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CD28 Costimulation Requirement for Interferon-y Secretion by Natural Killer T cells During Hepatitis B Virus Infection

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CD28 COSTIMULATION REQUIREMENT FOR INTERFERON-γ SECRETION BY NATURAL KILLER T CELLS DURING HEPATITIS B VIRUS INFECTION

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By

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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Paul James Rhodes Renick ENTITLED CD28 Costimulation Requirement for Interferon-γ Secretion by Natural Killer T cells During Hepatitis B Virus Infection BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science

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ABSTRACT

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Natural Killer T cells (NKT cells) are a unique subset of lymphocytes that express natural killer (NK) and T cell receptors (TCR). The NKT cell population includes four separate subclasses. This paper will focus on Category I NKT cells which possess a canonical TCR receptor (Va14Ja281) that recognizes only hydrophobic antigens presented by CD1d molecules. These cells are believed to play an important regulatory role in immunity. A variety of disease conditions, including cancer, infections and Type I diabetes, are controlled by NKT cells. NKT cells are also capable of secreting large quantities of cytokines, namely interleukin-4 (IL-4) and interferon-gamma (IFN-γ). This ability to switch between Th1 (IFN-γ) and Th2 (IL-4) cytokines emphasizes the immunological regulatory role that these cells play. The mechanisms by which NKT cells select the cytokines they secrete are not well characterized. Blocking of CD28 by monoclonal antibodies or mutation of the CD28 gene impairs NKT cell s ability to secrete IFN-γ in vitro. NKT cell of IFN-γ secretion plays a significant role in the clearance of Hepatitis B virus (HBV) in a HBV transgenic mouse model. Abrogating or blocking expression of CD28 should significantly impair the ability of NKT cells to clear HBV infection. This thesis suggests a series of in vitro and in vivo experiments designed to test the role of CD28 in IFN-γ secretion and HBV clearance in mice.

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ABBREVIATION LIST

α-GalCer	Alpha-Galactosylceramide
α-ManCer	Alpha-Mannosylceramide
APC	Professional antigen presenting cell
CD	Cluster of Differentiation
CD154	CD40 ligand (CD40L)
CD80	B7.1
CD86	B7.2
CTL	Cytotoxic lymphocyte
DC	Dendritic Cell
DN	Double Negative
DP	Double Positive
GPI	Glycosylphosphatidylinositol
HBV	Hepatitis B virus
IDDM	Insulin dependent diabetes mellitus
IFN-γ	Interferon gamma
ITAM	Immunoreceptor tyrosine kinase activation motif
iTCR	Invariant T Cell Receptor
ITIM	Immunoreceptor tyrosine kinase inhibition motif
KAR	Killer Activation Receptor
KIR	Killer Inhibitory Receptor
KO	Knockout
MCII	MHC Class II compartment
MHC	Major Histocompatability Complex
NK	Natural Killer
NKT cell	Natural Killer T cell
PAMP	Pathogen associated molecular marker
PRR	Pattern recognition receptor
Tc	Cytotoxic lymphocyte
TCR	T Cell Receptor
Th	T helper
Th1	T helper 1
Th2	T helper 2

INTRODUCTION

The immune system is made up of two components: the innate and the adaptive immune responses. Components of the innate immune system (e.g. macrophages, dendritic cells, and NK cells) activate and complement the adaptive immune system.

Cells of the innate immune system take up foreign antigens by germ-line encoded pattern recognition receptors (PRRs). The PRRs target groups of highly conserved pathogen associated molecular patterns (PAMP) such as lipotechoic acid and mannose derivatives (1), which are typically found in invading pathogens. These immune responses are low affinity and do not result generate immunological memory. The MHC and CD1 surface molecules of professional antigen presenting cells (APCs) of the innate immune system can combine foreign antigens (2, 3, 4, 5, 6) and activate CD8 and CD4 T cells. By secretion of cytokines (IL-12, IL-18 and IL-10), APCs can also interact and regulate T cells. These signals influence the differentiation of T cells into Th1 and Th2 CD4 T cells which regulate cellular and humoral immunity. The adaptive immune response targets specific pathogenic antigens with high affinity and results in the generation of memory cells. Unlike the innate immune system, the adaptive immune system s responses improve with each subsequent exposure to a specific pathogen.

A variety of immune cells occupy a position intermediate between the innate and adaptive immune systems. These cells play a role in regulating and directing the subsequent adaptive immune response. These immune cells are B-1 B cells, $\gamma\delta$-T cells and Natural Killer T cells (NKT cells).

Natural Killer T cells

NKT cells are a subset of lymphocytes, found in mice and humans (7) that possess characteristics of the both innate and adaptive immune cells. These cells are implicated in shaping immune responses to different pathogens. NKT cells express both T cell receptors (TCR) and natural killer (NK) receptors. There are 4 groups of these cells differing in TCR restriction; NK marker expression and surface marker expression (see Table 1).

Category I of NKT cells possesses an invariant TCR (iTCR) comprised of Vα14Jα281 chain paired with a Vβ8.2, Vβ7 or Vβ2 chain (8, 9, 7). The cells bearing iTCR are CD1d restricted and display either a CD4 co-receptor or no co-receptor (double negative). This type of NKT cell does not express CD8 surface molecule but usually express NK1.1 (10, 11).

Table 1- Categories of murine NKT cells (Adapted from Kronenberg et al 2002 (7))

Category	I	II	III	IV
Repertoire	Vα14-Jα18 Vβ8.2/7/2	Semi-diverse Vα3.2-Jα9/Vα8, Vβ8	Vα diverse Vβ diverse	Vα diverse Vβ diverse
Co-receptor	$CD4^+$ or DN	$CD4^+$ or DN	$CD8^+$, $CD4^+$ or DN	$CD8^+$, or $CD4^+$
Reactivity	α-GalCer	ND	Self-agonist	ND
Antigen-presenting molecule	CD1d	CD1d	MHC Class I	MHC Class I and MHC Class II
NK receptors	$DX5^-$ Mostly NK1.1	DX5 (?) Mostly $NK1.1^{+/-}$	$DX5^{+/-}$ Mostly $NK1.1^+$	$DX5^+$ Mostly $NK1.1^{+/-}$
Location	Thymus, liver, spleen and bone marrow	Thymus (?), liver, spleen and bone marrow (?)	Liver, spleen and bone marrow	Thymus (?), liver, spleen and bone marrow

No J region indicated T cell receptors are diverse, α-GalCer- α-galactosyl ceramide; DN, double negative; J joining region; ND, not determined; NK natural killer; V, variable region)

Type II NKT cells are CD1d-reactive and are double negative (DN) or CD4+ (12, 10). The TCR is classified as semi-diverse and express either Vα3.2Jα9.Vβ8 or Vα8/Vβ8 (7). The third category of NKT cells is CD1d-independent and display on their surface either CD4, or CD8 or no co-receptor (DN). Category III NKT cells, enriched in the bone marrow and spleen, express a na ve T cell phenotype and are thymus-independent in their development (12, 10, 13). The fourth population of NKT cell is characterized by the expression of CD49B (a ligand for DX5). Category IV NKT cells are believed to play a role in antigen-specific immune responses in irradiated skin and in suppression of Type I diabetes (7).

Category I NKT cells

This thesis focuses on Category I NKT cells (referred to hereafter as NKT cells). These cells are either $CD4^+$ or DN paired with an iTCR (Vα14Jα281 /Vβ8.2, 7 or 2) that is CD1-restricted. NKT cells possess an activated T cell phenotype (i.e. $CD69^+$, $CD44^{high}$, CD62L, $CD5^{high,}$ $CD45RB^{high}$ and IL-2R), which is also indicative of a cell type that can rapidly respond to environmental stimuli (8, 12). These cells express NK receptors and the most prevalent markers are NK1.1, CD122 and Ly49 membrane molecules. NKT cells are noted for their rapid secretion of high levels of IL-4. NKT cells secrete INF-γ as well as a wide variety of other Th1, Th2 and Th3 cytokines (8, 9, 7, 12). NKT cells are widely distributed through the body, but the ratio of NKT to T cells varies with tissue. In mice, NKT cells are most prevalent in the thymus (~15%), bone marrow (~25%) and thymus (~40%). NKT cells are least prevalent in the spleen (3%), lymph nodes (0.3%), blood (4%) and lungs (7%) (9 13, 12). Like conventional T cells, maintenance of NKT cells occurs independently of the thymus. The bone marrow plays a major role in replenishing NKT cells after activation-induced death (9, 14).

Table 2- Characteristics of murine and human category I NKT cells (Adapted from Kronenberg et al, 2002 (7))

Characteristics	Mouse	Human	Comment
Major Subset	CD4+, DN	CD4+, DN	Proportions vary
T cell receptor α-chain β-chain Expression level	 Vα14Jα281 Vβ8.2, 7,2 Intermediate	 Vα24JαQ Vβ11 Intermediate	 Homologous Homologous
Accessory molecules NK associated	NK1.1, CD122, Ly49	NKR-P1, CD122	Homologous (CD161)
Restriction element	CD1d	CD1d	Homologous
Cognate antigen	Glycolipid	Glycolipid	α-GalCer stimulates
Cytokine production IL-4 IFN-γ	Rapid high levels +	Rapid high levels +	Following TCR ligation Following TCR ligation
Frequency PBL	~1%	~0.1-0.5%	Move variable in humans

α-GalCer- α-galactosyl ceramide; PBL- peripheral blood lymphocytes

Human NKT cells

NKT cell subsets of humans have the same characteristics as murine NKT cells (See Table 2). Human type I NKT cells also have an iTCR comprised of Vα24JαQ paired with Vβ11. The human NKT cells also possess an invariant TCR comprised of the Vα24JαQ α chain

as well as a conserved β chain Vβ11. These iTCR are also CD1d restricted like their murine counterparts. The distribution in humans is not as clear, with most studies limited to the peripheral blood, but NKT cells are reported to be located in the liver (9). A major difference between human and murine NKT cells is that human category I NKT cells are separated into two distinct functional subsets; CD4+ and DN NKT cells.

The CD4 subset is polarized towards predominantly Th2 cytokine secretion (IL-4) with some Th1 cytokine secretion. These cells play a regulatory role in immune tolerance (15, 16, 17). The DN subset secretes Th1 cytokines (IFN-γ) and function in responding to infection and inflammation (15, 16, 17).

Chemokine receptors and NK receptors (Data from Lee et al, 2002 (15), Kim et al, 2002 (17) and Gumperz et al, 2002 (16))

NKT cell Subset	Exclusively CD4	Exclusively DN	Both CD4 and DN
Chemokine Receptors	CCR4	CCR1 CCR6 CXCR6	CCR1 CCR2 CCR5 CXCR3 CXCR4 CXCR6
NK markers		2B4 CD94 NKG2A	CD161 (CD4 lower frequency than DN) CD56

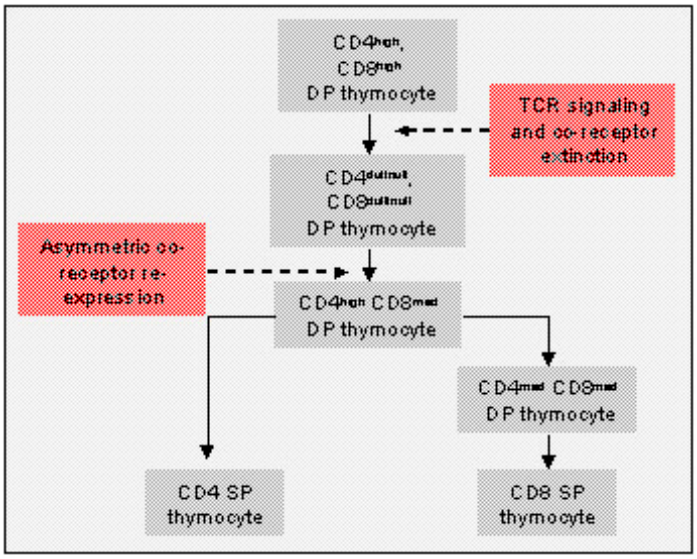
In addition, these subsets differ in chemokine and NK marker expression. Each subset possesses distinct chemokine receptors and T cell cytokine profiles (See Table 7) (18). CD161 and CD65 membrane markers are expressed by Human CD4 NKT cells (See Table 3) (18).

Murine and human NKT cells play similar roles in associated autoimmune disorders. Mouse NKT cell models exist for Type I diabetes, systemic erythematosis lupus, and systemic sclerosis (see NKT cells and Immunity). Consequently, mouse models of NKT cell responses are important in understanding the roles of human NKT cells in immune regulation. Human and mouse NKT cell iTCR and CD1d are so highly conserved between the two species that mouse CD1d is capable of activating human NKT cells and human CD1d is capable of activation of mouse NKT cells (5). This indicates that the evolutionary pressures that maintain the conserved nature of CD1d and iTCR are at least partially shared between humans and mice.

NKT CELL DEVELOPMENT

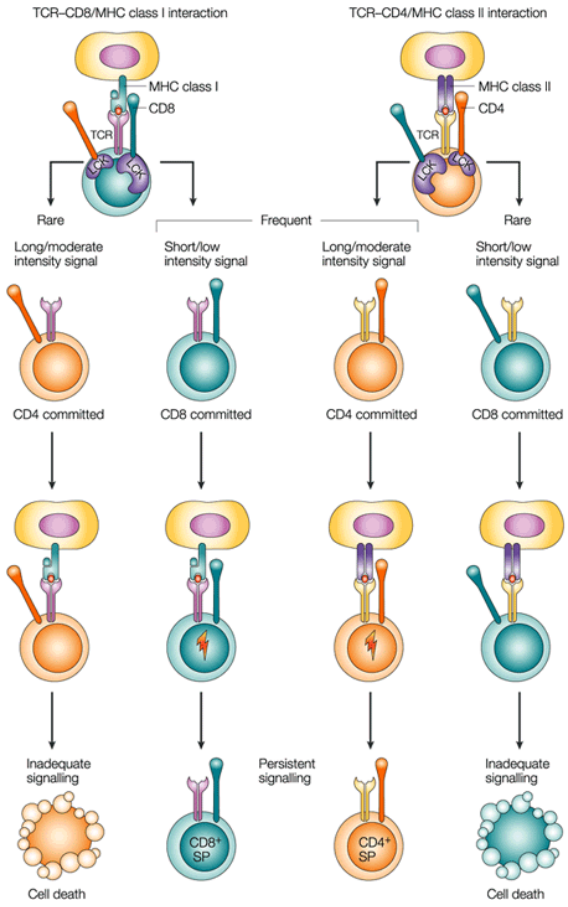
NKT cells develop in a manner similar to conventional T cells (7, 20, 21) and derive from common lymphoid progenitor cells that migrate to the thymus. Upon reaching the thymus, conventional T cells develop from double negative (DN) thymocytes to double positive (DP) thymocytes by successfully expressing TCR and co-receptors. Those DN thymocytes that do not successfully express TCR die of neglect (apoptosis). This selection is mediated by cortical epithelial cell and results in single positive thymocytes (SP) expressing either the CD4 or the CD8 co-receptor. The DP thymocytes undergo two selection events, positive and negative selection. Positive selection takes place in the cortex of the thymus with cortical epithelial cells. DP thymocytes that can recognize and loosely bind MHC class I or II survive and migrate to the medulla, while those that do not recognize MHC complexes undergo apoptosis. Negative selection occurs in the medulla of the thymus, eliminating auto-reactive thymocytes that tightly bind to MHC and self-antigen. These na ve T cells then migrate to the periphery. T cell maturation results in apoptosis of 95% of the thymocytes that enter the thymus from the bone marrow.

Figure 1- Asymmetric re-expression of CD4 and CD8
(Adapted from Lucas et al, 1996 (19))



T cell Co-receptor Selection

Figure 2- Strength of Signal Model (Adapted from Germain et al, 2002 (21))



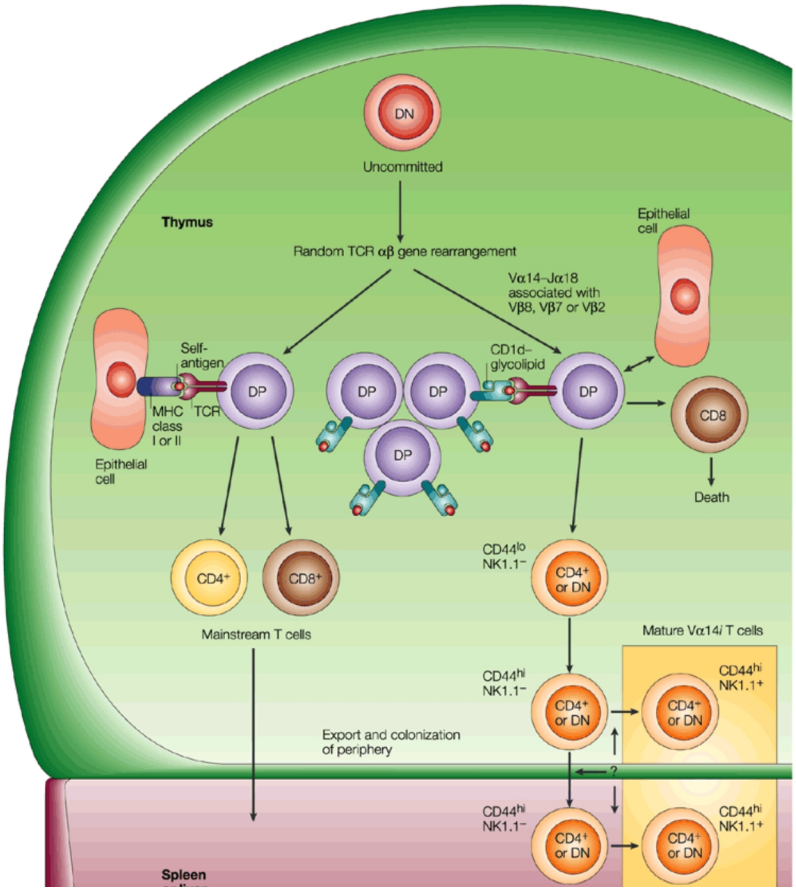
The mechanism of co-receptor selection by DN thymocytes is poorly understood. The instructive theory states that DP thymocytes receive a distinct signal from cortical epithelial cells to become committed to the CD4 or CD8 pathway, down-regulating the expression of opposing co-receptor. Stochastic development, maintains that the selection of lineage is a random genetic choice independent of external signals. Because of this genetic selection the opposing co-receptor is no longer expressed. Under this system some DP thymocytes will inappropriately select the wrong co-receptor and thus be eliminated by apoptosis. Several different types of studies were undertaken to test both theories (21).

The instructive theory is supported by rescue experiments in which thymocytes that selected the wrong co-receptors were not rescued by the transgenic expression of the appropriate MHC molecule. In these experiments, expression of one type of TCR (CD4 or CD8) was enforced throughout T cell development in addition to the randomly selected co-receptor. If the stochastic theory were correct, then T cells that chose an incorrect co-receptor should be rescued by this enforced expression. The end result would be CD4 and CD8 (DP) mature thymocytes. In the initial experiments, no mature DP T cells were generated (21). These observations support the instructive theory.

Data supporting stochastic development came from phenotypic analysis of MHC knock out (KO) mice. Two different KO mice were developed displaying two distinct DP thymocyte phenotypes: $CD4^{med}$, $CD8^{high}$ (MHC II -/-) and $CD4^{high}$, $CD8^{med}$ (MHC I -/-). These transitional states were not expected since the deficient MHC was not present to instruct development T cell development. The opposing co-receptor (CD4 in MHC I KO mice or CD8 in MHC II KO mice) expression was eventually extinguished (21). These observations supported the stochastic model.

Germain et al discovered that both CD4 and CD8 SP thymocytes derive from $CD4^{high}$, $CD8^{med}$ DP thymocytes and that co-receptor extinction is not linear (19). These data demonstrate that both co-receptors are lost on TCR signaling and the extent of that loss is determined by signal strength. Both of the co-receptors are then re-expressed in an asymmetric manner, first CD4 then CD8 giving rise to the observed $CD4^{high}$, $CD8^{med}$ DP thymocytes. After this re-expression, the inappropriate co-receptor is extingished (Figure 1). Germain et al have proposed a modification of the instructional development model and refer to this model as the strength of signal model (Figure 2) (21).

Figure 3- The NKT cell Developmental Pathway (Adapted from Kronenburg et al, 2002 (7))



According to the strength of signal theory, CD4 and CD8 commitment depends on the duration and intensity of the signaling from the TCR. Short duration signaling leads to CD8 commitment and long duration signaling leads to CD4 commitment. Selection of CD4 expression is based on the association of the tyrosine kinase Lck with this co-receptor (21). In this model some DP thymocytes will have TCR that weakly bind to MHC class II, resulting in selection of incorrect CD8 choice while other DP thymocytes will have TCR that bind strongly to MHC class II, resulting in incorrect CD4 choice. Both of these events would be fairly rare and cells exhibiting these choices undergo apoptosis. In experiments in which recombinant receptor studies in which the intracellular domains of both CD4 and CD8 were exchanged, lineage fate was controlled by the intracellular signaling end of the co-receptor. MHC II specific thymocytes in the absence of cells expressing CD4 developed into CD8 thymocytes. At low concentrations of phorbol ester or ionomycin, CD8 T cells were produced and at high concentrations of these drugs CD4 T cells were produced. Cross-linking experiments in which the TCR of double positive thymocytes were cross-linked with CD4 or CD8, or TCR generated single positive thymocytes with up to a 90% transition rate. Kinetic labeling studies of the selection efficiencies of CD4 and CD8 T cells resulted in selection rates of up to 40% for CD8 T cells and 90% for CD4 cells (22). These observations favor an instructive model over a stochastic model.

NKT cell Development vs. Conventional T cell Development

NKT cells follow a similar developmental pathway as T cells but with some differences (see Figure 3). Thymectomized neonatal mice show depletion of NKT cells (24). An intact thymic structure is also required (25). In mice with defective thymic structure (e.g. *aly*/*aly* mice), NKT cells are depleted, while conventional T cells are not. *Aly*/*aly* mice have a poorly defined cortico-medullary junction and no clearly defined boundary area. The medullary area is extremely small and the medullary epithelial cells are sparse and in the cortex there are thicker and more abundant reticular fibroblasts than with *aly*/+ mice. Vα14-Jα281 transcripts were only found in the thymus of mice and not in the bone marrow (a proposed alternative development site) (26). Within the thymus NKT cell precursors (NK1.1- CD4+) have been detected using CD1d-α–GalCer

tetramers (11). Unlike conventional T cells, NKT cells are selected by CD1d DP thymocytes (27, 28). CD8 expression results in apoptosis (9, 7). Following the strength of signal model, NKT cells that receive a frequent and long to moderate intensity signal probably develop into CD4 NKT cells, while those that receive a short/low intensity signal become double negative (DN) NKT cells. However, such events have not been observed and are only speculation. A variety of mutations affect the development of NKT cells (see Table 5). Deletion of CD1d results in depletion of NKT cells (29, 30, 31) and, like conventional T cells, NKT cells require successful expression of both pre α-chain and iTCR (32, 33). Unlike conventional T cells, NKT cells do not appear to undergo negative selection (7, 9) and these potential autoreactive cells are most likely kept in check by their KIR receptors (7).

Table 4 — NKT cell developmental mutations
(Adapted from Kronenburg et al, 2002 (7))

Genetically modified KO mice	Change in cell number			References
	T cells	NKT cells	NK cells	
Common γ-chain	--	--	--	(7)
IL2r	+++	--	--	(7), (9)
IL7	--	--	+++	(9)
IL15	+++	--	--	(7), (9)
GM-CSF	+++	--	+++	(34)
Ets1	+++	--	--	(35)
Fyn-/-	+++	--	+++	(36), (37), (38)
Lta/b-/-	+++	--	--	(39), (40)
Aly/aly	+++	--	--	(25)
Jα18-/-	+++	--	+++	(7), (9)
CD1d-/-	+++	--	+++	(29), (30) (31)
DnRAS/dnERK	--	+++	+++	(38)

The cytokines crucial for NKT cell development are IL-7 (9), (7), IL-15 (9, 7) membrane lymphotoxin (Ltα/β) (39, 40) and granulocyte macrophage colony stimulating factor (GM-CSF) (34). Mice in which expression of capthesin S gene is disrupted have impaired NKT cell selection and function (41). Expression of common γ-chain is also required for NKT cell development (7). Like conventional T cells; NKT cells require both RAG-1 and 2 for successful TCR expression (9). NKT cells, unlike conventional T cells, are also dependent on Fyn signaling (36, 37) for proper development yet are unaffected by dnRAS and dnErk knockouts (38) which are essential for conventional T cell development. Knockout of expression of the wing-helix-turn-helix transcription factor Ets-1 results in depletion of NKT cells (35).

NKT CELLS AND CD1D

The CD1 family is a group of nonclassical (Class Ib) MHC molecules originally discovered using monoclonal antibodies (42). This family of molecules presents hydrophobic antigens (lipids and peptides) (43) to the immune systems and has been referred to as the third antigen-presenting pathway. The discovery of this system also showed new roles for T cells in adaptive cellular immunity: the recognition of non-self glycolipid antigens and elimination of associated pathogens and targeting of altered self. This process of lipid antigen presentation closely parallels traditional peptide antigen presentation and utilizes similar intracellular compartments (44, 45, 41). CD1 genes show a lack of polymorphism (5, 3). This lack of diversity suggests that there are distinct evolutionary pressures to conserve these genetic sequences. CD1 molecules also have a role in presenting self-antigens to the immune system, suggesting they play a role in immune regulation and tumor surveillance (44).

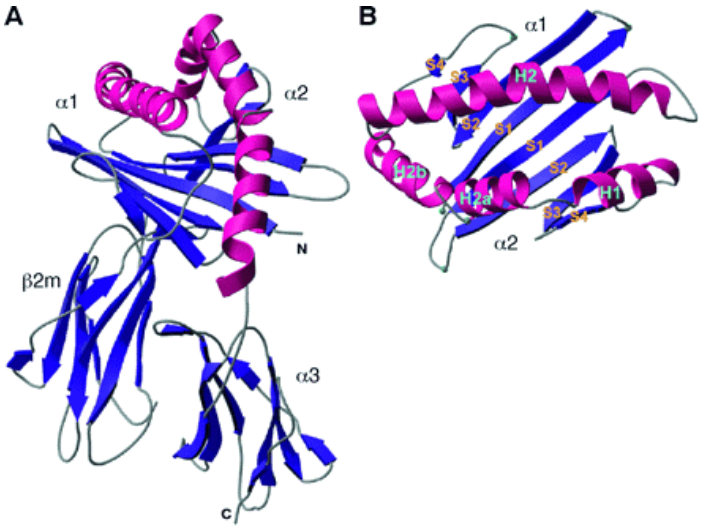
CD1 Characteristics and Intracellular Trafficking

The CD1 family of molecules is comprised of five isoforms each encoded by individual genes. These genes share many characteristics of MHC class I genes such as the intron/exon structure and their homology of the polypeptides to the MHC Class I gene products. Encoded by the CD1 genes are a leader peptide, 3 extracellular domains, a transmembrane region and carboxy terminus region. The isoforms are split into 2 groups: Group 1 is comprised of CD1a, b, c, e and Group 2 comprised of CD1d. The classification is based on homology of the nucleotide and amino acid sequence and is supported by expression and function of the groups (3). Based on the highly conserved nature of the $\alpha 3$ region coded on all CD1 molecules, the CD1 isoforms can also be considered members of immunoglobulin

superfamily (47). Secreted forms of CD1 have been characterized but the function of these soluble products in unknown (42). Mapping of the CD1 family has proven difficult due to multiple transcripts, poorly defined initiation sites and extensive alternative splicing (42). NKT cells recognize lipid antigens presented by CD1d molecules.

Group II (CD1d) molecules are expressed on all hematopoietic stem cells, gastrointestinal epithelial cells and most murine B cells, on human and murine thymocytes, on a subset of human T and B-lymphocytes and resting monocytes (3). While the CD1 family is structurally similar to the MHC class I molecules, antigen processing and presentation are similar to those of the MHC class II molecules.

Figure 4-The crystal structure of murine CD1d (Adapted from Zeng et al, 1997 (47))

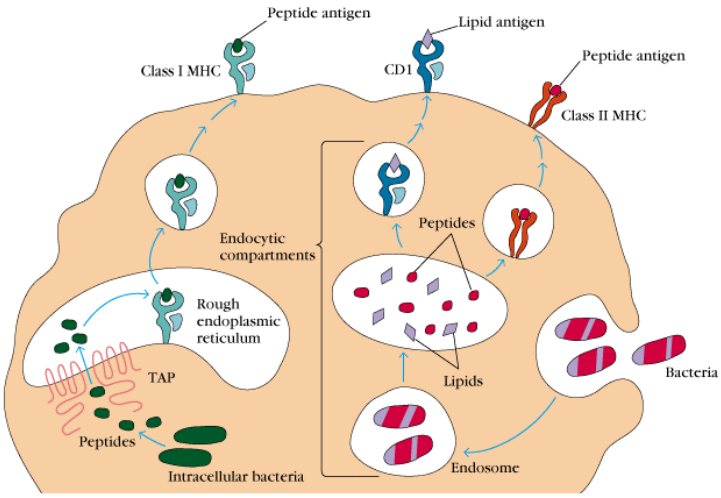


The crystal structure of murine CD1d has been determined and the organization and folding is similar to MHC class I molecules. CD1d is a heterotetrameric structure comprised of 3 ~50 kDa heavy chains (α chains) associated with β2 microglobulin (β2M). The association with β2m is essential for antigen presentation by CD1d (See Figure 4). Subtle differences between the CD1d structure and the MHC class I molecule exist, there is no single turn α1 H1 helix of class I molecules and CD1d has a different arrangement in the bulge of the β2m S4 strand compared to H-2K. The binding pocket of CD1d is extremely hydrophobic as determined by electrostatic potential mapping (47). The CD1d binding groove is the largest of any MHC antigen-presenting pocket whose structure has been determined. The binding groove of CD1d is narrower and has near constant width in contrast to the MHC Class I and II molecules (47). Proposed binding of glycolipid antigens occurs by insertion of the hydrophobic tail of the antigen

into the binding pocket leaving the polar head of the molecule exposed to interact with the TCR (47).

CD1d molecules enter the MHC II compartments (endosomes and lysosomes referred to as MCII) to bind to their respective antigens (see Figure 5). CD1d is found in LAMP-1+ compartments (late endosomes/lysosomes) (3, 45). Cells treated with either chloroquine or conacanmycin A show lose the ability to present lipid antigen. Both of these drugs neutralize the acidic pH of endosomes interfering with the antigen presentation by MHC II. The increase in the pH of the endosomes prevents the effective degradation of the pathogens into presentable molecules that can complex with MHC or CD1. CD1d possess a tyrosine-targeting motif on the C-terminus which interacts with a small group of cytosolic adapter proteins responsible for cellular sorting events. These proteins are referred to as AP-1 and AP-2. AP-1 is located at the cytoplasmic face of the trans-Golgi network and AP-2 is located mainly in the plasma membrane and is required for the internalization of proteins from clathrin coated pits and vesicles leading to the endocytic pathway (45). The adapter protein with which CD1d interacts is not known (6).

Figure 5- CD1 and MHC intracellular trafficking (Adapted from Goldsby et al, 2000 (48))



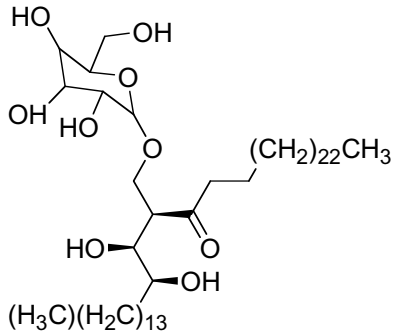
CD1 antigen-binding functionality depends in some manner on the invariant chain (Ii) (45, 18, 41). Removal of the cytoplasmic tail of CD1d causes redirection of CD1d to the cell surface, while this effect can be reversed by the co-expression of Ii. In Ii -/- mice CD1d was widely distributed to the cell surface and APCs for these mice do not optimally stimulate autoreactive NKT cell hybridomas (45). These observations suggest that a ligand must be optimally loaded. In immunoprecipitation with anti-CD1d antibody experiments, Ii was found with a fraction of CD1 molecules (45). Mice deficient in cathepsin S exhibit a reduced number of CD1 restricted NKT

cells. Cathespin S plays a role in the degradation of Ii, suggesting a link between CD1d and Ii (41).

Antigens presented by the CD1 family include glycolipids (diacylglycerols, sphingolipids, polyisoprenoids or mycolates) (49, 44, 50, 18). Lipid length plays a role in endosomal entry and antigen presentation by CD1b (51, 52). Typically long alkyl chains indicative of microbial glycolipids (e.g. a C_{80} alkyl chain) are delivered to the late endosomes several hours post uptake, but a shorter C_{32} chain on the same antigen is rapidly and inefficiently presented by surface CD1b. Dendritic cells (DCs) preferentially present long chain antigens with chain length determining T cell stimulation potential. Minimum antigen length for T cell activation is 12 carbons. These observations support the hypothesis that DCs utilize a specialized endosomal-loading pathway to promote preferential recognition of microbial glycolipids (52).

Currently, only mycobacterial antigens are known to be presented by CD1 molecules (CD1b and CD1c) (44, 5, 3, 53, 43, 54)(44). While activation of CD1 restricted NKT cells via CD1d antigen presentation has not been directly observed *in vivo*, several *in vitro* systems have demonstrated this effect (8, 9, 7, 47, 55).

Figure 6- The structure of alpha-galactosylceramide
(Adapted from Calabi et al, 2000 (42))



Currently, most *in vitro* systems study NKT cell activation using α-galactosylceramide (α-GalCer), a marine sponge derivative (5) (see Figure 6). While this substance does not represent a natural ligand for CD1d, it is able to activate NKT cells and may be an analog of undiscovered ligand (49). Following infection by bacteria, parasites and viruses, α-GalCer has stimulated NKT cells to respond (8, 7, 9). Alpha-galactosylceramide has also stimulated strong anti-tumor responses by NKT cells. CD1d:α-GalCer tetramers are used to quantify the numbers of NKT cells (56).

Another suspected CD1d ligand is glycosylphosphatidylinositol (GPI) but this has not been conclusively demonstrated (57, 58)

NKT CELLS AND IMMUNITY

Table 5- NKT cells and Immunity (Adapted from Godfrey et al 2000 (9))	
Immune Regulation	
Induction of Th2 response	Aid in generation by massive IL-4 secretion
Anterior chamber acquired immune deviation (ACAID)	NKT cells are required to generate negative effector CD8 T cells
Graft vs. Host Disease	NKT cells are required for prevention
Suppression of anti-tumor response	NKT cells are required to generate negative effector CD8 T cells
Autoimmunity	
Type I Diabetes	NKT cells are deficient in NOD mice, BB rats and human diabetics
Lupus	Decrease in NKT cells associated with pathogenesis (mice and humans)
Multiple sclerosis	Decrease in NKT cells associated with pathogenesis (mice and humans)
Systemic sclerosis	Decrease in NKT cells associated with pathogenesis (mice and humans)
Experimental autoimmune gastritis	Thymectomy in 3 day old mice depletes NKT cells
Anti-metastatic activity	
IL-12 mediated rejection	Low dose IL-12 activates NKT cells to direct anti-tumor responses
α-GalCer mediated rejection	Activates NKT cells to direct anti-tumor responses
Natural rejection	APCs activate NKT cells to direct anti-tumor responses in MCA induced tumor models
Infection	
Bacteria:	
Listeria	NKT cells aid in clearing infection
Mycobacteria	NKT cells needed for granuloma formation and switch to IFN-γ production
Psuedomonas aeroginosa	NKT cells serve as sentinel cells and activate effector cells
Eukaryotic Pathogens:	
Toxoplasma	NKT cells needed to generate CD8 effector T cells
Plasmodium	Inhibit parasite growth, debatable role in IgG formation
Cryptococcus neoformans	NKT cells recruited to the lungs and driver Th1 response to pathogen
Brugia pahangi	Source of IL-4 against 3rd stage larvae
Viruses:	
Diabetogenic encephalomyocarditis virus (EMCV-D)	NKT cell activation protects mice from infection and pathology
Hepatitis B	NKT cell activation inhibited viral replication

NKT cells play a role in immunity against a variety of bacterial, parasitic and viral infections. NKT cells may either direct or aid in anti-tumor responses. NKT cells drive of aid in the induction of tolerance (e.g. anterior chamber acquired immune deviation) and play an important role in immune regulation (e.g. Th2 induction).

Immune Regulation

The explosive secretion of IL-4 by NKT cells helps polarize T helper cell function and cytokine secretion towards a Th2 cell response and down-regulates any Th1 cell response. NKT Th1 cytokine secretion may not drive Th1 polarization but may activate other immune cells that can polarize the immune response to a Th1 profile. In this manner, NKT cells play a supporting role in Th1

induction. NKT cells are required for the induction of peripheral tolerance in the eye. Anterior chamber acquired immune deviation (ACAID) prevents damaging Th1 responses in the eye. Antigen in the eye is taken up by APCs that selectively recruit and activate NKT cells to produce TGF-β. TGF-β induces the generation and expansion of antigen-specific MHC Class I-restricted CD8 T regulatory (Tr) cells. These Tr cells secrete TGF-β and IL-10 that down-regulate Th1 mediated delayed hypersensitivity reactions (DTH) against the antigen (59, 9). The transfer of DN NKT cells to rats that underwent allogenic bone marrow transplantation prevented the induction of graft vs. host disease (60). NKT cells also can down regulate anti-tumor immune responses. DN NKT cell clones were able to suppress the generation of B16 melanoma specific CTLs without affecting the immune response against the tumors both *in vitro* and *in vivo* (61). The DN NKT cells were capable of directly lysing the tumor specific CTLs (61).

Autoimmunity

The absence of NKT cells appears to play a role in various autoimmune disorders (62). NKT cells are depleted in Type I diabetes (IDDM) in both humans and mice (9, 63). Genetic analysis of diabetic siblings compared to non-diabetic siblings revealed that the diabetic siblings show defective generation of NKT cells (64). Treatment of non-obese diabetic mice (NOD) mice with a-GalCer prevents the onset of IDDM. Transfer of NKT cells to transgenic diabetic mice from non-diabetic mice protects them from developing diabetes (63, 65, 66, 67).

Human patients with multiple sclerosis or systemic sclerosis patients exhibit decreased numbers of NKT cells in their peripheral blood (68, 69). Analysis of the thymocytes from the peripheral blood of systemic sclerosis patients revealed Vα24 T cells with alternate J regions. These cells show a five-fold increase over the number seen in non-afflicted individuals. These data were interpreted to mean that oligoclonal expansion of these cells coupled with a lack of NKT cells is responsible for the damage caused by systemic sclerosis (69).

Neonatal mice that undergo thymectomies in the 3rd day of life lack NKT cells and develop a variety of organ-specific autoimmune disorders (gastritis, thyroiditis and reproductive

organ disorders)(24). These mice also develop lymphopenia suggesting an increased pathogen load and loss of Th1 suppression.

In mouse models of lupus erythematoses, depletion of NKT cells gives rise to DN T cells display heterogeneous TCRs, no NK markers; these cells are restricted to MHC class I, suggesting a loss of immunoregulatory function (70). These DN T cells support the production of the pathogenic anti-DNA antibodies by synergeneic B-cells (69).

Anti-Cancer Responses

NKT cells play a role in tumor destruction in three murine cancer models. The three described models are the IL-12 mediated rejection model, the α-GalCer rejection model and the natural rejection model (71).

In IL-12 mediated tumor rejection, NKT cells are activated and anti-metastatic at low doses of IL-12 (71, 72, 73). NKT cells activate NK cells to lyse cancer cells; the roles of macrophages and CD8+ cytotoxic lymphocytes (CTLs) in this process are not known. Tumor lysis requires IL-12, perforin and IFN-γ. At high doses of IL-12, both NKT cells and NK cells are activated to produce IFN-γ (74), (71). CD1d and Rag deficient mice show reduced anti-cancer responses (75) These observations emphasize the role of NKT cells in IL-12 induced tumor rejection.

In α-GalCer mediated rejection, α-GalCer is presented by APCs to NKT cell resulting in activation of the NKT cell. This activation is enhanced by IL-12 secretion from the APC. In turn, the NKT cell secretes IFN-γ which then activates NK cells and further stimulates APC activity. The role of macrophages and CD8+ CTLs in this model is not clear. Tumor lysis is IL-12 and IFN-γ dependent but perforin independent (76, 77, 71, 78). NKT cells can directly lyse murine tumor cells in vitro (76).

The natural rejection model was discovered using methylcholanthrene (MCA) induced tumors. In this model, endogenous glycolipid antigen (possibly tumor derived) is presented to NKT cells by APCs. This model is similar to the α-GalCer mediated rejection model (IL-12 and

IFN-γ dependent) except perforin is required for tumor lysis. NK cells and CD8+ CTLs are activated by IFN-γ to kill the tumors. In this model, NKT cells are essential for protection compared to other models where NKT cells are protective only when endogenous IL-12 or is α-GalCer administered (78).

NKT Cells and Infection

NKT cells play a role in defense against several types of bacteria (8, 9, 7). NKT cells play a supporting role in clearing mycobacterial infection (7). NKT cells are present in tubercular granulomas, which do not form in NKT cell deficient mice (7, 9). In addition, NKT cells down regulate IL-4 production and up-regulate IFN-γ in response to mycobacterial infection. Despite these observations CD1d-deficient mice were not less susceptible to infection with mycobacteria, suggesting that NKT cells play an accessory role rather than driving the immune response against *Mycobacterium tuberculoses* (53). NKT cells also respond to *Listeria monocytogenes* (8, 9). CD4 NKT cells were down regulated in an IL-12 dependent manner with a concomitant increase in DN NKT cells secreting IFN-γ. In a separate study, Godfrey et al observed an increase in IL-4 secreting NKT cells in the spleen suggesting that DN IFN-γ secreting NKT cells respond at the site of infection while IL-4 secreting NKT cells migrate to the spleen to enhance antibody production (9). In mice, NKT cells play an important role in the clearance of *Pseudomonas aeroginosa* infection from the lungs (79). The inhibition of *Pseudomonas aeroginosa* infection was CD1d dependent, and was enhanced by α-GalCer treatment. In this model, the NKT cells served as sentinel cells, which responded to the infection by IFN-γ secretion, activating avelolar macrophages.

NKT cells have also been implicated in defense against various eukaryotic pathogens (parasites and fungi). In experimental murine malaria models, DN NKT cells responded to infection by IFN-γ secretion (similar to *Listeria*) (9). CD1d presentation of sporocyte altered GPI resulted in the stimulation of NKT cells and a Th2 response to infection (57). This response appeared to be severely inhibited in CD1d deficient mice (57). However, several different

laboratories have not been able to reproduce these data, so the issue is still contentious (58), (80). Treatment with α-GalCer does protect against malaria, implicating NKT cells in the response to this pathogen (81). In mice, DN NKT cell populations rapidly expand and accumulate in the spleen and draining lymph nodes during *Brugia pahangi* infection (within 24 hours). These DN NKT cells are a source of IL-4 against the 3rd stage larvae of *B. pahangi* (82). NKT cells also play a role in protecting mice against the fungal pathogen *Cryptococcus neoformans* (83). During infection with this pathogen, NKT cells are recruited to the lungs and drive the Th1 response against the fungus. NKT cell deficient mice showed a reduced Th1 response against this pathogen. In infection with *Toxoplasma gondii* NKT cells were implicated in generating CD8 effector cells (8, 9).

In defense against viruses, NKT cells have played a role in host defense in 2 mouse models. Treatment with α-GalCer leads to NKT cell activation and amelioration of diabetogenic encephalomyocarditis virus (EMCV-D) infection in mice (55). Both CD1d and Jα281 KO mice were not protected from infection. In the second model, replication of hepatitis B virus was inhibited by a-GalCer activation of NKT cells (84). Inhibition of viral replication was dependent on IFN-γ and IFN-α/β as determined with KO mice. Activated NKT cells secrete IFN-γ, which then activates NK cells to secrete antiviral cytokines.

NKT CELL NK AND T CELL RECEPTORS

NK Receptors Expressed by NKT cells

NKT cells express several families of NK receptors on their surfaces (see Table 4). NK receptors can be grouped into two types, killer activation receptors (KAR) and killer inhibition receptors (KIR). KAR possess an immunoreceptor tyrosine kinase activation motif (ITAM) delivering a signal to kill the targeted cell. When the KARs are activated various tyrosine kinase (PI3, Zap-70 and Syk kinases) are recruited and phosphorylated thus activating signal transduction pathways. This signaling results in the re-direction of the cellular secretion apparatus, stimulating release of granzyme/perforin that kills the target cell. Another effect of the activation of this receptor is the secretion of pro-inflammatory cytokines. KIR receptors recognize MHC Class I molecules. MHC I bind KIR resulting in the activation of SHP-1/2 phosphatases. These phosphatases dephosphorylate essential signal kinases (e.g. ZAP-70 and Fyn) interrupting the activation signaling cascades. This results in secretory killing mechanism being shut off. Consequently, these two receptors regulate NK killing by balancing both positive and negative signaling. Many of these receptors are among the Type II lectin superfamily and are located in a gene cluster in both mice and humans referred to as the NK gene complex (85, 86).

Table 6- NK receptors expressed by NKT cells (Adapted from Lanier et al, 1998 (85))

C-Type Lectins	Immunoglobulin Superfamily
NK1.1 (NKR-P1) Ly49 CD94/NKG NKG2D	CD16 Ly6

The most significant NK marker on NKT cells is NK1.1 in mice and humans (CD161). There are 3 isoforms reported in mice and a single analog in humans. These markers are members of the C-type lectin family and function as KAR receptors. The exception to the rule is the B isoform of NK1.1 that possesses an ITIM region thus serving as a KIR receptor. All of the 3 isoforms express the CxCP motifs that have been

shown in rats to interact with $p56^{lck}$ (85). This tyrosine kinase is also known to play a role in TCR signaling and cytokine secretion.

The Ly49 family is another major family of NK receptors expressed on NKT cells. There are multiple isoforms of this gene in mice coding for Isotypes A-I. Products of these genes display a wide diversity of cytoplasmic and extracellular domains, suggesting a multiplicity of roles in ligand binding and signal transduction. These receptors recognize H-2 class I molecules on possible target cells and inhibit cytotoxic activity upon recognition of self. These receptors possess ITIM motifs that inhibit activation of cytotoxic effector function. The exception is the Ly49H isoform that was recently implicated in the activation of NK cell cytotoxicity (87).

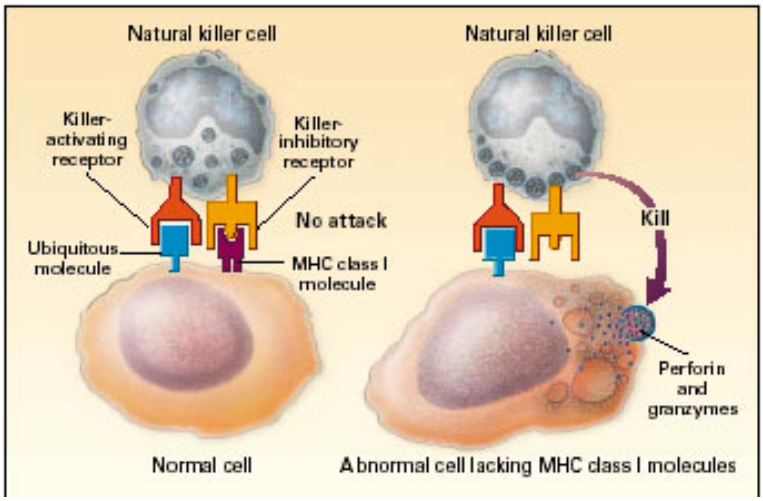
The NKG2 family is similar to the Ly49 family and has been implicated in the recognition of polymorphic HLA I molecules. These receptors, unlike other members of the Type II lectin family, are expressed as heterodimers on cell surfaces (86, 85). The exclusive partner molecule is CD94, encoded by a single gene with limited polymorphism. The role of CD94 is unknown; it has no cytoplasmic domain and thus no role in signal transduction. NKG2 proteins cannot be expressed unless paired with CD94, thus CD94 might serve as a chaperone (85). The NKG2 family is comprised of 5 genes (NKG2A, NKG2B, NKG2C, NKG2D/F and NKG2E) (86). The cytoplasmic domains of these proteins are different, suggesting diverse ligand and signal functions. NKG2D also varies in its cell surface expression and is expressed as a homodimer independent of CD94. It is comprised of 2 β sheets, 2 α helices, 4 disulfide bonds and a β strand which distinguishes it from other C-type lectin receptors (86, 88). Ligands for this receptor in mice are RaeI and H60 and in humans, MICA, MICB and ULBP (89). These ligands are typically induced and/or up regulated in response to cellular distress (86). Viver et al have proposed that NKG2D might serve a role in both NK cell activation and as a co-stimulatory molecule in T cells (86). Activation of NKDG2 in NK cells leads to cytotoxicity, proliferation, survival, cytokine production, chemokine production and tumor lysis. These observations support the hypothesis that NKG2D is a primary receptor for cytotoxicity and a co-stimulatory molecule for cytokine secretion (86, 90, 89).

CD16 (FcγRIII), one of the most extensively studied NK receptors, is a low affinity receptor for IgG. It is a member of the immunoglobulin superfamily and is expressed as a ~70 kDa glycoprotein. The cytoplasmic domain of CD16 has an ITAM, thus serves as a KAR receptor. The signal transduction pathways of CD16 are similar to the activation pathways of the T cell receptor (TCR). CD16 activation results in cytokine secretion, mediation of antibody-dependent cellular cytotoxicity (ADCC) and may signal apoptosis (85).

Three other receptors expressed on the surface of NKT cells have effector functions similar to CD16. These 3 receptors are Ly6, CD69 and CD44 and are reported to cause the lysis of Fc receptor bearing target cells (85).

Like NK cells, NKT cells are capable of lysing targeted cells through both the secretory and non-secretory pathways (92). Killing of targeted cells by the non-secretory pathway is by Fas/FasL interaction. This interaction signals the activation of caspases, resulting in apoptosis. Killing by the secretory pathway involves directed secretion of perforin and granzyme. Perforin polymerases in the target cell membrane creating a transmembrane pore to facilitate the entry of granzyme into the target cell. Granzyme is a serine protease responsible for caspase activation, which triggers apoptosis. It has recently been proposed that granzyme can cross cell membranes via receptor-mediated endocytosis and that perforin might allow escape from the endosome (92). It has also been suggested that granzyme might have non-caspase targets such as Bcl-2. Granzyme s ability to mimic Asp-ase activity of caspases suggests that downstream caspase targets might also be activated by granzyme (92). NKT cells recognize a target cell by the lack of an inhibitory

Figure 7- Secretory NK Killing Mechanisms (Adapted from Devles et al, 2001 (91))



signaling molecule on the surface of the target cell (See Figure 7). The lack of this molecule triggers mechanisms that re-orient the NKT cell s internal secretion machinery towards the target cell (93, 90, 94, 95, 96). Release of perforin and granzyme is thus directed only against the target cell.

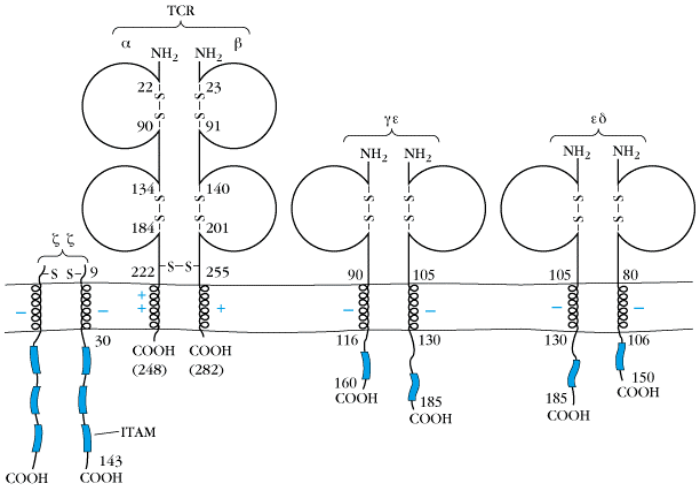
Invariant T Cell Receptor and Co-stimulatory Markers

NKT cells possess an activated T cell phenotype (i.e. $CD69^{+}$, $CD44^{high}$, CD62L, $CD5^{high,}$ $CD45RB^{high,}$ IL-2R), indicative of a cell type that can rapidly respond to environmental cues (8, 12).

NKT cells have a highly conserved T cell receptor (TCR) referred to as an invariant TCR (iTCR). The TCR receptor exists a complex with several other co-receptors, CD3, CD4 or CD8, CD28 and CD154 (CD40L). Activation occurs with the ligation of the iTCR and co-stimulatory signals received from CD28, CD40L or from soluble factors such as IL-12.

The TCR is comprised of a α chain and a β chain each, with variable, joining and constant regions. The variable regions of both chains make up the antigen-binding site. The structure of the TCR is similar to an immunoglobulin molecule (see Figure 8). The iTCR of NKT cells is comprised of specific α and β chains. In mice the α chain is Vα14Jα281 and this chain preferentially pairs with one of three β chains: Vβ8.2, Vβ7 or Vβ2.

Figure 8- The TCR/CD3 Complex (Adapted from Goldsby et al, 2000 (48))

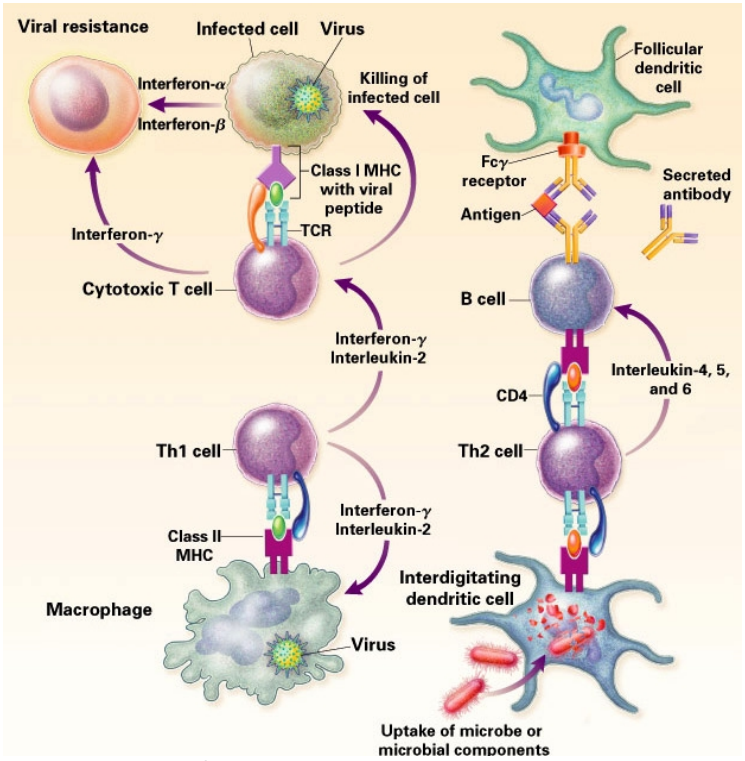


CD3 is closely associated with the TCR (and iTCR) and is essential for TCR signal transduction. CD3 is expressed as 3 dimers: ζζ, γε and a εδ (see Figure 8). Each of these dimers has an immunoreceptor tyrosine kinase signaling motif (ITAM) that interacts with the tyrosine kinases Lck, Fyn and Zap-70 to activate signal transduction cascades leading to cytokine

secretion. CD4 and CD8 are other important co-receptors associated with the TCR. These co-receptors help to stabilize binding between the MHC complexes and the TCR, and amplify signal transduction within the T cell. These receptors are members of the immunoglobulin superfamily and both CD4 and CD8 define the function of conventional T cells.

CD4+ T cells are T helper cells (Th) that can be divided into 2 categories: T helper 1 (Th1) and T helper 2 (Th2) (see Figure 9). Th1 T cells mediate cellular immunity and Th2 cells mediate humoral immunity (see Figure 2). CD4 T cells are restricted to recognizing antigen presented by MHC class II molecules. CD8 T cells are cytotoxic effector T cells (Tc or CTLs) that kill targeted cells by NK cell-like mechanisms. Tc cells are restricted to MHC class I. Other CD4+ cells that regulate tolerance induction are referred to as Th3 (98) and Tr cells (99). These cells differ in surface phenotype from Th1 and Th2 cells and are found in the oral cavity and gut. Recall that NKT cells are either CD4+ or double negative (DN) and they do not express CD8. CD4 on NKT cells serves to amplify signal transduction and stabilizing binding between CD1d and the iTCR. T cells require 2 signals for activation (48). The TCR-CD3 complex binding to the MHC-antigen complex provides one signal. A second co-stimulatory molecule provides a second signal. This second signal prevents uncontrolled T cell activation and thus prevents autoimmunity (100). Two important co-stimulatory molecules expressed by T cells are CD28 and CD154 (CD40L).

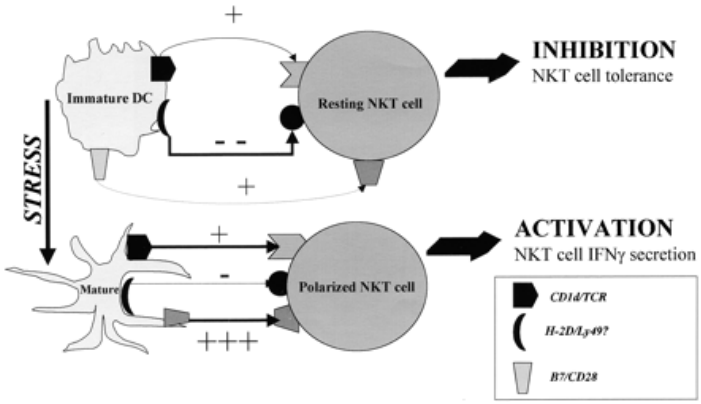
Figure 9- An overview of lymphocyte responses (Adapted from Delves et al, 2001 (97))



CD28/CD154 (CD40L)

CD28 is expressed on all CD4 T cells and most CD8 T cells. CD28, also a member of the immunoglobulin sperfamily, is expressed as a disulphide-linked homodimer. CD28 is involved in T cell proliferation, IL-2 production, prevention of T cell anergy, induction of the anti-apoptotic factor Bcl-x_L and differentiation of Th1/Th2 responses (102, 100, 103, 104, 105). CD28 also plays a role in stabilizing the generation of the immune synapse between T cells and APCs (106) serving a dual role as an adhesion and signaling molecule. The requirement for CD28 co-stimulation can be bypassed by high antigen concentrations or strong agonist peptides (102). Activated CD28 may interact with phophatase 2A (PP2A) and upon activation, the CD28 tyrosine kinase residues are phosphorylated by disassociation with PP2A. CD28 then activates phophatidylinositol 3- kinase (PI3K) induces intracellular signaling leading to cytokine production, proliferation and survival (103). In conventional T cells, activation of CD28 also activates cytotoxic lymphocyte associated molecule-4 (CTLA-4), which serves as an agonist for the CD28 ligand. The mechanism by which CTLA-4 inhibits TCR-CD3 signaling is unknown but it is believed that CTLA-4 interacts with SHP-2 phophatase and may dephosphorylate CD3 (103). CTLA-4 activation results in the inhibition of ERK and JNK kinases and the inhibition of NF-KB, AP-1and NF-AT activation. This CTLA mediated inhibition leads to the cell cycle arrest and the inhibition of cytokine secretion (103). The ligand for CD28/CTLA-4 is either B7.1 or B7.2, which are expressed on APCs. B7-2 is the dominant co-stimulatory ligand for CD28 during the initiation of immune responses. The expression of B7-1/B7-2 on APCs parallels the expression of CD28/CTLA-4 on T cells (104) suggesting that CD28/B7-2 primes the immune response, while CTLA-4/B7-1 is responsible for the termination of the immune response (102).

Figure 10-Proposed DC/NKT cell cross-talk (Adapted from Ikarashi et al, 2002 (101))



NKT cells do not express CTLA-4 (108). Regulation of NKT cell activation is controlled by NK KIR receptors. The exact KIR is not known but candidates include Ly49 and CD94/NKG2A (101). The ligands on the dendritic cells (DCs) that inhibit NKT cells are the H-2D^b murine self-recognition markers (Class I) (101). These self-markers inhibit IFN-γ production by NKT cells, thus inducing tolerance (101). Immature DCs are capable of inhibiting NKT cell activation but when subjected to stress (activation by antigen), express B7, which overcomes the H-2D^b-mediated inhibition. Immature DCs treated with anti-CD28 antibodies activated NKT cells (101). This proposed regulation is shown in Figure 10.

Table 7- Cytokines secreted by NKT cells (Adapted from Janeway et al, 1999 (107))

Cytokines:	Actions
Th1:	
IFN-γ	Macrophage activation, increased MHC expression and antigen processing, Ig class switching, inhibits Th2
GM-CSF	Stimulates growth and differentiation of myleomonocytic lineages
TNF-α	Local inflammation, endothelial activation
TNF-b	Killing, endothelial activation
IL-2	T cell proliferation
IL-3	Synergistic action in early hematopoiesis
Th2:	
Il-4	B cell activation, IgE switch, Th1 suppression
IL-5	Eosinophil growth, differentiation
IL-10	Inhibits Th1, stimulates MHC Class II, inhibits cytokine release
TGF-β	Inhibits growth, anti-inflammatory
Chemokines:	
Eotaxin	Chemoattractant for eosinphils, monocytes and T cells
MIP-1α	Chemoattractant for monocytes, NK and T cells (Th1>Th2), B cells and dendritic cell
MIP-1β	Chemoattractant for monocytes, NK, T cells and dendritic cell

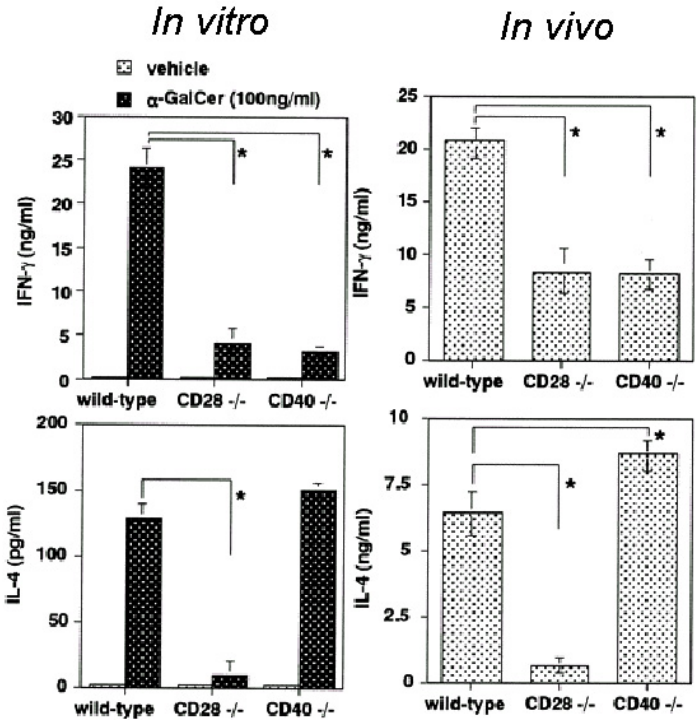
CD154 (CD40L) is a 32-39kDa member of the tumor necrosis factor (TNF) family and is expressed as a heteromultimeric complex (109, 110, 111). CD154 is expressed on activated mature T cells but not on resting T cells. CD154 is also found mainly on CD4 T cells although some CD8 T cells express CD154 (109). CD154 ligand is CD40, found on B cells, APCs and monocytes/macrophages. This signaling system is implicated in T cell priming, expansion and maturation into effector cells. CD40/CD154 plays a role in B cell proliferation, differentiation and immunoglobulin (Ig) production (109) (111). CD40/CD154 also plays a role in T cell tolerance, dendritic cell maturation and the differentiation of Th1/Th2 immune responses (109, 110).

Collectively, these receptors are located in specialized microenviroments referred to as rafts on the T cell surface (112) and all of the above receptors are recruited into the rafts by

CD28. These domains create specialized local conditions in the T cell membrane which optimizes signal transduction (113).

NKT cells secrete a variety of Th1, Th2 and Th3 cytokines (see Table 7). The switch mechanisms that govern the switch between cytokine secretion profiles are poorly understood. As with conventional T cells, NKT cells are activated by a two signal system. Disrupting expression of either CD28 or CD154 (CD40L) profoundly inhibits cytokine secretion by NKT cells. Blocking or genetic knockout of CD28, CD154 (CD40L) or both markedly decreased production of both IFN-γ and IL-4 secretion. These effects were observed both in vitro and in vivo (see Figure 11). Blocking expression or genetic knockout of CD28 and CD154 (CD40L) is marked by inhibition of anti-metastatic effects and impairment of α-GalCer serum responses (108, 76).

Figure 11- The Effects of CD28 and CD40 knockouts on IFN-γ and IL-4 secretion (Adapted from Hayakawa et al, 2001 (108))



THE ROLE OF NKT CELLS IN VIRAL INFECTION

Viral infection results in the activation of all components of the adaptive immune system. Effective T cell and B cell responses are required to clear viral infection. Mice with severe combined immunodeficiency (SCID) infected by a lytic virus (e.g. vaccina virus) rapidly develop fatal infections. Alternatively, when challenged by nonlytic virus (e.g. lymphocytic choriomeningitis virus) SCID mice develop persistent infections. Immunocompetent mice infected under the same conditions (viral dose and route) do not die or fall ill (106).

During the course of a viral infection, locally high levels of antigen can be generated within infected cells. The innate immune system can quickly respond to this threat by lysing the infected cell and processing viral antigens. These viral antigens are presented by APCs to the adaptive immune system resulting in T cell and B cell responses. Because effective T cell and B cell responses are required for viral clearance, NKT cells are in a unique position to aid in the immune response. NKT cells have been implicated in responding to viral challenge in separate viral models. NKT cells have been demonstrated to stop viral replication of Hepatitis B virus (HBV) (84) and activate NK cells to secrete anti-viral cytokines in the liver. NKT cells also protected mice from infection with diabetogenic encephalomycarditis virus (EMCV-D) (55). In both models, the α-GalCer was delivered by intravenous injection and the precise manner in which the NKT cells were activated could not be determined. α-GalCer may have been taken up and processed by an APC and then presented to NKT cells by CD1d. NKT cell iTCR activation requires 2 signals. The invariant TCR/CD3 complex interacts with CD1d on APCs to provide a primary signal. The second signal is dependent

on either soluble factors (e.g. IL-12) or stimulation of other surface expresses receptor such as CD28.

The ligands for CD28 and CTLA-4 are B7.1 (CD80) and B7.2 (CD86) that are expressed on a variety of APCs (100) (104). These two ligands function to play a role in the differentiation of the immune response. B7 co-stimulation modulates Th2 differentiation, transplant rejection, and initiation of autoimmune disease (104) (102) (114) (100). Paradoxically, some Th1 responses are strongly B7 dependent. B7 dependent Th1 responses include responses to vesicular stomatitis virus (VSV), mouse mammary tumor viruses (MMTV), alloantigens, soluble antigens and tumors (100). Based on these reports, CD28 could play a role in activation and polarization of NKT cell responses.

HYPOTHESIS

Ligation of CD28 triggers production of IFN-γ (Th1 polarization) in NKT cells responding to Hepatitis B virus infection.

AIM:

Determine if disruption of CD28 gene expression (i.e., gene knockout) in 1.3.32 HBV mice

1. Impairs IFN-γ secretion by NKT cells following challenge by HBV.
2. Inhibits IFN-γ secretion in a DC/NKT cell co-culture assay system.

Significance

Most current treatments for autoimmunity focus on disease symptoms rather than the abrogation of disease. Understanding the function of the immune system becomes paramount in both treating autoimmune disease and in avoidance of side effects of immunotherapy. NKT cells have been implicated in a variety of autoimmune disorders and manipulation of these cells could result in new therapies for these diseases.

Multi-resistant strains of bacteria, parasites and viruses exist in both clinical and community settings. Discovery and development of anti-infective drugs is difficult and effective drug-base anti-viral therapies are limited. By stimulating NKT cells, such infections could be eradicated without anti-infective pharmacophores.

Anti-cancer chemotherapy, while successful, has severe side effects. Among these side effects are severe immune suppression and inhibition of rapidly dividing non-cancerous cells (e.g.)hematopoietic stem cells), nausea, vomiting, diarrhea and fatigue. NKT cells are implicated in three models of anti-cancer response (71). Cancer treatment using NKT cells would not require anti-cancer drugs.

Another unique possibility for NKT cell based treatment would be manipulation of the CD1 antigen presenting system. As advocated by Bendelac, manipulation the CD1 antigen presenting system maybe be of value in developing vaccines for PAMPs (universal vaccines) and cancer (51). For example: the immune system (i.e. NKT cells) could be primed against LPS (a PAMP) in gram negative pathogens and thus protect the recipient against gram negative bacteria.

EXPERIMENTAL DESIGN

Ligation of CD28 helps to trigger INF-γ (Th1 polarization) in NKT cells during responses to HBV infection. The effects on CD28 knockouts mice would be assessed by *in vitro* co-culture assays measuring IFN-γ production and secretion. The role of CD28 in INF-γ secretion and inhibition of HBV replication would be measured by *in vivo* challenge studies. The experimental design is summarized in Table 8

Table 8- Experimental Design

Assay type:	Assay:	Comments:
In vitro assay	DC/NKT cell co-culture assay	Measures cytokine secretion in WT and CD28 knockout mice. Treatments would be vehicle, α-Gal, α-Man. Cytokine secretion is measured by ELISA.
In vivo assay	HBV transgenic mouse assay	Used for previous NKT cell work measuring IFN-γ secretion. Strains used for assay are WT, CD28, Jα281 and IFN-γ knockout. Inhibition of viral replication would be measured by RPA and southern blot, while IFN-γ secretion would be measured by ELISA.

Methods and Materials

Generation of transgenic mice

The mice used for this study would be derived from the previously described strain 1.3.32 (115), (84). This strain of mouse is derived from B6 mouse line and has a HBV transgene inserted into its genome. Hepatocytes of this mouse replicate the HBV virus at levels comparable to chronic hepatitis patients, but without any evidence of cytopathology. Knockout (KO) strains generated for this study would be CD28 KO (116), CD1d (116) and IFN-γ KO (84) strains. Each of these strains would be produced by insertion of neomycin resistance cassettes as described (118). Briefly, ES cells are harvested from the inner-cell mass of blastocysts in superovulating females and are genetically modified prior to re-insertion into the blastocysts to

produce chimeric animals containing the altered gene(s). First, the ES cells are cultured and the targeting vector is inserted by electroporation. All the knockout mice would have their respective genes disrupted by either insertion or replaced by a neomycin resistance cassette. The ES cells are then cultured in the presence of neomycin (the selection agent) so only the ES cells that have incorporated the neomycin resistance cassette survive. These clones are then re-introduced into blastocysts and re-implanted into pseudopregenant female mice. Coat color markers and Southern blot analysis is used to confirm that the chimeric mice contain the HBV transgene and to confirm the knockout of the different genes. Additional confirmation of the HBV transgene is determined by detection of hepatitis B e antigen (commercial kit from Abbott Laboratories, Chicago, IL). Mating of the chimeric offspring produces heterozygous mice and crossing these mice results in mice homozygous for the knockouts.

The IFN-γ HBV transgenic mice are viable (84) but it is unknown whether the CD28 KO HBV transgenic mice or the CD1d KO HBV transgenic mice would be viable. The combinations of both knockouts could result in a lethal phenotype that would not develop or eventually die. If these mice strains are not viable, the KO strains for CD28, CD1d and IFN-γ could all be purchased from the Jackson Laboratory (Bar Harbor, MA) with the wild type B6 parent and used in HBV infection studies.

Isolation of NKT cells

Liver NKT cells would be isolated as previously described (84) (117). Briefly, the liver would be pressed through a steel mesh, then suspended and washed in RPMI media. Mononuclear cells would be isolated by Ficoll-Isopaque density gradient centrifugation. Mononuclear cells would be collected from the interface, washed and analyzed by flow cytometry to isolate the NKT cells.

The NKT cells would be isolated using modification of the method of Bendelac et al (15) that isolated ~100% of human NKT cell population from peripheral blood. For the isolation of murine NKT cells, the three monoclonals used would be directed against NK1.1, α-GalCer:CD1d tetramers and finally Vα14. The rationale for the choice of monoclonal antibodies is as follows: NK1.1 is the main NK receptor expressed on NKT cells. α-GalCer:CD1d tetramers are specific

for CD1d restricted TCR (15) and Vα14 is only found on the TCR of NKT cells Once the NKT cells are isolated, they will be separated electromagnetically into CD4+ and DN populations of NKT cells.

Generation of dendritic cells

Dendritic cells (DCs) would be derived as described by Ikarashi et al (101). Bone marrow derived DCs would be propagated from BM progenitor cells in culture medium containing rmGS-CSF and rmIL-4 (R&D Systems). At 6 days of culture, DCs would be induced to mature by stimulation with anti-CD16 and anti-CD32 antibodies. CD16 mediates phagocytosis and antibody-dependent cell-mediated cytotoxicity. CD32 is a low affinity Fc receptor for aggregated immunoglobulin and immune complexes. Treatment with these anti-antibodies would simulate an infection causing the maturation of the immature DCs in the cell population. The DCs should up-regulate the expression of their surface receptors especially those associated with antigen presentation and immune cell activation (e.g. MHC I & II, CD1d, CD40, CD80 and CD86). These mature DCs would be isolated by flow cytometry gated for CD40, MHC Class II, CD1d, and CD80 or CD86 expression as described (101).

α-Galactosylceramide

α-GalCer would be prepared as described (84). α-GalCer in DMSO (10 ug/mL) would be diluted in 1xPhophate buffered saline to 2 ug/mL.

IFN-γ ELISA assay

IFN-γ would be quantified by using a commercially available ELISA kit from BD PharMingen (OptEIA Mouse IFN-γ Kit) (119).

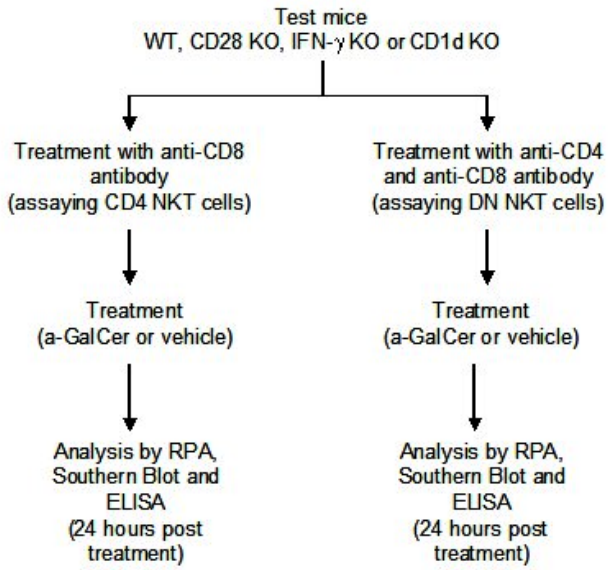
Ribonuclease protection assay/southern blot assay

Messenger RNA from NKT cells would be isolated by guanidinium isothiocyanate (GITC): phenol:chloroform extraction. This method lyses the cells, precipitates the cellular debris and DNA. The RNA would be isolated by ethanol precipitation resulting in a highly purified pool of cellular RNA. Using this pool of RNA, both RNA protection assays (RPA) and southern blot assays will be performed. RNase Protection assays are performed by hybridizing the sample RNA with digoxygenin labeled probes, then RNAase digestion (120). The protected samples would be resolved by PAGE electrophoresis and photographed on an image analysis system. A second RNA sample would be used to construct a cDNA library by RT-PCR. The DNA library would be digested with restriction enzymes (5 units/ mg DNA, 8 hours) and concentrated with an ethanol precipitation. Next the DNA would be separated on a 0.7% agarose 0.5x TBE gel at low voltage. The DNA would next be transferred onto a positively charged nylon hybridization membrane by downward capillary transfer (24 hours). Hybridization would be performed in a hybridization bottle. After pre-wetting the membrane with hybridization buffer for 2 minutes, fresh hybridization buffer with the radiolabeled probe as described (115). The membrane would then be wash once in 2x SSC buffer for 15 minutes and then in 0.1x SSC buffer plus 0.1% SDS for 4 hours. At the end of the incubation the membrane would be washed in 0.1x SSC buffer, air-dryed and glued 3MM paper. The membrane would be imaged using audioradiography and the viral DNA quantified. The probes used for both assays are described on the Mouse Genome Database (MGD) website or in Guidotti et al (115).

DC/NKT cell co-culture assay

Hepatic NKT cells would be cultured with mature DCs (described above) follow the previously described method (101). Prior to NKT cell addition, the DCs would be pulsed with 10-ng/mL α-GalCer or 10 ng/mL α-mannosylceramide (α-ManCer) to activate the DCs. Measurement of IFN-γ secretion would be by ELISA as described above. ELISA quantification of IFN-γ would confirm that α-GalCer is activating the NKT cells. Two separate assays would be performed, one with CD4 NKT cells and one with DN NKT cells

Figure 12-HBV Challenge Assay Flow Chart



Murine HBV challenge assay

The in vivo HBV challenge assay would be set up as described by Kakimi et al (84). The assay would use the wild type (WT) mice (positive control), IFN-γ KO mice (negative control), CD1d KO mice (-NKT cell control) and the CD28 KO mice (test strain). The assay would be performed as described (84). Briefly, the mice would be maintained in pathogen-free rooms with strict barrier conditions. Conventional T cell depletion would be accomplished by injection of anti-mouse CD4 mAb and anti-mouse CD8 mAb 24 hours prior to α-GalCer stimulation. This assay would determine the effects of CD28 KO on DN NKT cells. To determine the effect of CD4 NKT cells, the anti-CD4 monoclonal antibody would not be injected into a set of the α-GalCer treated CD28 KO mice, CD1d KO mice, IFN-γ KO mice and WT control mice. The mice would then be dosed with 2 ug IV of a-GalCer and sacrificed at 1 day or PBS vehicle control. The livers would be extracted and frozen (-80°C) for later RPA and southern blot analysis. The serum levels of IFN-γ would also be determined by ELISA assay (above). The flow chart for the procedure is shown in Figure 12.

Test Design and Predicted Results

In vitro IFN-γ secretion by co-culture of DC and NKT cells

The co-culture experiments would be performed as described in the methods section. Mature dendritic cells would be pulsed with α-GalCer and then cultured with NKT cells.

NKT cell Type:	Treatment:	IFN-γ secretion:
Wild Type	Vehicle	---
	α-GalCer	+++
	α-ManCer	---
CD28 Knockout	Vehicle	---
	α-GalCer	---
	α-ManCer	---

DC/NKT cell co-culture assays

These experiments would measure the ability of stimulated NKT cells to secrete IFN-γ in vitro. Both CD4 and DN NKT cells would be assayed and the IFN-γ levels secreted by each would be compared. The assays could be executed two ways. The first would involve extraction of NKT cells from WT mice, blocking CD28 with blocking anti-CD28 mAbs (108), and executing the co-culture assay. The second option is to extract NKT cells from both the WT and the CD28 KO mice and then performs the co-culture experiments. Data between the parent and the mutant could be compared. The test treatments would be as follows: vehicle (1xPBS), α-GalCer, α-ManCer (negative control). These treatments would be administered to both WT and CD28 blocked/KO NKT cells. The vehicle treated and α-ManCer NKT cells should have little to no IFN-γ secretion while the WT NKT cells should secrete high levels of IFN-γ. The results of the *in vitro* work are summarized in Table 9. Recent work by Benlagha et al (121) suggests that murine NKT cells might undergo a Th2 (CD4/DN NKT cells) to Th1 (CD4 mainly) conversion as they migrate to the periphery. If this were the case the CD4 NKT cells would secrete higher levels of IFN-γ than DN NKT cells (121).

In vivo inhibition of HBV replication

The impact of CD28 knockout on IFN-γ and Th1 polarization by NKT cells would be assessed using HBV transgenic mice. The experiment would consist of a single intravenous dose of α-GalCer to activate NKT cells and determining the effects of the mutations on IFN-γ secretion. The assay would use wild type (WT) mice (positive control), IFN-γ KO mice (negative control), CD1d KO (NKT cell negative control) and the CD28 KO mice (test strain). Anti-CD4 and anti-CD8 monoclonal antibodies would be added to deplete conventional T cells in the mice. By treating the mice with these antibodies only the DN NKT cells could be assayed.

To determine the affect of CD28 KO on CD4 NKT cells, the anti-CD4 monoclonal antibody would not be added to a second set of mice. If anti-CD4 monoclonal antibody is added to the test treatment the CD4 NKT cells would be depleted with the conventional CD4 T cells.

The second experiment would be set up the same as the DN experiment with one difference. In order determine the level of IFN-γ secretion by CD4 NKT cells, a baseline IFN-γ measurement against CD1d KO mice would be performed. This base line measurement would determine the amount of IFN-γ secretion by both conventional T cells.

In addition, there would be a WT vehicle control mouse (dosed with 1x PBS) in both experiments. One day after activation of NKT cells by α-GalCer the mice would be sacrificed, levels of viral replication and IFN-γ transcription assessed by RPA and Southern Blot analysis. ELISA would be used to determine the serum levels of IFN-γ.

Based on previous data (84) generated with DN NKT cells, the WT vehicle control mice should show low to no levels of IFN-γ as detected by ELISA, RPA or Southern Blot. There should also be high levels of viral replication as measured by RPA and Southern Blot. The WT mice + α-GalCer should show inhibition of viral replication and have elevated levels of IFN-γ. There should be elevated levels of IFN-γ transcription as measured by RPA and Southern blot. The CD28 mice should be similar to the vehicle control mice, with no to low levels of IFN-γ compared to WT treated mice, as well as no to low levels of IFN-γ transcription. Finally, IFN-γ KO mice should be similar to the vehicle control, displaying uninhibited viral replication, undetectable levels of IFN-γ secretion and replication.

The results of the CD4 NKT cells would have to be determined by subtracting the baseline IFN-γ secretion of conventional T cells from the WT positive control. The results should mirror the DN NKT cells. If murine NKT cells do undergo a Th2 (CD4/DN NKT cells) to Th1 (CD4 mainly) conversion, CD4 NKT cells would secrete higher levels of IFN-γ than DN NKT cells (121). The predicted results of the *in vivo* work are summarized in Table 10.

Table 10 — Predicted *in vivo* results of HBV Transgenic mouse studies

Test treatments		IFN-γ		Viral Replication inhibition	
Treatment	Blocking mAb	ELISA	RPA	RPA	Southern
WT + vehicle	CD4 & CD8	---	---	---	---
	CD4	---	---	---	---
WT + α-GalCer	CD4 & CD8	+++	+++	+++	+++
	CD4	+++	+++	+++	+++
CD28 KO + α-GalCer	CD4 & CD8	---	---	---	---
	CD4	---	---	---	---
IFN-γ KO + α-GalCer	CD4 & CD8	---	---	---	---
	CD4	---	---	---	---
CD1d KO + α-GalCer	CD4 & CD8	---	---	---	---
	CD4	+	+	+	+

The IFN-γ KO HBV transgenic mice have been previously described (84). The CD28 and CD1d KO HBV transgenic mice have not been reported in the literature. The effects of both CD28 and CD1d knockouts in this HBV background are unknown. It is possible that these knockouts could be lethal to these mice. If these mouse strains are not viable, the hypothesis will have to be tested in non-HBV transgenic mice KO mice (see Methods and Materials). The experiment could be run as described except that the mice would be inoculated with a lethal dose of HBV and then stimulated with α-GalCer. The results should be the same as described for the HBV transgenic mice.

CONCLUSIONS

CD28 knockout should impair IFN-γ secretion and thus block the NKT cell mediated response to HBV infection. The loss of α-GalCer induced antimetastatic effect in CD28 KO mice is paralleled by a loss in IFN-γ secretion (108). IFN-γ plays a crucial role in the clearance of HBV infection (84). The NKT cell IFN-γ secretion (Th1 polarization) is responsible for inhibiting viral replication and for activating NK cells to aid in viral clearance.

Previous work with HBV transgenic mice has illustrated the importance of NKT cell IFN-γ secretion and Th1 polarization in response to HBV infection (84). The inhibition of HBV replication was demonstrated to be dose and time dependent (84). The described system provides a unique opportunity to determine the role of co-receptors in IFN-γ and Th1 polarization of NKT cells in response to viral infection. Work by Hayakawa et al (108) has illustrated the central role of CD28 in IFN-γ secretion and thus Th1 polarization. These previous studies demonstrated that inhibiting INF-γ secretion abolishes the anti-metastatic responses of NKT cells. There are currently no reports in the literature studying infection in NKT cell KO mice. CD28 stimulation results in transcriptional regulation of the IFN-γ gene. This has been demonstrated in conventional T cells (108). The effects of CD154 (CD40L) and IL-12 blocking on IFN-γ production also emphasize the role of CD28. CD28 co-stimulation stabilizes expression of CD154 (CD40L) on conventional T cells thus facilitating CD40-CD154 (CD40L) interactions. In turn, CD40-mediated activation up-regulates CD80/CD86 (B7.1/B7.2) expression on DCs and enhances CD28-CD80/86 interactions (108). The concept that CD28 might serve to stabilize and create a microenvironment favorable for signaling has been advanced by Bromely et al (122).

Study of co-stimulatory receptors in NKT cells could lead to technologies and therapies that could selectively stimulate NKT cell cytokine secretion. Such technologies would have broad applications in immune regulation, infection treatment and cancer treatment.

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