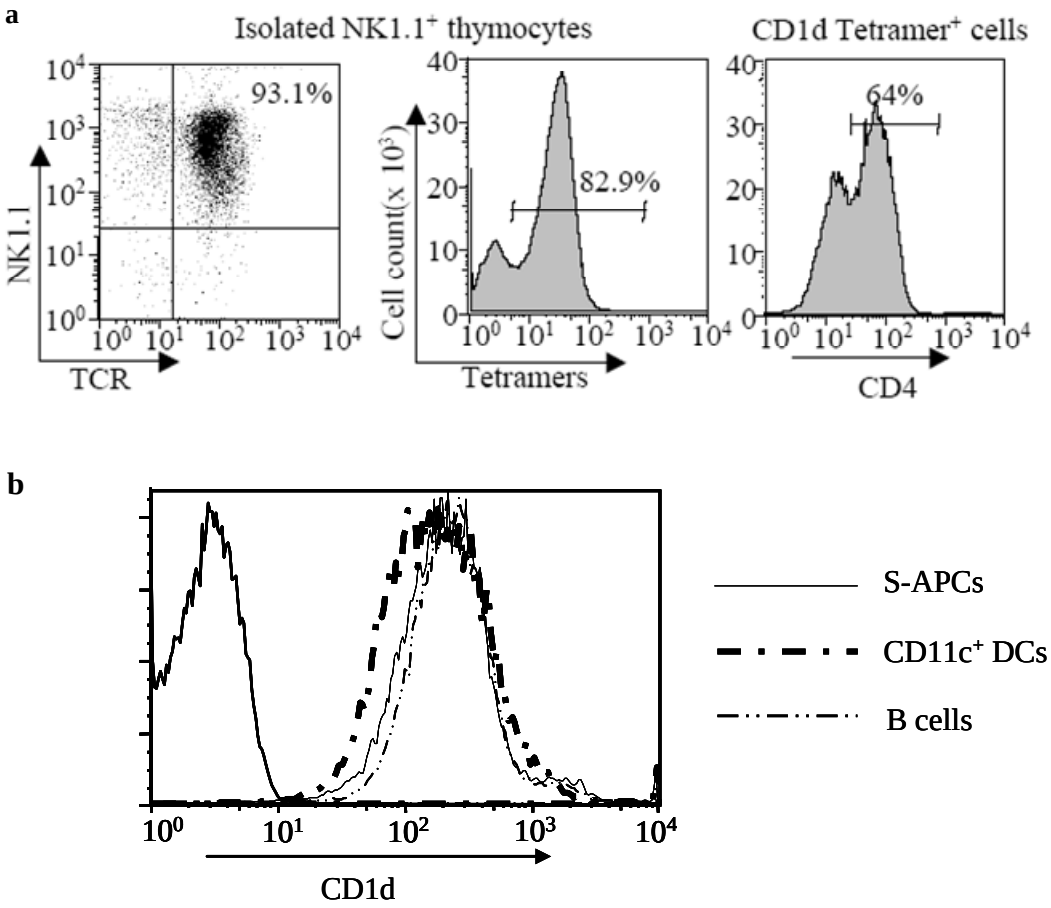

Figure 5 Thymic NKT Cells and Subsets of APCs (a) Isolated NK1.1^{+}TCR^{+} thymic NKT cells (left). Most of these cells were αGalCer/CD1d tetramer positive (middle). More than 60% of the tetramer positive cells were CD4 positive (right). (b) CD1d expression on S-APCs, DCs and B cells.

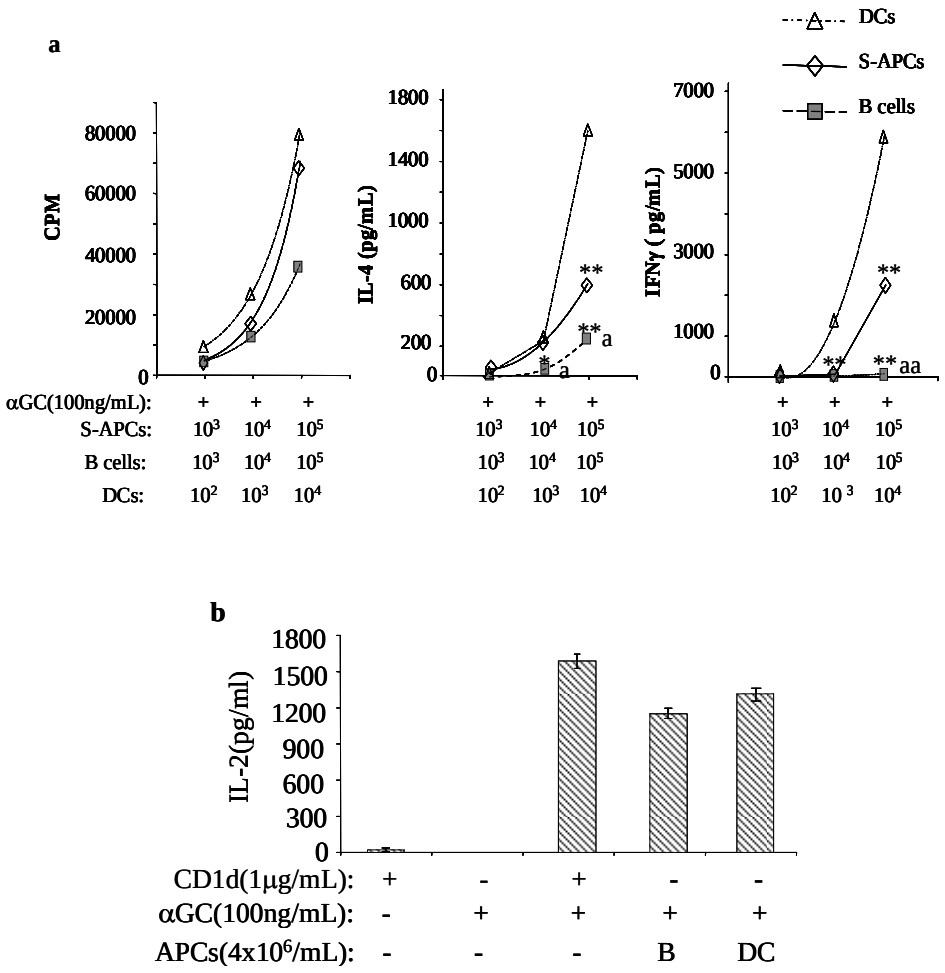

Figure 6 NKT Cells are activated differently by subsets of APCs. (a) Proliferation (left) and cytokine production (right) of thymic NKT cells activated by αGalCer in the presence of S-APCs, DCs or B cells. Two-way ANOVA demonstrated a significant effect of both APC subsets ($P < 0.01$) and APC concentrations ($P < 0.05$) on IFN-γ and IL-4 production. One-way ANOVA followed by Student Newman-Kuels post hoc test was conducted at each APC concentration, $^{**}p< 0.01$ or $^{*}p< 0.05$ versus DC group, $^{aa}p<0.01$ or $^{a}p<0.05$ versus S-APC group (b) IL-2 production of DN32.D3 hybridoma NKT cells ($1x10^6$/mL) activated by soluble αGalCer (100 ng/mL) in the presence of CD1d (1 μg/mL) or subsets of APCs. The data are expressed as the mean ± S.D. of triplicate wells. One representative experiment of three is shown.

3.12 DCs potentiate NKT Cells to be efficiently activated by low Levels of αGalCer.

To understand the role of DCs in the effective activation of NKT cells, we compared the response of NKT cells to αGalCer presented by CD1d monomers or DCs *ex vivo*. We isolated $NK1.1^+$ thymocytes, and activated these NKT cells by immobilized CD1d monomers that had been loaded with αGalCer (100 ng/mL). We found that the cytokine production by activated NKT cells was CD1d monomer dose-dependent. Significant amounts of cytokines were only detected when the concentration of CD1d monomers was higher than 6 μg/mL (Fig.7a). A high concentration of αGC (100 ng/mL) was also required for the activation of thymic NKT cells. Thymic NKT cells were also activated by plate-bound anti-CD3 antibody when the coating concentrations of anti-CD3 antibody were over 2 μg/mL (Fig. 7b). Clearly, thymic NKT cells can be activated by TCR ligation in the absence of co-stimulatory signals if the strength of signals from TCR ligation was sufficient.

In contrast to the requirement of high concentrations of αGalCer loaded CD1d monomers for NKT cell activation, when thymic NKT cells were activated by αGalCer that had been loaded on DCs (1×10^5 cells/mL), a significantly higher amount of cytokines were detected even when the concentration of αGalCer was as low as 1 ng/mL, and IFN-γ production almost peaked when the loading concentration of αGalCer was 10 ng/mL (Fig.7c). Thus, when presented by DCs the minimum concentration of αGalCer required for the activation of NKT cells was much lower than by CD1d monomers. If we use a lower numbers of αGalCer/DCs (2.5×10^4 cells/mL) in the cultures, in order to reach similar levels of cytokine production, increased αGalCer concentrations were required

(Fig.7c), suggesting that the number of DCs in the cultures can compensate for a low concentration of αGalCer for activation of NKT cells.

To test whether αGalCer loaded on DCs alone was responsible for efficient activation of NKT cells, DCs were first loaded with a high concentration of αGalCer (100 ng/mL) and then these αGalCer loaded DCs were fixed by 0.06% glutaraldehyde. As expected, unfixed αGalCer/DCs strongly activated NKT cells to produce high levels of IL-4 and IFN-γ. However, the fixed αGalCer/DCs did not trigger any IFN-γ production, whereas only a very low level of IL-4 was detected. This result suggested that αGalCer presented by DCs alone was not sufficient for full activation of NKT cells. Interestingly, when fresh isolated DCs (unfixed DCs and not loaded with αGalCer) were added into the culture containing fixed αGalCer/DCs, the cytokine production, especially IFN-γ production was restored (Fig.8). When increased numbers of unfixed DCs were added into the cultures, the cytokine production also increased. It was clear that the activation and IFN-γ production of NKT cells depended on both fixed αGalCer/DCs and unfixed DCs, suggesting that unfixed DCs provide additional signals to trigger NKT cells to be activated by αGalCer on the fixed DCs.

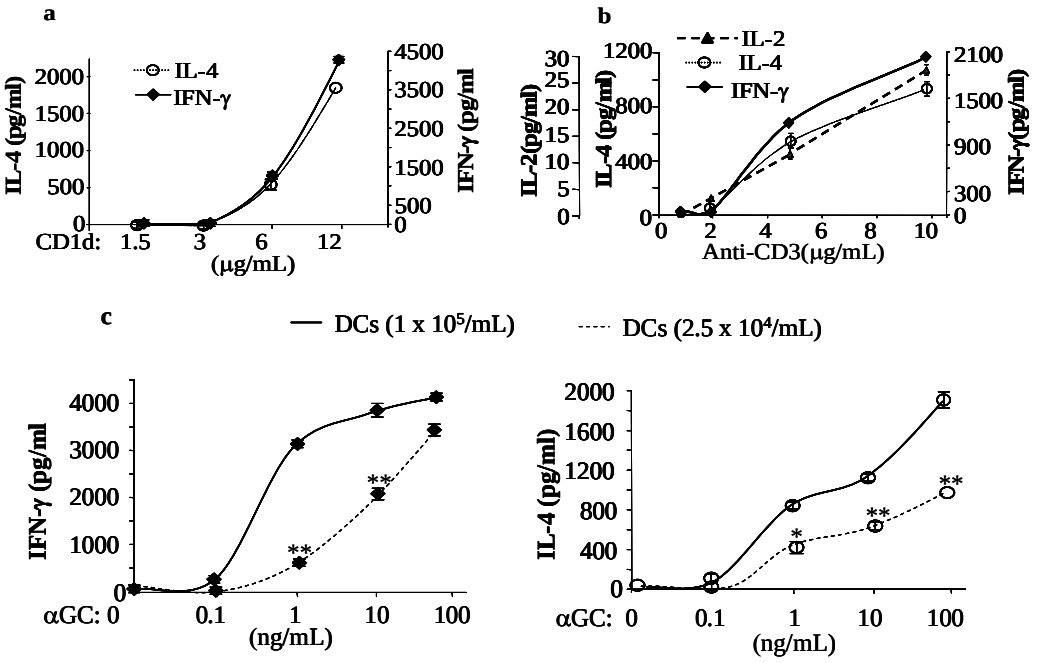

Figure 7 DCs trigger efficient Activation of NKT Cells in Response to low Concentrations of αGalCer (1). (a)Various concentrations of CD1d monomers were loaded with αGalCer (100 ng/mL) to coat culture plates. Thymic NKT cells ($1x10^6$/mL) were incubated with plate-bound αGalCer/CD1d complex for 48 hours, and cytokine production was measured. IFN-γ and IL-4 production increased in a CD1d monomer dose-dependent fashion ($p<0.01$, one-way ANOVA). (b)Cytokine production of thymic NKT cells ($1x10^6$/mL) activated by various concentrations of plate-bound anti-CD3 antibody for 48 hrs. IFN-γ, IL-4 and IL-2 production increased in an anti-CD3 antibody dose-dependent fashion ($p<0.01$, one way ANOVA). (c)Production of IFN-γ (left) and IL-4 (right) by thymic NKT cells ($1x10^6$/mL) activated by either a low ($2.5x10^4$/mL) or high ($1x10^5$/mL) number of DCs loaded with αGalCer at the indicated concentrations. Two-way ANOVA demonstrated a significant effect of both DC ($P < 0.05$) and αGalCer concentrations ($P < 0.01$) on IFN-γ and IL-4 production. Dunnett's test was conducted at each αGalCer concentration, $^{**}p< 0.01$ or $^{*}p< 0.05$ versus high concentration DC group. The data are expressed as the mean $\pm$ S.D. of triplicate wells. One representative of at least three independent experiments is shown.

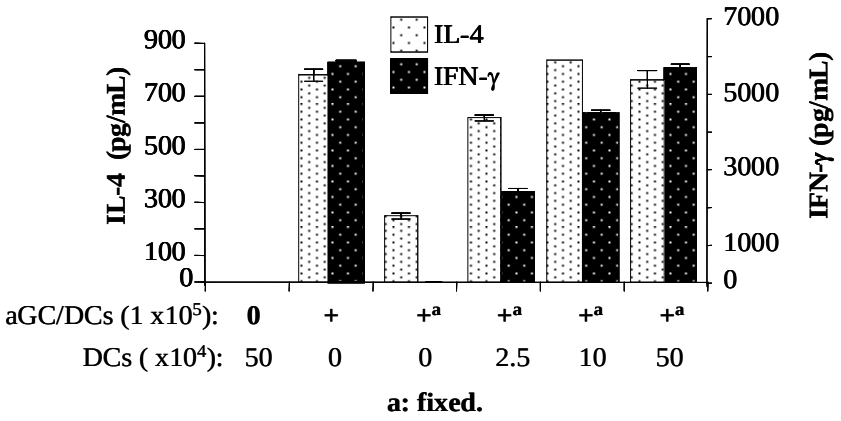

Figure 8 DCs trigger efficient Activation of NKT Cells in Response to low Concentrations of αGalCer (2). αGalCer (100 ng/mL) loaded DCs ($1x10^5$/mL) were fixed with glutaraldehyde before co-culturing with thymic NKT cells ($1x10^6$/mL) in the presence or absence of the indicated numbers of fresh DCs. IFN-γ production of thymic NKT cells increased in a DC dose-dependent fashion ($p<0.05$, one-way ANOVA). The data are expressed as the mean $\pm$ S.D. of triplicate wells. One representative of at least three independent experiments is shown.

3.13 The Potentiation of NKT Cells is Cell-contact dependent and DC-specific.

The next question to answer was how DCs potentiated NKT cells to respond to a low concentration of αGalCer. In order to test the additional signals provided by DCs, we developed a new culture system. In this system, αGalCer was first loaded on immobilized CD1d, and freshly isolated DCs from naïve mice were then added into the cultures. We used αGalCer loaded CD1d monomers to mimic stimulatory signals of exogenous lipid antigens since αGalCer can be recognized by most NKT cells that express Vα14 TCR, and also, αGalCer can be presented directly by CD1d molecules to NKT cells without endosomal uptake and processing (60, 128). Since αGalCer was loaded on plate-bound CD1d monomers, function of antigen presentation was thus separated from other functions of DCs added into the cultures (Fig.9). When a low concentration (1 μg/mL) of plate-bound αGalCer/CD1d was used, thymic NKT cells could not be activated. However, the addition of freshly isolated DCs into the cultures of NKT cells in the presence of the low concentration of plate-bound αGalCer/CD1d (1 μg/mL) resulted in significant amounts of both IL-4 and IFN-γ produced by NKT cells (Fig.10a). The cytokine production was αGalCer-dependent, since no cytokine was produced by NKT cells in the presence of DCs and αGalCer unloaded CD1d monomers (Fig.10a). The levels of cytokine production of NKT cells increased proportionally to numbers of DCs in the cultures, indicating that the stimulatory signals from DCs were cumulative. DCs alone, even at high numbers in the culture, could not activate NKT cells (Fig.10a). Like splenic DCs, bone marrow-derived immature DCs were also able to potentiate NKT cells for activation by αGalCer/CD1d (Fig. 10b).

We further found that the potentiation of NKT cells by DCs requires cell-cell contact, since separation of NKT cells from DCs using a transwell plate prevented the activation of NKT cells (Fig.10c). It was possible that the activation of NKT cells was due to cross-presentation of αGalCer taken up by DCs from plate-bound αGalCer/CD1d. To test this possibility we incubated DCs with plate-bound αGalCer/CD1d overnight, and then took out the DCs. The addition of these DCs into the cultures of thymic NKT cells failed to trigger NKT cell activation, indicating that NKT cell activation was not due to the cross presentation of αGalCer by DCs (Fig.10d).

Next, we examined if the ability to potentiate NKT cells was unique to DCs. Either B cells or S-APCs were added into the thymic NKT cell cultures in the presence of a low concentration of αGalCer/CD1d monomers. B cells only triggered low levels of IL-2, but neither IFN-γ nor IL-4 production (Fig. 11a). In contrast to B cells, the addition of S-APCs into the cultures resulted in the production of significant amounts of cytokines, especially IFN-γ (Fig. 11a). It was possible that DCs contained in S-APCs played a major role for the activation of NKT cells. Since it was suggested that splenic and thymic NKT cells displayed substantial differences in their phenotypes, requirements for costimulatory signals, and cytokine profiles (127), we then tested the splenic NKT cell activation in the same system. Our results showed that splenic NKT cells were similarly activated by a low concentration of αGalCer/CD1d in the presence of DCs but not B cells. However, splenic NKT cells produced more IFN-γ but less IL-4 (Fig.11b). This part of study clearly showed that DCs or S-APCs increased the sensitivity of NKT cells in response to plate-bound αGalCer/CD1d complex, whereas B cells were unable to do so.

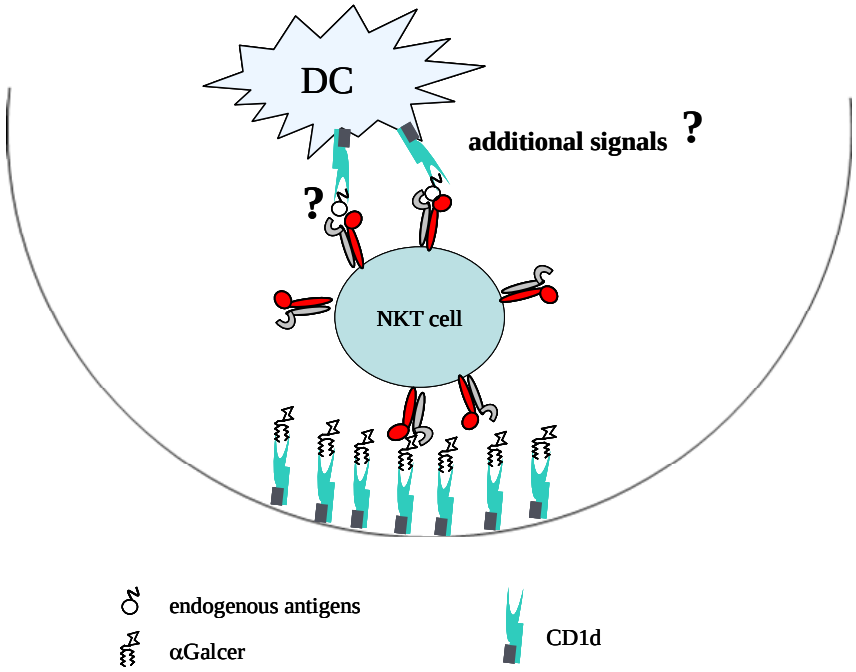

Figure 9 Overview of the New System. Exogenous lipid antigens (αGalCer) are presented by plate-bound CD1d. Thymic NKT cells are activated by plate-bound αGalCer/CD1d monomers with different subsets of APCs (antigen presenting cells, including DCs, B cells and S-APCs).

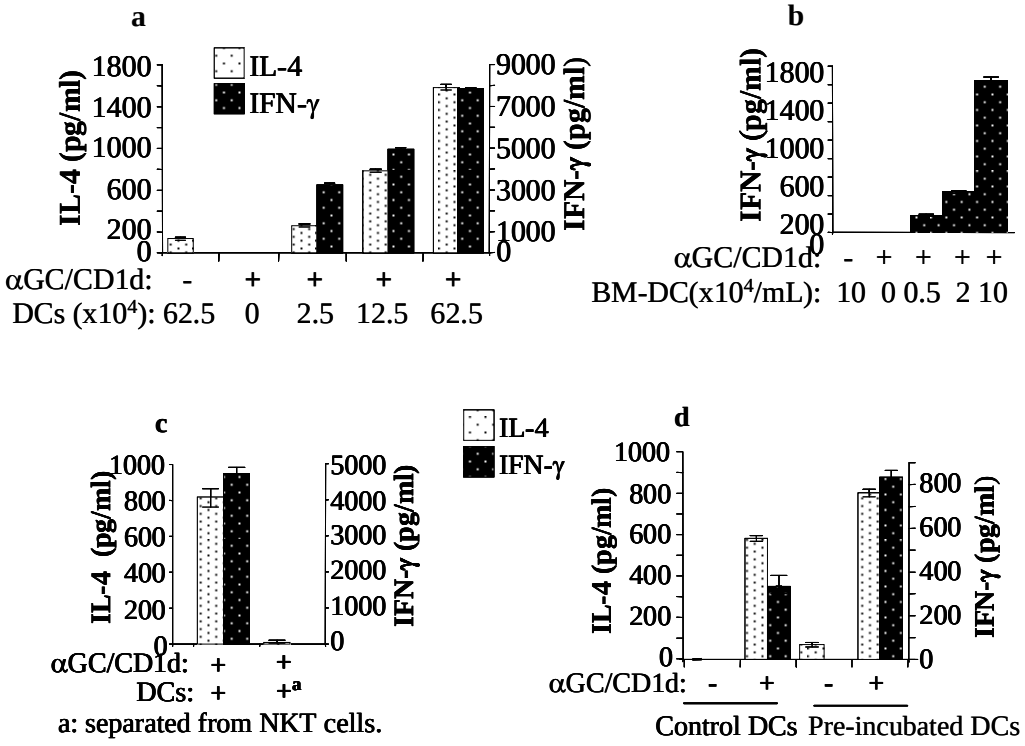

Figure 10 Contact-dependent cumulative Signals from DCs potentiate NKT Cells. (a)Cytokine production of thymic NKT cells ($1x10^6$/mL) activated by plate-bound αGalCer (100 ng/mL)/CD1d (1 μg/mL) in the presence of indicated numbers of splenic $CD11c^+$ DCs. IFN-γ and IL-4 production of thymic NKT cells increased in a DC dose-dependent fashion ($p<0.01$, one-way ANOVA). (b)IFN-γ production by thymic NKT cells ($1x10^6$/mL) activated by plate-bound αGalCer (100 ng/mL)/CD1d (1 μg/mL) in the presence of indicated numbers of bone marrow-derived DCs. IFN-γ production of thymic NKT cells increased in a BM-DC dose-dependent fashion ($p<0.01$, one-way ANOVA). (c)Cytokine production by thymic NKT cells ($1x10^6$/mL) activated by plate-bound αGalCer (100 ng/mL)/CD1d (1 μg/mL) in the presence of DCs ($1x10^5$/mL) or DCs that were separated from thymic NKT cells using a Transcell plate. (d)Cytokine production by thymic NKT cells ($1x10^6$/mL) activated by control DCs and DCs ($1x10^5$/mL) that had been pre-incubated with plate-bound αGalCer/CD1d (2 μg/mL) in the presence or absence of plate-bound αGalCer/CD1d (1 μg/mL). The data are expressed as the mean ± S.D. of triplicate wells. One representative experiment of at least three is shown.

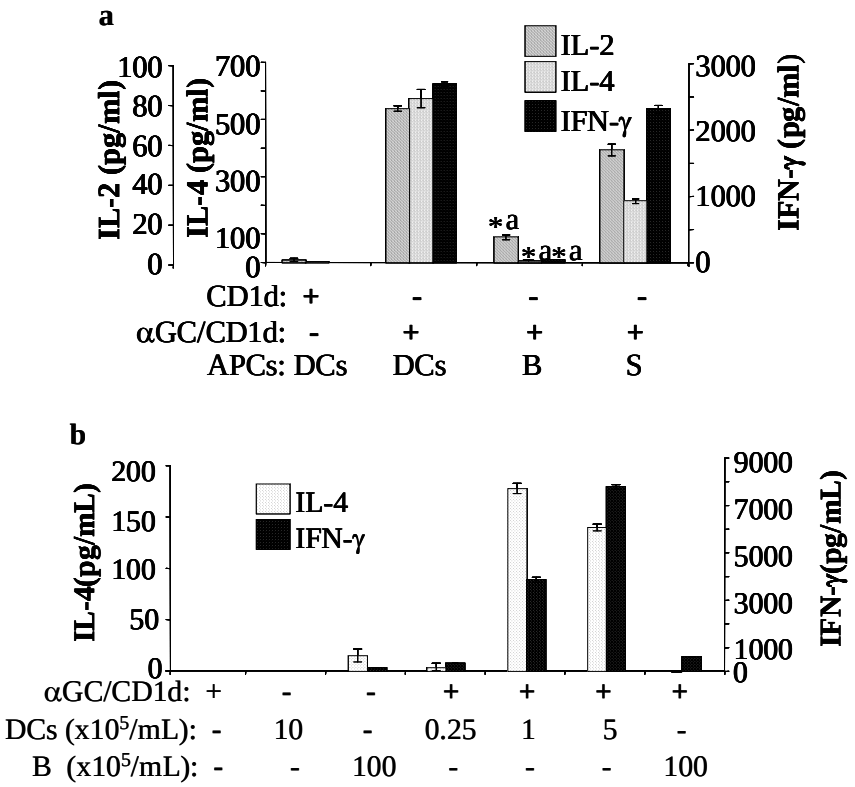

Figure 11 Potentiation of NKT Cells is DC-specific. (a)Cytokine production of thymic NKT cells ($1x10^6$/mL) activated by plate-bound αGalCer (100 ng/mL)/CD1d (1 μg/mL) in the presence of DCs ($1x10^5$/mL), B cells ($1x10^6$/mL) or S-APCs ($1x10^6$/mL). *$p<0.01$ versus corresponding DC group, $^a p<0.05$ versus corresponding S-APC group. (One-way ANOVA, Student Newman-Kuels post hoc test). (b)Cytokine production by splenic T cells ($2.5x10^6$/mL) activated by plate-bound αGalCer (100 ng/mL)/CD1d (1 μg/mL) in the presence or absence of indicated numbers of DCs or B cells. IFN-γ production of splenic T cells increased in a DC dose-dependent fashion ($p<0.01$, one-way ANOVA). The data are expressed as the mean ± S.D. of triplicate wells. One representative experiment of at least three is shown.

3.14 The Potentiation is CD1d-dependent, but Co-stimulatory Molecules, Co-receptors and IL-12 are not required for Potentiation of NKT Cells.

To determine the role of co-stimulatory signals for potentiation of NKT cells, we added anti-CD28, anti-CD40L or both antibodies into cultures of thymic NKT cells in the presence of plate-bound αGalCer/CD1d complex. We also purified thymic NKT cells from CD28 gene deficient C57BL/6 mice, and activated these NKT cells by a low concentration of plate-bound αGalCer/CD1d (1 μg/mL). The addition of either anti-CD28, or anti-CD40L or both of the antibodies did not induce any detectable cytokine production (Fig.12a), whereas $CD28^{-/-}$ NKT cells produced significant amounts of IFN-γ, albeit at lower levels than NKT cells from WT mice, in the presence of αGalCer/CD1d and DCs (Fig. 12b). Thus co-stimulatory signals from CD28 and CD40 pathways were not able to replace the function of DCs required for the potentiation of NKT cells, although they might modulate the levels and profile of cytokines produced by the activated NKT cells.

In addition to B7 and CD40, Many other surface molecules on APCs may confer costimulatory signals. To test the possible role of other co-stimulatory factors of DCs for potentiation of NKT cells, DCs were isolated from CD1d gene-deficient C57BL/6 ($C57BL/6^{CD1d-/-}$) mice. Compared with normal DCs, CD1d gene deficient DCs expressed similar levels of MHC class I, MHC class II and CD40, CD80 molecules, but not CD1d molecules on the cell surface (Fig.13a). These DCs therefore were likely to provide every costimulatory signal. On the other hand, CD1d on DCs was not required for presentation of αGalCer in our experimental design. Therefore, $CD1d^{-/-}$ DCs were expected to

potentiate NKT cells when added into the cultures with αGalCer/CD1d monomers. However, when cultured with thymic NKT cells in the presence of a low concentration of CD1d/αGalCer, $CD1d^{-/-}$ DCs did not trigger any cytokine production of thymic NKT cells (Fig.13b). The result showed that $CD1d^{-/-}$ DCs could not potentiate NKT cells to be activated by αGalCer, and clearly, the potentiation of NKT cells by DCs is CD1d-dependent. We further defined the effects of fixation on potentiation and antigen presentation and found that fixed DCs could not potentiate NKT cells. In contrast, NKT cells were activated by fixed αGalCer/CD1d in the presence of fresh DCs (Fig.13c). These results showed that fixation had no effect on CD1d antigen presentation, but abolished potentiation. It was possible that potentiation required the mobility of certain molecules on the surface or within DCs, and inhibition of mobility of these molecules disrupted their function during potentiation.

Since the majority of NKT cells from the thymus were $CD4^{+}$, we then isolated thymic NKT cells from CD4 gene deficient C57BL/6 mice. When cultured with DCs in the presence of CD1d/αGalCer, $CD4^{-/-}$ thymic NKT cells could be similarly activated as control thymic NKT cells, and produced large amounts of IFN-γ (Fig.13d). Therefore, the CD4 expression had no effect on potentiation and activation of NKT cells. Taken together, the potentiation of NKT cells is CD1d-dependent, but does not relay on co-stimulatory and co-receptor molecules.

It has been shown that IL-12 produced by DCs had a role in activation of NKT cells (129). To define the role of IL-12 in potentiation of NKT cells, IL-12 was added into the cultures of NKT cells in the absence or presence of plate-bound αGalCer/CD1d. Addition

of IL-12 alone, even at a high concentration (20 ng/mL), did not activate NKT cells. In contrast, a low concentration of IL-12 (2 ng/mL) was able to trigger cytokine production of NKT cells activated by αGalCer/CD1d, although IL-4 production was lower than that in the presence of DCs (Fig. 14a). Nevertheless, IL-12 produced by DCs was not required for the potentiation of NKT cells but was rather an enhancer for IFN-γ production, since NKT cells were activated by αGalCer/CD1d in the presence of DCs from IL-12 gene deficient mice (Fig.14a). Furthermore, DCs might also be a potential source of IFN-γ production during NKT cell activation. To eliminate the possibility that the IFN-γ is produced by DCs, DCs from IFN-γ gene deficient mice were added into the thymic NKT cell cultures in the presence of αGalCer/CD1d. Clearly, the result showed that addition of these DCs also drove the activation of NKT cells, suggesting there was no role of DC-derived IFN-γ for the potentiation of NKT cells (Fig.14b).

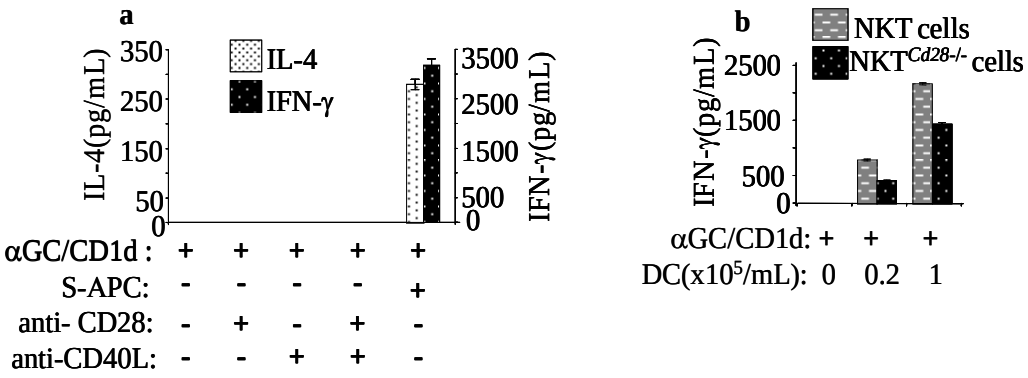

Figure 12 Potentiation of NKT Cells does not require Co-stimulatory Signals. (a)Cytokine production by thymic NKT cells ($1x10^6$/mL) activated by plate-bound αGalCer (100 ng/mL)/CD1d (1 μg/mL) in the presence of anti-CD28 (5 μg/mL), anti-CD40L (5 μg/mL) or both antibodies. Fresh S-APCs ($5x10^5$/mL) were added in control cultures. (b)IFN-γ production by thymic NKT cells ($1x10^6$/mL) from WT and $CD28^{-/-}$ C57BL/6 mice activated by plate-bound αGalCer (100 ng/mL)/CD1d (1 μg/mL) in the presence of the indicated numbers of DCs. The data are expressed as the mean ± S.D. of triplicate wells. One representative experiment of at least three is shown.

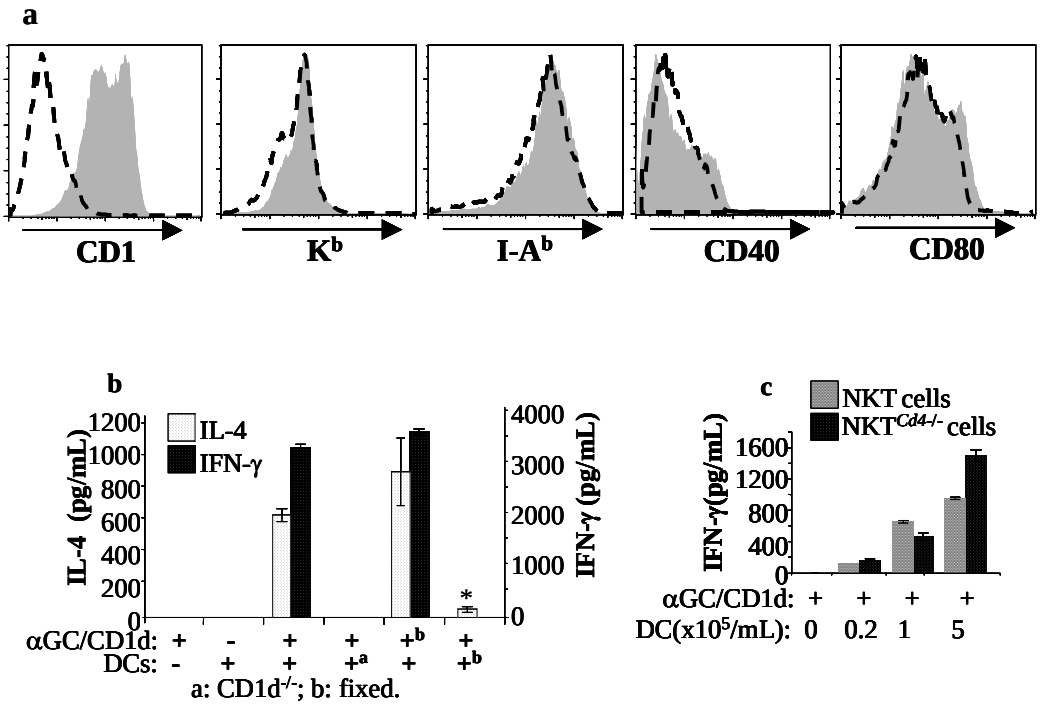

Figure 13 Potentiation of NKT Cells requires CD1d expressing DCs. (a)FACS analysis of CD1d, MHC and co-stimulatory molecules expressed on DCs from WT (shaded area) and CD1d gene deficient (dot line) C57BL/6 mice. (b)Cytokine production of tymic NKT cells ($1x10^6$/mL) activated by plate-bound αGalCer (100 ng/mL)/CD1d (1 μg/mL) in the presence of DCs ($1x10^5$/mL) isolated from WT or $CD1d^{-/-}$ C57BL/6 mice. As indicated, in some cultures, plate-bound αGalCer/CD1d or DCs were fixed using glutaraldehyde before the addition of NKT cells. *P < 0.01 versus "αGC/CD1d + DC" group and "fixed αGC/CD1d + DC" group (One way ANOVA, Student Newman-Kuels post hoc test). (c)IFN-γ production by thymic NKT cells ($1x10^6$/mL) from WT and $CD4^{-/-}$ C57BL/6 mice activated by plate-bound αGalCer (100 ng/mL)/CD1d (1 μg/mL) in the presence of the indicated numbers of DCs. The data are expressed as the mean ± S.D. of triplicate wells. One representative experiment of at least three is shown.

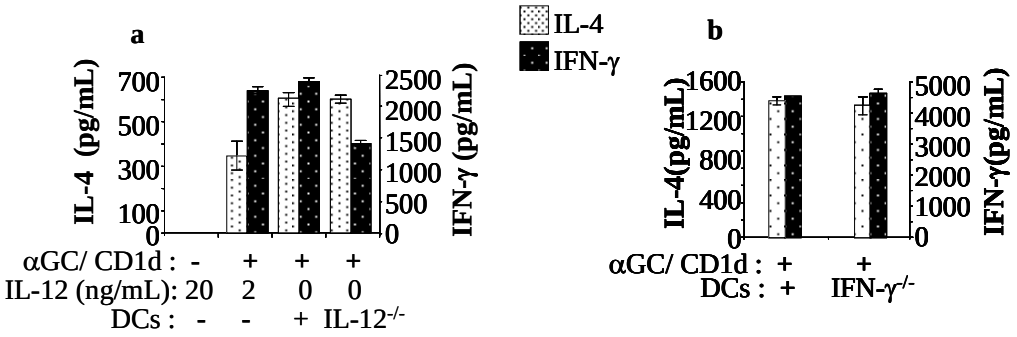

Figure 14 Potentiation of NKT Cells does not require DC-derived IL-12 and IFN-γ. (a, b)Cytokine production by thymic NKT cells ($1x10^6$/mL) activated by plate-bound αGalCer (100 ng/mL)/CD1d (1 μg/mL) in the presence of the indicated concentrations of IL-12 or DCs ($1x10^5$ /mL) from WT, IL-$12^{-/-}$, or IFN-$\gamma^{-/-}$ C57BL/6 mice. The data are expressed as the mean ± S.D. of triplicate wells. One representative experiment of at least three is shown.

3.15 Endosomal Pathways and endogenous Lipid Antigens in DCs are required for Potentiation and IFN-γ Induction of NKT Cells.

The requirement of unfixed CD1d-expressing DCs implies the involvement of a dynamic process in DCs for potentiation of NKT cells. Since many endogenous lipid antigens are displayed on the surface of DCs by CD1d trafficking through endosomes, it was possible that endogenous lipid antigens processed in DCs played a role for potentiation of NKT cells. To investigate this possibility, we interrupted the endosomal pathways required for processing of endogenous lipid antigens in DCs using specific inhibitors. Isolated DCs were incubated with either 1) concanamycin A (CMA), an inhibitor of the vacuolar adenosine triphosphatase/H+ pump; 2) bafilomycin A1 (Baf), which interferes with the uptake of macromolecules in late lysosomes (128); 3) a combination of DL-thero-1-phenyl-2-palmitoylamino-3-morpholine-1-propanol (PPMP) and threo-1-phenyl-2-decanoylamino-3-morpholine-1-propanol hydrochloride (PDMP), which inhibits β-glucosylceramide synthase (130); or 4) deoxygalactonojirimycin (DGJ), an inhibitor of α-galactosidase (128). The drug-treated and control DCs were then added into cultures of NKT cells in the presence of plate-bound αGalCer/CD1d. Thymic NKT cells could not be activated by αGalCer/CD1d in the presence of DCs treated with CMA, Baf, or PPMP+PDMP (Fig. 15a). In the presence of DGJ treated DCs, IFN-γ production of NKT cells was markedly reduced, but IL-2 and IL-4 production was not affected (Fig. 15a). To test if the effects of the treatments on DCs were NKT cell-specific, we purified DCs from NOD mice and $CD8^+$ T cells from 8.3-NOD mice. 8.3 $CD8^+$ T cells could be activated by recognizing specific peptides presented by DCs (131). Then DCs from NOD mice were

incubated with different inhibitors for 4 hrs before used as APCs to activate 8.3-T ($CD8^+$) cells. We found that the control and drug-treated DCs presented peptide antigens and activated $CD8^+$ T cells at similar levels (Fig.15b), showing that treatment of endosomal inhibitors did not interrupt APC functions of DCs for conventional T cells, and also suggesting that the effects of the treatment on DCs were NKT cell specific and did not have global negative effects. In addition, the treated DCs were still able to present αGalCer to and activate NKT cells, but the activation efficiency was low, even with a high concentration of αGalCer (Fig. 16).

Since isoglobotrihexosylceramide (iGb3) has been proposed as a major endogenous lipid antigen (52) displayed by DCs, we next tested whether iGb3 displayed on DCs contributed to the potentiation of NKT cells. To do this, we blocked iGb3 using different concentrations of inhibitor isolectin B4 (IB4) that specifically binds with iGb3. Our result showed that both IFN-γ and IL-4 production of NKT cells activated by plate-bound αGalCer/CD1d was markedly reduced in proportion to increased IB4 concentrations (Fig. 17a). The role of iGb3 in the activation of NKT cells in response to αGalCer was further determined by using DCs from HexB (ß-hexosaminidase) gene deficient mice that are unable to synthesize iGb3 (52). These DCs could not potentiate NKT cells when added into thymic NKT cell cultures in the presence of immobilized αGalCer/CD1d complex (Fig. 17b). Furthermore, although these DCs were able to present αGalCer at high concentrations to activate NKT cells, the cytokine production by NKT cells was significantly reduced in comparison with control DCs, especially when αGalCer concentrations were low (Fig. 17c), suggesting endogenous lipid iGb3 displayed on the

surface of DCs played a role in the activation of NKT cells by exogenous lipid antigens. Importantly, when $HexB^{-/-}$ DCs were coated with a low concentration (20 ng/mL) of iGb3 that alone could not activate NKT cells and then added into the cultures, cytokine production of NKT cells was significantly improved when concentrations of αGalCer was low (Fig.17c), providing strong evidence in supporting the important role of iGb3 in the potentiation of NKT cells.

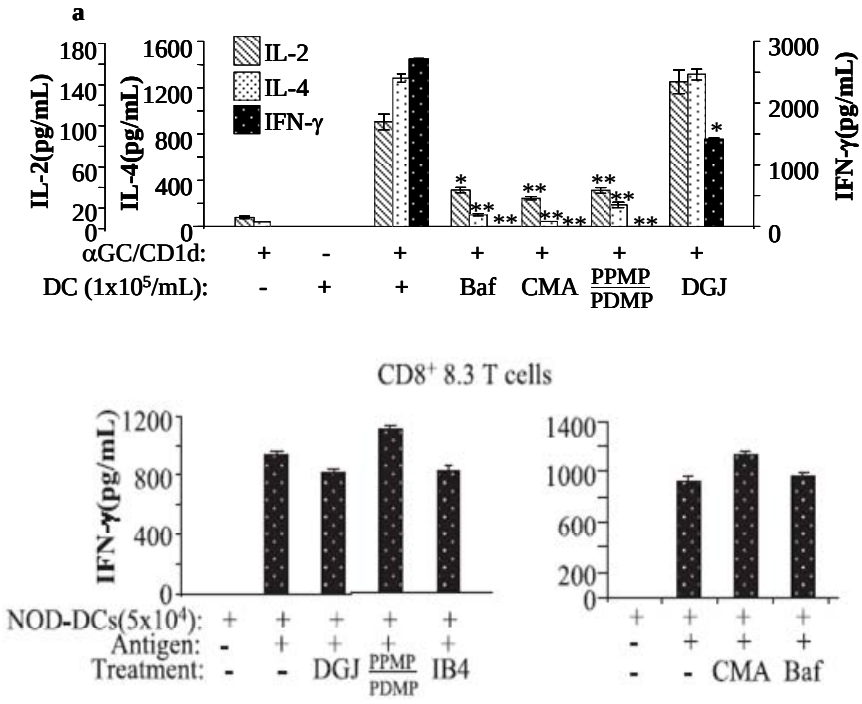

Figure 15 Endosomal endogenous Lipid Antigens in DCs are required for efficient Activation of NKT Cells (1). (a)Cytokine production by thymic NKT cells ($1x10^6$/mL) activated by plate-bound αGalCer/CD1d in the presence of fresh DCs or DCs treated with specific inhibitors as indicated. (One way ANOVA followed by Dunnett' test, $^{**}p < 0.01$ or $^{*}p<0.05$ versus control DC group) (b)IFN-γ production by 8.3 ($CD8^+$) T cells ($2.5x10^5$/mL) isolated from TCR transgenic NOD mice. These T cells were activated by their specific peptide ligand NRP-A7 (100 ng/mL) presented by fresh DCs or DCs ($5x10^4$/mL) treated with inhibitors for lipid antigen processing as described. The data are presented as the mean ± S.D. of triplicate wells. One representative experiment of more than three independent experiments is shown.

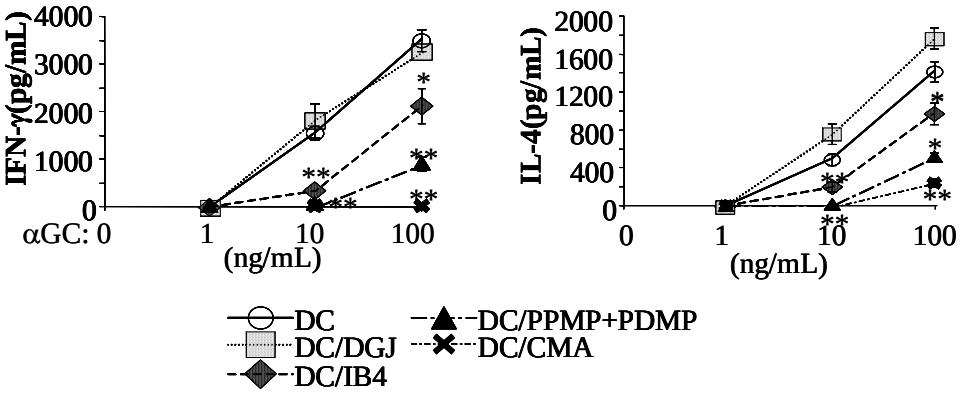

Figure 16 Endosomal endogenous Lipid Antigens in DCs are required for efficient Activation of NKT Cells (2). Cytokine production by NKT cells activated by DCs and treated DCs ($1x10^5$/mL) loaded with indicated concentrations of αGalCer. The concentration of IB4 used to block iGb3 was 800 ng/ml. Two-way ANOVA demonstrated a significant effect of both drug-treatment of DCs ($P < 0.01$) and αGalCer concentrations ($P < 0.01$) on IFN-γ and IL-4 production. Dunnett's test was conducted at each αGalCer concentration, $^{**}p<0.01$ or $^{*}p<0.05$ versus control DC group. The data are presented as the mean ± S.D. of triplicate wells. One representative experiment of more than three independent experiments is shown.

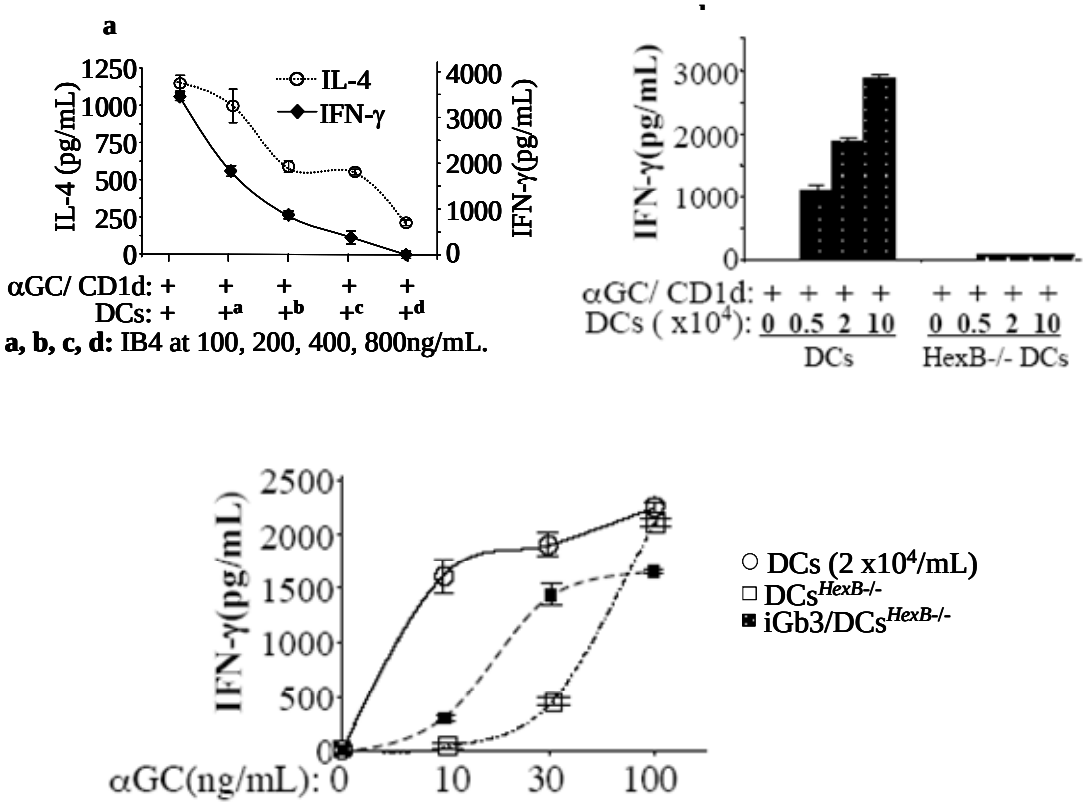

Figure 17 iGb3 displayed by DCs is critical for Potentiation of NKT Cells. (a) Cytokine production by thymic NKT cells ($1x10^6$/mL) activated by plate-bound αGalCer/CD1d in the presence of fresh DCs or DCs ($1x10^5$/mL) bound with the indicated concentrations of IB4. IFN-γ and IL-4 production of thymic NKT cells decreased in an IB4 dose-dependent fashion ($p<0.01$, $p<0.05$, one-way ANOVA). (b)IFN-γ production by thymic NKT cells ($1x10^6$/mL) activated by plate-bound αGalCer/CD1d (1 μg/mL) in the presence of the indicated numbers of WT or $HexB^{-/-}$ $CD11c^+$ DCs. (c)IFN-γ production by thymic NKT cells ($1x10^6$/mL) activated by various concentrations of αGalCer presented by DCs, $HexB^{-/-}$ DCs, or $HexB^{-/-}$ DCs loaded with 20 ng/ml iGb3. Two-way ANOVA demonstrated a significant effect of both types of DCs ($P < 0.01$) and αGalCer concentrations ($P < 0.05$) on IFN-γ and IL-4 production. One-way ANOVA followed by Student Newman-Kuels post hoc test was conducted at each αGalCer concentration, $^{**}p<0.01$ or $^{*}p<0.05$ versus control DC group, $^{aa}p<0.01$ or $^{a}p<0.05$ versus $DC^{Hexb-/-}$/iGb3 group. The data are presented as the mean ± S.D. of triplicate wells. One representative experiment of more than three independent experiments is shown.

3.16 Vα14 NKT Cells preferentially form stable Conjugates with CD1d expressing DCs.

The activation of T cells requires the formation of stable conjugates and immune synapses (ISs) between T cells and APCs. The conjugate formed between T cells and DCs is due to the contacts and engagements between TCRs and peptide/MHC complexes, and the stability of conjugate formation depends on density and affinity of peptide/MHC to specific TCRs (132, 133). Therefore, conjugate formation between T cells and DCs is an important factor contributing to the activation of T cells. To determine whether potentiation of NKT cells by DCs was associated with conjugate formation between these cells, we incubated purified thymic NKT cells with DCs labeled with anti-MHC class II I-A^b antibody for different periods of time (0 min, 20 min, 40 min, 60 min). MHC class II labeling should not affect contacts between NKT and DCs. The cell mixtures were then harvested and the conjugate formation between DCs and NKT cells was analyzed using flow cytometry. We found that in the absence of exogenous lipid antigen αGalCer, small numbers of conjugates were quickly formed. The number of conjugates reached a peak level within 40 min after incubation. When NKT cells were incubated with B cells, the conjugate formation was detected with a much lower frequency than with DCs. Importantly, NKT cells did not conjugate with $CD1d^{-/-}$ DCs (Fig.18a,b), indicating that the conjugate formation was CD1d-dependent, and possibly required involvement of endogenous lipid antigens. Furthermore, these data indicated that even in the absence of exogenous lipid antigens, DCs contacted and engaged with NKT cells to form conjugates albeit with a low frequency.

We then examined conjugate formation in the presence of αGalCer. In these experiments, DCs and B cells were loaded with different concentrations of αGalCer before incubation with NKT cells. With either αGalCer-loaded DCs or B cells, NKT cells formed stable conjugates, and the frequency of conjugates was αGalCer dose-dependent. However, the formation of conjugates between DC and NKT cell was much more rapid and frequent than that of B cell and NKT, especially when the concentration of αGalCer was low (Fig. 18c). Therefore, when the concentration of exogenous lipid antigens was low, stability of conjugates depended on nature of APCs, whereas high concentrations of exogenous antigens reduced the differences between subsets of APCs for their ability to engage with NKT cells.

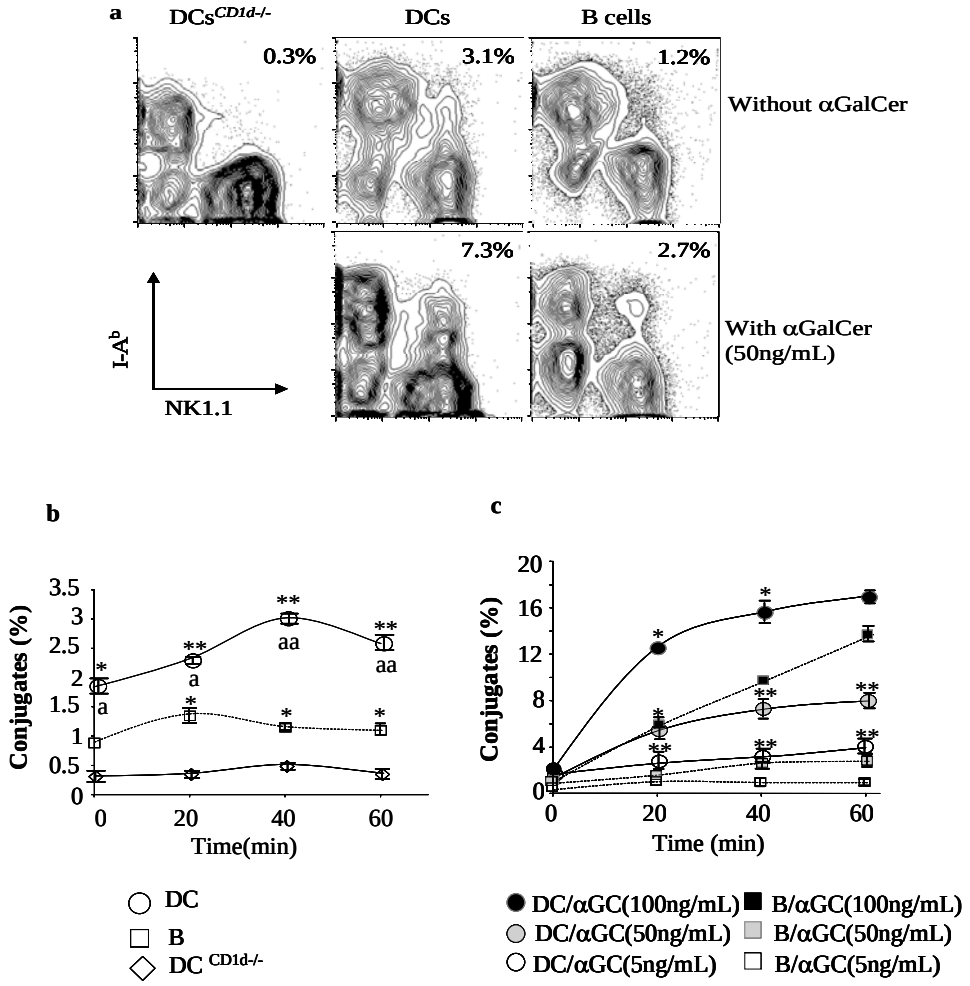

Figure 18 Different Ability of DCs and B Cells to Form CD1d-dependent Conjugates with NKT Cells. (a)FACS profiles of conjugates formed between NKT cells and DCs, $CD1d^{-/-}$ DCs or B cells ($10x10^6$/mL) 40 min after incubation with or without preloaded αGalCer (50 ng/mL). (b)Formation of conjugates between NKT cells and DCs, $CD1d^{-/-}$ DCs or B cells ($10x10^6$/mL) at different time points in the absence of αGalCer. Two-way ANOVA demonstrated a significant effect of subsets of APCs ($P < 0.01$) ($P < 0.05$) on conjugate formation between APCs and thymic NKT cells. One-way ANOVA followed by Student Newman-Kuels post hoc test was conducted at each time points, $^{**}p<0.01$ or $^{*}p<0.05$ versus $DC^{CD1d-/-}$ group, $^{aa}p<0.01$ or $^{a}p<0.05$ versus B cell group. Similar results were obtained in three independent experiments. (c)Formation of conjugates between NKT cells and DCs or B cells ($10x10^6$/mL) preloaded with various concentrations of αGalCer. Two-way ANOVA demonstrated a significant effect of subsets of αGalCer-loaded APCs ($P < 0.01$) on conjugate formation between APCs and thymic NKT. Dunnett's test was conducted at each time points between B/αGC and DC/αGC group, $^{**}p<0.01$ or $^{*}p<0.05$ versus the corresponding αGalCer-loaded B cell group. Similar results were obtained in three independent experiments.

3.17 Vα14 NKT Cells are potentiated by B Cells overexpressing Wild-type CD1d

Since the potentiation of NKT cells is CD1d dependent, it is possible that an increase in CD1d expression on B cells could improve the ablility of B cells to potentiate NKT cell activation. To test this possibility, A20 B cell lines, including A20, A20-CD1d and A20-CD1d-TD were used. A20 is a B cell lymphoma expressing similar levels of CD1d molecules as B cells (Fig.19a). A20-CD1d cells are A20 B cells transfected with wild type CD1d, whereas A20-CD1d-TD is A20 cell line transfected with CD1d molecules with a truncated N-terminal cytoplasmic tail. CD1d-TD presented αGalCer equivalently to CD1d, but its endosomal trafficking was severely reduced (51, 60). Both A20-CD1d and A20-CD1d-TD similarly expressesed high levels of CD1d on the cell surfaces (Fig.19a). When NKT cells were cultured with B, A20, A20-CD1d, or A20-CD1d-TD cells in the presence of plate-bound αGalCer/CD1d, we found that similar to B cells, A20 could not potentiate NKT cells to be activated, however, NKT cells were strongly activated by αGalCer/CD1d if A20-CD1d were added into the culture. In contrast, NKT cells produced significantly reduced IL-4 and much less IFN-γ when they were co-cultured with A20-CD1d-TD cells in the presence of αGalCer/CD1d (Fig.19b). These data suggested that increased levels of CD1d expression enabled B cells to potentiate NKT cells, and endosomal trafficking of CD1d was required for this process.

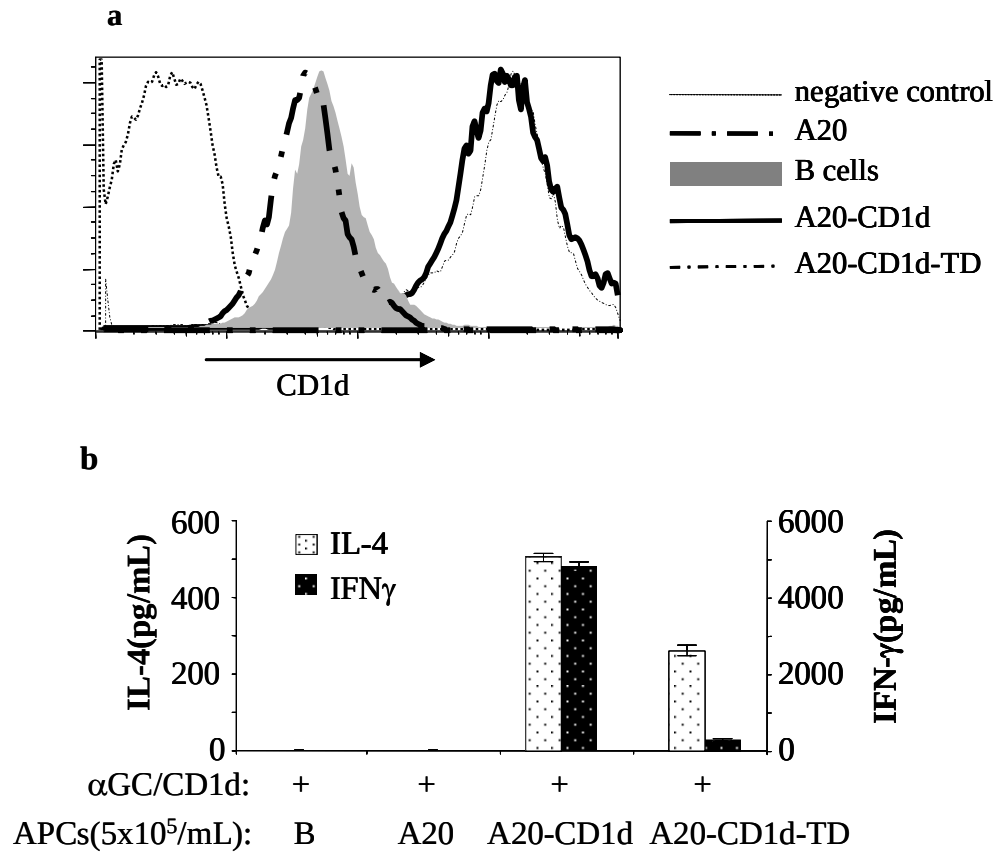

Figure 19 B Cells overexpressing CD1d potentiate NKT Cells for Activation. (a) CD1d expression by isolated B cells, A20 cells and transfected A20 cells overexpressing CD1d or CD1d-TD. (b)Cytokine production by thymic NKT cells ($1x10^6$/mL) activated by plate-bound αGalCer/CD1d (1 μg/mL) in the presence of identical numbers ($5x10^5$/mL) of B cells, A20, A20-CD1d or A20-CD1d-TD cells. The data e presented as the mean $\pm$ S.D. of triplicate wells. One representative experiment of more than three independent experiments is shown. There were significant differences of IFN-γ ($p<0.01$) and IL-4 ($p<0.05$) production between A20-CD1d group and A20-CD1d-TD group unparied T test).

3.18 DCs enhanced the Activation of NKT Cells mediated by B Cells as APCs

Since iGb3 displayed on DCs is important for the potentiation of NKT cells, we then loaded B cells with iGb3 before using them as APCs to present αGalCer, to determine whether increased iGb3 enhanced the function of B cells in the activation of NKT cells. However, NKT cells only produced slightly increased IFN-γ at a high concentration of αGalCer presented by iGb3-loaded B cells (Fig. 20a), suggesting that the weak ability of B cells to activate NKT cells was not due to a low level of iGb3 on their surface.

One explanation for the poor ability of B cells to activate NKT cells was that B cells had inhibitory effect on the activation of NKT cells (119). To test whether B cells inhibited the activation of NKT cells, different concentrations of B cells were loaded with a high concentration (100 ng/mL) of αGalCer before added into the thymic NKT cell cultures. Only weak cytokine production of NKT cells was triggered by αGalCer/B cells and IFN-γ production couldn't be detected until the concentration of B cells reached $10x10^5$ cells/mL, and with the increasing of B cell concentrations, IFN-γ production increased (Fig. 20b). These results showed that B cells had a weak ability to activate NKT cells, and the weak ability might be partially improved by increasing the number of B cells interacting with NKT cells. Interestingly, addition of small numbers of DCs significantly increased IFN-γ production by NKT cells. With the increase of DCs concentrations, the IFN-γ production increased. However, addition of DCs increased IL-4 production only if the B cell numbers were low, whearas IL-4 concentrations in the culture decreased rapidly along with the increased numbers of B cells (Fig.20b). The decreased IL-4 production was likely due to the increased binding, internalization and consumption of

IL-4 by large numbers of B cells (134, 135). These results showed that NKT cells were effectively activated by αGalCer/B cells after potentiation by DCs, and that optimal activation of NKT cells depended on both the levels of exogenous Ags and DC-mediated potentiation. Moreover, the timing of DC potentiation was also critical for optimal activation of NKT cells. Simultaneous addition of DCs and αGalCer/B cells activated NKT cells. However, the IFN-γ production of NKT cells was significantly reduced when NKT and αGalCer/B cells were cultured for 6 hours before the addition of DCs. In contrast, when DCs were cultured with NKT cells for 6 hours before the addition of αGalCer/B cells, the activation of NKT cells was enhanced (Fig.21). Therefore, the DC-mediated potentiation has to occur before or simultaneously at initial exposure of NKT cells to a non-self Ag for the optimal and efficient activation.

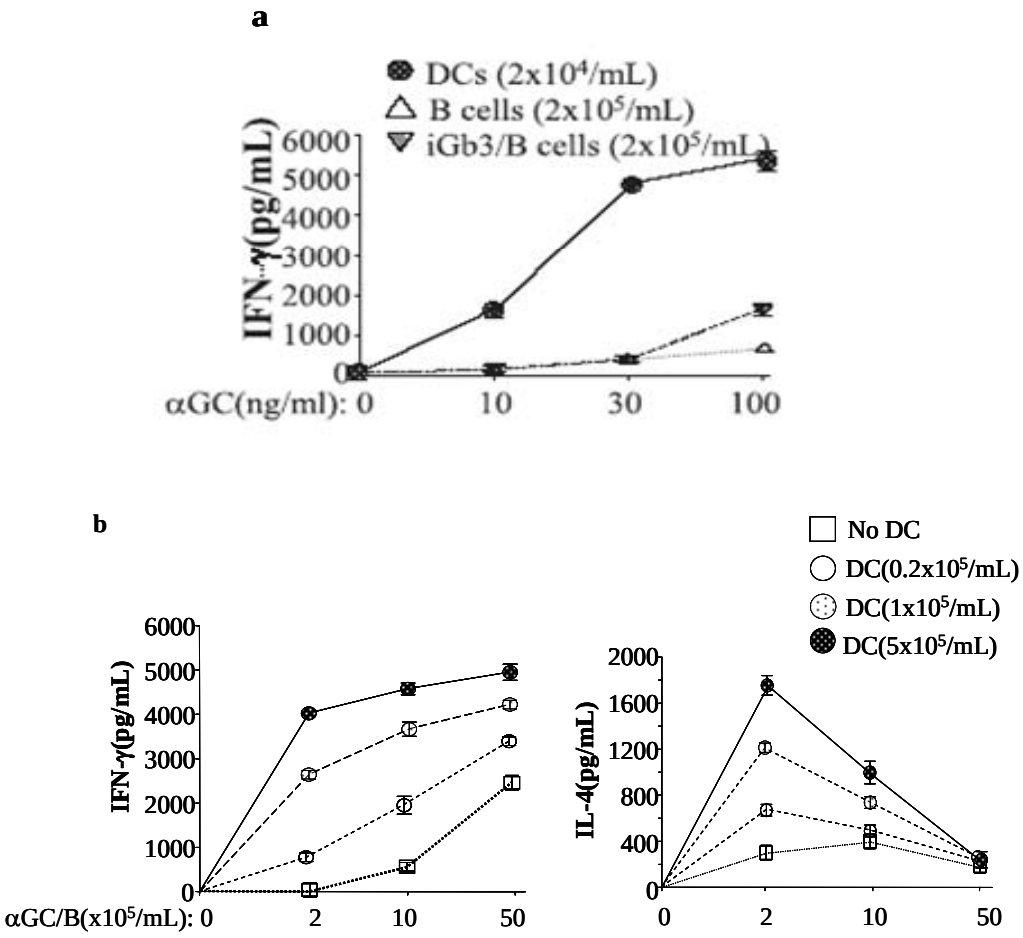

Figure 20 DCs enhanced the Activation of NKT Cells mediated by B Cells as APCs (1). (a)IFN-γ production of thymic NKT cells ($1x10^6$/mL) activated by different concentrations of αGalCer in the presence of DCs, B cells and iGb3-loaded B cells. Two-way ANOVA demonstrated a significant effect of both subsets of APCs ($P < 0.01$) and αGalCer concentrations ($p<0.05$) on IFN-γ production. One-way ANOVA followed by Student Newman-Kuels post hoc test was conducted at each αGalCer concentration, *$p<0.01$ versus DC group. (b)Cytokine production of thymic NKT cells ($1x10^6$/mL) activated by increased numbers of B cells loaded with αGalCer (100 ng/mL) in the presence or absence of different numbers of DCs ($0.2x10^5$/mL, $1x10^5$/mL, $5x10^5$/mL). Two-way ANOVA demonstrated a significant effect of DC ($P < 0.05$) and B cell concentrations ($p<0.05$) on IFN-γ and IL-4 production. One-way ANOVA followed by Student Newman-Kuels post hoc test was conducted at each concentration of B cells, IFN-γ production of thymic NKT cells increased in a DC dose-dependent fashion when B cell concentrations were $2x10^5$/mL ($p<0.01$), $10x10^5$/mL ($p<0.05$) and $50x10^5$/mL ($p<0.05$). IL-4 production of thymic NKT cells increased in a DC dose-dependent fashion when B cell concentrations was $2x10^5$/mL ($p<0.01$). The data are presented as the mean $\pm$ S.D. of triplicate wells. One representative experiment of three independent experiments is shown.

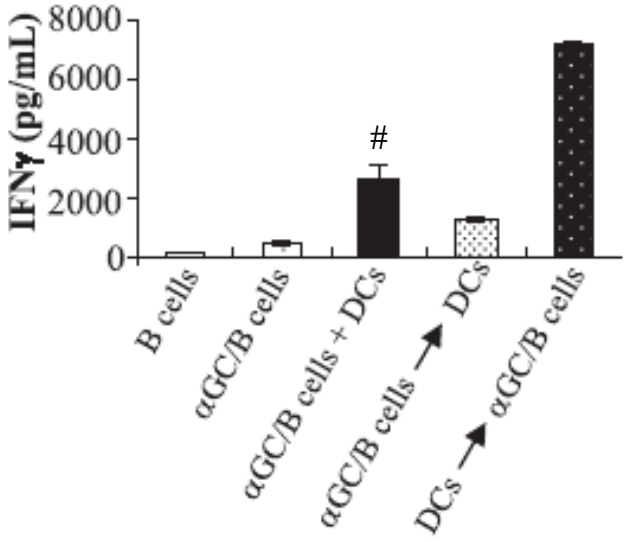

Figure 21 DCs enhanced the Activation of NKT Cells mediated by B Cells as APCs (2) IFN-γ production of thymic NKT cells ($1x10^6$ /mL) activated by αGalCer/B cells ($10x10^5$/ml) and DCs ($1x10^5$/ml) added at different time points. $^{\#}P<0.05$ versus B cell and αGC/B cell group, *P< 0.05 versus all the other groups (One way ANOVA, Student Newman-Kuels post hoc test). The data are presented as the mean ± S.D. of triplicate wells. One representative experiment of three independent experiments is shown.

3.2 The Regulation of NKT Cells by Costimulatory Signals

3.21 Costimulatory Signals deficient Mice have similar Cytokine-producing NKT Cells but produce less Cytokines in the Serum

Because previous studies got controversial results about the role of costimulatory signals on NKT cell activation, and the controversial results were obtained by using different methods in different animal models, we first examined both the cytokine production in the serum and frequencies of cytokine-producing cells in the spleen after in vivo αGalCer stimulation, by ELISA and intracellular staining respectively. It is known that in vivo injection of αGalCer into mice can trigger rapid and robust cytokine production; however, cytokine levels in serum change rapidly in a narrow time window. We therefore tested the optimal time point for detection of both cytokines secreted in serum and synthesized in NKT cells after αGalCer injection. To clarify this, WT (C57BL/6) mice were injected (I.P.) with αGalCer first. One hour or three hours later, mice were bled and their spleens were removed. We then measured cytokine levels in serum and frequencies of cytokine-producing NKT cells in the spleen, and found that at the three hour point, high levels of both IFN-γ and IL-4 in the serum were detected by ELISA, and higher percentages of cytokine-producing NKT cells were also detected (Fig.22). We used αGalCer/CD1d tetramer staining to identify NKT cells, and found that 3 hours after αGalCer injection, only NKT cells, but not other T cells, were cytokine-producing cells (Fig.23). Therefore, this time point was used through out the following studies. We first compared effects of αGalCer injection on WT and $CD28^{-/-}$ mice. Three hours after αGalCer injection, there were significant portions of IFN-γ or IL-4 producing NKT cells in the spleen of WT mice

(Fig.24a). When IFN-γ and IL-4 producing NKT cells in spleens of $CD28^{-/-}$ mice were examined, we found that the frequencies of IFN-γ producing NKT cells in these mice were almost identical to that in WT mice, whereas the frequencies of IL-4 producing cells in these mice were slightly lower than those in WT mice (Fig.24a). In a sharp contrast, cytokine levels in the serum of $CD28^{-/-}$ mice were significantly lower than WT mice (Fig.24b). Therefore, our results were consistent with previous studies that reported different effects of costimulatory signals using different measurements and mice models. CD80 (B7.1) and CD86 (B7.2) are only two ligands for CD28. To determine which ligand preferentially delivered costimulatory signals to NKT cells, we further analyzed cytokine production in response to αGalCer stimulation of CD80 or CD86 deficient mice. The result showed that both CD80 and CD86 single gene deficient mice produced IFN-γ and IL-4 normally in response to αGalCer injection (Fig.25). However, similar to $CD28^{-/-}$ mice, $CD80/CD86^{-/-}$ mice produced a very low level of cytokines in the serum (Fig.24b), although frequencies of cytokine-producing NKT cells in the spleen were relatively normal (Fig.24a). Together, these results demonstrated an indispensable role of CD28/B7 engagement for cytokine secretion by NKT cells, whereas either B7.1 or B7.2 molecule delivered sufficient costimulatory signals to NKT cells.

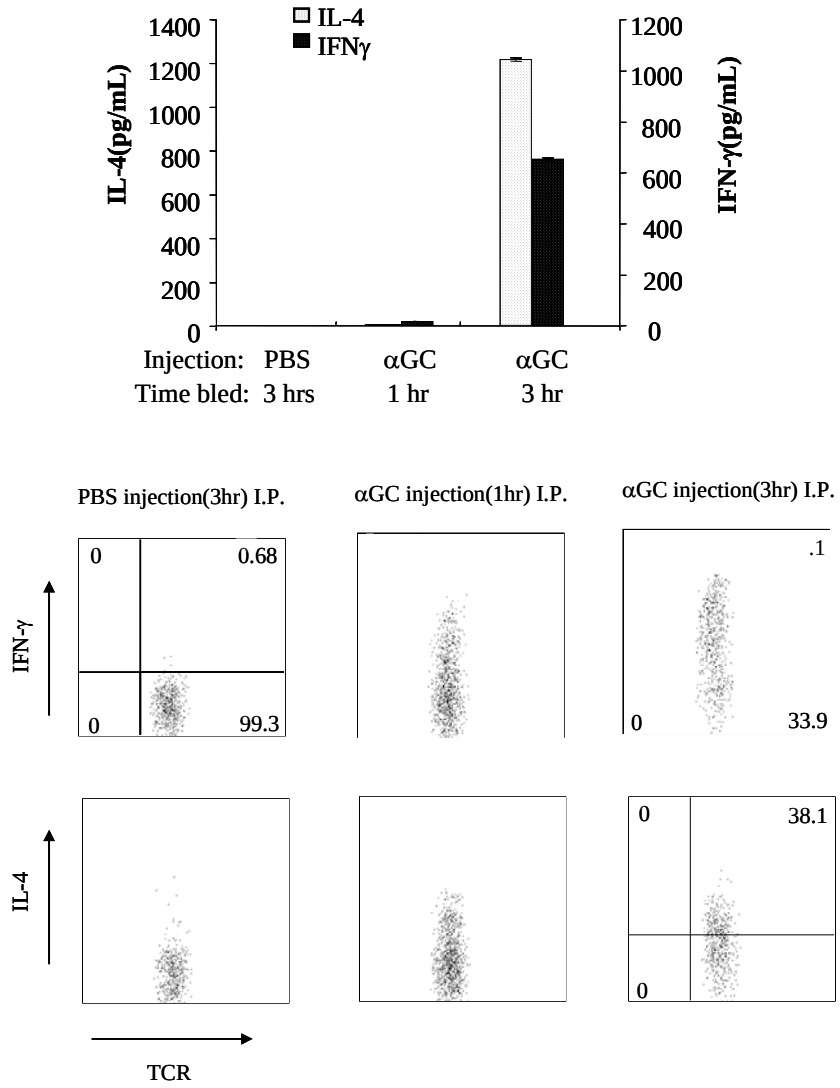

Figure 22 NKT Cell Activation at different Time Points after in vivo αGalCer Stimulation. WT C57BL/6 mice were injected I.P. with PBS or αGalCer (5 μg/mouse), 1 hr or 3 hrs later mice were bled. Serum cytokine production was tested by ELISA (upper) and frequencies of intracellular cytokine-producing cells in the spleen were tested by intracellular staining (lower). Similar results were obtained in two independent experiments.

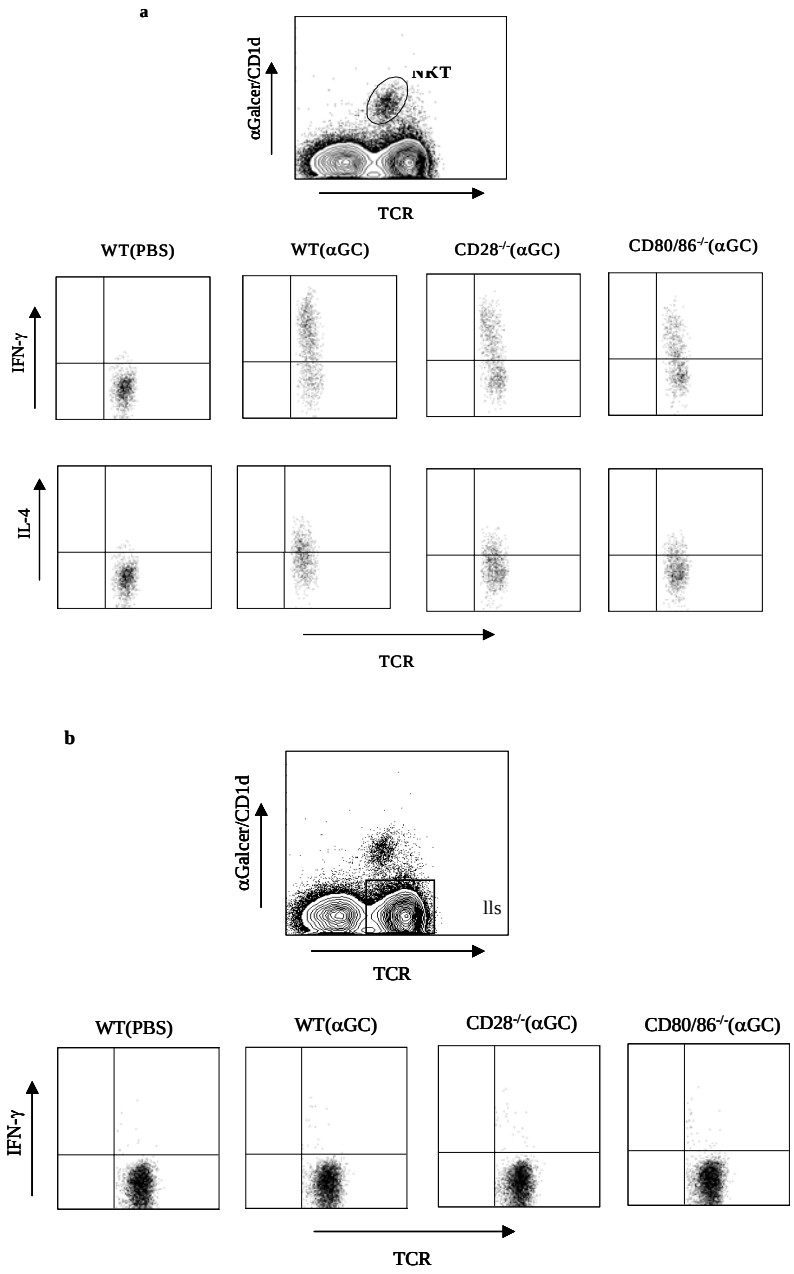

Figure 23 Profiles of intracellular Cytokine Production after αGalCer Stimulation. FACS profiles of intracellular cytokine production of mice injected I.P. with PBS or αGalCer (5 μg/mouse) for 3 hrs. Mice are WT, $CD28^{-/-}$ and $CD80/86^{-/-}$ C57BL/6 mice. 3 hrs after PBS or αGalCer injection, $thy1.2^{+}$ cells were purified from spleen-derived lymphocytes, and then cultured for 2 hrs in GolgiStop; surface-labeled with CD1d/αGalCer tetramer and TCR; and then fixed and permeabilized for intracellular staining with Abs to IL-4 or IFN-γ. NKT cells (a) and T cells (b) were electronically gated and analyzed for their expression of IFN-γ or IL-4.

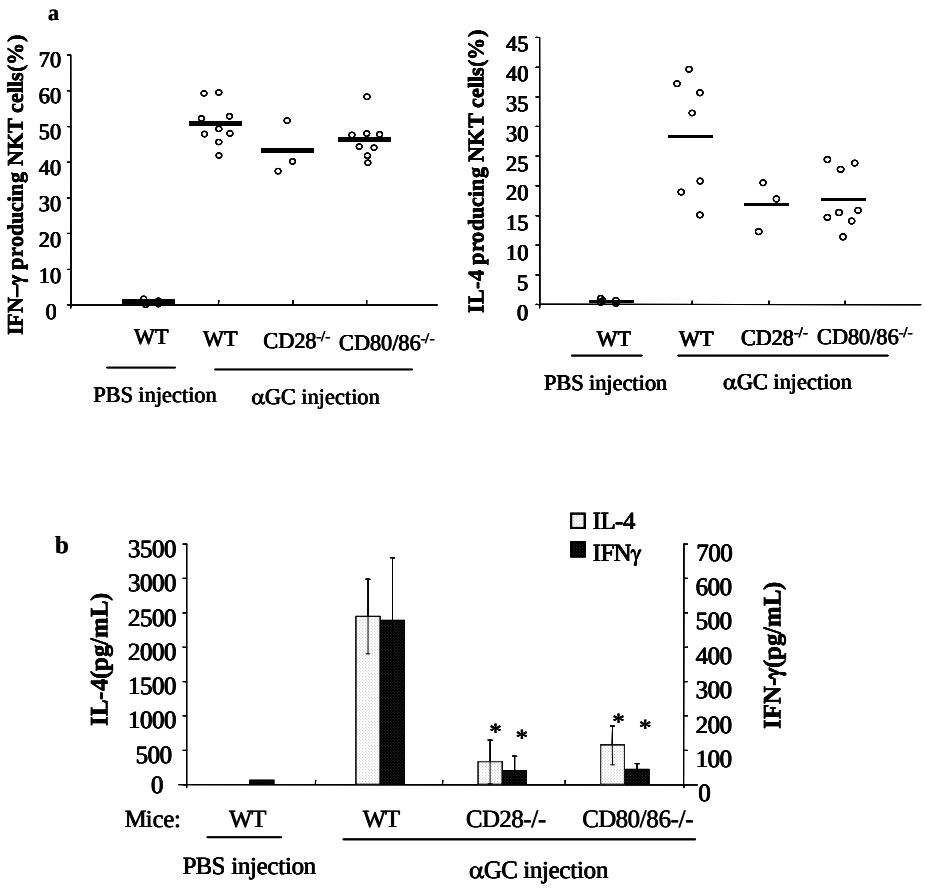

Figure 24 CD28-costimulatory Signals influence Activation of NKT Cells by in vivo αGalCer Stimulation. (a)Frequency of cytokine-producing cells in the spleen of mice (WT, $CD28^{-/-}$ and $CD80/86^{-/-}$ CL57/BL6 mice) 3 hrs after PBS or αGalCer (5 μg/mouse) I.P .injection from multiple experiments is shown; (d)Serum IFN-γ and IL-4 levels in WT, $CD28^{-/-}$, and $CD80/86^{-/-}$ C57BL/6 mice 3 hrs after PBS or αGalCer (5 μg/mL) I.P. injection. Data are represented as the mean ± S.D of three to five mice in each group. (One way ANOVA followed by Dunnett's post hoc test, $*P < 0.01$ versus WT group).(e)Serum IFN-γ and IL-4 levels in WT, $CD80^{-/-}$ and $CD86^{-/-}$ C57BL/6 mice 3 hrs after PBS or αGalCer (5 μg/mL) I.P. injection. Data are represented as the mean ± S.D of three to five mice in each group.

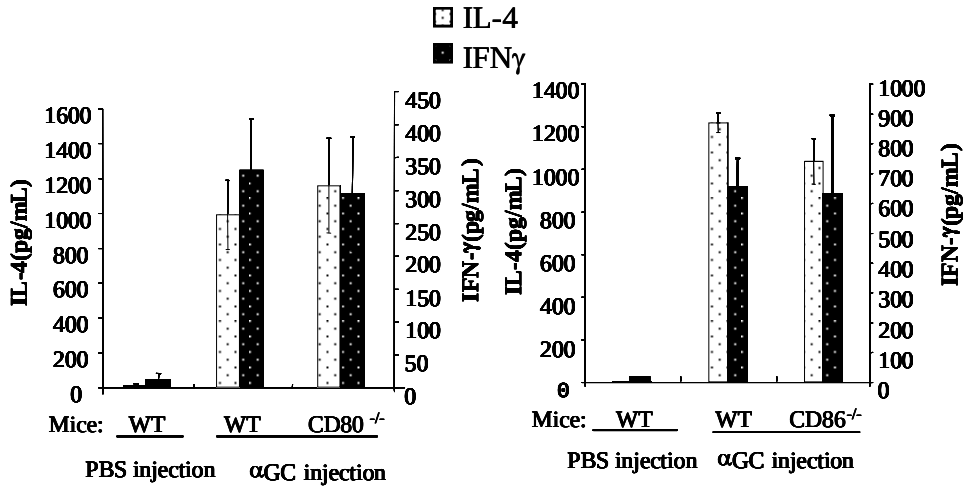

Figure 25 Either CD80- or CD86-costimulatory Signals is sufficient for NKT Cell Activation by in vivo αGalCer Stimulation. Serum IFN-γ and IL-4 levels in WT, $CD80^{-/-}$ and $CD86^{-/-}$ C57BL/6 mice 3 hrs after PBS or αGalCer (5 μg/mL) I.P. injection. Data are represented as the mean ± S.D of three to five mice in each group.

3.22 NKT Cell Development in the Thymus is defective in CD28-costimulatory Signal Deficient Mice (Conventional T cell Development is not affected)

It is possible that low levels of cytokine production in $CD28^{-/-}$ mice were associated with low numbers of NKT cells resulting from abnormal development or homeostasis of NKT cells in the absence of costimulatory signals. Since NKT cells develop in the thymus, and distribute in the peripheral organs, we then compared the frequencies and absolute numbers of NKT cells in thymus and spleen of WT and $CD28^{-/-}$ mice. We found that the frequencies of both splenic and thymic NKT cells were significantly reduced in $CD28^{-/-}$ mice as compared to those in WT mice (Fig.26a), although the development of conventional T cells was not affected by this deficiency (Fig.27a). The absolute number of thymic NKT cells was also severely decreased in $CD28^{-/-}$ mice. However, in spleen, similar numbers of NKT cells were detected in $CD28^{-/-}$ and WT mice (Fig.26a). These results suggested that CD28 costimulatory signals were specifically required for NKT cell development in the thymus, but not for homeostasis, nor for survival and accumulation in the periphery. We further analyzed NKT cell development and distribution in $CD80^{-/-}$ and $CD86^{-/-}$ mice and found that the frequencies and absolute numbers of both splenic and thymic NKT cells in these mice were similar to those in WT mice (Fig.26b and Fig.26c). Therefore, neither CD80 nor CD86 was indispensable for the NKT cells development. However, severely reduced numbers of NKT cells were found in the thymus but not spleen in $CD80/86^{-/-}$ mice (Fig.26d). These results indicated that either B7.1 or B7.2 delivered sufficient costimulatory signals in the development of NKT cells. Importantly, the development of conventional T cells was also normal in $CD80/86^{-/-}$ mice, showing that the requirement of CD28/B7 costimulatory signals was specific for NKT

cell lineage (Fig.27a). We further examined NKT cells in CD40 gene deficient mice and found that the frequencies and absolute numbers of splenic and thymic NKT cells in $CD40^{-/-}$ mice are normal (Fig.26e), indicating that in contrast to CD28/B7 costimulatory signals, CD40/CD40L costimulatory signals are not required for NKT cell development.

We then wanted to know whether CD28 costimulatory signals were required for a specific stage during development of NKT cells. To do this, we examined NKT cell population in WT, $CD28^{-/-}$ and $CD80/86^{-/-}$ mice at different ages. When mice were 2 weeks old, very low percentages and absolute numbers of NKT cells were found in both thymus and spleen of $CD28^{-/-}$ and $CD80/86^{-/-}$ mice as compared to WT mice (Fig. 28a). Interestingly, when mice had grown up to 6 weeks old, low numbers of NKT cells were only found in thymus, but not in spleen of $CD28^{-/-}$ and $CD80/86^{-/-}$ mice (Fig. 28b). These results demonstrated that the CD28/B7 costimulatory signals were required from early stages for development of NKT cells in thymus. In contrast, these results also suggested that the homeostasis of NKT cells in the periphery did not require costimulatory signals, and NKT cells in $CD28^{-/-}$ or $CD80/CD86^{-/-}$ mice may undergo stronger homeostastic proliferation than those in WT mice thus compensated the developmental deficiency. The strong homeostasis of NKT cells in $CD28^{-/-}$ and $CD80/CD86^{-/-}$ mice might be associated with increased cellularity in lymphoid organs which is due to the deficiency of a specific $CD4^{+}CD25^{+}$ FoxP3 expressing regulatory T cells (Treg cells) (Fig. 27b), thus enhanced homeostasis of NKT cells in these mice might also be associated with defective Treg cells (136, 137).

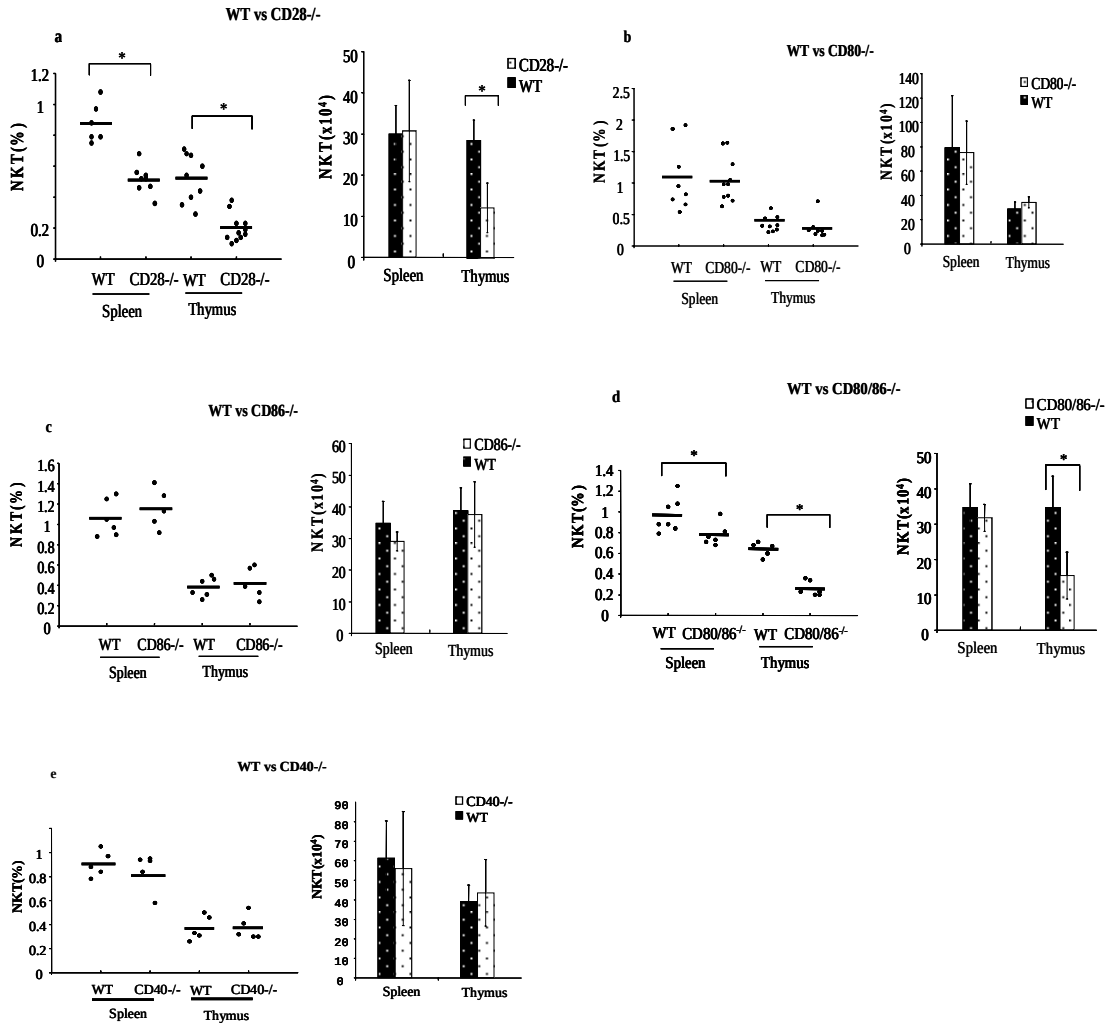

Figure 26 CD28-costimulatory Signal deficient Mice have impaired NKT Cell Development in the Thymus. (a)(b)(c)(d)(e) Percentages and absolute numbers of thymic and splenic NKT cells in WT and $CD28^{-/-}$, $CD80^{-/-}$, $CD86^{-/-}$, $CD80/86^{-/-}$ and $CD40^{-/-}$ C57BL/6 mice from multiple experiments are shown. The data represented the mean $\pm$ S.D. (5-11 mice/group). All the mice were 6 to 8 weeks old. *$p<0.01$ (unpaired T test).

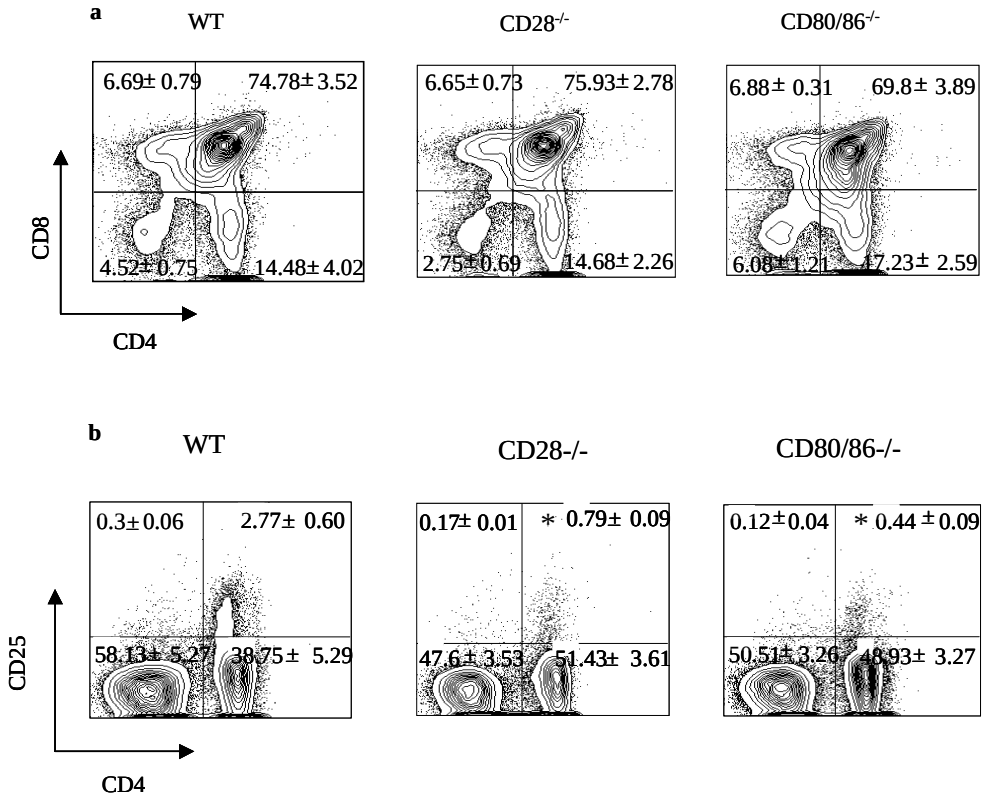

Figure 27 CD28-costimulatory Signal deficient Mice have normal conventional T Cell but impaired CD4^{+}CD25^{+} Treg Cell Development in the Thymus. (a)The profile of conventional T cells development in the thymus of WT, CD28$^{-/-}$ and CD80/86$^{-/-}$ C57BL/6 mice. The data represented the mean ± S.D. (3-5 mice/group); (b)Treg cells distribution in the spleen of WT, CD28$^{-/-}$ and CD80/86$^{-/-}$ C57BL/6 mice. The data represented the mean ± S.D. (3-5 mice/group). *P < 0.01 versus WT (unpaired T test).

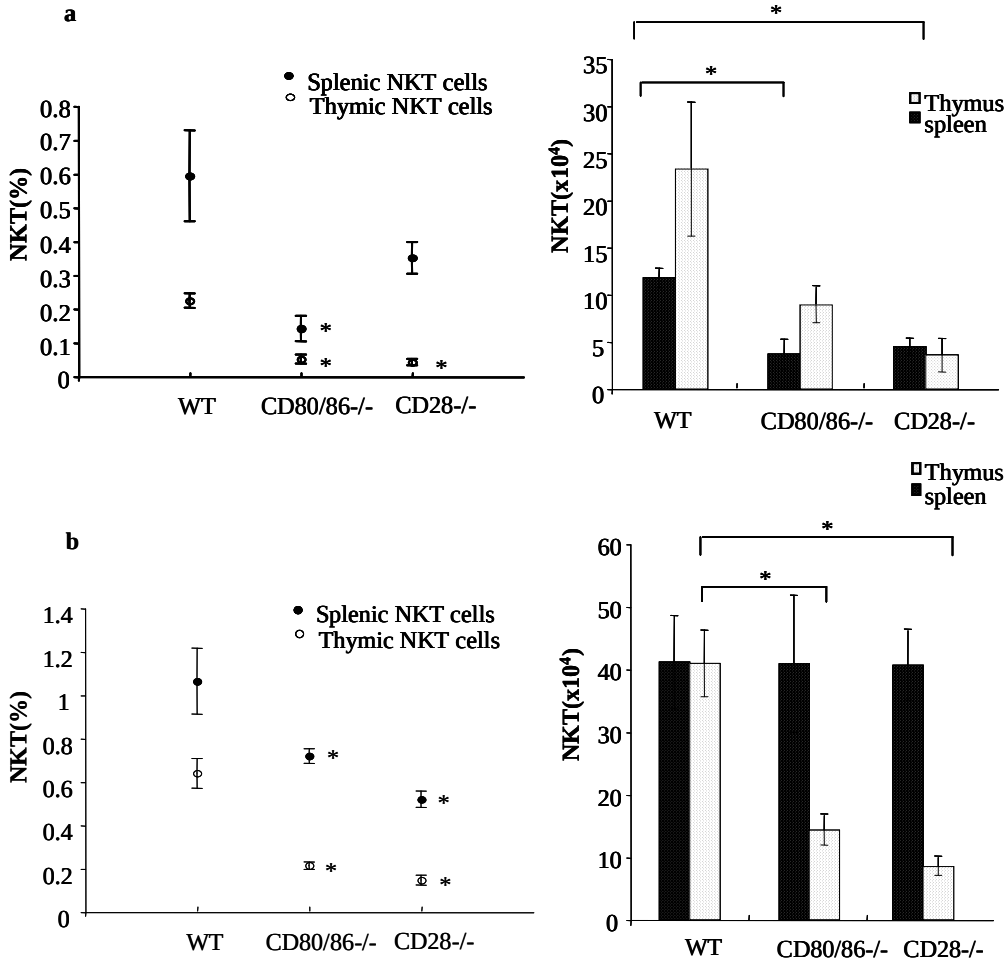

Figure 28 NKT Cell Development in WT and CD28/B7 Costimulatory Signal deficient Mice at different Ages. (a)The percentage and absolute number of thymic and splenic NKT cells in 2 weeks old WT, $CD28^{-/-}$ and $CD80/86^{-/-}$ C57BL/6 mice. The data represented the mean $\pm$ S.D. (3-5 mice/group). *P < 0.01 versus WT group (One way ANOVA, Dunnett's post hoc test); (b)The percentage and absolute number of thymic and splenic NKT cells in 6 weeks old WT, $CD28^{-/-}$ and $CD80/86^{-/-}$ C57BL/6 mice. The data represented the mean $\pm$ S.D. (4 mice/group). *P < 0.01 versus WT group (One way ANOVA, Dunnett's post hoc test).

3.23 Costimulatory Signals may regulate the Cytokine Release of NKT Cells

By studying the activation of NKT cells from $CD28^{-/-}$ or $B7^{-/-}$ mice, we found that despite the normal numbers of peripheral NKT cells, and intact intracellular frequencies of IFN-γ producing NKT cells, the capacity of NKT cells from $CD28^{-/-}$ or $B7^{-/-}$ deficient mice to produce cytokines was impaired, as detected by a striking decrease in cytokine levels in the serum after αGalCer injection. It was possible that the low levels of cytokines in the serum of $CD28^{-/-}$ and $CD80/86^{-/-}$ mice resulted from defective APC function in these mice. To test this possibility, DCs were purified from WT, $CD80^{-/-}$, $CD86^{-/-}$ and $CD80/86^{-/-}$ mice. After loaded with αGalCer, their ability to induce cytokine production of WT thymic NKT cells was examined in vitro. These DCs all expressed similar levels of CD1d on the cell surface (Fig.29a). Clearly, DCs from either $CD80^{-/-}$ or $CD86^{-/-}$ mice could trigger high levels of both IFN-γ and IL-4 production by thymic NKT cells as those from WT mice. However, DCs from $CD80/86^{-/-}$ mice could only trigger very low levels of IFN-γ and IL-4 production by thymic NKT cells (Fig.29b). To determine whether deficiency of costimulatory function was responsible for the inability of DCs from $CD80/CD86^{-/-}$ mice to trigger cytokine release of activated thymic NKT cells, soluble anti-CD28 antibody was added into the cultures. Addition of αCD28 antibody drastically increased the cytokine production by thymic NKT cells (Fig.29c), clearly demonstrating that the defect of costimulatory signals of DCs resulted in failure of cytokine release by activated NKT cells.

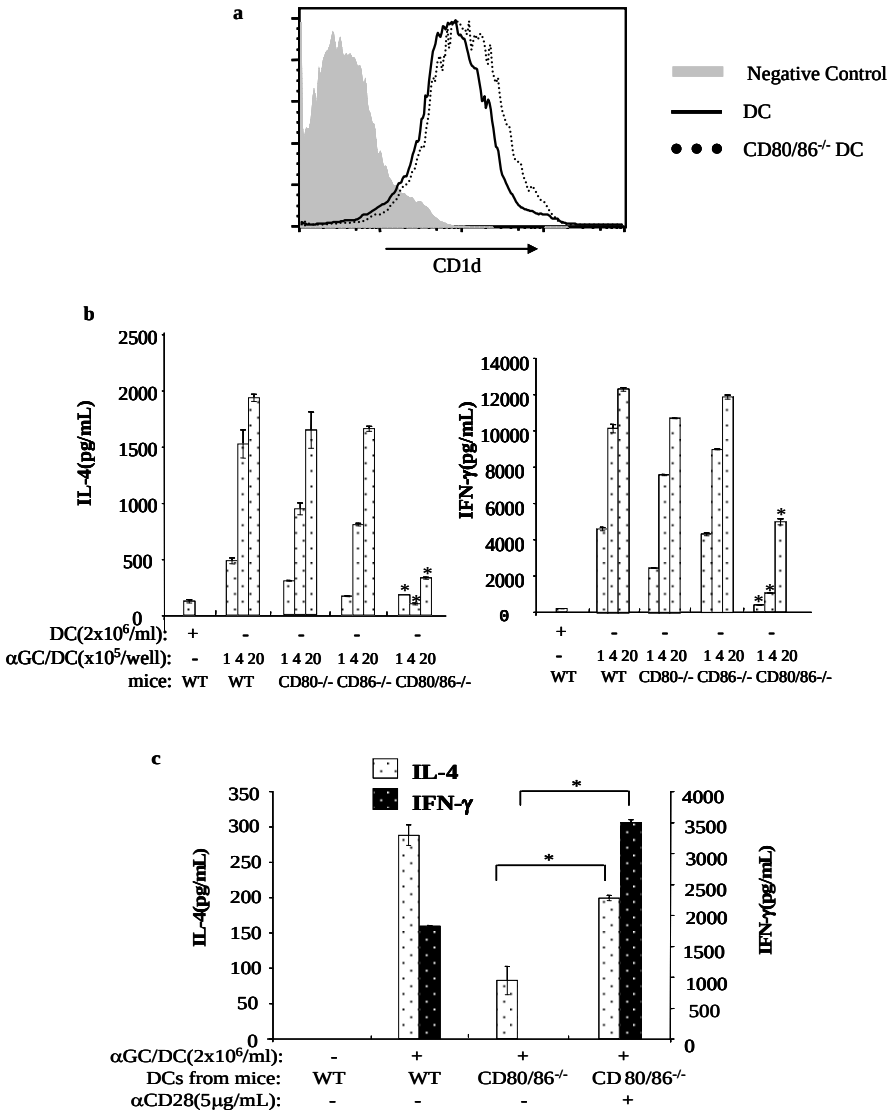

Figure 29 CD28/B7 Costimulatory Signals are required for the Cytokine Release of Thymic NKT Cells. (a)CD1d expression on DCs from WT, $CD28^{-/-}$ and $CD80/86^{-/-}$ C57BL/6 mice; (b)Cytokine production of thymic NKT cells ($1x10^6$/mL) activated by increased numbers of DCs loaded with αGalCer (100 ng/mL). The DCs were from WT, $CD80^{-/-}$, $CD86^{-/-}$ or $CD80/86^{-/-}$ C57BL/6 mice. Two-way ANOVA demonstrated a significant effect of mice strain ($P < 0.01$) and DC concentrations ($p<0.01$) on IFN-γ and IL-4 production of thymic NKT. One-way ANOVA followed by Dunnett's test was conducted at each concentration of DCs, *$p<0.01$ versus the corresponding concentration WT DC group (c)Cytokine production of thymic NKT cells ($1x10^6$/mL) activated by αGalCer (100 ng/mL) loaded WT DCs or $CD80/86^{-/-}$ DCs ($2x10^6$/ml) in the absence or presence of αCD28 antibody (5 μg/mL). (One-way ANOVA; *$p<0.05$; Student Newman-Kuels post hoc test). The data are expressed as the mean $\pm$ S.D. of triplicate wells. One representative experiment of at least three is shown.

CHAPTER FOUR: DISCUSSION

4.1 The Regulation of NKT Cells by CD1d-dependent Lipid Antigen Autoreactivity

It has been known for years that DCs induce strong activation and cytokine production of NKT cells, and efficient NKT cell activation is important to trigger the subsequent anti-infectious and antitumor responses. As antigen presentation cells for NKT cells, DCs, macrophages and B cells express similar levels of CD1d, co-receptors and co-stimulatory molecules on the cell surface, however, the ability to activate NKT cells efficiently is unique to DCs, although the nature of this ability has not been resolved (119, 121).

By comparing the activation of NKT cells triggered by αGalCer/CD1d monomers and by αGalCer/DCs, we found that αGalCer alone when presentated by CD1d monomers could activate NKT cells, but high concentrations of both αGalCer and monomers were required. However, DCs triggered NKT cell activation by a very low concentration of αGalCer. Further analysis revealed that it was the presence of DCs, rather than other APCs that was responsible for highly efficient activation of NKT cells, and the highly efficient lipid antigen presentation was unlikely due to a high density of CD1d on the surface of DCs, since other APCs, such as B cells, expressed CD1d at similar levels but were unable to mediate effective activation of NKT cells. Our results further showed that the presence of DCs was required for the activation of NKT cells by a low level of αGalCer/CD1d. These observations propose a model in which DCs potentiate NKT cells for quick and effective responses to exogenous lipid Ags. Soluble factors are not required for the potentiation since Vα14 NKT cells can be potentiated by DCs in the absence of DC-derived IL-12 or IFN-γ, although in vivo cytokines produced by DCs certainly

enhance the activation of NKT cells as described in other studies (129). Moreover, the potentiation of NKT cells requires direct contact with DCs, but the costimulatory signals alone could not replace this contact. Furthermore, potentiation of NKT cells is CD1d-dependent and requires intact endosomal pathways for processing of endogenous lipid antigens. Disruption of specific endosomal pathways or fixation abolished DCs' ability to potentiate NKT cells. Although inhibitor-treated or fixed DCs, as well as fixed CD1d monomers were still able to bind and present αGalCer, they could only trigger a weak activation of NKT cells. These results support the view that the unique NKT-stimulatory nature of DCs requires the display of endogenous lipid Ags on their surfaces.

Correlating with their ability for potentiation, DCs could conjugate with NKT cells even in the absence of αGalCer in a CD1d-dependent manner. In the presence of αGalCer, the formation of conjugates between DCs and NKT cells was rapid and stable, leading to the formation of ISs and effective activation. These results indicate that potentiation of NKT cells by DCs is mediated by engagement between Vα14 TCRs and endogenous lipid Ag-CD1d complexes, thus strongly support a role of endosomal endogenous lipid antigens displayed by DCs in efficient activation of NKT cells. The formation of conjugates in the absence of αGalCer provided additional evidence for the existence of Ag-independent engagement between DCs and NKT cells. In contrast to DCs, B cells were unable to potentiate, nor to form stable conjugates with NKT cells in the presence of αGalCer. Although B cells are able to present αGalCer, the activation of NKT cells by αGalCer/B cells was inefficient. This is not due to the inhibitory effects of B cells, since the presence of a small number of DCs significantly enhanced IFN-γ productions of NKT cells when

activated by large numbers of αGalCer/B cells. Clearly, once NKT cells were potentiated by DCs, they could be effectively activated by αGalCer/B cells. The potentiation of NKT cells by A20-CD1d cells demonstrated a critical role of CD1d-autoreactivity for efficient activation of NKT cells, and the inability of A20-CD1d-TD cells to potentiate NKT cells revealed that high levels of surface CD1d expression did not ensure efficient activation of NKT cells unless CD1d molecules traffic through endosomes. These results support a view that endogenous lipid antigens displayed by B cells are at lower levels than that of DCs, perhaps due to low activity of endosomal pathways for lipid processing. Increased CD1d trafficking through endosomes bring more endogenous lipid antigens on the surface of A20-CD1d cells that in turn become capable to potentiate NKT cells for efficient activation.

Our results provide a novel explanation for the rapid activation of NKT cells in vivo. It is conceivable that frequent contacts with DCs through CD1d-dependent autoreactivity maintain a high sensitivity of NKT cells against exogenous lipid antigens, and the stimulation from endogenous lipid antigens may also contribute to the presence of constitutive cytokine mRNAs in NKT cells (138). These mRNAs may be transcribed in response to these frequent DC contacts. In support of this idea, Ag-independent activation events have been observed in naive T cells making contact with DCs (139). Nevertheless, cytokine production by NKT cells is not detectable in the absence of exogenous lipid Ags, possibly because maturation of cytokine mRNAs and subsequent translation require TCR ligation with a higher threshold of avidity (138). It is important to emphasize that full activation of NKT cells cannot be achieved by DC contacts until NKT cells encounter exogenous antigens such as αGalCer or microbial antigens with a high affinity to Vα14

TCRs. When the concentration of high affinity exogenous lipid antigens is very low, two types of signals from Vα14 TCRs that engage with both exogenous and endogenous antigens are required for activation. Two-signal-dependent activation may ensure effective responses of NKT cells against weak exogenous Ags without overreaction to endogenous Ags. Because the activation of NKT cells do not require the engagement of Vα14 TCRs with exogenous and endogenous antigens presented by the same cell, activation of NKT cells in vivo may be determined by the summation of signals from engagement of Vα14 TCRs with endogenous and exogenous antigens presented by different APCs, such as DCs and B cells. In fact, engagement of single T cells with multiple APCs has been observed in vivo and productive ISs may be preferentially formed towards strongest stimulus (140, 141). It is interesting to note that IL-12 could replace the signal from either endogenous or exogenous Ags to serve as a secondary signal for activation of NKT cells (27, 92), whereas IL-12 alone was not sufficient.

Among endogenous lipid, iGb3 displayed by DCs was proposed to be important for autoreactive interaction and potentiation of NKT cells. Blocking iGb3 almost abolished the ability of DCs to potentiate NKT cells, and DCs from *Hex*B gene deficient mice failed to mediate efficient activation of NKT cells. Deficient development of NKT cells in *Hex*B gene deficient mice indicated a critical role of iGb3 in positive selection of NKT cells (52). Therefore, iGb3 is an important endogenous lipid Ag that plays a critical role in both the development and modulation of NKT cells. However, our data suggested that iGb3 may not be the only endogenous lipid antigens that potentiate NKT cells, and other endosomal lipids are also involved in the potentiation of NKT cells. This is supported by a recent study that has shown that NKT cells from mice deficient in iGb3 synthase have

normal development and function (54). Although the identity of these endosomal lipid antigens remains unknown, our results indicated that at least some of these antigens were required for potentiation of NKT cells. It was shown that the activity of β-glucosylceramide synthase is crucial for endogenous lipid antigens with a structure similar to α-glucosylceramide (α-GlcCer) (130). We have found that inhibition of this enzyme disabled DCs to potentiate NKT cells. Our results also suggest that, in addition to potentiation of NKT cell activation, specific endogenous lipid antigens also affect cytokine profile of NKT cells. DGJ inhibits the activity of α-galactosidase that is required for the processing of some glycolipid Ags, but not the processing of iGb3 (52, 128). DGJ-treated DCs were still able to potentiate NKT cells, but IFN-γ production was selectively reduced. The inhibition of α-galactosidase might reduce presentation of the specific endogenous antigens on DCs, resulting in the decreased IFN-γ production of NKT cells. Furthermore, when B cells were preloaded with iGb3, their APC function for NKT cells was barely improved, suggesting a low level of iGb3 alone was not responsible for the poor activity of B cells in NKT cell activation.

The findings of this study are paralleled by similar events in other aspects of cellular immunity. Recognition of certain endogenous peptide Ags by $CD4^+$ or $CD8^+$ T cells increases their sensitivity to foreign Ags (142-144). CD1d-dependent potentiation similarly enhances the sensitivity of NKT cells against exogenous lipid Ags. However, CD1d-dependent potentiation is specific for DCs, whereas no specific types of APCs have been identified for autoreactive engagement of conventional T cells. A recent study showed that NKT cells that had never been exposed to autoreactivity of DCs were

hyperreactive to αGalCer loaded DCs (39). This hyperreactivity also supported DCs-specific potentiation of NKT cells. Furthermore, interactions between CD4 or CD8 coreceptors and self peptide-MHC complexes are required for enhanced sensitivities of conventional T cells to foreign peptide Ags (143, 144). Although the majority of Vα14 NKT cells express CD4, and CD1d molecules are more MHC class I-like, the data on NKT cells from $CD4^{-/-}$ mice, together with the facts that few NKT cells express CD8 and many NKT cells are $CD4^{-}CD8^{-}$ double negative, indicate that coreceptor-CD1d interaction is not involved in the potentiation of NKT cells. Thus, the molecular mechanism of CD1d-dependent potentiation of NKT cells may be unique, and remains to be characterized.

4.2 The Regulation of NKT Cells by Costimulatory Signals

Costimulatory molecules positively augment T cell receptor (TCR) signaling by generating important signals through ligand engagement, so they play critical roles in activation and induction of effector function of conventional T cells (122). However, due to controversial results from previous studies, as well as the unique nature of NKT cells, the role of costimulatory molecules in regulation of NKT cells was unclear. Peripheral NKT cells display a phenotype of activated memory T cells with high CD44 and CD69 expression, and can promptly produce large amounts of Th1 cytokines (IFN-γ, TNF) as well as Th2 cytokines (IL-4, IL-10, IL-13) in response to TCR ligation (74). The mechanism of the quick cytokine production is unique to NKT cells since naïve thymic, splenic, and hepatic NKT cells all have transcriptionally active IL-4 and IFN-γ gene loci. Upon TCR stimulation, there will be immediate translation of preformed cytokine mRNA

and rapid cytokine secretion (145). Therefore, unlike conventional T cells, costimulatory signals are not required for de novo synthesis of cytokine mRNAs in NKT cells after TCR ligation.

Our data clarified the conflicting conclusions from previous studies and showed that the capacity of NKT cells from CD28 or B7 (CD80/86) deficient mice to produce cytokine was impaired, as detected by a striking decrease in cytokine levels in the serum in these mice after αGalCer stimulation. However, the defect of cytokine production is not due to the blockage of induction and translation of cytokine genes since normal frequencies of intracellular IL-4 and IFN-γ producing NKT cells were detected in the spleen of $CD28^{-/-}$ and $CD80/86^{-/-}$ mice after αGalCer stimulation. Moreover, it was proposed that the serum cytokine production is mainly from splenic NKT cells (120), and our data clearly showed that the absolute splenic NKT cell number of CD28/B7 deficient mice was comparable to that of WT mice, further suggesting that NKT cells in $CD28^{-/-}$ or $CD80/CD86^{-/-}$ mice are unable to secrete translated cytokines into serum. Whether this defect is intrinsic to NKT cell or acquired during thymic development remains to be determined. However, cytokine secretion of activated NKT cells from WT mice was regulated by CD28/B7 engagement, indicating a role of costimulatory signals in cytokine secretion of activated NKT cells. Therefore, if the defect is not intrinsic to NKT cells of costimulation deficient mice, the cytokine production in the serum would be restored by co-injection of αGalCer together with anti-CD28 antibody into $CD80/86^{-/-}$ mice. Within lymphocytes, signal-induced secretion of cytolytic molecules seems to undergo the following pathway: secreted molecules packed into vesicles first, and then they are transported to the synapse,

where vesicles dock at the plasma membrane. In response to agonistic signals, the fusion of secretory and plasma membrane lipid bilayers occurs, resulting in rapid release of the content (cytokines) into the exocytic space (146, 147). It remains to determine at which step costimulatory signals are required for the process of cytokine secretion.

It was surprising that our study demonstrated the requirement of CD28/B7 costimulatory signal pathway in NKT cell development in the thymus since $CD28^{-/-}$ and $CD80/86^{-/-}$ mice showed significantly decreased frequencies and numbers of NKT cells in the thymus as compared to WT mice. The defective development of NKT cells was found to occur from early stages, since reduced NKT cells were clearly detected in the thymus and spleen of mice as young as two weeks old. Very low numbers of NKT cells in spleen of these young mice also indicated that reduced numbers of thymic NKT cells was not due to increased emigration from thymus to the periphery in $CD28^{-/-}$ and $CD80/86^{-/-}$ mice. The B7.1 (CD80) and B7.2 (CD86) are the only two ligands for CD28. In general, B7.2 (CD86) is thought to be more widely expressed and at higher levels than B7.1 (CD80), and is also the more important costimulator of T cell activation (148). However, with respect to NKT cell development and activation, there is no difference between B7.1 and B7.2 molecules, it seems that they deliver similar levels of costimulatory signals to NKT cells, and either one is sufficient for normal NKT cell development and activation.

Another important finding is that the requirement of costimulation for development was specific for NKT cell lineage since $CD28^{-/-}$ and $CD80/86^{-/-}$ mice have normal conventional T cell development in the thymus. However, it is not known why NKT cells are different from conventional T cells for this requirement during development. Murine NKT cells share the same progenitor population with conventional T cells in the thymus

until the $CD4^+CD8^+$ DP stage. NKT cells that derive from a $CD4^+CD8^+$ DP thymic subset can be detected in the thymus 1 week after birth. At this stage, the interaction of CD1d with the Vα14-Jα18 TCR triggers both positive selection and NKT lineage commitment. In concordance with these activities, CD28 is also found to be expressed at the highest levels in DP thymocytes (149). NKT cells in the thymus undergo a stepwise developmental progression characterized by expression of different surface markers (Fig.2). It remains to be clarified at which stage the developmental block happens. Functionally, immature NKT cells produce only IL-4, and they acquire the ability for IFN-γ production in later maturation stages (stage3) (150). In the first part of my study, we observed IFN-γ production by thymic NKT cells from $CD28^{-/-}$ mice (Fig.12b), suggesting the existence of mature NKT cells in these mice. Therefore, the deficiencies of NKT cells in $CD28^{-/-}$ and $CD80/86^{-/-}$ mice may have more to do with the homeostasis and survival of mature NKT cells instead of a block in development.

One unique feature of NKT cells is the expression of NK receptors on their surface from early development stage. It has been known that thymic NKT cells express substantially higher frequencies of NK receptors than peripheral NKT cells (114, 116, 151). The majority of Ly49 receptors contain the immunoreceptor tyrosine-based inhibitory motif in their cytoplasmic domains and function as inhibitory receptors. The expression of NK inhibitor receptors, including Ly49 and CD94/NKG2 receptors, which are known for their inhibitory effects on NK cell activation, were detected in mature NKT cells (stage 3) (152). Upon interaction with their ligands, MHC class I molecules, Ly49 receptors transmit inhibitory signals that counteract activating signals, potentially influencing NKT cell development and function. It has been demonstrated that enforced transgenic

expression of Ly49 receptors, including inhibitory type Ly49A and even receptor considered as activating type Ly49D, dampened NKT cell development (151, 153). In accordance with this, we have found in an ongoing study that in TAP-1 (transporter-1) deficient mice, which have a deficient MHC class I (ligand for Ly49 receptors) and Qa-1b (ligand for CD94/NKG2 receptors) expression, thymic NKT cells were present at greatly increased frequencies (Fig.30), implying that development of NKT cells would be enhanced in the absence of inhibitory effects mediated by engagement between MHC class I or non-classical MHC class I and NK receptors.

In addition to the development, activation and function of NKT cells may also be influenced by mostly inhibitory effects mediated by NK receptors, including Ly49 and CD94/NKG2 as showed in some studies (114, 117, 154). In contrast, it has been demonstrated that under stressed conditions, DCs acquired a B7/CD28 dependent activator signals that were dominant over the Ly49/H-2D inhibitory pathway, allowing CD1d-restricted NKT cell activation toward a Th1 predominant pattern (155). Thus, Ly49 and CD94/NKG2-mediated signals regulate the activation of NKT cells by modulating the strength of TCR signaling and CD28 costimulatory signaling. Taken together, it is possible that Ly49 or CD94/NKG2 receptors and activation signals from CD28 attenuate or augment perceived TCR signals respectively, potentially influencing NKT cell development and function.

Our study is the first to show that CD28-B7 costimulatory signals are critical for NKT cell development in the thymus, and our results strongly prompt a new view of the role of costimulation in NKT cell development. According to this view, under the normal condition, the positive signals delivered by CD28 overcome the inhibitory signals from

Ly49 and/or other NK receptors, and exceeds the threshold required for selection and expansion of mature NKT cells in the thymus. In the absence of the positive signals delivered by CD28 in gene deficient mice, the inhibitory signals from NK receptors are predominant, and severely block homeostasis and survival of mature NKT cells in the thymus. It is possible that removal of NK/MHC engagement will eliminate the requirement for costimulatory signals during development of NKT cells.

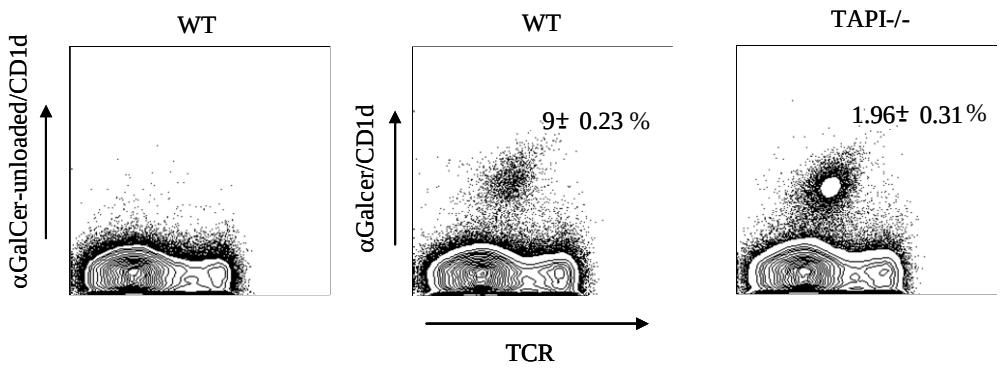

Figure 30 Thymic NKT Cell Development of WT and TAPI deficient Mice. The percentage of thymic NKT cells in WT and TAPI deficient C57BL/6 mice. The data represented the mean ± S.D. (3-5 mice/group). There was a significant difference between WT and TAPI deficient mice ($p<0.05$, unpaired T test).

CHAPTER FIVE: SUMMARY AND FUTURE DIRECTION

In this study, we have investigated two important factors that regulate development and activation of NKT cells. First, we showed that endogenous lipid antigens displayed specifically by DCs could potentiate NKT cells for effective activation by low doses of exogenous lipid antigens. We provided strong and clear evidence for a critical role of autoreactivity of NKT cells against endogenous lipid antigens in the activation induced by exogenous lipid antigens. Importantly, DC-specific autoreactivity is only able to potentiate, but not to activate NKT cells. It is possible that activation of NKT cells requires additional signals, such as co-stimulatory signals, to overcome negative regulation from other receptors. Although potentiation of NKT cells does not require co-stimulatory signals or other soluble factors from DCs, the results in the second part of my study suggested that CD28-costimulatory signals might be required for cytokine secretion, even though NKT cells could be activated to synthesize cytokine proteins in the absence of co-stimulatory signals. In addition, our study is the first to show that CD28/B7 costimulatory signals are required specifically for the NKT cell development in the thymus. However, the mechanisms through which co-stimulatory signals influence the development and cytokine release of NKT cells remain to be identified.

In the future study, we will investigate the mechanisms underlying the requirement of CD28/B7 signal pathway for NKT cell development and cytokine secretion. We will first determine whether the defect in cytokine secretion is intrinsic to NKT cells in the absence of CD28 costimulatory signals. We will isolate NKT cells from CD28 deficient mice and test their ability to secret cytokines when activated in the culture with different mitogens. Similarly, NKT cells from CD80/CD86 double deficient mice will be activated in the

presence of CD28 ligand, such as anti-CD28 antibody, to determine whether these NKT cells produce and secrete cytokines normally under these conditions. Because cytokine secretion of T cells is known to undergo specific pathways, these pathways will be examined in NKT cells from CD28 deficient mice using FACS analysis and confocal imaging to determine whether CD28 deficiency disrupts the process of a specific pathway or assembly of secretive machinery. One of the characteristics of NKT cells is that they express NK receptors, some of which are known to deliver negative regulatory signals. It is possible that CD28/B7 signals are required to overcome the negative signals by NK receptors during NKT cell development and cytokine secretion. We will test this hypothesis by using CD28/TAP1 double deficient mice. CD28/TAP1 deficient mice do not express MHC class I molecules that are the major ligands for NK receptors due to TAP deficiency. Therefore NKT and NK cells will be released from negative regulation from NK receptors in these mice. If NK receptor mediated negative regulation inhibits development of NKT cells in the absence of CD28 signals, a restoration of normal development of NKT cells in the thymus may be expected in CD28/TAP1 double deficient mice. Furthermore, CD28 signals may not be required for cytokine secretion by activated NKT cells from TAP1 or CD28/TAP1 deficient mice. These studies may further reveal the unique characteristics of NKT cells, and enhance our understanding of NKT cell biology, as well as the mechanisms of NKT cells mediated immune regulation.

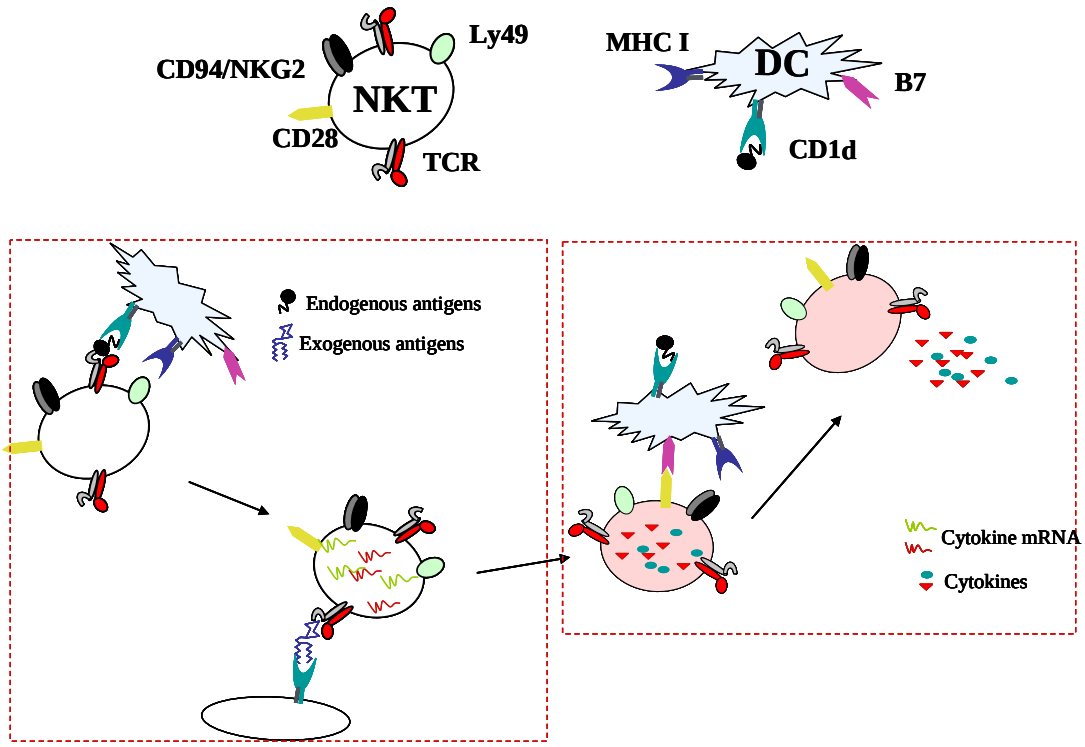

Figure 31 Regulation of NKT Cell Activation. In this thesis, I have studied two factors that regulate the activation of NKT cells. First, we found that DCs are able to potentiate NKT cells by displaying specific patterns of endogenous lipid antigens. The potentiated NKT cells can be efficiently activated by a small amount of exogenous lipid antigens and synthesize cytokine proteins. CD28/B7 costimulatory signals are not required at this stage. However, as the secondary activation signals, CD28/B7 signals are required for cytokine secretion by the activated NKT cells, since cytokine release is impaired in CD28/B7 signal deficient mice. In addition, CD28/B7 signals are also important for the normal development or maturation of NKT cells. It is possible that CD28/B7 signals are required to overcome the inhibitory signals from NK receptors (Ly49 or CD94/NKG2) expressed on NKT cells.

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