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THE DIFFERENTIAL REQUIREMENT FOR VITAMIN D AND VITAMIN D RECEPTOR IN iNKT CELL DEVELOPMENT

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ABSTRACT

CD1d-reactive natural killer T (NKT) cells with an invariant T cell receptor Vα14 rearrangement are a unique subset of lymphocytes, which play important roles in immune regulation, tumor surveillance and host defense against pathogens. Vitamin D is a nutrient/hormone that has been shown to regulate conventional T cell responses, but it remains unclear whether it regulates iNKT cell development and function. Here we determined the effect of both the vitamin D receptor (VDR) and the vitamin D ligand ($1,25(OH)_2D_3$) on iNKT cell development. The data show that expression of the vitamin D receptor (VDR) as well as vitamin D is required for normal development while only the VDR is required for normal function of iNKT cells. Moreover, the receptor and ligand regulate iNKT cell development by different mechanisms. The iNKT cells from VDR KO mice are intrinsically defective and lack T-bet expression. VDR KO iNKT cells fail to express NK1.1 although they express normal levels of CD122. Extrinsic factors that impact iNKT cell development and function in the VDR KO mice included a failure of the liver to support homeostatic proliferation and reduced thymic expression of CD1d and other factors important for optimal antigen presentation in the thymus. In addition, VDR KO iNKT cells were intrinsically defective even when wild type antigen presenting cells were used to stimulate them. Similar to what was shown in VDR KO mice, fewer iNKT cells were identified in the $1,25(OH)_2D_3$-deficient mice. However, $1,25(OH)_2D_3$-deficiency did not affect the function of the remaining iNKT cells. In addition, $1,25(OH)_2D_3$-deficiency did not affect the development of iNKT cells in the thymus, while VDR KO iNKT cells were blocked at the $CD44^+/NK1.1^-$ stage of development.

The 1alpha-hydroxylase (encoded by Cyp27B1) is an enzyme required for production of $1,25(OH)_2D_3$. Cyp27B1 KO mice are $1,25(OH)_2D_3$ deficient because they cannot convert vitamin D to the active form. Cyp27B1 ko/+ breeders were fed vitamin D deficient diets. At three weeks of age WT and Cyp27B1 KO littermates were weaned and fed diets that are vitamin D-deficient for the next 5 weeks. Analysis of iNKT cells from these mice showed that both the WT and Cyp27B1 KO littermates had very few iNKT cells. In fact the percentage of iNKT cells in the liver of vitamin D deficient mice was very low 1.2-1.8% and lower than in the VDR KO mice (6%). Early exposure to vitamin D in utero is required for iNKT cell development and iNKT cells failed to recover in either WT or Cyp27B1 KO littermates that were $1,25(OH)_2D_3$ fed from the age of 3-8 wks. Taken together, the data indicate that both the VDR and vitamin D are required for iNKT cell development. Furthermore, there are differences in the role of the VDR and the vitamin D ligand in iNKT cell development. In addition, the data suggest a critical role for vitamin D early during neonatal development or the first 3 weeks post-birth in inducing iNKT cells.

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ABBREVATION

NKT	natural killer T cells
VDR	vitamin D receptor
TCR	T cell receptor
DP	double positive
Th	T helper
DN	double negative
IL	interleukin
IFN	interferon
NOD	nonobese diabetic
MRL(lpr)	murine lupus model
AHR	airway hyperresponsiveness
DCs	dendritic cells
TNF	tumor necrosis factor
GM-CSF	granulocyte/macrophage colony-stimulating factor
HBV	hepatitis B virus
KO	knockout
APC	antigen-presenting cells
VDRE	vitamin D response element
iNOS	inducible nitric oxide synthase
Rag	recombination activating gene
RIPA	Radio-Immunoprecipitation Assay

HRP	horseradish peroxidase

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Chapter 1

Introduction

Natural killer T (NKT) cells are a particular subset of T cells that express both T cell receptor (TCR) and NK cell lineage markers such as NK1.1. The majority of NKT cells express semi-invariant TCR, Vα14-Jα18. Those NKT cells are called iNKT cells. iNKT cells diverge from conventional T cells during TCR engagement by CD1d expressing double positive (DP) thymocytes. They play important regulatory roles in a variety of immune responses including tumor surveillance and host defense against pathogens. Vitamin D is a fat soluble nutrient, originally characterized as a key regulator for maintaining bone and calcium homeostasis. Recently it has been shown to play roles in immune responses. It has been shown that vitamin D regulates T cell function enhancing T helper (Th) 2 cell development while interfering with Th1 cell development. Vitamin D functions through binding to vitamin D receptor (VDR). However, it is not known whether vitamin D and the VDR play a role in iNKT cell development and function. In addition, it is not clear the mechanisms by which vitamin D and the VDR regulates iNKT cell development. A better understanding of the mechanisms by which NKT cells are controlled is essential to understanding how these cells function and might be controlled in both health and disease.

The VDR and 1,25$(OH)_2D_3$ in immune response

1.1 The development and function of iNKT cells

1.1.1 Definition of iNKT cells

Originally NKT cells were defined as lymphocytes that express both a TCR and NK1.1 (1). However, only three common mouse strains (B6, NZB, and SJL) express a form of NK1.1. In addition other T cells including virus-specific CD4 and CD8 T cells express NK1.1 (2). So this definition is not satisfactory. More recently, NKT cells have been defined as cells that usually have an invariant Vα14-Jα18 TCR and are reactive to α-galactosylceramide (αGalCer) presented by CD1d (3). They are called invariant (i) NKT cells or type I NKT cells. The former term is used here. The semi-invariant TCR expressed on iNKT cells always combines with Vβ8.2, Vβ7 or Vβ2 in mice. In humans, iNKT cells express an invariant Vα24 rearrangement and react to αGalCer presented by human CD1d (4, 5). Type II NKT cells are also CD1d dependent, but they express more diverse TCR Vα chains and there is no specific reagents to directly identify them.

In mouse, iNKT cells account for ~0.5% of the T cell population in the blood and peripheral lymph nodes, ~5% T cells in the spleen, mesenteric, and pancreatic lymph nodes, and about 50% T cells in the liver. In the thymus iNKT frequency is about 0.5%, but they represent 5% or so of emigrants found in the spleen (6, 7). The expression of both CXCR6 and CXCL16 is important for survival of iNKT cells in liver (8).

In mice iNKT cells are classified into 2 subsets: CD4 and CD8 double negative (DN) or $CD4^+$ while in human: they are either $CD4^+$, DN or $CD8^+$. Mature $CD4^+$ and $CD4^-$ NKT cells function differently. In human, a T-helper (T_H0)-type cytokine profile (interleukin (IL)-4 and interferon (IFN)-γ) is produced by $CD4^+$ NKT cells while T_H1-type cytokine (IFN-γ) production bias by $CD4^-$NKT cells (9, 10). Mouse $CD4^-$NKT cells from liver are more potent at protecting against tumors than $CD4^+$ NKT cells (11). The developmental relationship between $CD4^+$ and $CD4^-$ iNKT cells remains elusive. It has been shown that $CD4^-$ iNKT cells develop later than $CD4^+$ iNKT cells (6, 12, 13), and they are found with an immature and mature phenotype (12, 14). This suggests that $CD4^-$ iNKT cells are derived from $CD4^+$ iNKT cells but it is not clear about the precise timing of this branch point.

1.1.2 iNKT cell function

NKT cells play a pivotal role in various immune response (15). So far, iNKT cell cells have been shown to be both benefical and harmful depending on different diseases. Beneficial functions of iNKT cells include protection against infectious dieases, inhibition of tumor growth, and protection against autoimmune diseases. The harmful side of iNKT cells are that they are pathogenic in asthma and atherosclerosis (16) (Figure **1-1**).

iNKT cells have cytotoxic function which is dependent on TCR recognition of cognate antigen. However, the hallmark of iNKT cells is rapid and copious cytokine production upon activation. The most thoroughly demonstrated cytokines produced by

iNKT cells are IL-4 and IFN-γ. In addition, iNKT cells produce many other cytokines including IL-2, tumor necrosis factor (TNF), IL-5, IL-13 and granulocyte/macrophage colony-stimulating factor (GM-CSF) (17).

iNKT cells are important in bacterial, parasite and virus infection. Granuloma formation hinders the dispersal of tuberculosis bacilli during tuberculosis infection. In a granuloma model using deproteinated bacterial cell wall, iNKT cells were identified as the major cell type in early granulomas and iNKT cell-deficient mice failed to form granulomas, indicating their primary role in the formation of granulomas (18). The example of anti-parasite activity might be its anti-malaria activity. iNKT cells not only directly inhibit the development of intrahepatic stage of malaria but also enhance memory response elicited by malaria vaccines, including irridated sporozoites and a recombinant circumsporotzoite protein (19). iNKT cell-deficient, CD1d-deficient, and IFN-γ-deficient mice did not show αGalCer-mediated protection against malaria infection (20). iNKT cells are protective for virus infection as well. It is well known that IFN-γ and IFN-α/β prevent hepatitis B (HBV) replication (21). Activated iNKT cells produced IFN-γ and IFNα/β and prevented HBV replication when HBV transgenic mice were injected with αGalCer (22). Most of the antiviral activity of αGalCer is mediated by cytokines produced by iNKT cells since antiviral activity of αGalCer disappeared in mice deficient for receptors of either IFN-γ or IFNα/β (22). Recent studies suggest that viral evasion might relate to counter measures against CD1d or iNKT cells. Kaposi sarcoma-associated herpes virus encodes two modulators of immune recognition, MIR1 and MIR2, which

down-regulated CD1d expression (23). Therefore, iNKT cells might be good candidates for the control of infectious diseases.

The antitumor activity of iNKT cells has been well documented. iNKT cells were decreased or functionally hyporeactive in mice and humans with cancer (24, 25). In a tumor transplant model, tumors were induced by subcutaneous injection of a fibrosarcoma tumor line originated from methylcholanthrene-inoculated Jα18-deficient mice. Jα18-deficient mice showed faster tumor growth than WT mice. And tumors were prevented by transfer of purified iNTK cells into Jα18-deficient host (26). Anti-tumor activity required CD1d expression and the presence of $CD8^+$ T cells in the host (26). In another study, it was shown that the growth of the CD1d-transfected tumor cell line cells was inhibited by iNKT cells (27). All these data suggest that iNKT cells play protective roles in tumor immune responses.

Role of iNKT cells in autoimmue disease can be exemplified by its role in type I diabetes and lupus. Nonobese diabetic (NOD) mice develop spontaneous autoimmune type I diabetes caused by the destruction of pancreatic islet cells mediated by a Th1 immune responses. Bach and his colleagues showed that NOD mice had greatly reduced numbers of iNKT cells and the remaining iNKT cells could not make IL-4 (28). Diabetes development in NOD mice can be corrected by transfer of iNKT cells from nondiabetic (BALB/c×NOD) F1 mice (29, 30). Patients with type I diabetes had fewer iNKT cells than nondiabetic persons. Even diabetic siblings of identical twin sets had fewer iNKT cells than their nondiabetic twins (31). Similar to what was seen in NOD mice, in murine lupus model (MRL/lpr mouse) iNKT cell numbers started to decrease 3-4 weeks of age before the onset of disease and completely disappeared at around 10 weeks of age.

Transfer of iNKT cells into MRL/lpr mice delayed onset of disease development (32). These data suggest a strong connection beween the reduction of iNKT cells and autoimmue diseases.

The harmful effect of iNKT cells on immune response comes from data that have suggested that iNKT cells are required for the development of allergen-induced airway hyperresponsiveness (AHR, a murine model for asthma) and atherosclerosis. CD1d- and Jα18-deficient mice were reported to show decreased AHR compared to WT mice in the alum ovalbumin model of AHR (33, 34). In patients with persistent asthma, around 60 percent of the pulmonary $CD4^+$ T cells were found to be iNKT cells (35). NKT cells recognize lipid antigen presented by CD1d. In addition, iNKT cells were reported to be important in the development of atherosclerosis as well. NKT cell deficient CD1d knockout (KO) mice have reduced development of atherosclerosis (36-39). The activation of iNKT cells by exogenous administration of αGalCer brought about a 50-100% increase in atherosclerotic lesions in apoE KO mice (36-38).

Three distinct pathways that elicit iNKT cell effector function are responsible for regulatory function of iNKT cell in immune response. In the first pathway, iNKT cells recognize directly the CD1d-restricted lipids derived from lipopolysaccharide-negative bacteria. The presentation of microbial lipids by CD1d induces NKT cells to up-regulate CD40L, Th1 and Th2 cytokines and chemokines. The cross-linking of TCR and antigen-CD1d complex also enhances expression of CD40, B7.1 and B7.2 on dendritic cells (DCs) and production of IL-12 by DCs. The increased production of IL-12 in turn promotes NKT cells activation and cytokine production. The up-regulation of DC co-stimulatory

properties result in optimal activation of T cell responses and cytokines stimulate cellular immune effectors in a bystander way (40, 41).

In the second pathway, microbial Toll-like receptor ligands including lipopolysaccharide and single-stranded RNA activate the secretion of inflammatory cytokine by antigen-presenting cells (APC)s and those cytokines in turn induce the production of IFN-γ by iNKT cells independent of CD1d recognition (42). In the third pathway, iNKT cells recognize unknown endogenous antigens presented by CD1d. For example, in human, iNKT cells cross-link $CD1d^+$ monocytes that produce GM-CSF and IL-13. The production of GM-CSF and IL-13 in turn promote the differentiation of monocytes into immature DCs (42).

Activation of iNKT cells has beneficial or deleterious effects on a variety of immune responses depending on types of distinct diseases. Favorable effects of iNKT cells are exemplified by their roles in anti-infection, anti-tumor and autoimmune diseases surveillance. However, iNKT cells have been shown to be important for the development of AHR and atherosclerosis, suggesting that iNKT cells are pathogenic in AHR and atherosclerosis. Regulatory roles of iNKT cells in different immune responses can be ascribed to distinct pathways that elicite iNKT cell activation.

1.1.3 iNKT cell development

iNKT cells first appear in the thymus at day 5 after birth and do not reach significant levels until at least 3 weeks of age (12). They arise from DP thymocytes and are positively selected by CD1d-expressing DP thymocytes (13, 43, 44). The first

detectable iNKT cells are $CD24^{high}$ and $CD4^{intermediate}CD8^{intermediate}$ and then become $CD4^{high}CD8^{neg}$ (Figure **1-2**). These developmental intermediates appear right after positive selection since they are absent in the CD1d-deficient thymus, and present at very low frequency in regular thymus (14). The pre-selection DP cells cannot be detected by tetramer in wild type mice because of the rarity of random Vα14-Jα18 rearrangement and low TCR levels at this stage. Interestingly, iNKT cell development was interrupted in retinoic acid (RA)-related orphan receptor (ROR)γt knockout (KO) mice. RORγt is a transcription factor induced in DP thymocytes and is essential for prolonged survival before the Vα to Jα rearrangement (13, 45).

As iNTK cells progress to the mature $CD24^{low}$ stage, they go through three more stages: first a $CD44^{low}NK1.1^{neg}$ stage (naïve), then a $CD44^{high}NK1.1^{neg}$ stage (memory), and finally a $CD44^{high}NK1.1^{pos}$ stage (NK) (6, 12). They experience massive expansion after the naïve stage (first stage) and acquire a memory phenotype (46). This massive expansion is very important for the innate role of NKT cells, which require high numbers and memory properties for prompt and effective response. In addition, during these stages, DN iNKT cells arise by down-regulation of CD4 expression in ~30-50% of the cells, as shown in cell transfer experiments (14). It is not clear what factors determine DN iNKT lineage development since DN cells are shown to share the same TCR repertoire as the $CD4^{+}$ subset. A majority of the $CD44^{high}NK1.1^{neg}$ cells emigrate to peripheral tissues where they stop proliferating and up-regulate expression of NK1.1. The $NK1.1^{-}$ to $NK1.1^{+}$ stage of NKT cell transition is also accompanied by the up-regulation of expression of other NK lineage markers, including NKG2D, CD94/NKG2A, Ly49C, C/I and G2 (44, 47, 48). Interestingly, some of the $CD44^{high}NK1.1^{neg}$ cells do not emigrate

and proceed to terminal maturation in the thymus instead. These mature iNKT cells become long-lived resident cells of the thymus.

These developmental stages relate to precisely defined functional changes. The $CD44^{low}NK1.1^{neg}$ cells produce IL-4 exclusively upon TCR stimulation in vitro, while the $CD44^{high}NK1.1^{neg}$ cells are producers of both IL-4 and IFN-γ and the $CD44^{high}NK1.1^{pos}$ produce more IFN-γ (6, 12).

Positive selection of iNKT cells requires their ligation to glycolipid antigens presented by CD1d expressed on DP thymocytes. This process is different from positive selection of conventional T cells, as conventional T cell selection requires TCR recognition of peptide antigens presented by thymic cortical epithelial cells in the context of MHC class I or II molecules. This raises the question of why thymic epithelial cells can not select NKT cells although they also express CD1d (1). One possible explanation is that DP thymocytes may promote some of the unique characteristic associated with NKT cells. This is reflected by the results that when $CD4^+$ T cells are selected by interaction with MHC II on DP thymocytes, they adopt some characteristic reminiscent of NKT cells (49). The presence of CD1d alone is not sufficient for NKT cell selection. CD1d needs to be able to recycle through the intracellular endosomal and lysosomal pathway (16). So far, several molecules have been shown to be critical for antigen presentation by CD1d molecules including cathepsin L, adaptor protein complex 3 and saposins (50-52). Recently it has been shown that some viruses escape immune surveillance in part by reducing surface CD1d expression and recycling (53).

Thymic selection of NKT cells is also affected by the TCR Vβ domain. The TCR Vβ domain influences thymic NKT cell selection by CD1d complexed with endogenous ligand (s). As mentioned above, mouse NKT cells normally co-express Vβ8, Vβ7 and Vβ2 with Vβ8 accounting for more than 50%. When endogenous ligand concentration was limited and TCRα-chain avidity was reduced in vivo, the thymic selection of Vβ7 NKT cells was favored over Vβ8.2 NKT cells. In addition, Vβ8.2 NKT cells tend to have the highest affinity for CD1d-αGalCer and are underrepresented in the CD1d transgenic mice (54, 55). Therefore, it is suggested that Vβ usage may play a role in the negative selection of NKT cells.

1.1.4 Factors that regulate NKT cell development

NKT cells are developmentally and functionally distinct from conventional T cells, which suggest that NKT cells and conventional T cells are regulated by different signaling pathways. Consistent with this, the lack of expression of particular molecules differentially influences NKT and conventional T cell development. The most well-studied factors will be discussed (Figure **1-3**).

The importance of CD1d in iNKT cells has been well documented. CD1d is a nonclassical MHC class I-like molecule that associates with β2-microglobulin (56). As mentioned above, interaction with CD1d is essential for NKT cell positive selection. In addition, CD1d is required for NKT cell maturation. When transferred into CD1d-

deficient mice, most of $NK1.1^-$ NKT cells did not mature both within and outside thymus, remaining NK1.1 negative (7).

It has been shown that IL-15 is critical for the $NK1.1^-$ to $NK1.1^+$ stage of NKT cell maturation (57, 58). IL-15 also seems crucial for normal NKT cell turnover and homeostasis (59, 60). NKT cell deficiency observed in some other mutant mice may be ascribed to a lack of IL-15 induced signaling, including T-bet KO mice. In T-bet KO mice, NKT cells were stalled at $NK1.1^-$ stage and had reduced level of the IL-15R, CD122 (61).

The effect of GM-CSF on the development of NKT cells has been controversial. However, GM-CSF is critical for normal cytokine secretion by NKT cells. In the absence of GM-CSF, cytokines including IL-4 and IFN-γ were accumulated in the cytoplasm of NKT cells and very little was secreted outside of cells (62).

Lymphotoxin (LT) has been demonstrated to be important for iNKT cell development. LT-deficient mice have reduced number of NKT cells in the periphery. Decreased number of peripheral NKT cells were observed in both LTβ-deficient and LTβ receptor deficient mice (63). In contrast, LTα deficient mice have reduced NKT cell numbers both in the thymus and periphery (63, 64).

NF-κB-family members have been reported to be essential for iNKT cell development. The requirement of NF-κB for iNKT cell development is both NKT cell intrinsic (via the NF-κB pathway) and extrinsic (in thymic stromal cells). An example of the intrinsic requirement of NF-κB for iNKT cell development is that mice expressing an IκBα (inhibitor of NF-κBα) dominant-negative transgene had almost no NKT cells (65).

Defects of NF-κB in stromal cells also inhibit iNKT cell development. RelB deficiency in stromal cells resulted in severely reduced NKT cell numbers in mice (66, 67).

The SLAM-SAP-FYN pathway is important for iNKT cell development as well. In the absence of Fyn, NKT cell development was selectively inhibited. In 2005, three studies showed that lack of signaling lymphocytic activation molecule (SLAM)-associated protein (SAP) led to completely abrogated iNKT cell development (68-70). Recently, it is reported that mice lacking signaling pathways that associate with both Slamf1 and Slamf6 had about 95% reduction in iNKT cell development (71, 72).

In addition to these well-studied factors, more recently at least 2 more molecules were shown to be critical for iNKT cell development. One is PLZF (promyelocytic leukemia zinc finger; *Zbtb16*), a member of the BTB/POZ-ZF family of transcription factors which includes the CD4 lineage-specific c-Krox (Th-POK, *Zbtb7b*). PLZF is specifically expressed in iNKT cells and PLZF KO mice had about 70 fold reduction of iNKT cells in both thymus and periphery (73, 74). The other is Egr2 (early growth response), a target of nuclear factor of activated T cells (NFAT). It is demonstrated that Egr2 was specifically required for iNKT cell selection, survival and maturation (75).

1.1.5 iNKT cell ligands

It is not clear what the nature and source of different lipids that bind naturally to CD1d are. However, combinations of genetic, cell biological and chemical approaches have shed light on some key NKT ligands.

The marine sponge αGalCer was the first ligand identified to be able to activate specifically iNKT cells (76). αGalCer is extracted from a marine sponge and has very high activity in vitro and in vivo (77, 78). It has been used broadly in various functional assays and to generate the first CD1d tetramers specific for mouse and human iNKT cells (79).

The role of isoglobotrihexosylceramide (iGb3) as a self antigen is controversial. Compared to αGalCer, iGb3 is a relatively weak agonist ligand, however, it can easily activate mouse and human iNKT cells (80, 81). The most convincing evidence supporting iGb3 as a self agonist comes from β-hexosaminidase B-deficient mice. β-hexosaminidase B is a lymosomal glycosphingolipid degrading enzyme. In mammalian cells, β-hexosaminidase removes β-linked GalNAc residues on glycosphingolipids of the ganglio-series (GalNAc β1,4 Gal), globo- and isoglo-series glycoplipids (GalNAc β1,3 Gal), and GlcNAc residues of the lacto-series (GlcNAc β1,3 Gal) (82). One of reactions catalyzed by β-hexosaminidase B is degradation of iGb4 to iGb3. β-hexosaminidase B-deficient mice exhibited a 95% reduction in thymic iNKT cells. In addition thymocytes from β-hexosaminiedase B KO mice could not stimulate Vα14 NKT cells (83). However, iNKT cell numbers are reduced in multiple mouse models of glycosphingolipid (GSL) storage diseases including Sandhoff and Fabry, which would not primarily affect iGb3 formation (84, 85). In addition, no iGb3 was present in the thymus even in the mouse model where α-glactodsidase (responsible for degrading iGb3) was deficient (84). Results showing that iGb3 is not the physiological ligand of iNKT cells come from iGb3 synthase deficient mouse. Through the action of iGb3 synthase, iGb3 and poly iGb3 are

formed. Mice lacking iGb3 synthase had normal iNKT cell development and function (86). Moreover, iGb3 could not be detected in humans except at the dorsal root ganglion (87). Therefore, it is less likely that iGb3 is an endogenous ligand for iNKT cells.

Lipids from microbes have been shown to activate specifically iNKT cells. Two studies showed that closely related structures that take the place of lipopolysaccharide (LPS) were found in the cell wall of *Sphingomonas* (a Gram-negative, LPS-negative member of the class of α-proteobacteria) (88, 89). These glycosphingolipids contribute to the strong stimulation of NKT cells and clearance of infection (90-92). The most abundant glycosphingolipids have only one sugar, α-anomerically branched to the ceramide backbone (GSL)-1. Less abundant but more complicated glycosphingolipids include GSL-2,-3 and -4 (93).

2.1 Vitamin D

Vitamin D is a fat soluble nutrient that is essential for maintaining calcium and phosphate homeostasis (94). Vitamin D is inactive and converted to 1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), the active form of vitamin D by 1α-hydroxylase. $1,25(OH)_2D_3$ binds to the vitamin D receptor (VDR) and plays a regulatory role in gene expression through recruiting co-activators or co-repressors. It appears to be an important regulator of immune response.

1.2.1 Production and metabolism of vitamin D

Vitamin D is found in two forms: vitamin D_3-cholecalciferol, occurring in humans and animals; and vitamin D_2-ergocalciferol, occurring in plants. In the form of vitamin D_3, it is produced from 7-dehydrocholesterol in the skin under ultraviolet light. Alternatively, vitamin D (both vitamin D_2 and vitamin D_3) can be provided by diet. Both vitamin D_2 and vitamin D_3 undergo the same activation process and there is little evidence that active forms of vitamin D_2 and vitamin D_3 differ in their mode of action (94). Therefore, only the metabolism of vitamin D_3 will be discussed (Figure **1-4**).

Vitamin D_3 produced in the skin and ingested through diet binds to vitamin D binding protein (DBP) and enter the liver where it is converted to 25 hydroxyvitamin D_3 ($25(OH)D_3$) by 25-hydroxylase, one of the enzymes encoded by gene CYP27A1 (95, 96). $25(OH)D_3$ is the major circulating form of vitamin D3 and one of the best biomarkers to determine the vitamin D status of patients and populations. $25(OH)D_3$ is transported to the kidney where it is hydroxylazed again to $1,25(OH)_2D_3$, the active form of vitamin D_3. This reaction is catalyzed by 1α-hydroxylase encoded by the CYP27B1gene. CYP27B1 had been shown to be expressed in cultures of skin, bone, colon, and parathyroid gland cells (97). Both $25(OH)D_3$ and $1,25(OH)_2D_3$ can be hydroxylyzed and inactivated by 25(OH)-24-hydroxylase encoded by the gene CYP24A1. The primary excretion route of vitamin D is via the bile and into the feces. It can also be metabolized to water soluble metabolites and excreted in the urine. Vitamin D3 may be metabolized by immune cells (98, 99).

The metabolism of vitamin D3 is controlled by those 3 enzymes mentioned above. The synthesis of $25(OH)D_3$ seems to be loosely controlled while the synthesis of $1,25(OH)_2D_3$ is concisely regulated by the level of plasma $1,25(OH)_2D_3$ and calcium. The 1α-hydroxylase is induced by the hormone parathyroid hormone (PTH) and down-regulated by increases in $1,25(OH)_2D_3$ (100). It is shown that expression of the 1α-hydroxylase is ablated 2-4 h after vitamin D-deficient rats were exposed to $1,25(OH)_2D_3$ (101). As for expression of the 24-hydroxylase, the gene is silent in the absence of vitamin D and expression of it is induced by $1,25(OH)_2D_3$ working through the VDR (102).

1.2.2 Role of vitamin D in calcium homeostasis

Calcium levels are tightly regulated by many hormones including vitamin D, PTH and calcitonin in humans and animals (Figure **1-5**). The classic function of vitamin D is to maintain calcium and phosphate homeostasis. There are calcium-sensing proteins in the parathyroid gland. When calcium levels in plasma are lower than normal, the secretion of PTH is stimulated. PTH then increases tubular re-absorption of calcium in kidney and activates 1α-hydroxylase. The renal re-absorption of calcium requires the presence of both $1,25(OH)_2D_3$ and PTH (103, 104). Activated 1α-hydroxylase produces $1,25(OH)_2D_3$, which triggers active intestinal calcium transport in the small intestine, a process that takes several days. Osteoblasts are also activated, either stimulate osteoclasts to reabsorb bone, and/or to activate the reverse transport of calcium from the bone fluid compartment to the plasma compartment. This process relies on the presence

of PTH (105). The final result is that calcium is mobilized from the skeleton into the plasma compartment by vitamin D and PTH and serum calcium increases. The rise in calcium level shuts down the parathyroid sensor, thus turning off the secretion of PTH and completing the feedback loop (105). These pathways act synergistically to maintain constant calcium levels.

1.2.3 The VDR and signaling by $1,25(OH)_2D_3$-VDR

The VDR is a member of a superfamily of nuclear receptors (106). Within this family, the VDR, retinoic acid, thyroid hormone, and peroxisome proliferator activator receptor (PPAR) receptors show the highest similarity (107). Like other nuclear receptors, the VDR can be divided into several domains. The two major functional domains are N-terminal zinc finger DNA binding domain and C-terminal ligand binding domain. The DNA-binding domain is located between amino acids 90-130, followed by the C-terminal ligand binding domain between 130 and 423 amino acids. At the N-terminus is a truncated A/B domain, the function of which is not clear (108).

The VDR gene in the mouse has two promoters and two exons that are non-coding. The gene has a number of AP1 (activation protein 1) sites and no TATA box. In addition, there is no vitamin D receptor response element (VDRE) in the promoter, but there might be VDREs in the intron between exons 2 and 3 as well as between exons 3 and 4 (109). These VDREs are believed to act as enhancer elements and induce VDR expression in response to calcium.

The expression of the VDR can be found in large amounts in some tissue while not in some tissues at any meaningful amounts (109, 110). The duodenum expresses the highest level of the VDR with colon, kidney and skin expressing very little. The VDR protein is not found in liver, adult heart muscle, skeletal muscle, smooth muscle, or most of the brain. Maximal expression of the VDR in kidney and parathyroid glands requires calcium and $1,25(OH)_2D_3$ (111). Higher level of dietary calcium alone has little effect on the VDR expression, but higher level of dietary calcium plus $1,25(OH)_2D_3$ result in markedly elevated expression of the VDR (112).

$1,25(OH)_2D_3$ functions by binding to the VDR. In order to bind the VDRE in target genes, the VDR needs to form a heterodimer with the retinoid X receptor (RXR) (113, 114). The VDREs, found primarily in the promoters of target genes, consist of two repeats of the half-site sequence AGGTCA separated by three unspecified nucleotides (115). The mechanism by which $1,25(OH)_2D_3$ exerts its regulatory effect on target gene expression is well known. Firstly the binding of $1,25(OH)_2D_3$ to the VDR causes the rejection of a co-repressor and formation of heterodimer with RXR (116). Then the altered VDR binds the 3' segment of the VDRE and RXR the 5' segement together with several other proteins including CBP/p300, pCAE and SRCs that have histone acetylase activity. This results in an alteration of the chromatin structure. Next, some of the ancillary proteins leave and other proteins such as RNA polymerase II bind the receptor complex to start transcription. A bending of DNA and a phosphorylation is associated with RNA polymerase II binding (117). This finally causes stimulation or suppression of transcription by vitamin D. Over 50 genes are shown to be up-regulated and 50 down-regulated by intrajugular administration of $1,25(OH)_2D_3$ to vitamin D deficient rats (118).

1.2.4 Immunomodulatory role of vitamin D

It is well known that vitamin D has immunomodulatory effects (119, 120). This was firstly suggested by the identification of the VDR in peripheral blood mononuclear cells (121, 122). In addition, it has been demonstrated to play a role in autoimmune diseases. $1,25(OH)_2D_3$ has been shown to exhibit marked effects on innate and adaptive immune responses as well. .

Vitamin D deficency has been shown to predispose animals to a lot of autoimmune diseases. Serum levels of $25(OH)_2D_3$ are significantly decreased in patients with type I diabetes and systemic lupus erythematosus (SLE) (123, 124). $1,25(OH)_2D_3$ has also been shown to be inversely correlated with disease activity in patients with rheumatoid arthritis (125). Moreover, vitamin D deficiency accelerates intestinal inflammation in mice that spontaneously develop inflammatory bowel diseases (IBD) (126, 127). Vitamin D deficiency is common in patients with IBD even when the disease in remission (128, 129). Another example is multiple sclerosis (MS). Similar to IBD, vitamin D deficiency is also common in patients with MS (130, 131).

$1,25(OH)_2D_3$ has been shown to be effective in blocking the progression of Th1-mediated autoimmune diseases including arthritis, experimental autoimmune encephalomyelitis (EAE), and type I diabetes in animals. In these animal models, Th1 cells were shown to be responsible for the development of these diseases. Administration of $1,25(OH)_2D_3$ reduced clinical EAE severity within 3 days (132-134). This was due in part to inhibited production of chemokines and inducible nitiric oxide synthase (iNOS) by $CD4^+$T cells as a result of increased apoptosis of $CD4^+$T cells induced by $1,25(OH)_2D_3$

(132, 133). In mouse models of type I diabetes, treatment of non-obese diabetic mice with $1,25(OH)_2D_3$ alleviates the symptoms of and delays onset of the disease while vitamin D deficiency in early life exacerbates it (135-138). In another report, it was shown that $125(OH)_2D_3$ treatment significantly decreased the incidence of diabetes in female NOD mice compared to controls (139). $1,25(OH)_2D_3$ treatment rescued the suppressor function of T cells in the NOD mice (139). When $1,25(OH)_2D_3$ treated splenocytes were transferred into irradiated NOD mice that had passively received diabetic splenocytes, they were able to prevent the development of diabetes in those mice (139). Normal T cell function and prevention of type I autoimmune disease require signaling via $1,25(OH)_2D_3$ and the VDR.

The beneficial effect of vitamin D on autoimmue diseases can be reflected by its role in immune cells. The effects of $1,25(OH)_2D_3$ on the immune system can be inhibitory and stimulatory depending on distinct cell types. An example of inhibitory effect of $1,25(OH)_2D_3$ on innate immune response is that it inhibits DC differentiation, maturation and immunostimulatory capacity in vitro (119, 140, 141). Treated DCs display reduced expression of MHC II molecules and of CD40, CD80 and CD86 (119, 140, 141). In addition, the VDR-deficient mice appear to have decreased numbers of mature DCs in skin-draining lymph nodes (141). $1,25(OH)_2D_3$ reduces the production of IL-12 and enhances the synthesis of IL-10 by DCs (142). These events lead to decreased T cell activation. Moreover, these DCs are able to induce IL-10-producing $CD4^+CD25^+$ regulatory T cells. One mechanism by which IL-12 is down-regulated is by interfering with the nuclear factor NF-κB pathway. $1,25(OH)_2D_3$ affects both activation of NF-κB pathway as well as its binding to IL-12p40 promoter (143, 144).

In contrast, $1,25(OH)_2D_3$ exerts stimulatory effects on other immune cells such as monocytes. For example, it was shown to stimulate human monocyte proliferation in vitro at physiological concentrations (145), as shown in human myeloid leukemia HL60 cells. This is mediated by activation of MAPK (mitogen-activated protein kinase) signaling and by up-regulated expression of C/EBPβ (CAAT/enhancer-binding protein β) expression (146, 147). Moreover, $1,25(OH)_2D_3$ increases secretion of IL-1 and the bacterial peptide cathelicidin by monocytes and macrophages (148). It improves phagocytic and oxidative burst ability as well (148, 149). During differentiation from monocytes to macrophages, cells may acquire the ability to synthesize more $1,25(OH)_2D_3$ (144).

$1,25(OH)_2D_3$ exerts inhibitory effects on some adaptive immune cells in vitro, including B cells and T cells. Chen et al have shown that $1,25(OH)_2D_3$ induces apoptosis of activated B cells, impeding their ongoing proliferation without influencing their initial cell division (123). Although it has modest effects on up-regulation of genes involved in B cell differentiation, it significantly inhibits generation of plasma cells and post-switch memory B cell (123, 150). It is suggested that the inhibitory role of $1,25(OH)_2D_3$ in B cells is by up-regulation of p27 expression. However, since there are conflicting reports concerning the expression of the VDR by B cells (123, 151), it is very likely that effects of $1,25(OH)_2D_3$ on B cells are indirectly mediated through the effects it has on APCs function (152).

In addition to its inhibitory effects on B cells, $1,25(OH)_2D_3$ treatment of T cells decreases their activation and proliferation and alters cytokine expression profile (150, 153-155). It also inhibits $CD8^+$ T cell-mediated cytotoxicity (156). IFN-γ and IL-2

production is impeded in $CD4^+$ T cells by different mechanisms. The inhibited IFN-γ transcription results from binding of VDR/RXR to a silencer region in the promoter while the reduced IL-2 production is a result of delayed initiation or progression of transcription (157). The IL-2 promoter has a positive regulatory NFAT1 site where a complex containing T cell-specific transcription factors NFATp and AP1 can bind (158). $1,25(OH)_2D_3$ obstructs IL-2 transcription through impeding complex formation of $1,25(OH)_2D_3$/VDR/RXR with NFAT1/AP1. The anti-proliferative effect results partially from the decreased production of IL-2, since proliferation is rescued by adding exogenous IL-2 (153). The most pronounced effects of $1,25(OH)_2D_3$ on T cells are observed on memory T cells consistent with the higher expression of the VDR in effector and memory T cells compared with naïve T cells (151, 159). Furthermore, $1,25(OH)_2D_3$ increases non-specific T cell suppressor activity as $1,25(OH)_2D_3$ treated T cells show enhanced ability to repress primary mixed-lymphocyte reactions and cytotoxic T cell response (156).

Moreover, $1,25(OH)_2D_3$ plays a critical role in T-cell differentiation. Overall, the net result of $1,25(OH)_2D_3$ on T cells is to block the initiation of T_H1-cell cytokines while promoting T_H2-cell response. This effect is mediated both by indirectly reducing IFN-γ production as well as directly increasing IL-4 production (119). As discussed above, $1,25(OH)_2D_3$ diminishes IL-12 production by DCs, which is a cytokine promoting T_H1-cell response. This in turn elevates the influence of $1,25(OH)_2D_3$ on T-cell differentiation (142, 143). Furthermore, $1,25(OH)_2D_3$ also inhibits T_H17-cell response partially as a result of its capability to decrease the production of IL-6 and IL-23 (160, 161). Finally, it

enhances differentiation and expansion of forkhead box protein 3(FOXP3)$^{+}$ regulatory T (T_{Reg}) cells (161-163).

To conclude, 1,25$(OH)_2D_3$ has been shown to be an important regulator of immune response in addition to its role in calcium homeostasis. Expression of both 1α-hydroxylase and the VDR can be found in distinct immune cells. However, the role of the VDR and 1,25$(OH)_2D_3$ in iNKT cell development and function remains unclear.

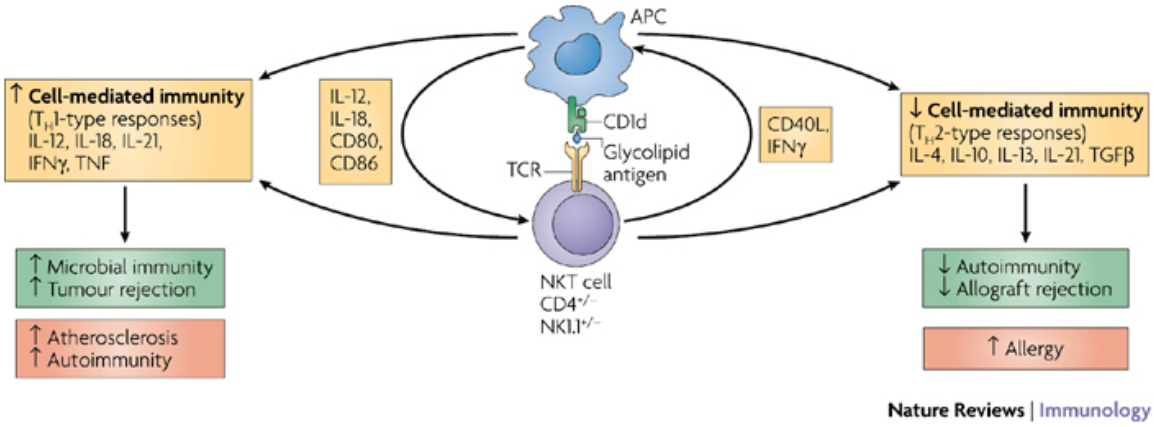
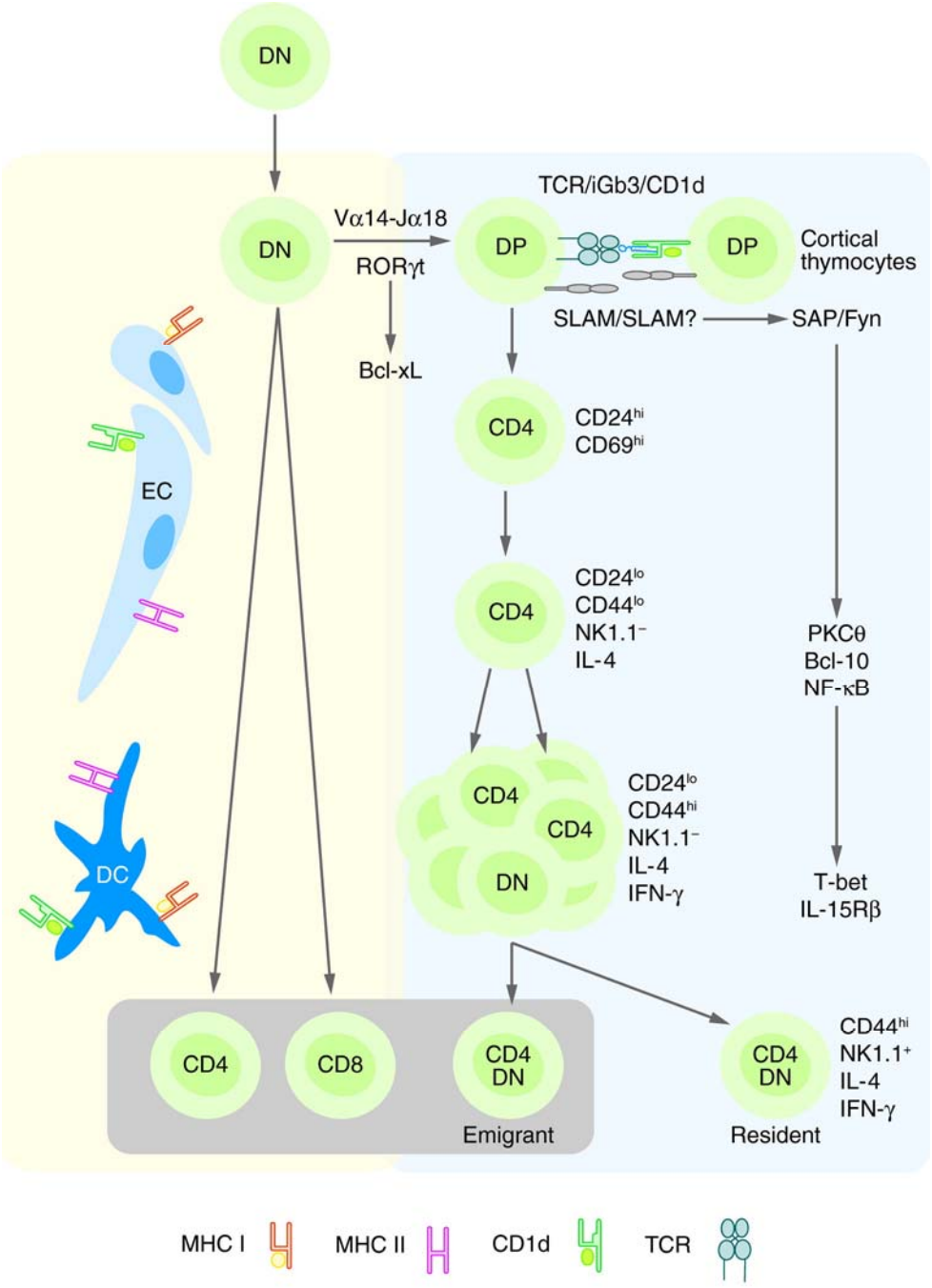


Figure **1-1**: iNKT cell function. The activation of iNKT cells by glycolipid leads to rapid production of cytokines and the expression of co-stimulatory molecules, which in turn results in the activation of antigen-presentating cells (APCs) and many other bystander cells (including NK, T and B cells; not shown). The effect of cytokine production is very broad, ranging from enhanced cell-mediated immunity to suppressed cell-mediated immunity. CD40L, CD40 ligand; IFN-γ, interferon-γ; IL, interleukin; TCR, T-cell receptor; TGF-β, transforming growth factor-β; TNF, tumor-necrosis factor. (Nature reviews, 2007, 7: 505-518)



Bendelac A, et al. 2007.
Annu. Rev. Immunol. 25:297–336

Figure **1-2**: Development of thymic iNKT cells. iNKT cell precursors diverge from conventional T cell development at $CD4^+CD8^+$ double positive (DP) stage. Upon expression of Vα14-Jα18 TCRa chain, iNKT cell precursors interact with endogenous ligands presented by CD1d expressed on other DP thymocytes. DP precursors down-regulate CD8 to generate $CD4^+$ cells. Later a subset down-regulates CD4 to produce

CD4-CD8 double negative (DN) cells. Different from conventional T cells, iNKT cell precursors divide several rounds and acquire a memory/effector phenotype before they emigrate to peripheral. A minor subpopulation of iNKT cells remain and mature in the thymus. Up-regulation of NK lineage markers usually occurs in the peripheral tissues. EC, epithelial cell; DC, dendritic cells. (Annu. Rev. Immunol, 2007, 25: 297-336)

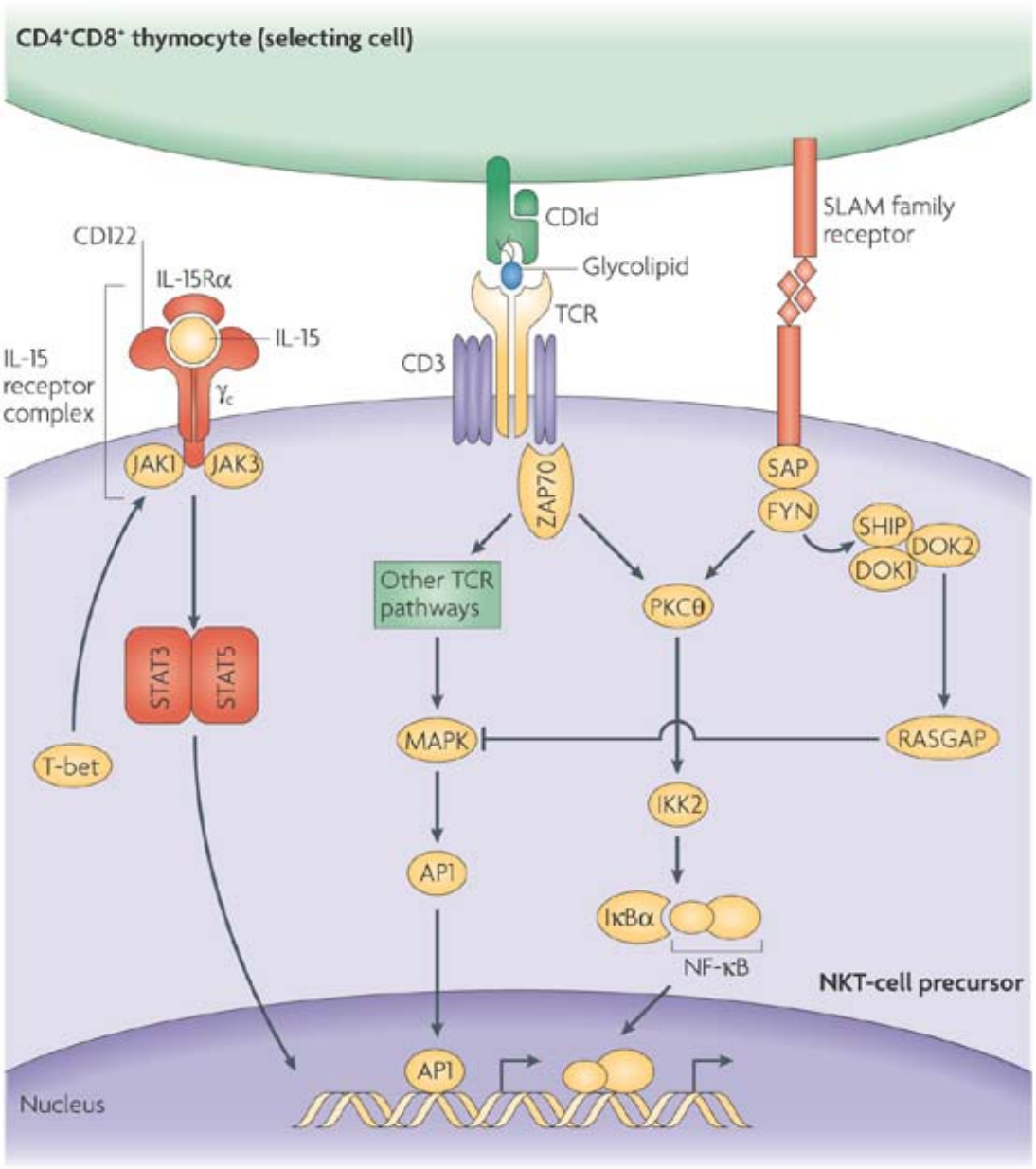


Figure **1-3**: Factors that regulate iNKT cell development. iNKT cell development and maturation requires a range of signaling events, which are not full understood. The three main intrinsic pathways that link most of the known mutants that influence iNKT cell development are described. These include the SLAM-SAP-FYN pathway, the T-cell receptor (TCR)-signaling cascade (particularly the classical nuclear factor-κB (NF-κB) pathway and interleukin-15 (IL-15) pathway. AP1, activating protein 1; γ, common cytokine-receptor γ-chain; DOK, docking protein; IκBα, inhibitor of NF-κB; IKK2, IκB kinase 2; JAK, Janus kinase; MAPK, mitogen-activated protein kinase; PKCθ, protein

kinase θ; RASGAP, RAS GTPase-activating protein; SAP, SLAM-associated protein; SHIP, SRC-homology-2-domain-containing inositol-5-phosphatase; SLAM, signaling lymphocytic activation molecule; STAT, signal transducer and activator of transcription; ZAP70, ξ-chain associated protein kinase of 70 kDa. (Nature Reviews, 2007, 7: 505-518)

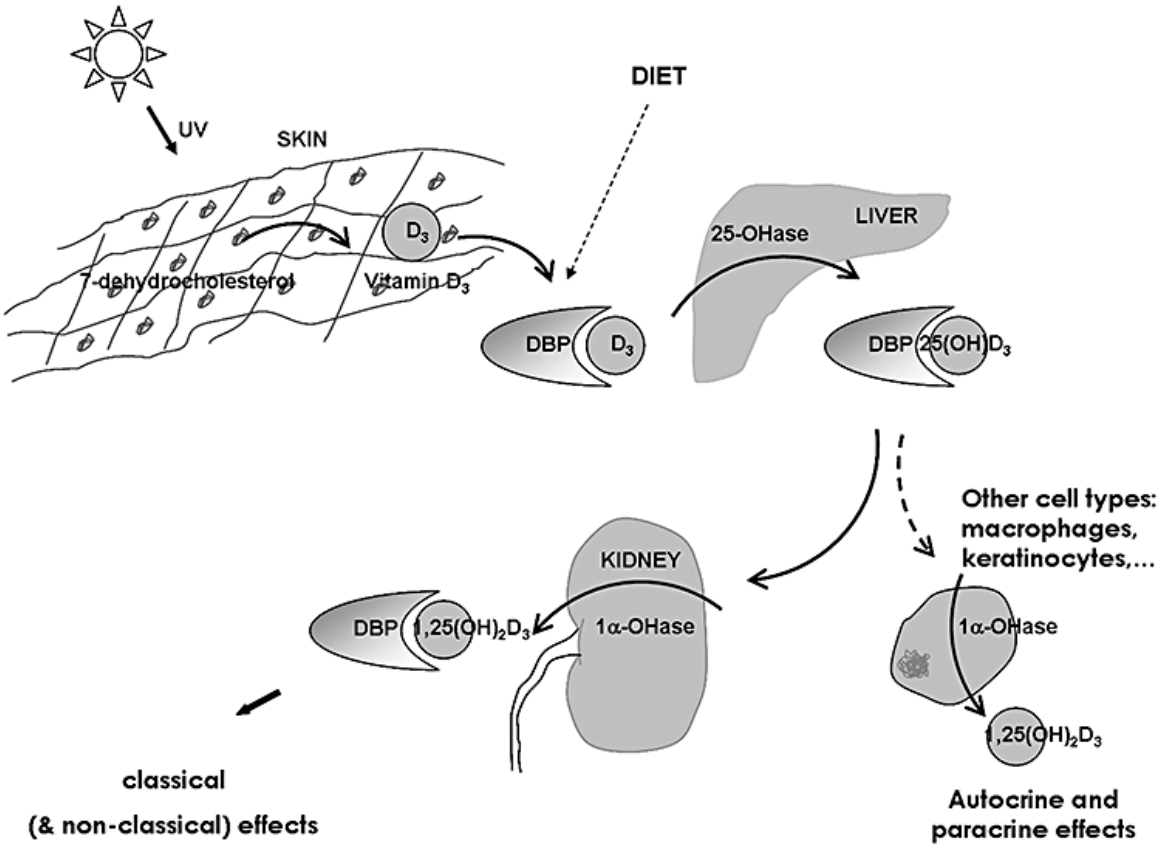


Figure **1-4**: Overview of vitamin D metabolism. Vitamin D is produced from 7-dehydrocholesterol when exposed to sunlight, while a small amount is from the diet. To become biologically active, two hydroxylation steps are necessary. The first hydroxylation take place in the liver and the second in the kidney producing 25-hydroxyvitamin D3 25(OH)D_3 and 1,25(OH)$_2D_3$ respectively. In blood, vitamin D3 binds to vitamin D binding protein (DBP). (Nutrition Reviews, 2008, 66: S125-S134).

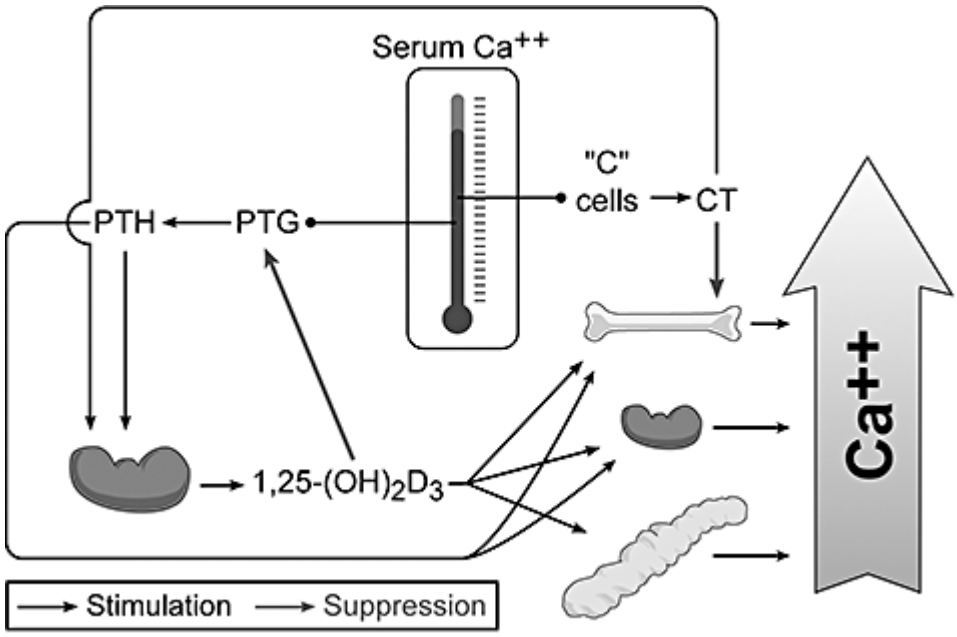


Figure **1-5** Physiologic regulators of the vitamin D endocrine system in calcium homeostasis. Decrease in the calcium level in the blood induces PTH secretion, which in turn leads to elevated production of $1,25(OH)_2D_3$. $1,25(OH)_2D_3$ elicit the calcium mobilization on three sites. PTH, parathyroid hormone; PTG. (Nutrition reviews, 2008, 66: S73-S87).

Chapter 2

Material and Methods

2.1 Mice and diet

Age- and sex-matched VDR KO and WT C57BL/6 mice were produced at the Pennsylvania State University (University Park, PA). VDR KO mice were a gift from M. Demay (Harvard University, Cambridge, MA). Cyp27B1 KO and WT littermates were a kind gift from Dr. Hector DeLuca (University of Wisconsin, Madiso, WI). For some experiments mice were fed synthetic diets that either includes 50 ng of $1,25(OH)_2D_3$ per day ($1,25D_3$) or does not include $1,25(OH)_2D_3$ as we have described previously (164). All the mice were used at 6-8 week of age and kept in pathogen-free conditions. All experimental procedures received approval from the Office of Research Protection's Institutional Animal Care and Use Committee (Pennsylvania State University, University Park, PA)

2.2 αGalCer stimulation

αGalCer (Axxora, San Diego, CA) was dissolved in PBS containing 0.5% Tween 20, heated to 80°C for 10 min, and sonicated for 5 min on ice. Mice were given an i.p. injection of 2μg of αGalCer or vehicle (PBS and 0.5% Tween 20). Blood was collected from the retro-orbital plexus for serum isolation at different time points after injection.

2.3 Flow Cytometry

Single-cell suspensions of thymus, spleen and liver were prepared. Liver was perfused with PBS via the portal vein until the liver was opaque and pressed through nylon screening cloth (PGC Scientifics Corp, Frederick, MD). Total liver cells were resuspended in a 40% Percoll solution (Sigma, St Louis, MO) underlaid with an 80% Percoll solution. After centrifugation, mononuclear cells were washed with HANKs' balanced salts twice (Sigma).

Cells were stained with phycoerythrin (PE) labeled CD1d- αGalCer tetramers (gift of the NIH Tetramer Facility, Atlanta, GA). mAbs used in this study for flow cytometry include PE labeled anti-NK1.1 (PK136), PE-Cy5-labeled anti-TCRβ (H57-597), PE labeled anti-CD1d (1B1), PE-Cy5-labeled anti-CD44 (IM-7), Fluorescein isothiocyanate (FITC)-labeled anti-CD3 (17A2), FITC-labeled anti-CD4 (L3T4), Allophycocyanin (APC)-labeled anti-CD62L (MEL-14), AlexaFluor ® 488-labeled anti-IL-4 (11B11), PE-Cy7-labeled anti-IFN-γ (R46A2), FITC-labeled anti-Annexin V, FITC BrdU flow kit, FITC-labeled anti-CD45.2 (104), FITC-labeled anti-CD45.1 (A20), FITC-labeled anti-CD122 (TM-b1), FITC-labeled anti-Ly49C (5E6), FITC-labeled anti-NKG2A/C/E 20d5, FITC-labeled anti-Vβ8 (F23.1), FITC-labeled anti-Vβ7 (TR310), FITC-labeled anti-CD24 (M1/69), APC-labeled anti-Annexin V and PE-Cy7-labeled anti-CD8α (53-6.7) (BD Pharmigen, San Diego, CA). PE-Texas Red-labeled anti-CD4 (L3T4) were purchased from Invitrogen (San Diego, CA). For intracellular staining, mice were i.p injected with 2μg αGalcer. 2 hours later, splenocytes and lymphocytes isolated from liver were cultured in the presence of brefeldin A (Sigma) for 6 hours. Alternately

lymphocytes isolated from the liver were cultured in the presence of αGalCer (100nM) and Brefeldin A overnight. Surface markers were stained, the cells fixed with 4% paraformadehyde (Sigma), permeabilized with 0.1% saponin (Sigma) and the following antibodies were added; AlexaFluor 488-labeled anti-IL-4 (11B11) or FITC-labeled anti-IFNγ, or PE-Cy7-labeled anti-IFN-γ, (BD Pharmingen). The isotype controls were FITC-labeled or Alexa Fluor 488-labeled mouse IgG1 (MOPC21) (BD Pharmingen).

2.4 In vitro proliferation and cytokine ELISA

Splenocytes (5 x 10^5) were stimulated for 72 h with various concentrations of αGalCer (0.1–100 nM) or medium alone. For some cultures 100 nM of 1,25(OH) $_2D_3$ dissolved in ethanol or the equivalent amount of ethanol was added to *in vitro* cultures. Proliferation was measured by adding [^{3}H]thymidine (1 μCi/well; MP biomedical, Solon, OH) for the last 12 h of the culture. Cells were harvested, and [^{3}H]thymidine incorporation was determined using a Microbeta Trilux scintillation counter (LKB Wallac, Turku, Finland). Supernatants and serum levels of IL-2, IL-4 and IFN-γ were measured by ELISA (BD Pharmingen) according to manufacturer's instructions.

APC; including purified splenic DCs (98% CD11c$^+$), thymocytes, and recombination activating gene (Rag) KO splenocytes, were incubated alone or with 100 ng/ml of αGalCer and 5x10^4 of the NKT cell hybridoma DN32D3 (gift of Dr. Albert Bendelac, University of Chicago, Chicago, IL) or purified liver iNKT cells for 24 h. IL-2 content of the supernatants was measured by ELISA (BD Pharmingen).

2.5 Antigen Presentation

Thymocytes from WT and VDR KO or Cyp27B1 KO mice were incubated with 5×10^4 DN32D3 NKT cell hybridoma (gift of Dr. Albert Bendelac, University of Chicago, IL) for 24 h, and IL-2 in the supernatant was measured by ELISA (BD Pharmingen).

2.6 Retroviral CD1d expression

Thymocytes from WT and VDR KO mice were infected with the control retrovirus or CD1d-expressing retrovirus (gift of Dr. Mithchell Kronenberg, La Jolla Institute for Allergy and Immunology, San Diego, CA) generated as described (165). 24h later infected thymocytes were incubated with 5×10^4 DN32D3 for 48 h. IL-2 content of the supernatants were measured by ELISA.

2.7 Quantitative mRNA analysis

Thymoctyes or iNKT cells purified from thymus ($TCR\beta^+$ $tetramer^+$) were used to isolate mRNA using the RNeasy Mini kit (Qiagen, Valencia, CA) and reverse-transcribed (Promega, Madison, WI). Real-time quantitative PCR was performed with DNA engine OpticonII (Bio-Rad, Hercules, CA) using iQ^{TM}SYBR Green (Bio-Rad). The primers for Vα14-Jα18 have been described previously (67).

2.8 BrdU incorporation assay

Mice were injected ip with 100µl of 10 mg/ml BrdU dissolved in PBS at the onset of experiments. At the same time, mice were fed with 0.8 mg/ml BrdU supplemented with 5% glucose and water was changed every other day. At day 9 thymocytes, splenocytes and mononuclear cells from livers were stained with a BrdU flow kit (BD Pharmingen) following the manufacturer's instructions.

2.9 Bone marrow transplantation

Bone marrow cells were harvested from WT (CD45.1) and VDR KO or Cyp27B1 KO (CD45.2) and transferred into sublethally (950 rad) irradiated WT or VDR KO or Cyp27B1 KO mice. For competitive BM transplantation, 1:1 mixtures of WT (CD45.1) BM cells and VDR KO or Cyp27B1 KO (CD45.2) BM cells were transferred into WT (CD45.1) mice. Reconstituted iNKT cell populations were analyzed 8 or more weeks after transfer.

2.10 Western Blot

Thymocytes were isolated from adult WT and VDR KO mice and solubilized in Radio-Immunoprecipitation Assay (RIPA) buffer containing protease and phosphatase inhibitors. Proteins were separated by SDS-PAGE and transferred onto nitrocellulose membranes. Membranes were incubated with rabbit anti-mouse CD1d (eBioscience, San

Diego, CA) followed by anti-rabbit Ig-horseradish peroxidase (HRP) (Santa Cruz Biotec, Santa Cruz, CA) and detected using the ECL system (Amersham, Piscataway, NJ).

2.11 Immunofluorescence microscopy.

Fresh thymocytes were adhered to poly-L-lysine treated coverslips. After fixation/permeabilization with 4% formaldehyde and 0.1% saponin, cells were costained with FITC-conjugated rat anti-mouse LAMP-1, 20H2-biotin anti-CD1d, revealed by PE-Texas-red Streptavidin (BD Pharmingen). Coverslips were mounted onto slides using the SlowFade kit (Molecular Probes, Carlsbad, CA). Samples were visualized under an Olympus IX70 inverted system fluorescence microscope equipped with appropriate filters (Hitech Instruments; Edgemont, PA). Images were captured using a SPOT camera (Diagnostic Instruments, Sterling Heights, MI).

2.12 Statistical analysis

Statistical analyses were performed using PRISM software (GraphPad, La Jolla, CA). Cell percentage and numbers were compared by ANOVA. P values of 0.05 or less were considered statistically significant.

Chapter 3

Requirement of the VDR for for iNKT cell development and function

Chapter adapted from the manuscript entitled "**The vitamin D receptor is required for iNKT cell development.**"

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3.1 Introduction

Natural killer T (NKT) cells are a subset of T lymphocytes that play an important regulatory role in several models of autoimmunity, infection and cancer (166, 167). NKT cells participate in the innate immune system and have been shown to be very early producers of high amounts of cytokines including IL-4 and IFN-γ (168). *In vivo,* NKT cell activation has been shown to alter the outcome of autoimmunity reducing or exacerbating disease symptoms depending on when in the disease process they are stimulated (169). A role for NKT cells in the development of autoimmune disease has been proposed.

NKT cells are thymically derived lymphocytes that express the TCR and receptors of the NK lineage including NK1.1, NKG2D and members of the Ly-49 family (1, 170, 171). The majority of mouse NKT cells express a semi-invariant TCR composed of the Vα14-Jα18 rearrangement and are selected in the thymus through the interaction with CD1d expressed on CD4/CD8 double positive (DP) thymocytes (15, 44, 172). These Vα14 invariant NKT cells (iNKT) are present in the thymus, bone marrow (BM), spleen and liver and are reactive with the glycolipid α-galactosylceramide (αGalCer) presented in the context of CD1d (76). iNKT cells were shown to recognize a lysosomal sphingolipid as an endogenous ligand (83) and recently it has been shown that iNKT cells can recognize exogenous ligands from *Sphingomonas* and *Borelia burgdorferi* (90, 173). iNKT cells develop from DP Vα14-Jα18 TCR thymocyte precursors that lack expression of NK markers and then undergo robust proliferation and upregulation of CD44 before terminal maturation that is accompanied by the expression of NK1.1, Ly-49 and CD122

(6, 44). The final steps of iNKT cell development require the transcription factor T-bet and cytokine signaling initiated by IL-15 (174).

CD1d is a nonclassical MHC class I-like molecule that associates with β2-microglobulin (56). CD1d molecules can present exogenous and endogenous ligand to iNKT cells. CD1d trafficking through endosomal compartments is necessary for presentation of ligands to iNKT cells (175, 176). In the thymus CD1d expression of endogenous antigens by DP thymocytes is critical for normal development of iNKT cells (44). The importance of CD1d in iNKT cell development is illustrated by the fact that CD1d null mice lack iNKT cells (177).

Vitamin D has been well characterized as a regulator of bone and mineral metabolism. 1α,25-dihydroxyvitamin D3 ($1,25(OH)_2D_3$) is the active form of vitamin D and functions through binding to the vitamin D receptor (VDR) (178).The VDR is a member of the steroid thyroid super family of nuclear receptors. Various cells of the immune system express the VDR and $1,25(OH)_2D_3$ has been shown to be an important regulator of T cell function (178). $1,25(OH)_2D_3$ treatment has been shown to suppress animal models of autoimmune diseases while VDR KO mice are more susceptible to inflammatory bowel disease (178). I*n vitro* studies showed that $1,25(OH)_2D_3$ treatment increased the production of IL-4 by Th2 cells and decreased the production of IFN-γ by Th1 cells (179). Other $CD4^+$ T cell populations including Th2 and Treg cells have been shown to express the VDR and to be $1,25(OH)_2D_3$ targets (180).

Here we show that iNKT cells fail to develop in the absence of the VDR. A blockade in iNKT cell development results in few iNKT cells in the periphery of VDR KO mice and in addition homeostatic proliferation of iNKT cells is impaired in the liver.

$1,25(OH)_2D_3$ treatment increased the function of WT iNKT cells but not development. CD1d expression in the VDR KO thymus was reduced and as a result VDR KO thymocytes showed decreased stimulation of an iNKT cell hybridoma that could be partially rescued by retroviral CD1d expression. The VDR is required for normal expression of CD1d in the thymus and the data suggest that the VDR KO thymus is not optimal for antigen presentation and selection of iNKT cells. In addition the iNKT cells from VDR KO mice are intrinsically defective and lack T-bet expression. We've identified multiple VDR dependent pathways in the development of iNKT cells.

3.2 Results

3.2.1 Diminished responses of VDR KO mice to αGalCer

WT NKT cells express the VDR and activation of iNKT cells with αGalCer caused a significant increase in mRNA for the VDR (Figure **3-1**A). Splenocytes from WT and VDR KO mice were isolated and stimulated *in vitro* with increasing concentrations of αGalCer (Figure **3-2**A). Proliferation and production of IFN-γ was maximal for WT iNKT cells at 10nM αGalCer. Maximal amounts of IL-4 were produced between 0.1-10 nM αGalCer (Figure **3-2**A). Stimulation of VDR KO splenocytes with equivalent amounts of αGalCer resulted in significantly less proliferation, IL-4 and IFN-γ production (Figure **3-2**A). Feeding WT mice $1,25(OH)_2D_3$ (1,25D3) for 1 wk increased the production of both IL-4 and IFN-γ by splenocytes cultured *ex vivo* with αGalCer (Figure **3-2**B). Furthermore, addition of 100 nM $1,25(OH)_2D_3$ *in vitro* to WT cultures

with αGalCer increased IFN-γ secretion 10-fold higher than control (Figure **3-1**). To investigate whether vitamin D affects *in vivo* iNKT cell function, VDR KO, WT and $1,25D_3$ fed mice were injected with αGalCer. The level of IFN-γ peaked 6h after activation and then decreased and disappeared by 48h (Figure **3-2**C). IL-4 peaked early after αGalCer injection (2h) and disappeared by 12h post-injection. Feeding mice 1,25D3 for 1 wk prior to αGalCer injection increased IFN-γ and IL-4 production but did not alter the kinetics of the response (Figure **3-2**C). VDR KO mice produced significantly less IFN-γ and IL-4 than both the WT and 1,25D3 fed WT mice and the kinetics were similar across the three groups (Figure **3-2**C).

3.2.1 Reduced numbers of NKT cells in VDR KO mice.

The numbers of NKT cells in the thymus, BM, spleen, and liver of WT, and VDR KO mice were determined using NK1.1 and CD3 staining. The percentage of NK1.1/CD3 positive NKT cells were significantly less in the thymus and liver of VDR KO mice compared to WT mice while the BM and spleen of WT and VDR KO mice showed similar NK1.1/CD3 NKT percentages (Figure **3-3**A, Figure **3-4**). $1,25(OH)_2D_3$ treatment had no effect on NKT cell numbers in any tissue (Figure **3-4**). The percentages of iNKT cells (defined as TCRβ and CD1d-αGalCer tetramer positive) were significantly lower in VDR KO thymus, and liver compared to WT mice (Figure **3-3**B). In addition, there was a significant decrease in iNKT cells in the spleen of VDR KO mice compared to WT spleen. VDR KO spleens and livers contain a higher proportion of NKT cells than

WT mice that are not reactive with αGalCer (Figure **3-4**). Expression of the TCR Vα14-Jα18 chain was significantly less in thymocytes from VDR KO mice (Figure **3-3**C).

iNKT cells consist of two defined populations: a $CD4^+$ and a $CD4^-CD8^-$ double negative (DN) population. When the absolute numbers of iNKT cells were examined there was a decrease in both the $CD4^+$ and DN iNKT cells in the periphery of VDR KO mice (Figure **3-3**D). It should be noted that the percentage and absolute numbers of conventional T cells and NK cells were not different in WT and VDR KO mice (Figure **3-3**A and data not shown). Therefore, VDR deficiency results in a specific defect in the numbers of iNKT cells and not other T cells or NK cell subsets.

3.2.3 VDR KO iNKT cells are hyporesponsive.

To determine whether VDR KO iNKT cells are functionally different from WT iNKT cells; cytokine production from iNKT cells was assessed at the single-cell level by intracellular staining. WT and VDR KO mice were injected with αGalCer and animals were then sacrificed after 2h. iNKT cells were gated by flow cytometry and then analyzed for intracellular IL-4 and IFN-γ. At 2h post αGalCer injection 3% of the iNKT cells from the spleen of VDR KO mice made IL-4 and 22% made IFN-γ (Figure **3-3**E). Conversely, 15% of the spleen derived WT iNKT cells produced IL-4 and 51% produced IFN-γ (Figure **3-3**E). A similar result was found when iNKT cells were analyzed from the liver in that at least twice as many WT iNKT cells produced IL-4 and IFN-γ than VDR KO iNKT cells (Figure **3-3**E and Figure **3-1**). This was a reproducible result and

significantly more iNKT cells from WT mice made both IFN-γ and IL-4 compared to the iNKT cells from VDR KO mice (Figure **3-1**).

3.2.4 Defective NKT cell maturation in the absence of the VDR.

Immature iNKT cells have rearranged the Vα14-Jα18 TCR and are therefore tetramer and TCR β positive. The steps in iNKT cell development are 1) $CD44^{low}/NK1.1^{-}$ followed by 2) $CD44^{high}/NK1.1^{-}$ and leading to 3) $CD44^{high}/NK1.1^{+}$ (6, 44). Analysis of thymic iNKT cells showed that VDR KO iNKT cells did upregulate CD44 expression but that the majority of the iNKT cells failed to express NK1.1 (Figure **3-5**A). The difference between mature $CD44^{high}/NK1.1^{+}$ cells in WT and VDR KO is more obvious when the cell numbers of the different iNKT cell subpopulations were compared (Figure **3-5**B). The majority of iNKT cells in the VDR KO mouse were $CD44^{high}/NK1.1^{-}$ NKT cells whereas the majority of iNKT cells in WT mice were $CD44^{high}/NK1.1^{+}$NKT cells (Figure **3-5**B). In addition, the data show that there are fewer of the iNKT cell early thymic precursors or $CD44^{low}/NK1.1^{-}$ in the VDR KO mice (Figure **3-5**B). By contrast, VDR KO mice had higher than normal numbers of $CD44^{high}/NK1.1^{-}$ iNKT cells in the thymus (Figure **3-5**B). The absence of mature $CD44^{high}$ $/NK1.1^{+}$ NKT cells in the VDR KO mouse also occurred in the periphery (data not shown) and is consistent with the complete absence of T-bet expression in VDR KO iNKT cells but normal levels in the other VDR KO thymocytes (Figure **3-5**C). The predominately $CD44^{high}/NK1.1^{-}$ iNKT cells from VDR KO mice had decreased expression of Ly49C but normal levels of CD122 and NKG2A/C/E compared to WT iNKT cells (Figure **3-5**D).

3.2.5 Reduced homeostatic proliferation in the liver of VDR KO mice.

iNKT cell apoptosis was measured in thymocytes from WT and VDR KO mice. VDR KO and WT iNKT cells had similar percentages of annexin V^+ cells suggesting that iNKT cells from VDR KO mice were not dying due to increased apoptosis (Figure **3-6**A). Homeostatic proliferation of iNKT cells from VDR KO mice was also examined using a BrdU incorporation assay. A similar percentage of WT and VDR KO iNKT cells in the thymus and spleen incorporated BrdU (Figure **3-6**B). However, a significantly smaller percentage of iNKT cells in the liver of VDR KO mice incorporated BrdU than iNKT cells in the liver of WT mice (Figure **3-6**B). Decreased homeostatic proliferation of iNKT cells in the liver of VDR KO mice may contribute to the lower numbers of iNKT cells in this organ.

3.2.6 VDR expression in the thymus is required for normal iNKT cell development.

Reciprocal BM transplants were successful as judged by the complete reconstitution of the thymus of recipients with the donor BM (WT-WT, 98% donor, WT-VDR KO 99% donor, and VDR KO-WT 99% donor). A small (0.16%) and consistent number of iNKT cells were recovered from WT recipients of WT BM (WT-WT, Figure **3-7**A&B). Others have shown similar levels of iNKT cell reconstitution in the thymus following BM reconstitution (14, 67). The same numbers and percentage of iNKT cells were recovered from VDR KO recipients of WT BM (WT-VDR KO, Figure **3-7**A & B). Conversely, WT recipients of VDR KO BM had significantly reduced iNKT cell reconstitution of the thymus (0.07%), compared to either recipient of WT BM (Figure **3-**

7A & B). WT BM transplantation into either WT or VDR KO recipients resulted in similar percentages of donor derived iNKT cells in the spleen of recipients (Figure **3-8**). VDR KO BM into WT recipients resulted in half as many donor derived iNKT cells in the liver and a small but significant decrease in donor derived iNKT cells in the spleen compared to the WT-WT combination (Figure **3-8**). The liver of WT-VDR KO recipients showed very low reconstitution with donor derived iNKT cells and the result resembled that from the VDR KO-WT instead of the WT-WT combination (Figure **3-8**).

Liver mononuclear cells from the recipient mice were stimulated *in vitro* with αGalCer for intracellular cytokine staining. The liver mononuclear cells were 82-92% of donor origin. The staining was done by gating on donor iNKT cells and quantitating cytokine producing cells. Thirteen percent of the iNKT cells from WT-WT liver were IL-4 positive and 43% were IFN-γ positive. In the WT-VDR KO or VDR KO-WT combination the percentage of IL-4 and IFN-γ producing donor derived iNKT cells was 50% or less than the WT-WT response (Figure **3-9**). The data suggest that a defect in VDR KO hematopoetic cells results in decreased numbers of iNKT cells that repopulate the thymus and periphery of WT mice. In addition, there are hematopoetic and nonhematopoetic effects on iNKT cell development of liver iNKT cells such that either combination WT-VDR KO and VDR KO-WT results in fewer donor derived liver iNKT cells.

Expression of CD1d on DP thymocytes is required for iNKT cell development. Reconstitution efficiency in the reciprocal BM transplants above showed that following BM transplantation 98-99% of the thymocytes were of donor origin. To investigate whether reduced iNKT cell precursor frequency or altered antigen presentation caused

the reduced iNKT cell numbers in VDR KO mice, mixed BM chimeras were performed. CD45.1 WT mice were reconstituted with 1:1 mixture of CD45.1 WT BM and CD45.2 VDR KO BM. Approximately 50% of the thymocytes were from the CD45.2 VDR KO BM (Figure **3-7**C). The frequency of iNKT cells derived from VDR KO BM (CD45.2, 0.075%) was comparable to that from WT BM (CD45.1, 0.073%) and the total of the CD45.1 and CD45.2 iNKT cells (0.15 %) was the same as shown when WT BM was transplanted into WT or VDR KO mice (Figure **3-7**A). Analysis of the spleen and liver showed a 50/50 split in each of the donor populations in the peripheral tissues as well (Figure **3-8**). The data suggest that antigen presentation may be responsible in part for the defect in iNKT cells in the VDR KO mice.

3.2.7 VDR mediated CD1d expression in the thymus.

Thymoctyes from VDR KO and WT mice were used as stimulators of the CD1d restricted NKT cell hybridoma. As expected WT thymocytes stimulated the NKT cell hybridoma to produce IL-2 (Fig **3-10**A). The use of VDR KO thymocytes as stimulators of the NKT cell hybridoma resulted in significantly less IL-2 production (Figure **3-10**A). Notably, the frequency of CD1d positive as well as the mean fluorescence intensity of CD1d expression was significantly lower on VDR KO thymocytes compared to WT thymocytes (Figure **3-10**A&B). The intracellular distribution of CD1d in WT and VDR KO thymocytes was determined using lysosome-associated membrane protein-1 (LAMP-1) as a marker. The degree of CD1d colocalization with LAMP-1 was comparable in VDR KO and WT thymocytes (Figure **3-11**A). To determine whether increasing CD1d

expression in VDR KO thymocytes would have the cells act like WT thymocytes, a retroviral vector that expresses CD1d was used to infect WT and VDR KO thymocytes and produce additional CD1d (Figure **3-10**C). Retroviral expression of CD1d had no effect (compared to infection with the empty vector) on the stimulatory ability of WT thymocytes for the NKT cell hybridoma (Figure **3-10**C). Conversely, retroviral expression of CD1d in the VDR KO thymocytes upregulated CD1d expression and induced higher IL-2 production from the NKT cell hybridoma (Figure **3-10**C). The CD1d transfected VDR KO thymocytes were still not as good as WT thymocytes for stimulation of the NKT cell hybridoma.

DC were purified from the spleens of WT and VDR KO mice and CD1d expression was measured and shown to be similar on the WT and VDR KO DC (Figure **3-10**D). CD1d expression on hepatocytes from WT and VDR KO mice was also shown to be similar (Figure **3-11**B). αGalCer or control pulsed DCs were used as APC for the NKT cell hybridoma. Media pulsed DC did not induce IL-2 production while both the WT and VDR KO DC pulsed with αGalCer were good and equivalent stimulators of NKT cell derived IL-2 (Figure **3-10**E). Lastly, iNKT cells were purified from VDR KO and WT mice and cultured with Rag KO splenocytes as APC. Rag KO mice do not have NKT cells and therefore αGalCer treatment of Rag KO splenocytes did not induce cytokine production (data not shown). iNKT cells from WT mice were induced to produce IL-4 and IFN-γ following culture with Rag KO splenocytes and αGalCer (Figure **3-10**F). Equal numbers of VDR KO iNKT cells produced significantly less IFN-γ than the WT iNKT cells and undetectable IL-4 (Figure **3-10**F).

3.3 Discussion

The data demonstrate a critical role for the VDR in directing maturation of iNKT cells. In the absence of the VDR, both thymic and peripheral numbers of iNKT cells are significantly diminished because the iNKT cells are halted at a stage prior to the final step in maturation. iNKT cell development largely occurs in the thymus with the final step in maturation (NK1.1 expression) occurring in both the thymus and the periphery (181). This process has been shown to depend on the transcription factor T-bet and T-bet KO mice have iNKT cells that are blocked at the $CD44^{high}/NK1.1^{-}$ stage (61). The iNKT cells in the VDR KO mouse fail to express T-bet. Other factors are important in iNKT cell development including IL-15 and relB; however, IL-15 KO and relB KO mice show blocks at earlier stages of iNKT cell development (67, 174). CD122 expression was normal on the VDR KO iNKT cells suggesting that like the T bet KO iNKT cells, VDR KO iNKT cells are responsive to IL-15 but NK1.1 negative. Expression of the VDR in the hematopoietic compartment is required for T bet expression in iNKT cells and conversion to the NK1.1 expressing mature cell type.

iNKT cells differ from conventional T cells in that resting iNKT cells have transcriptionally active IL-4 and IFN-γ gene loci (182) while conventional T cells do not. Even the precursors of iNKT cells, $NK1.1^{-}$ thymic iNKT cells, transcribe and translate their IL-4 locus (6, 182, 183). It has been suggested that iNKT cells acquire this effector property during thymic development at the stage when they diverge into a distinct lineage from conventional T lymphocytes (184). Not only do VDR KO mice have fewer iNKT cells but the remaining iNKT cells produce less IL-4 and IFN-γ. Therefore, there may be

additional defects at the iNKT cell commitment stage that result in the diminished cytokine production by VDR KO iNKT cells.

The decreased numbers of iNKT cells in the VDR KO thymus are not due to increased cell death or reduced basal proliferation rates. However, in the liver homeostatic proliferation of iNKT cells was less in the VDR KO mouse. WT BM transplantation into the VDR KO mouse resulted in normal numbers of iNKT cells in the thymus and spleen but significantly fewer in the liver (compared to WT recipients). The failure of WT iNKT cells to repopulate the liver of VDR KO mice is likely due to decreased homeostatic proliferation and suggests that a signal important in this process is missing in the VDR KO liver.

Reciprocal bone marrow transfers suggested that the VDR must be expressed in BM for normal positive selection of iNKT cells in the thymus. However, the precursor frequency for iNKT cells was not different in the VDR KO thymus as determined by competitive BM transplantation; since the percentage of VDR KO and WT iNKT cells in the repopulated WT thymus were the same. In addition, VDR expression did not affect Vα14-Jα18 rearrangement since the Vα14-J18α transcript levels are comparable between VDR KO and WT $TCR^{low/-}$CD1d-αGalCer-DP thymoctyes (data not shown). We therefore conclude that the iNKT cell precursor frequency is not different in the VDR KO and WT mice.

Positive selection of iNKT cells is mediated by CD1d-expression on DP thymocytes (1). Analysis of CD1d expression and function showed lower surface CD1d expression and impaired antigen-presentation by VDR KO thymocytces. CD1d transfected VDR KO thymocytes were better than VDR KO thymocytes but still not as

good as WT thymocytes for stimulation of the NKT cell hybridoma. It seems possible that other factors important for endogenous ligand loading onto CD1d (Niemann-Pick type C2 protein) might be defective in the VDR KO thymus (185). CD1d endosomal trafficking was not altered in the VDR KO mice. Reduced surface expression and function of CD1d in the VDR KO thymus is not a global result of the VDR on CD1d expression since the peripheral DC from VDR KO mice had normal CD1d expression and functioned normally as antigen presenting cells. In addition, iNKT cells develop normally in the CD1d heterozygous mice despite reduced CD1d in the thymus and the periphery (186, 187).

The VDR is a member of the steroid hormone superfamily of nuclear receptors that act as ligand inducible transcription factors. The ligand for the VDR is the active form of vitamin D, which is a nutrient/hormone with profound effects on the immune system. The absence of the VDR leads to diminished iNKT cell numbers in both the thymus and the periphery. The remaining iNKT cells don't fully mature, express NK1.1 or T-bet. VDR deficiency resulted in reduced thymic CD1d expression that impaired the ability of thymocytes but not peripheral DC to act as APC. The defects in VDR KO iNKT cells stem from both intrinsic and extrinsic requirement for VDR expression in the developmental pathway of iNKT cells.

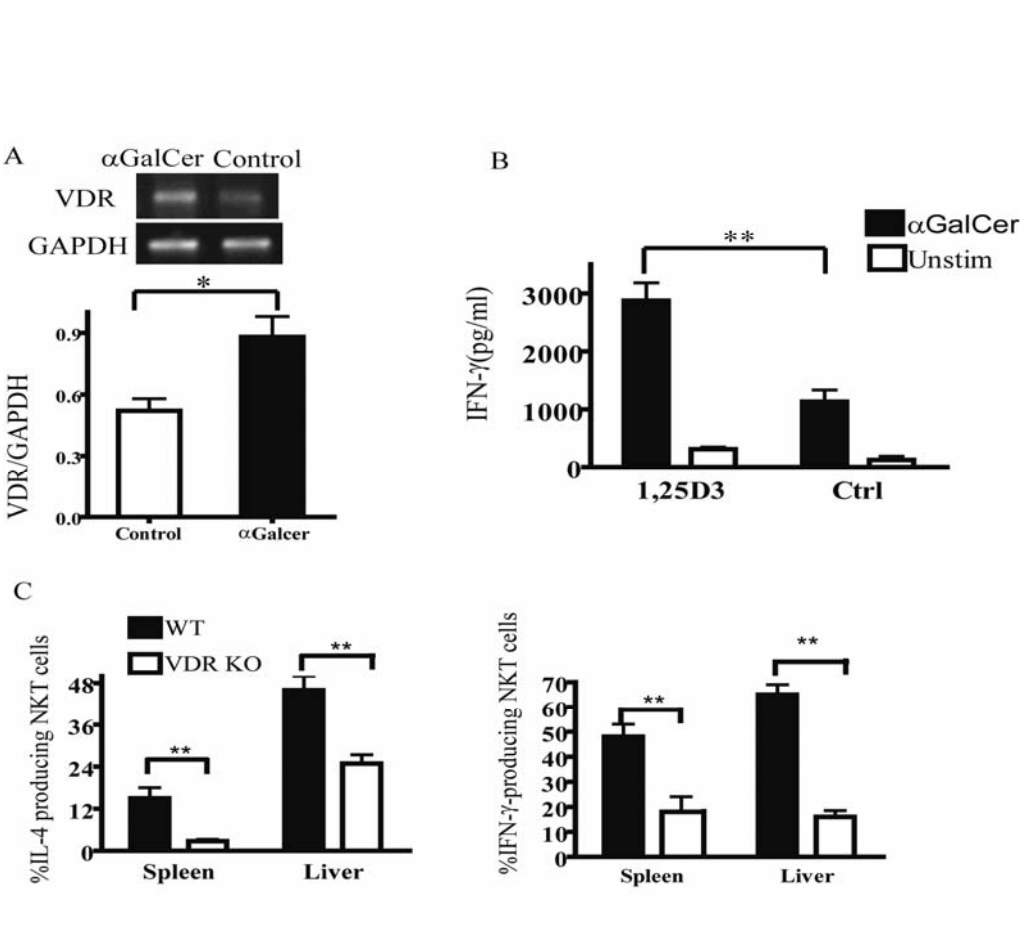

Figure **3-1**: VDR expression and cytokine production by the VDR and $1,25(OH)_2D_3$. (A) RT-PCR analysis of VDR expression in WT thymocytes and iNKT cells. Purified iNKT cells were stimulated with αGalCer or media alone (control) and VDR expression was measured by RT-PCR. The upper panel shows agarose gel electrophoresis of the products of the VDR and GAPDH PCR. Graph shows the mean ± SEM of two independent experiments (*p<0.05). (B) Splenocytes from either WT mice treated *in vitro* with 100 nM $1,25(OH)_2D_3$ (1,25D3) or control treated (equivalent amount of ethanol, Ctrl) and stimulated with αGalCer. Unstimulated splenocytes did not produce IFN-γ (Unstim). Graphs represent mean ± SD from triplicate samples. Data are one of three individual experiments (**, p<0.01). (C) Pooled data showing the percentage of cytokine-producing iNKT cells. Graphs represent mean ± SEM of three independent experiments. Left panels show percentage of IL-4-producing iNKT cells. Right panels show IFN-γ-producing iNKT cells (**p<0.01)

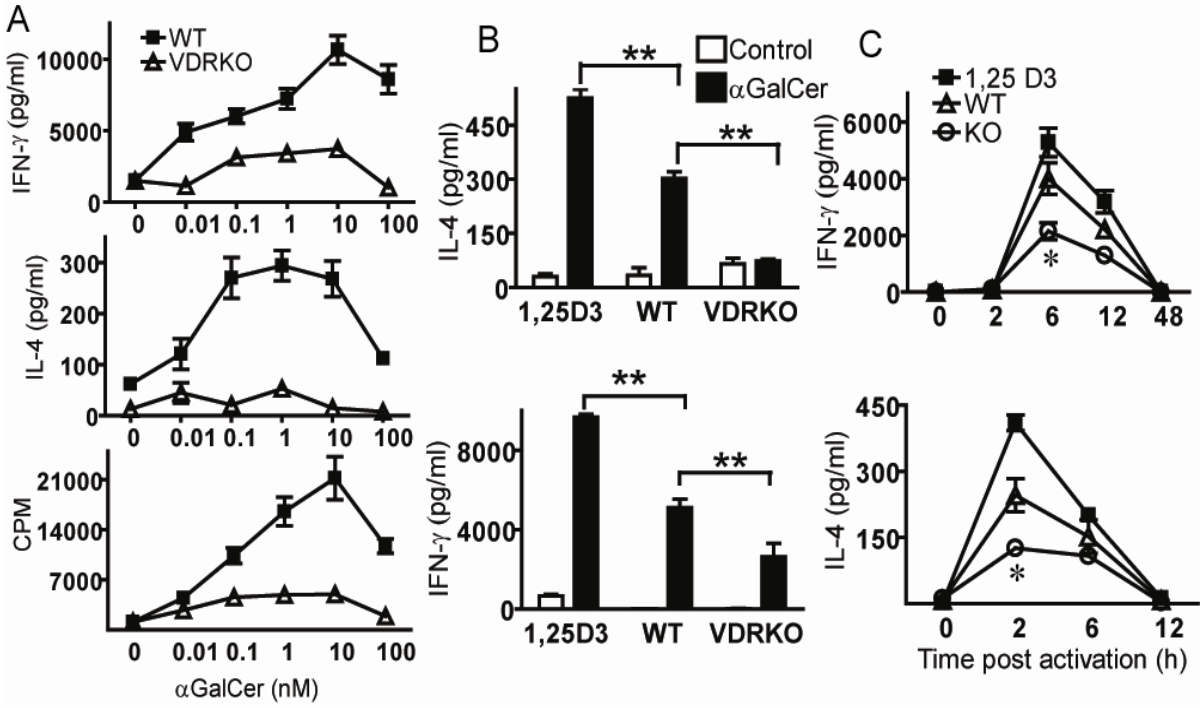

Figure **3-2**: iNKT cells from VDR KO mice are hyporesponsive. (A) Splenocytes from WT and VDR KO mice were stimulated with different concentrations of αGalCer *in vitro*. IFN-γ, IL-4 and proliferation were measured. Graphs represent mean ± SD from triplicate samples. Data is one representative experiment of three. (B) *In vitro* IL-4 and IFN-γ production by splenocytes stimulated with 10nM αGalCer. The splenocytes were from $1,25(OH)_2D_3$ fed WT (1,25D3), WT and VDR KO mice. Data is from the mean ± SEM of 3 experiments. (C) Serum cytokine production in WT and VDR KO mice induced by systemic administration of αGalCer. Levels of IFN-γ and IL-4 in the serum were determined at different times following injection (n=15 per group. *p<0.05, **p<0.001).

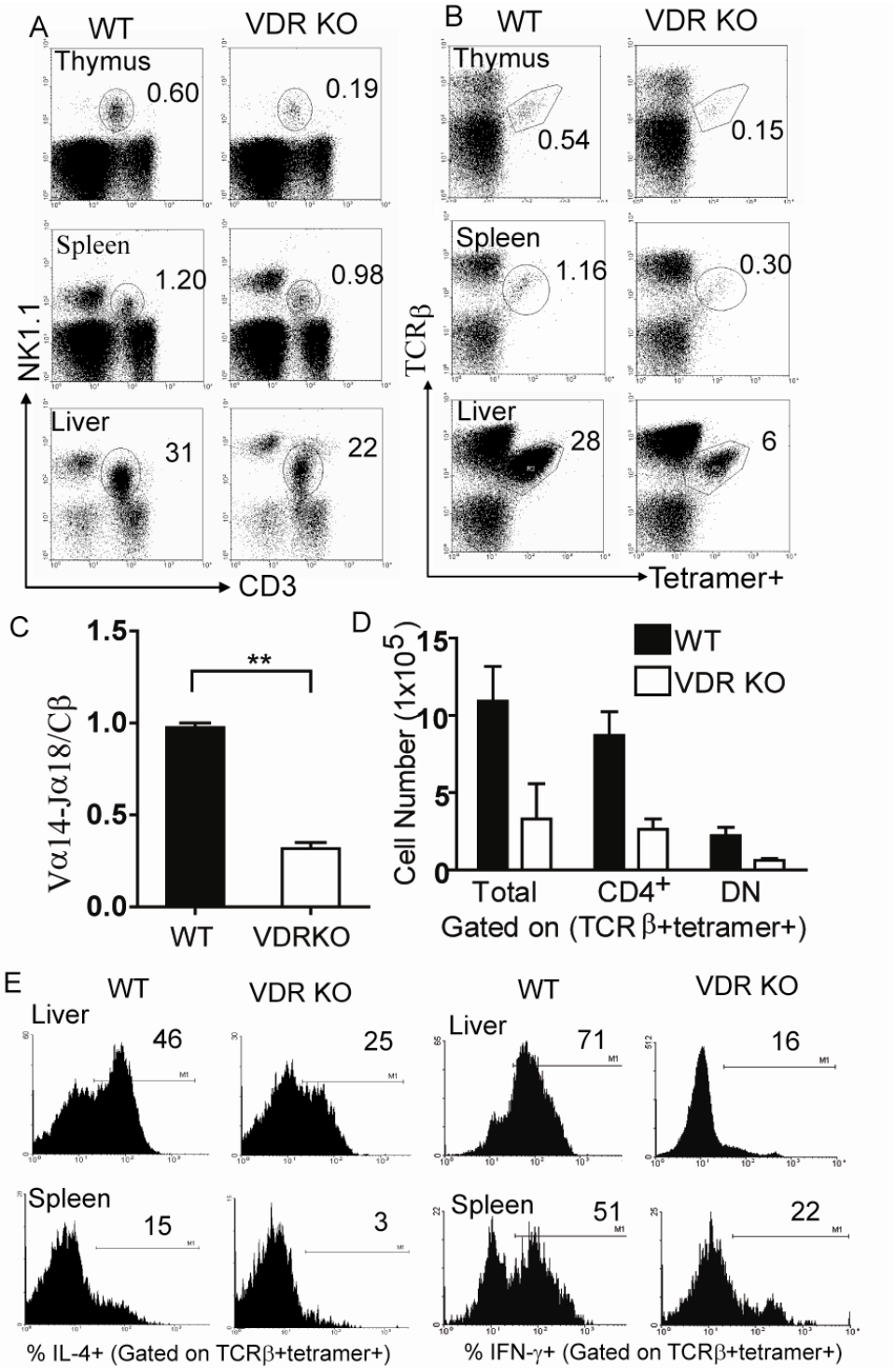

Figure **3-3**: Reduced NKT cell development in VDR KO mice. (A) Dot plots showing percentage of NK1.1 and CD3 positive NKT cells in VDR KO and WT controls. Data show one representative of 25 mice per group. Numbers indicate percentage of NK1.1 and CD3 double positive cells. Figure **3-4** shows the means ± SEM for NK1.1 and CD3 double staining (B) Dot plot showing iNKT cells (TCRβ and CD1d-αGalCer double

positive). Data show one representative of 20 mice per group. Numbers indicate percentage of iNKT cells in the circled gate. (C) Real-time PCR analysis of the TCR Vα14-Jα18 expression in WT and VDR KO mouse thymocytes. Expression of the TCR constant β chain was used as an amplification control. Data are the mean of three independent experiments ± SEM. **$p<0.001$. (D) The absolute number of total iNKT, $CD4^+$ iNKT and DN iNKT (TCRβ and tetramer double positive) cells in the spleen were compared between WT and VDR KO mice (n=15). (E) Frequency of cytokine-producing iNKT cells in WT and VDR KO mice. Mice were injected with αGalCer in vivo followed by intracellular cytokine staining ex vivo as described in the methods. Histograms show production of IFN-γ and IL-4 by iNKT cells. Data from one representative of 10 mice.

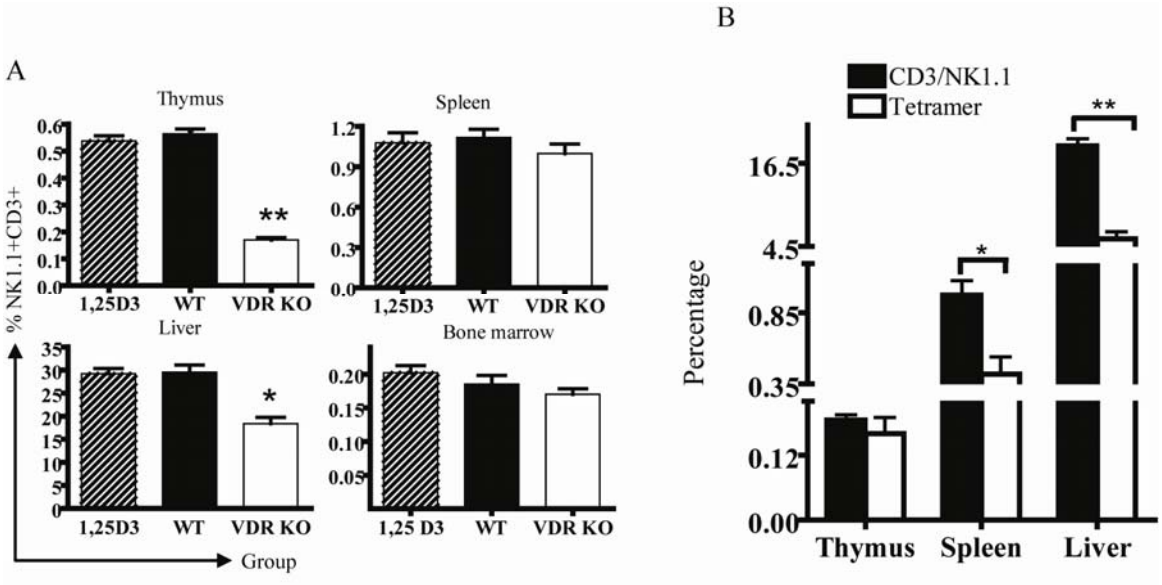

Figure **3-4**: Comparison of NKT and iNKT cell development. (A) Pooled data of iNKT cell percentage in different organs from 1,25D3, WT, VDR KO mice. Values are the mean ± SEM of 25 individual mice per group (* $p<0.05$; ** $p<0.01$). (B) Comparison of the NK1.1/CD3^{+} double positive and TCRβ/tetramer positive staining in the VDR KO host (* $p<0.05$; ** $p<0.01$).

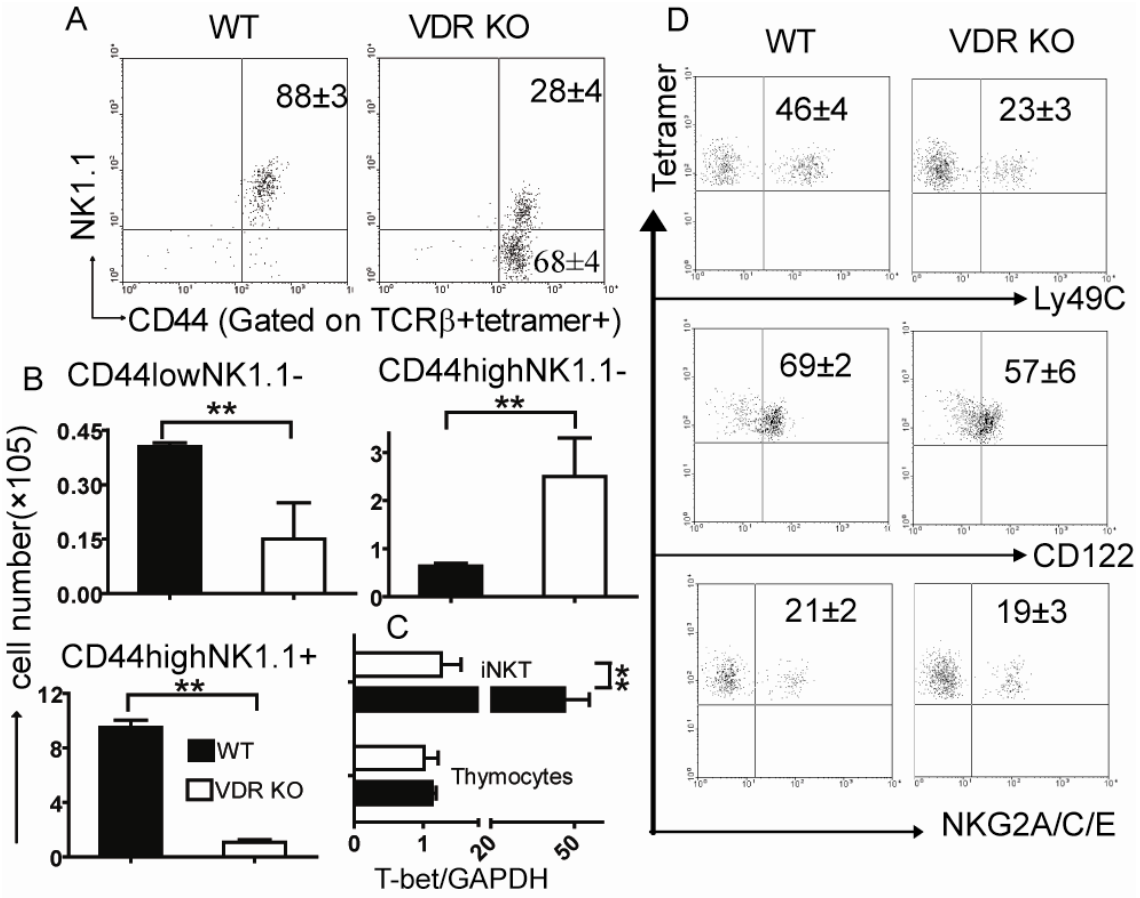

Figure **3-5**: VDR KO iNKT cells fail to mature fully. (A) Dot plots showing expression of CD44 and NK1.1 on TCRβ and CD1d-αGalCer tetramer double positive thymocytes. Data from one representative of 10 mice is shown. (B) Absolute numbers of $CD44^{low}NK1.1^{-}$, $CD44^{high}NK1.1^{-}$ and $CD44^{high}NK1.1^{+}$ iNKT cells in the thymus of WT and VDR KO mice (n=10). (C) Real-time PCR analysis of T-bet expression in thymocytes and thymic iNKT cells from WT and VDR KO mice. Expression of GAPDH was used as an amplification control. Data are the mean of two independent experiments ± SEM. (**, p<0.001). (D) Tetramer positive thymocytes were analyzed for Ly49C, CD122, and NKG2A/C/E expression. Data from one representative of 6 mice is shown.

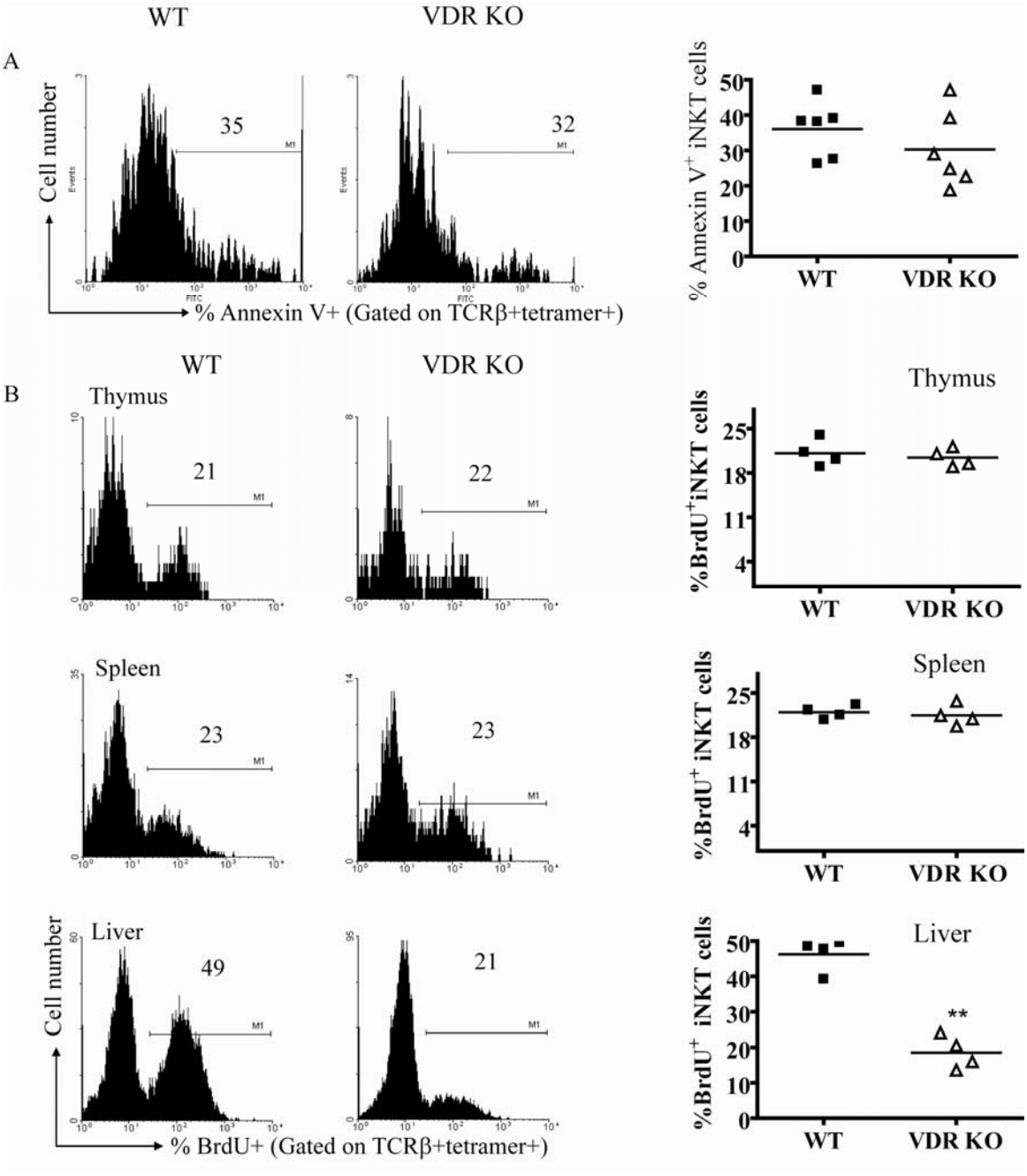

Figure **3-6**: Normal iNKT cell apoptosis and homeostatic proliferation in the absence of the VDR. (A) Annexin V staining of iNKT cells from the thymus of WT and VDR KO mice. The data is one representative of six mice per group. Graphs in the right panels show the 6 individual values and the mean (line) of the data. (B) BrdU incorporation by iNKT cells from WT and VDR KO mice on day 9. Histograms of one representative of 4 mice per group are shown. Right panels show the individual values and the mean (line) of the data (*p<0.05).

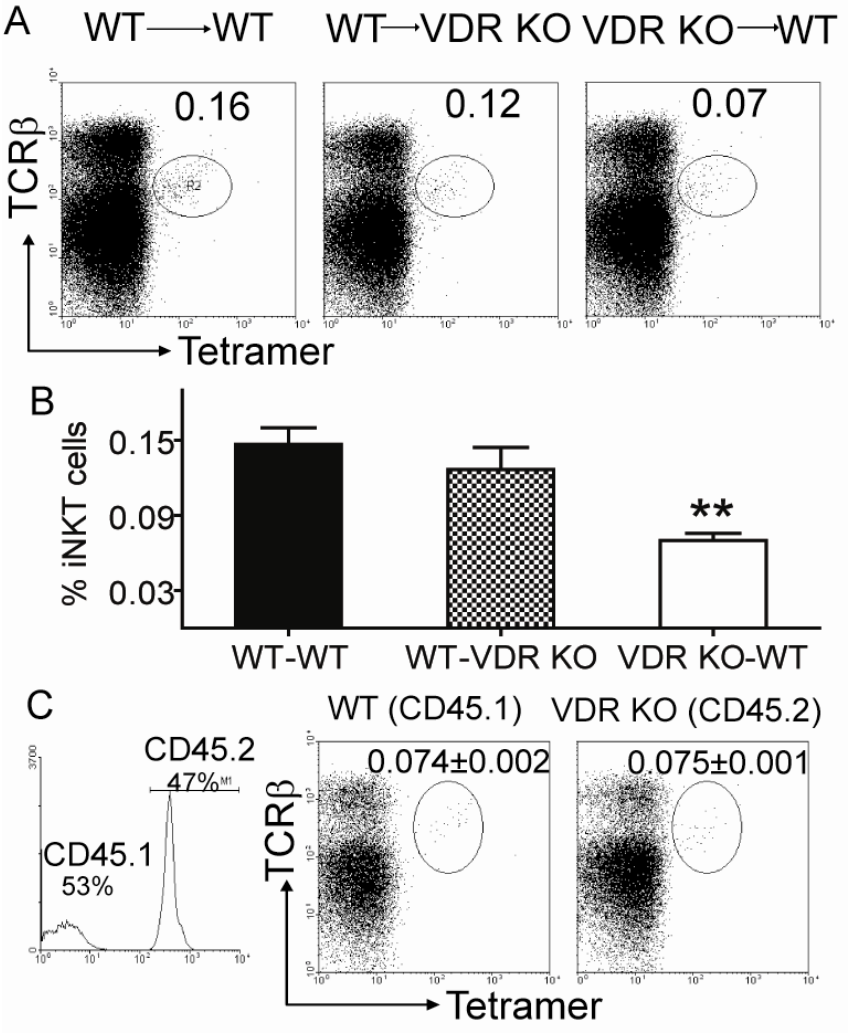

Figure **3-7**: iNKT cell development requires VDR expression in the hematopoetic compartment. (A) Reciprocal BM transplants were done using WT (CD45.1) and VDR KO (CD45.2) mice (donor BM→recipient). The success of the transplant was determined by staining for donor cells in the recipient mice. Percentage of donor iNKT cells in the thymus is shown. Data shown is one representative of 4 mice per group and two independent experiments. Figure **3-8** shows the percentage of donor iNKT cells in liver and spleen of the recipient mice (mean ± SEM of 8 mice). (B) Pooled data showing the % of donor iNKT cells recovered in the thymus of reciprocal BM transplantation experiments. Data are the mean ± SEM of 8 mice (**, $p<0.01$). (C) Competitive BM chimeras were generated using a 1:1 ratio of WT CD45.1 and VDR KO CD45.2 BM and WT CD45.1 recipients. Thymocyte chimerism was checked by flow cytometry (left) and shown to be 52.7% of WT CD45.1 origin and 47.3% of VDR KO CD45.2 origin. The data shown is from one representative of five mice (mean ± SEM) and one of two experiments.

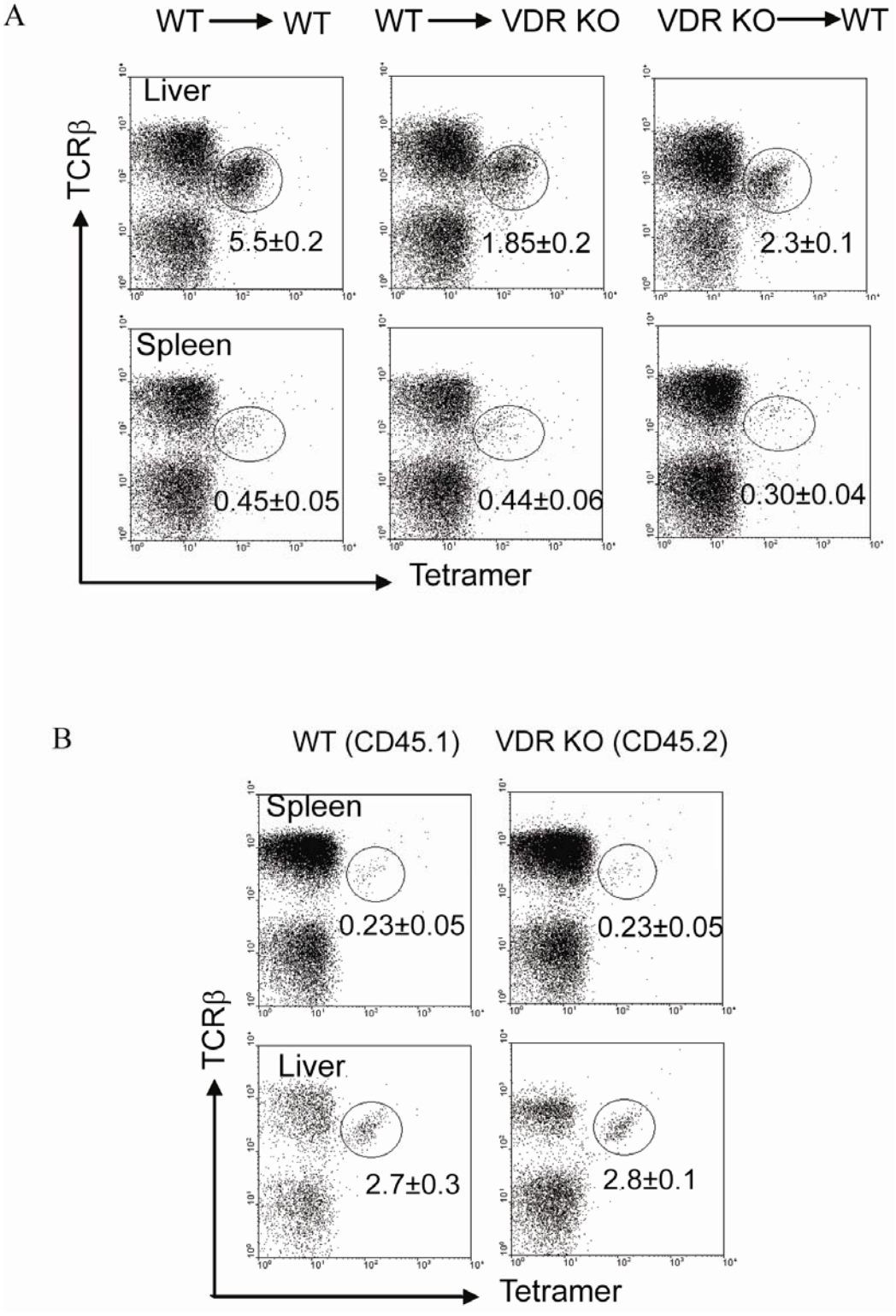

Figure **3-8**: BM reconstitution of the spleen and liver of transplanted mice. (A) Dot plots

show percentage of donor iNKT cells in the liver and spleen of recipient animals. Data are one representative of four mice per group of two independent experiments (donor BM →recipient, n=8 total). Values are the mean ± SEM. Values in the liver of WT-VDRKO and VDR KO-WT are significantly less than WT-WT ($p<0.05$). Values in the spleen of WT-WT recipients are significantly higher than VDR KO-WT recipients ($p<0.05$). (B) Percentage of iNKT cells of each of the 2 donor phenotypes in the liver and spleen of recipient WT mice. The data shown are from one representative of five mice and one of two independent experiments. Values are the mean ± SEM.

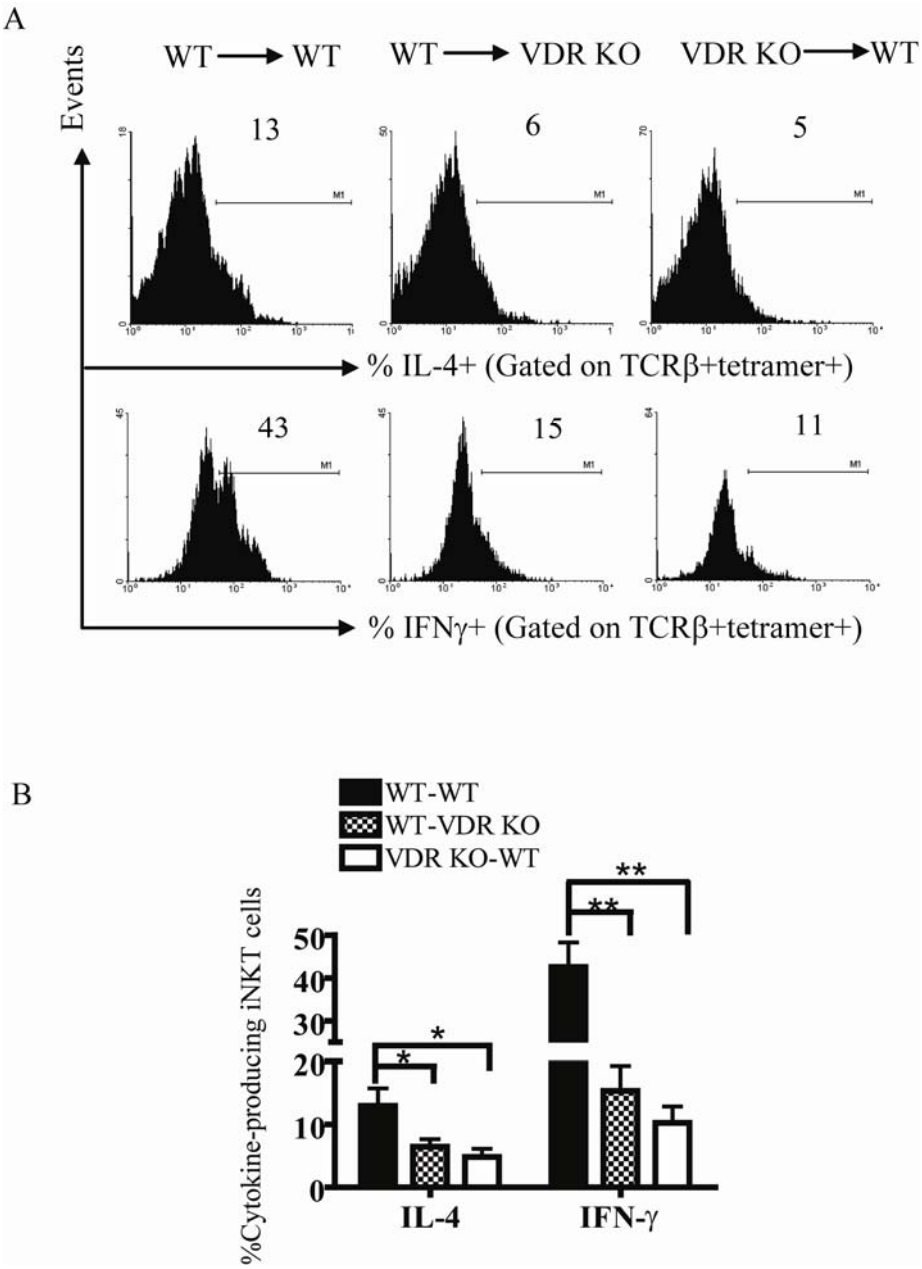

Figure **3-9**: Functional analysis of transplanted iNKT cells in recipient mice. (A) Frequency of cytokine-producing iNKT cells. Mononuclear cells from the liver of recipient mice were stimulated *in vitro* with αGalCer and intracellular staining was performed as described in the methods. Histograms show percentage of IL-4 (upper panels) and IFN-γ (lower panels) by iNKT cells of donor origin. Data shown are one representative of four mice per group and one of two individual experiments. (B) Pooled data of cytokine-producing iNKT cells. Graphs represent mean ± SEM of two independent experiments (donor BM-recipient). (* $p<0.05$; ** $p<0.01$).

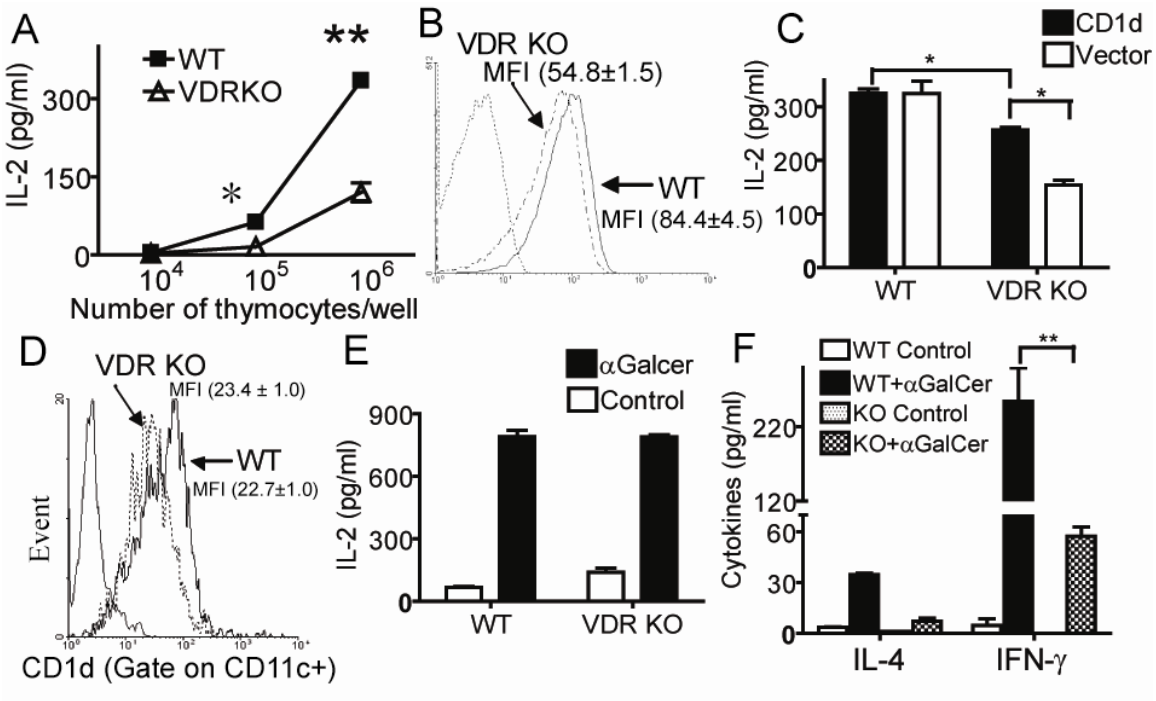

Figure **3-10**: Low CD1d expression and reduced antigen presentation in the thymus of VDR KO mice. (A) Equal numbers of the CD1d-restricted hybridoma NKT cell were incubated in the presence of different concentrations of thymocytes from WT and VDR KO mice and IL-2 production by the NKT cell hybridoma was measured. Results shown are from one representative of four independent experiments. (B) Thymocytes isolated from WT and VDR KO mice were analyzed by flow cytometry for CD1d expression. Mean fluorescent intensity (MFI) ± SEM. Data shown are from one representative of 15 mice. (C) Thymocytes from WT and VDR KO mice were infected with a CD1d-expressing retrovirus (dark bar) or empty vector (blank bar) and used to stimulate the NKT cell hybridoma for IL-2 production. Graph represents the mean ± SD from triplicate samples. Data shown are one individual experiments of two (*p<0.05). (D) CD1d expression on purified VDR KO and WT DC (MFI ± SEM). (E) Purified DC from WT and VDR KO spleens were pulsed with αGalCer or media alone and cocultured with CD1d-restricted hybridoma cells for IL-2 production. Graph represents the mean ± SD from triplicate samples. Data shown are one representative experiment of two. (F) iNKT cells from WT and VDR KO (KO) mice were purified and cultured with Rag KO splenocytes in media only (Control) or with αGalCer. Graph represents the mean ± SD from triplicate samples (**p<0.001).

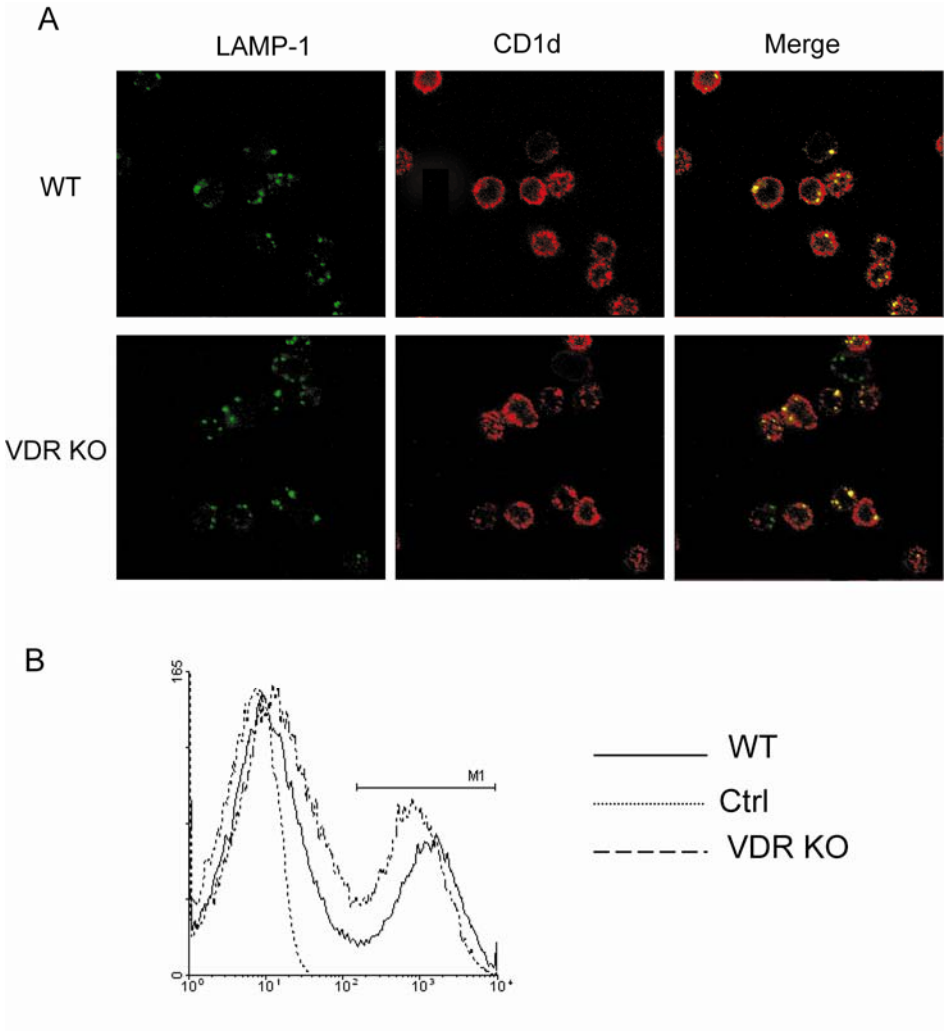

Figure **3-11**: Co-localization of CD1d and LAMP-1 and CD1d expression on hepatocytes. (A) WT and VDR KO thymocytes were analyzed for colocalization of LAMP-1 and CD1d by confocal fluorescence microscopy. Data shown are from one representative experiment of three. (B) Expression of CD1d on WT and VDR KO hepatocytes.

Chapter 4

$1,25(OH)_2D_3$ is required for iNKT cell development

4.1 Introduction

iNKT cells are a separate lineage of T cells which have a few unique characteristics including the expression of NK cell lineage markers, the constitutive expression of activation markers and greatly restricted TCR diversity (16). The iNKT cells have an identical TCRα chain (Vα14-Jα18 in mouse) which pairs with variable Vβ8, Vβ7, and Vβ2 chains (16, 188). This TCR contributes to specificity to the non-MHC-encoded self molecule CD1d. In comparison to classic MHC molecules, CD1d binds and presents conserved self and foreign glycolipid antigen.

In addition the iNKT cells function distinctly. A case in point is that they secrete large quantities of various cytokines rapidly after activation through the TCR (16). The rapid secretion of cytokines, the conserved usage of TCRβ and the ability to modulate indirectly the function of a variety of cell types have brought about the appreciation that iNKT cells bridge innate and adaptive immune responses (189). The wide range of cytokines including IL-4 and IFN-γ produced by iNKT cells enables them to function seemingly opposingly in distinct immune responses. For example, iNKT cells have been shown to increase immune responses to tumors, while they also have been demonstrated to prevent autoimmunity (17).

The cellular stages of iNKT cell development have been well-studied and use of a tetramerized version of CD1d loaded with glycolipid allows for the direct and precise

detection of iNKT cells (79, 190, 191). iNKT cells diverge from conventional T cells at the DP stage (6, 13). As iNKT cell precursors progress into mature iNKT cells, they undergo three distinct stages of development. Different from conventional T cells, immature iNKT cells (lacking NK1.1 and CD44 expression, "stage 1") remain in the thymus, immediately go through several rounds of cell division, and activate basal transcription of IL-4 (6). This expansion is followed by the up-regulation of CD44 and the β chain of the IL-15 receptor (CD122) which requires the expression of the transcription factor T-bet ("stage 2") (12, 48). A fraction of stage 2 iNKT cells remain in the thymus and differentiate into stage 3 while the rest of them egress from thymus to peripheral tissues where they up-regulate the expression of a variety of NK cell lineage receptors including NK1.1, NKG2D, CD94 and Ly49 isotypes ("stage 3") (44, 47, 48). Peripheral iNKT cells reside in the liver, spleen, lymph nodes, bone marrow, lung, and gut, each of which has about the same number of iNKT cells. The homeostasis of iNKT cells is regulated by IL-15 (59, 60).

Vitamin D ingested from the diet is inactive and converted to 25-hydroxy vitamin D3 ($25(OH)D_3$) in the liver. $25(OH)D_3$ is the major circulating form of vitamin D_3. The second hydroxylation is catalyzed by the 25-hydroxyvitamin D_3-1α-hydroxylase (1α-hydroxylase) encoded by the CYP27B1 gene (192-196). The second hydroxylation occurs primarily in the kidney to form active vitamin D or 1,25dihydroxyvitamin D_3 ($1,25(OH)_2D_3$). The activity of 1α-hydroxylase in the kidney is tightly regulated by distinct factors including parathyroid hormone (PTH) and calcium levels. PTH, calcitonin, low calcium level, and low phosphate levels induce the expression of 1α-hydroxylase,

while $1,25(OH)_2D_3$, high calcium, and high phosphate inhibit the 1α-hydroxylase expression.

$1,25(OH)_2D_3$ binds to the vitamin D receptor (VDR) and plays an important role in immune responses in addition to its classic function of maintaining calcium and phosphate homeostasis. Immune cells express the VDR and $1,25(OH)_2D_3$ has been shown to affect both innate and adaptive immune cells. $1,25(OH)_2D_3$ enhances regulatory T cell differentiation and IL-4 production by T helper (Th)2 cells. $1,25(OH)_2D_3$ has been shown to inhibit maturation and differentiation of dendritic cells. Moreover, we have shown that the VDR is required for both iNKT cell development and function (197). However it is not known whether $1,25(OH)_2D_3$ plays a role in iNKT cell development.

Heterozygous mice were used as breeders and fed vitamin D sufficient diets; therefore, both wild type (WT) and Cyp27B1 knockout (KO) littermates were exposed to $1,25(OH)_2D_3$ in utero and during lactation. At weaning the Cyp27B1 KO and WT littermates were continued on vitamin D sufficient diets. Since Cyp27B1 KO mice don't express the enzyme (1α-hydroxylase), they are not able to produce the active form of vitamin D ($1,25(OH)_2D_3$ deficient) while WT mice can.

Cyp27B1 KO mice had fewer iNKT cells. However, in contrast to the VDR deficient mice, the remnant iNKT cells are fully mature and function normally. The reduced numbers of iNKT cells in Cyp27B1 KO mice is not due to increased cell death rates. Bone marrow transplants suggest that the presence of $1,25(OH)_2D_3$ is required for precursors of iNKT cells.

4.2 Results

4.2.1 Selective defect of iNKT cell development in Cyp27B1 KO mice

TCRβ and αGalCer loaded-CD1d tetramer were used to specifically identify iNKT cells. Cyp27B1 KO mice had significantly lower frequency of iNKT cells in the thymus, spleen, and liver in comparison to WT littermates (Figure **4-1**). There was 0.50% of iNKT cells in WT thymus, whereas only 0.17% of iNKT cells were found in 1,25(OH)2D3-deficient thymus with similar trend in the liver. $1,25(OH)_2D_3$-deficient mice had 6% iNKT cells in the liver in comparison to 25% in the WT mice. This is similar to what is seen in the VDR KO mice (Figure **4-1**), and these mice had 0.15% of iNKT cells in the thymus. Consistent with the decreased percentage of iNKT cells in the Cyp27B1 KO mice, the absolute numbers of iNKT cells were significantly reduced compared with WT littermates as well (data not shown). It should be noted that Cyp27B1 KO mice had normal numbers of other lymphocyte population in the thymus, spleen, and liver including T cells, B cells, NK cells, dendritic cells and macrophage. This result indicates that $1,25(OH)_2D_3$ deficiency selectively affected iNKT cell numbers.

4.2.2 Normal function of iNKT cells in the absence of $1,25(OH)_2D_3$

To look at the function of iNKT cells in the absence of $1,25(OH)_2D_3$, WT and Cyp27B1 KO mice were injected i.p with αGalCer, and production of IL-4 by liver iNKT cells was analyzed ex vivo at the single cell level by intracellular staining. The frequency of IL-4-producing iNKT cells was similar in Cyp27B1 KO compared to WT mice (Figure

4-2). Around 61 ± 4 % of WT iNKT cells and 64 ± 3 % of Cyp27B1 KO iNKT cells produced IL-4. This is different from what was seen in the VDR KO mice (Figure **4-2**). The VDR KO mice have a significantly lower fraction of IL-4-producing iNKT cells than WT mice or Cyp27B1 KO mice did (Figure **4-2**). The result indicated that $1,25(OH)_2D_3$-deficient iNKT cells functioned normally, although there were fewer iNKT cells found in $1,25(OH)_2D_3$-deficient mice.

4.2.3 iNKT cells develop and mature normally in the absence of $1,25(OH)_2D_3$

During iNKT cell development in the thymus, iNKT precursors that express Vα14-Jα18 TCR interact with CD1d expressed on double positive (DP) thymocytes and are selected to enter the iNKT cell lineage (44). To investigate whether the reduced numbers of iNKT cells result from altered antigen-presentation by CD1d, CD1d expression on thymoctyes was examined. As shown in Figure **4-3**A, Cyp27B1 KO thymocytes had lower levels of expression of CD1d in comparison to that from WT mice. In order to determine antigen-presentation function in Cyp27B1 KO mice, thymocytes from Cyp27B1 KO and WT mice were used as stimulator of the iNKT cell hybridoma and IL-2 production was analyzed by ELISA. Thymocytes from Cyp27B1 KO mice were as potent as that from WT mice to stimulate the production of IL-2 by the iNKT cell hydridoma (Figure **4-3**B). The data suggest that although Cyp27B1 KO thymocytes exhibit lower levels of CD1d, their antigen-presentation function is normal. The reduced numbers of iNKT cells were not a result of impaired antigen-presentation in Cyp27B1 KO mice.

As iNKT cell undergo the process of maturation, they progress through $CD44^{low}$ and $CD44^{high}$ stages and finally up-regulate the expression of NK1.1 molecules. The expression of other NK lineage marker including CD122, Ly49C, and NKG2/A/C/E are also up-regulated (44, 61, 198). Expression of CD44/NK1.1 on thymic and splenic iNKT cell was analyzed to determine the maturation of iNKT cells in Cyp27B1 KO mice. The data showed that the percentages of immature and mature iNKT cells from thymus and spleen were comparable between Cyp27B1 KO and WT littermates (Figure **4-4**A). In addition, Cyp27B1 KO iNKT cells successfully up-regulated the expression of CD122, Ly49C, and NKG2/A/C/E (Figure **4-4**B).

4.2.4 Normal cell death rate in Cyp27B1 KO littermates

To investigate whether the reduced numbers of iNKT cells in $1,25(OH)_2D_3$-deficient mice were due to increased cell death rate, thymocytes from WT and Cyp27B1 KO mice were isolated. Apoptosis of thymic iNKT cells was measured by staining with annexin V, TCRβ and CD1d-αGalCer tetramer. The results showed that the percentage of annexin V positive iNKT cells in Cyp27B1 KO mice was equivalent to that in WT mice. In WT mice, about 36% of iNKT cells were annexin V positive. Similar results were observed in Cyp27B1 KO mice, showing about the same amounts of iNKT cell undergoing apoptosis in Cyp27B1 KO and WT mice. The data indicated that decreased numbers of Cyp27B1 KO iNKT cells were not a result of increased cell death rates (Figure **4-5**).

4.2.5 Intrinsic defect of iNKT cell precursors in the absence of $1,25(OH)_2D_3$

To study whether the defective iNKT cell development were intrinsic or related to environmental defects, reciprocal bone marrow transplants were performed. WT (CD45.1) or Cyp27B1 KO (CD45.2) bone marrow cells were transferred to lethally irradiated WT littermates. 12 weeks later, donor derived iNKT cells were analyzed. As shown in Figure **4-6**, bone marrow cells from WT littermates were able to repopulate iNKT cells in WT mice, while bone marrow cells from Cyp27B1 KO littermates failed to repopulate iNKT cells in WT littermates to the same extent as that from WT littermates. The frequency of iNKT cells derived from Cyp27B1 KO bone marrow cells was 2-3 fold less and significantly lower than that from WT bone marrow cells.

WT mice were lethally irradiated and reconstituted with a 1:1 mix of WT (CD45.1) and Cyp27B1 KO (CD45.2) bone marrow cells. Although WT and Cyp27B1 KO bone marrow were able to repopulate peripheral organs in the competitive chimeras to the same extent as WT couterparts did, iNKT cell derived from Cyp27B1 KO bone marrow were selectively reduced in the thymus, spleen and liver (Figure **4-7**A, B). In the thymus, only about 28% of iNTK cells were derived from Cyp27B1 KO bone marrow while 72% of iNTK cells were from WT bone marrow. Similar results were seen in the spleen and liver of chimeric mice (37% vs 61% in the spleen; 41% vs 60% in the liver; Cyp27B1 KO vs. WT). However, the percentage of $CD4^+$T cells that originated from Cyp27B1 KO bone marrow was equivalent to that from WT bone marrow in all these organs (data not shown). In addition, splenic $CD4^+$T cells derived from Cyp27B1 KO bone marrow were able to produce comparable levels of IL-4 and IFN-γ compared to that

from WT bone marrow, further suggesting that $1,25(OH)_2D_3$ does not play a role in T cell development (Figure **4-8**). $1,25(OH)_2D_3$-deficiency results in a cell-intrinsic and highly specific iNKT cell developmental defect.

iNKT cells derived from Cyp27B1 KO bone marrow produced as much IFN-γ and IL-4 as WT derived cells did upon TCR stimulation. The percentage of iNKT cells derived from WT and Cyp27B1 KO bone marrow that produce either IL-4 or IFN-γ was equvilaent (Figure **4-8**). The result confirms that although Cyp27B1 KO donor bone marrow cells accounted for fewer iNKT cells, the Cyp27B1 KO derived iNKT cells function normally.

4.2.6 Redistribution of Cyp27B1 KO iNKT cells to peripheral lymph nodes

iNKT cells are usually present in the thymus, spleen, and liver, while there are fewer iNKT cells in peripheral lymph nodes. Equivalent or higher percentages of iNKT cells derived from Cyp27B1 KO mice were found in the lymph nodes of the competitive chimeras compared to that from WT (43% Vs 58% on average) (Figure **4-7**B). Furthermore, iNKT cells derived from Cyp27B1 KO bone marrow cells showed significantly higher levels of expression CD62L than that by WT counterparts, which is a homing receptor for high endothelial venules in lymph nodes (Figure **4-9**). The difference in percentage of cells expressing CD62L between Cyp27B1 KO derived iNKT cells and WT derived iNKT cells was most pronounced in the thymus (12.4% vs 1.9%, on average), but also significantly different in spleen and lymph nodes. Collectively, the data suggest

that decreased numbers of iNKT cells of Cyp27B1 KO origin might be a result of redistribution of iNKT cells toward lymph nodes.

4.3 Discussion

Although $1,25(OH)_2D_3$ and the signaling through the VDR have been shown to be an important regulator of conventional T cell function, the role of $1,25(OH)_2D_3$ in iNKT cell development and function has not been elucidated. We have shown here that the active form of vitamin D3, $1,25(OH)_2D_3$ is required for the development but not the function of iNKT cells. The absence of $1,25(OH)_2D_3$ resulted in reduced numbers of iNKT cells. However, the remaining iNKT cells successfully up-regulated expression of NK1.1, CD122, Ly49C and NKG2A/C/E. In addition, equivalent proportions of cells that are able to produce IL-4 in the absence of $1,25(OH)_2D_3$. Interestingly, $1,25(OH)_2D_3$-deficient iNKT cells accumulated in the lymph nodes rather than in the thymus, liver, and spleen. The reciprocal and chimeric bone marrow transplants showed that the presence of $1,25(OH)_2D_3$ was essential for iNKT cell precursors in the bone marrow. These data collectively show that $1,25(OH)_2D_3$ is a critical regulator of iNKT cell development.

$1,25(OH)_2D_3$ is probably not involved in the positive selection of iNKT since the antigen-presentation function of thymocytes from Cyp27B1 KO mice was normal. In addition, $1,25(OH)_2D_3$-deficient mice did develop many T cells expressing conventional TCRs. Moreover, the remaining iNKT cells in Cyp27B1 KO mice progressed from immature iNKT cells to mature iNKT cells and showed no developmental block. $1,25(OH)_2D_3$-deficiency likely affected the proliferation of iNKT cells in thymus, spleen,

and liver, and reduced numbers of iNKT cells were probably due to decreased turnover of iNKT cells in those organs. Another possibility is that reduced frequency of iNKT cells in the spleens and liver was the result of decreased export of iNKT cells from the thymus in the Cyp27B1 KO mice. However, the decreased export of iNKT cells from thymus to the periphery wouldn't account for the diminished numbers of iNKT cells in the thymus itself.

There was no indication that $1,25(OH)_2D_3$ -deficient iNKT cells were dying as there was comparable proportion of iNKT cells that were annexin V positive between WT and Cyp27B1 KO mice. However, it is possible that there was more cell death since apoptotic cells are rapidly removed from the thymus. Another possibility is that $1,25(OH)_2D_3$-deficient iNKT cells were retained in the thymus because of a failure to express chemokine receptors essential for their retention in the thymus. Moreover, it is demonstrated that $NK1.1^-$ iNKT cells are more prone to emigrate from the thymus than $NK1.1^+$ iNKT cells (12). Since $1,25(OH)_2D_3$-deficient iNKT cells can up-regulate NK1.1 expression, the chances that iNKT cells in Cyp27B1 KO mice leave the thymus more efficiently are slim.

$1,25(OH)_2D_3$-deficient iNKT cells were able to make IL-4 as Cyp27B1 KO iNKT cells undergo maturation process to the extent as WT iNKT cells. $1,25(OH)_2D_3$-deficient iNKT had similar ratios of immature to mature iNKT compared to WT mice. Another possibility is that development and function of iNKT cells are controlled by different gene programs. It has been shown that granulocyte/macrophage colony-stimulating factor (GM-CSF) is required for normal function of iNKT cells but not required for iNKT cell development (62).

The requirement for $1,25(OH)_2D_3$ in iNKT cell development is intrinsic as indicated by reciprocal and chimeric bone marrow transplants. In addition, the data also suggest that the presence of $1,25(OH)_2D_3$ is essential for precursors of iNKT cells as well as for the normal turnover of iNKT cells as discussed above. In mixed bone marrow chimeras, iNKT cells derived from Cyp27B1 KO bone marrow were as frequent as their WT counter-parts in the lymph nodes while they were significantly outnumbered in the thymus. This could be due to the higher levels of the homing receptor CD62L expressed by $1,25(OH)_2D_3$-deficient iNKT cells, suggesting that the reduced numbers of iNKT cells may partly result from redistribution of iNKT cells to lymph nodes. $1,25(OH)_2D_3$ has been shown to enhance the expression of chemokine receptor 10 by T cells while it suppressed gut-homing receptor $\alpha4\beta7$ and CCR9 expression (99).

There are differences in function, maturation, and mechanisms underlying lack of iNTK cells between VDR-deficient and $1,25(OH)_2D_3$-deficient mice. VDR-deficient iNKT cells were hyporesponsive, while $1,25(OH)_2D_3$-deficient iNKT cells were capable of producing cytokines. The remaining iNKT cells in VDR KO mice are stalled at a stage before maturation, while those in $1,25(OH)_2D_3$-deficient mice successfully up-regulated expression of NK1.1 and other NK lineage markers. Bone marrow transplants indicated that VDR affected antigen-presentation and $1,25(OH)_2D_3$ influneced iNKT cell precursors. The disparity between VDR-deficient and $1,25(OH)_2D_3$-deficient mice might be due to the higher levels of $25(OH)D_3$ in the $1,25(OH)_2D_3$-deficient mice. Physiological doses of $25(OH)D_3$ are not effective, but high doses of $25(OH)D_3$ are curative for rickets, probably because of the ability of $25(OH)D_3$ to bind and activate the VDR when present in excess (112). $1,25(OH)_2D_3$-deficient mice were vitamin D sufficient except that they

could not convert $25(OH)D_3$ to $1,25(OH)_2D_3$. Therefore, it is very likely the $1,25(OH)_2D_3$-deficient mice had high levels of $25(OH)D_3$ and high levels of $25(OH)D_3$ bound to and activated the VDR.

$1,25(OH)_2D_3$ is the active form of vitamin D and functions through the VDR. Here we revealed a new essential role of $1,25(OH)_2D_3$ in iNKT cell development. We show that $1,25(OH)_2D_3$-deficiency results in reduced iNKT cell numbers. The reduced numbers of iNKT cells are likely due to decreased cell turnover and redistribution of iNKT cells to lymph nodes. The requirement for $1,25(OH)_2D_3$ in iNKT cell development is cell intrinsic. Overall, our findings pinpoint a critical role of $1,25(OH)_2D_3$ in iNKT cell development.

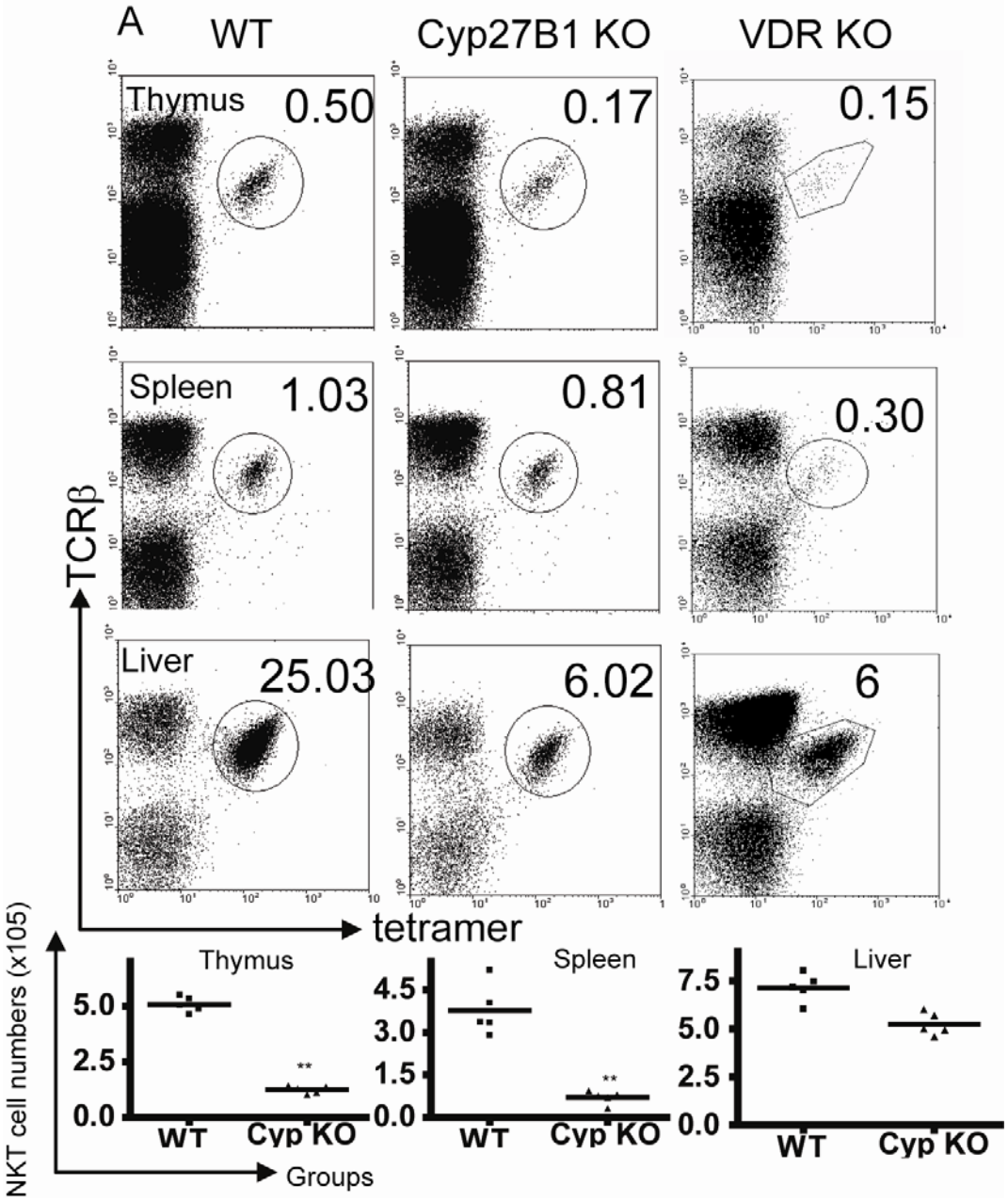

Figure **4-1**: Reduced numbers of iNKT cells in Cyp27B1 KO mice.(A) The frequency of iNKT cells in the thymus, spleen and liver. iNKT cells were gated (as shown) as $TCR\beta^{+}$ αGalCer-CD1d $tetramer^{+}$. Data are representative of ten mice. (B) Total number of iNKT cells in the thymus, liver and spleen of WT and Cyp27B1 KO mice (n=8).

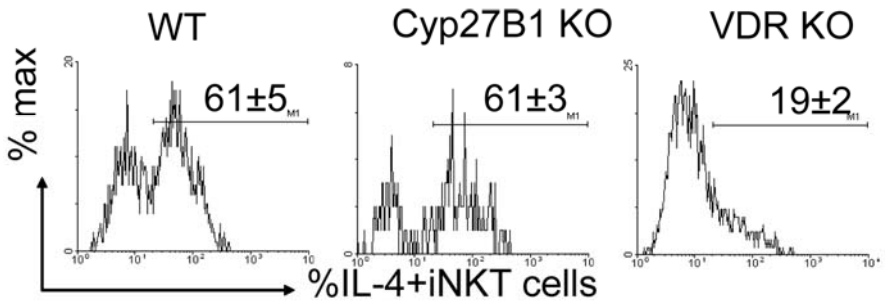

Figure **4-2**: $1,25(OH)_2D_3$-deficient iNKT cells function normally. Mice were injected with αGalCer in vivo followed by intracellular staining ex vivo as described in the methods. Histograms show production of IL-4 by iNKT cells from one representative of 10 mice per group. Mean ± SEM values percentage of IL-4 positive iNKT cells.

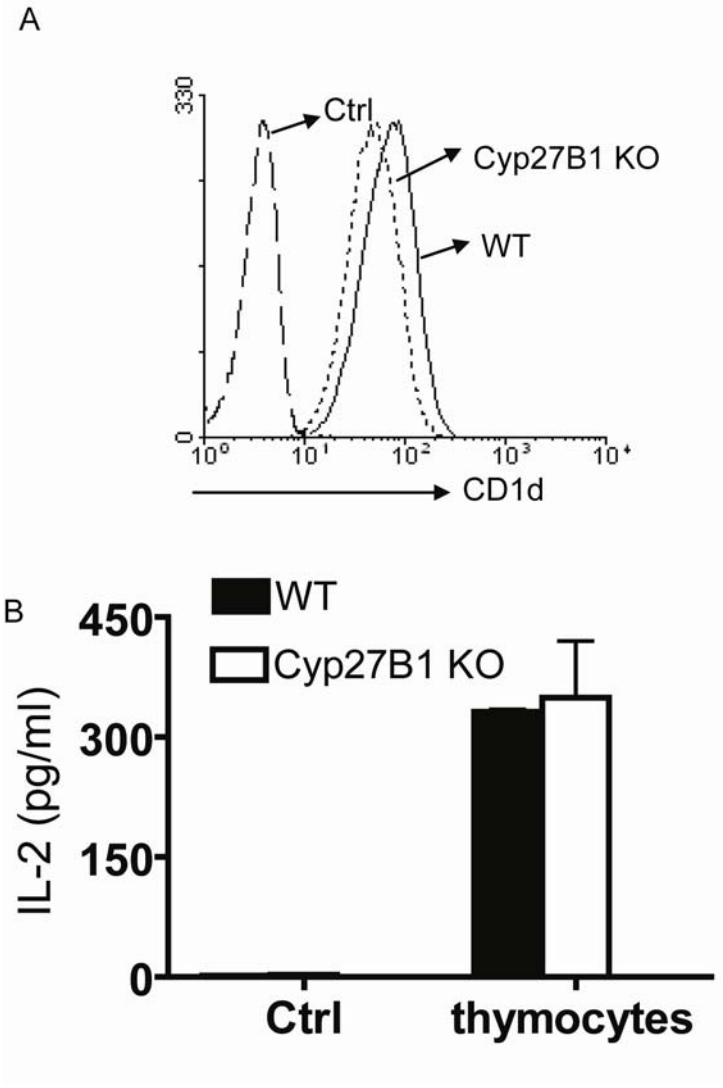

Figure **4-3**: Normal antigen-presentation in $1,25(OH)_2D_3$-deficient mice. (A) Thymocytes isolated from WT and Cyp27B1 KO mice were analyzed by flow cytometry for CD1d expression. Data shown are from one representative of 7 mice. (B) Equal numbers of the CD1d-restricted NKT hybridoma cell were incubated in the presence of thymocytes from WT and Cyp27B1 KO mice and IL-2 production by the NKT cell hybridoma was measured. Results shown are from one representative of three independent experiments.

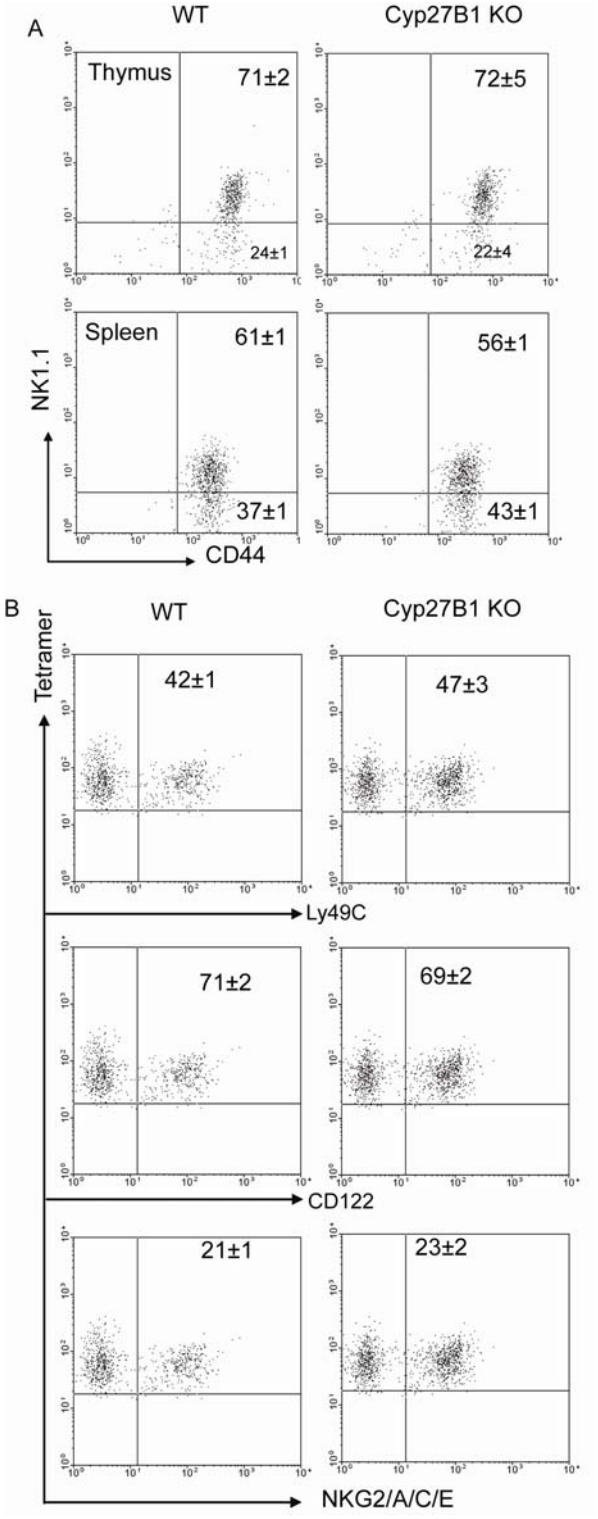

Figure **4-4**: Cyp27B1 KO iNKT cells mature fully. (*A*) Dot plots showing expression of CD44 and NK1.1 on TCRβ and CD1d-αGalCer tetramer double-positive thymocytes and

splenocytes. Data shown are results from 10 mice pooled together (means ± SEM). (B) Tetramer-positive thymocytes were analyzed for Ly49C, CD122, and NKG2A/C/E expression. Data shown are data pooled from 8 mice (means ± SEM).

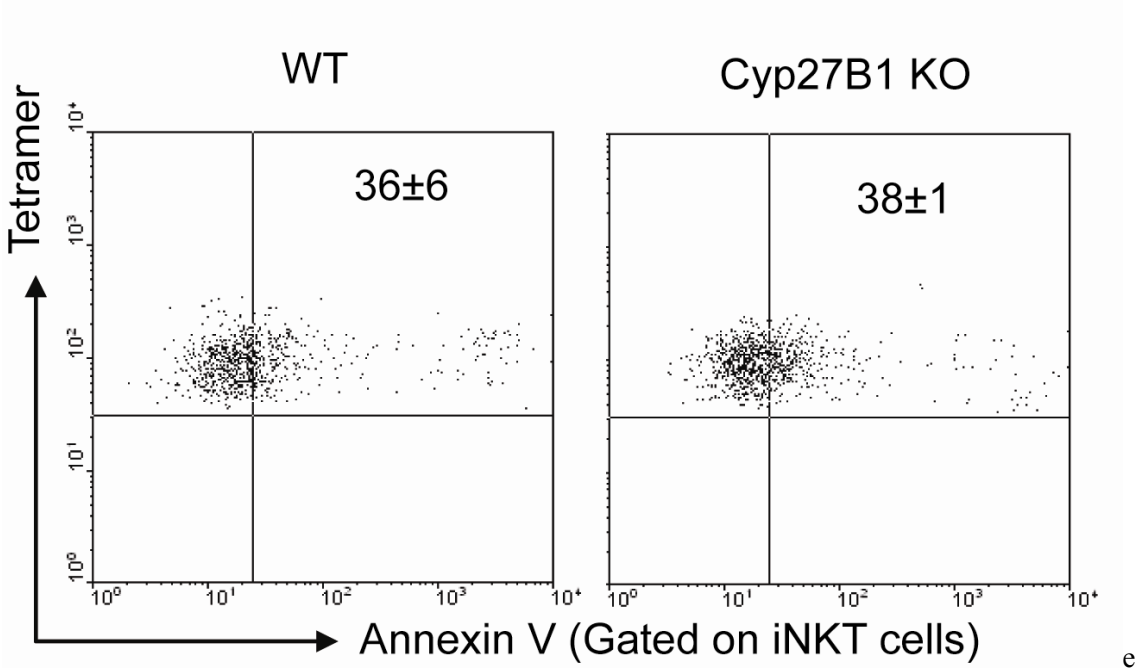

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Figure **4-5**: Cyp27B1 KO mice had normal cell death. Dot plots showing expression of annexin V on TCRβ^{+}tetramer^{+} thymocytes. Pooled data from 7 mice are shown (mean ± SEM).

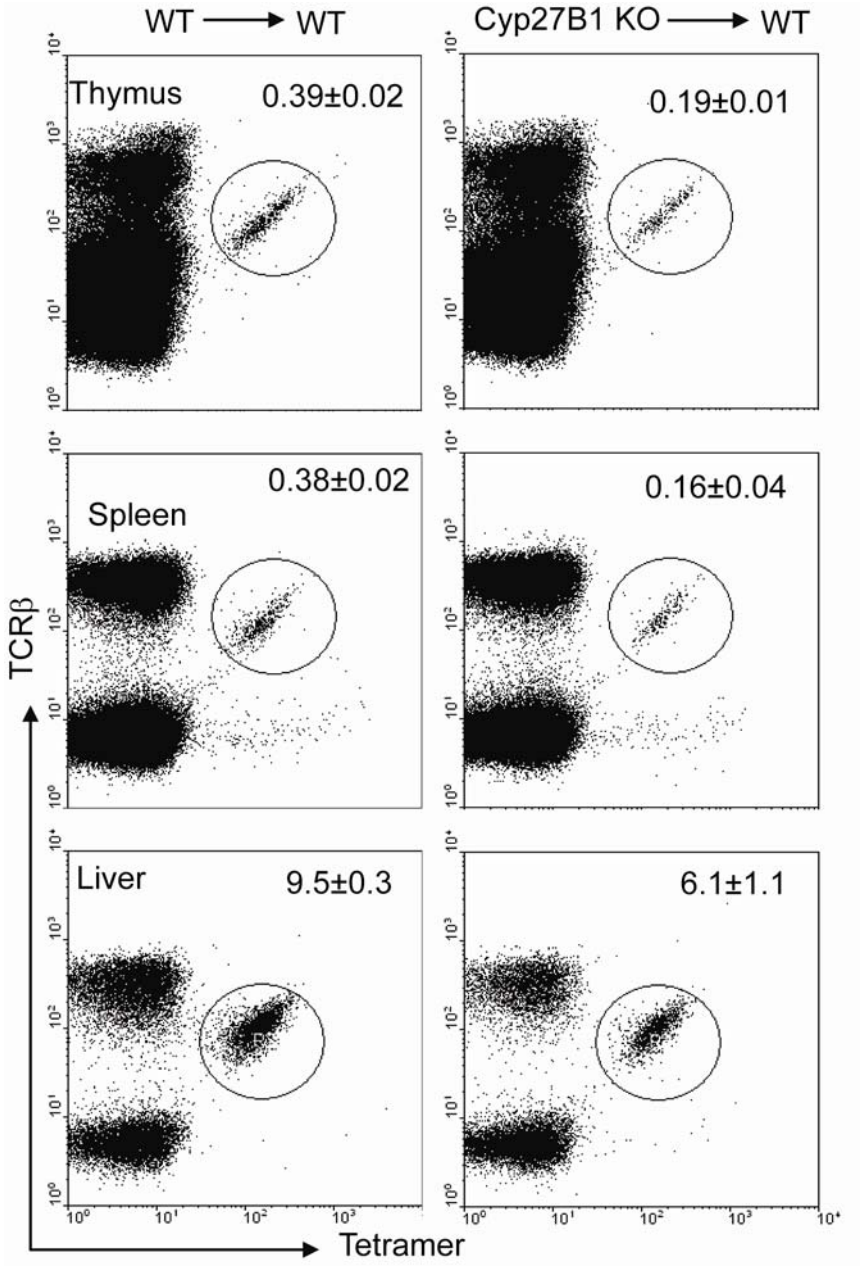

Figure **4-6**: $1,25(OH)_2D_3$ is required for hematopoetic cells. Reciprocal BM transplants were done using WT (CD45.1) and Cyp27B1 KO (CD45.2) mice (donor BM→recipient). Percentage of donor derived iNKT cells in the thymus and spleen and liver is shown. Data shown are pooled data of 5 mice (means ± SEM).

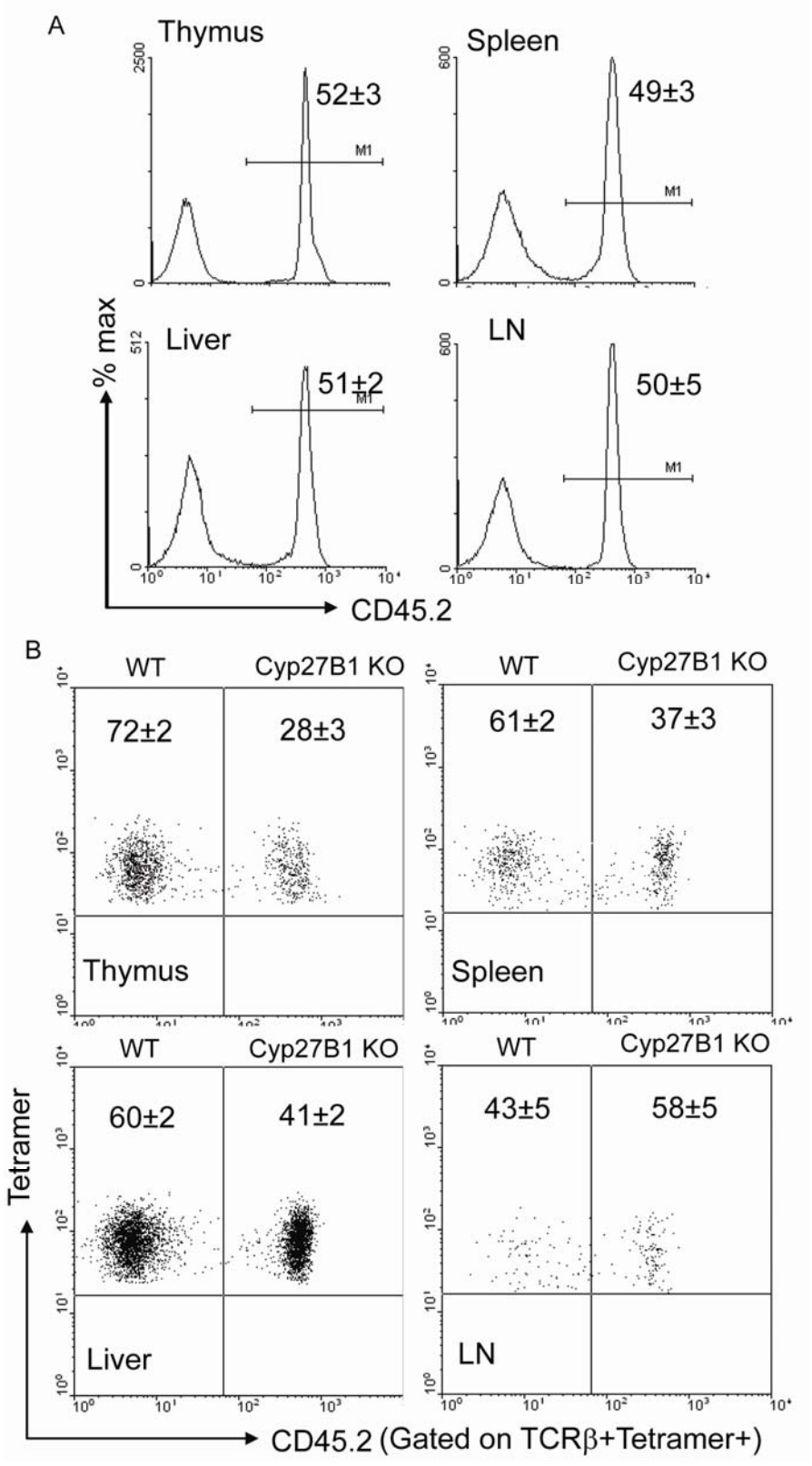
A
Thymus
52±3
M1
Spleen
49±3
M1
Liver
51±2
M1
LN
50±5
M1
% max
CD45.2
B
WT
Cyp27B1 KO
72±2
28±3
Thymus
61±2
37±3
Spleen
60±2
41±2
Liver
43±5
58±5
LN
Tetramer
CD45.2 (Gated on TCRβ+Tetramer+)

Figure **4-7**: Intrinsic defect of Cyp27B1 KO iNKT cells. Competitive BM chimeras were generated using a 1:1 ratio of WT CD45.1 and Cyp27B1 KO CD45.2 BM and WT CD45.1 recipients. (A) Lymphocyte chimerism was checked by flow cytometry (left) and shown to be half of WT CD45.1 origin and half of Cyp27B1 KO CD45.2 origin. Data shown is one representative of 4 mice per group and two independent experiments. (B) Dot plots showing percentage of donor-derived iNKT cells in the thymus, spleen, liver, and lymph nodes. The mean shown is from 5 mice (mean ± SEM).

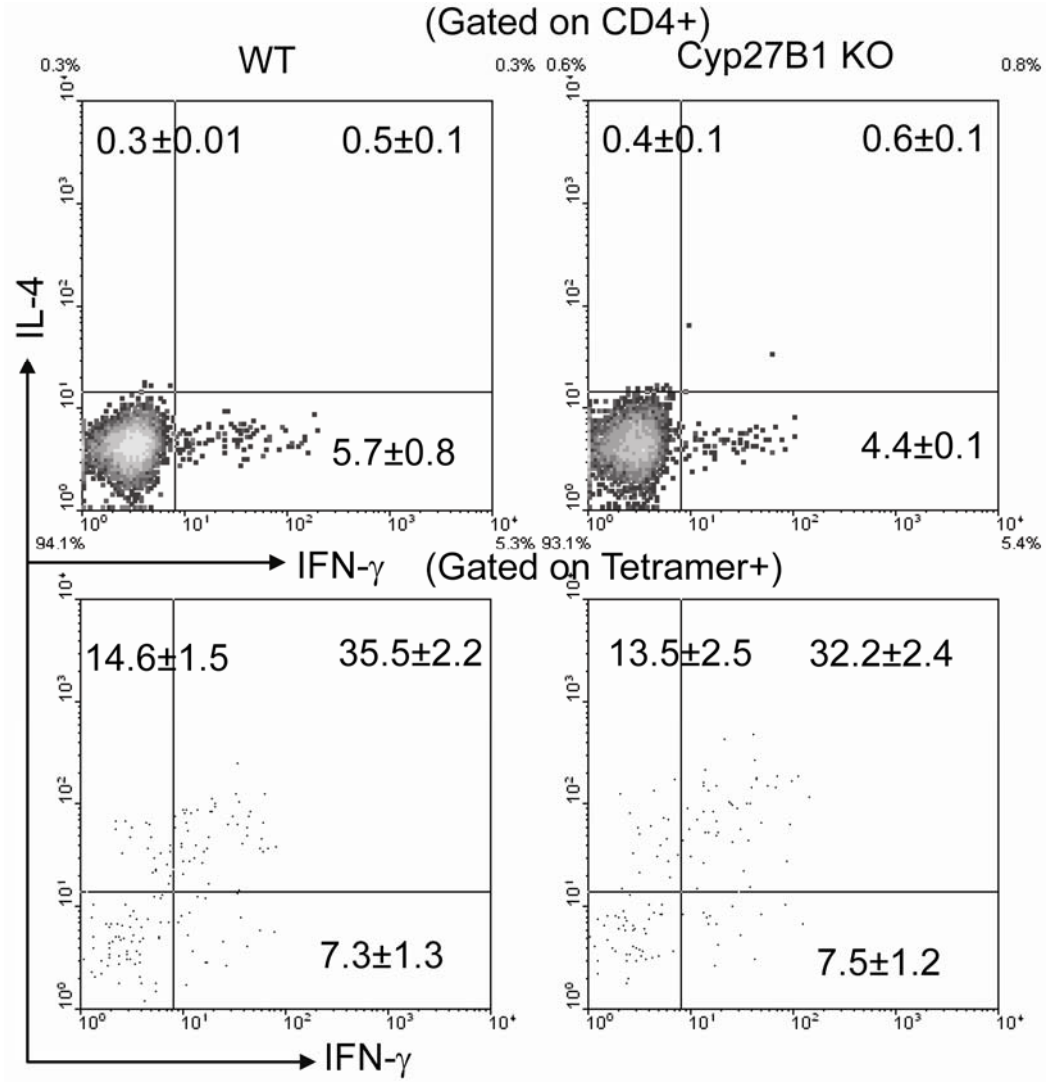

Figure **4-8**: T and iNKT cell derived from Cyp27B1 KO BM were as potent as WT counterparts in producing cytokines when they were activated. Competitive BM transplants were generated as in Figure **4-7**. Whole splenocytes isolated from chimeric recipients were stimulated with PMA and ionomycine followed by intracellular staining as described in methods. Dot plots showing percentage of cytokine-producing $CD4^+$ T and $tetramer^+$ cells of donor origin. Data shown is pooled from 5 mice (means ± SEM).

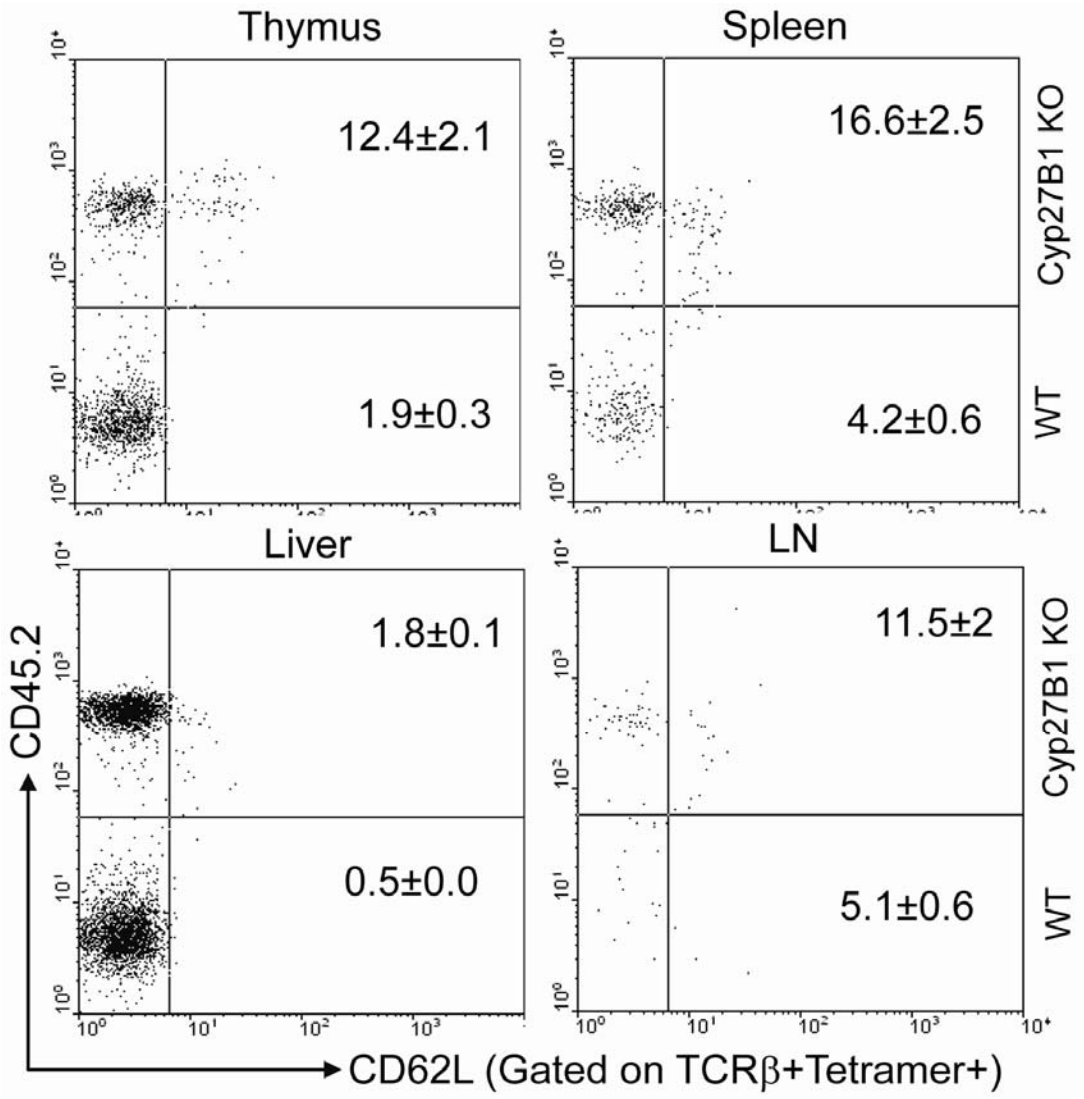

Figure **4-9**: Redistribution of Cyp27B1 KO iNKT cells to lymph nodes. Competitive BM chimeras were generated as in Figure **4-7**. Expression of CD62L on TCRβ$^+$tetramer$^+$ cells was analyzed by flow cytometry. Dot plots showing percentage of CD62L$^+$ iNKT cells. Data shown is pooled from 5 mice (means ± SEM)

Chapter 5

Early exposure to $1,25(OH)_2D_3$ is required for normal iNKT cell development

5.1 Introduction

NKT cells constitute a separate lineage of lymphocytes, characterized by the co-expression of NK cell surface markers and TCR. The majority of mouse NKT cells express a semi-invariant TCR consisting a Vα14-Jα18 rearrangement and are selected through interaction with CD1d molecules on DP thymocytes (44, 172, 199-204). These NKT cells are called iNKT cells and play important roles in immune regulation, tumor surveillance, and host defense against pathogens (15). Their TCR structure combines preferentially Vα14-Jα14 with Vβ8, Vβ7, or Vβ2 (205, 206). Different from the invariant TCRα chain, these TCRβ chains show a great diversity of CDR3 junctions allowing for the recognition of the self antigen, the microbial cell wall α-glycuronosylceramides, and their mimic α-GalCer (90, 91, 207, 208). In addition, it is reported that a fraction of NKT cells are able to recognize mycobacterial phosphatidylinositolmannosides (209). These iNKT cells are present in thymus and peripheral tissues including liver and spleen.

iNKT cells are derived from DP thymocytes (13, 44, 45, 210). They are positively selected by CD1d-expressing DP thymocytes which alone are able to mediate positive selection of iNKT cells (172, 187, 204, 211). Negative selection of iNKT cells can be induced by strong activation signals provided by thymic dendritic cells or DP cortical

thymocytes (54, 187). Ectopic over-expression of CD1d leads to a similar reduction in frequency of $V\beta8^+$ and $V\beta7^+$ iNKT cells (54). In another report, it was shown that thymic selection of $V\beta7^+$ iNKT cells is favored when endogenous ligand concentration or TCRα-chain avidity is suboptimal, suggesting an endogenous ligand-driven negative selection (212).

Understanding of the developmental pathway of iNKT cells has been elusive. The earliest precursor identified by CD1d tetramer so far was a rare, immature heat stable antigen $(HSA)^{high}$, DP^{low} , followed by a $CD4^{high}CD8^{neg}$ stage (14, 16). These $HSA^{high}DP$ and $CD4^+$ stages are not cycling and express a biased Vβ usage (14). iNKT cell precursors then progress to HSA^{low} $CD44^{low}$ thymocytes followed by $CD44^{high}$ memory-type stage cells (6). $CD44^{high}$ NKT cells migrate to the periphery where they increase expression of NK lineage markers including NK1.1, Ly49 and CD94/NKG2A (6, 7, 12). The function of iNKT cells change in parallel with phenotypic events with mature iNKT cells producing both IL-4 and IFN-γ upon TCR stimulation (6, 12).

Vitamin D is a fat soluble vitamin with its role in calcium and phosphorus homeostasis well characterized (94). Food-derived or skin-released vitamin D is inactive and is transported to liver where it is converted to $25(OH)D_3$, the major circulating form. $1,25(OH)_2D_3$ is the active form of vitamin D and produced in the kidney, catalyzed by the enzyme 1α-hydroxylase, the product of CYP27B1 gene (192, 193, 195, 213). $1,25(OH)_2D_3$ exerts its functions through binding to the VDR, a nuclear receptor.

One of the non-classical roles of vitamin D is as an important regulator of immune response. Vitamin D has been shown to influence both innate and adaptive

immune response. In addition, immune cells express the VDR. We showed previously that the VDR is required for iNKT cell development and function (197). In the previous chapter, we show that $1,25(OH)_2D_3$-deficiency resulted in decreased numbers of iNKT cells while the remaining iNKT cells functioned normally. However, it is not clear what the effect of vitamin D deficiency will have on iNKT cell development.

In this study, Cyp27B1 KO/+ breeders were feed vitamin D-deficient diets as described (214). Weaning mice continued on vitamin D-deficient diet. So the littermates (WT and Cyp27B1 KO) were dietary vitamin D-deficient (D-). Vitamin D-sufficient WT mice were used as controls (D+). iNKT cells were analyzed at 6-8 week of age.

Here we show that dietary vitamin D-deficiency in utero resulted in dramatically reduced numbers of iNKT cells both in WT and Cyp27B1 KO littermates. Like $1,25(OH)_2D_3$-deficient iNKT cells, iNKT cells from vitamin D-deficient WT and Cyp27B1 KO mice were able to produce the same amount of cytokines compared to vitamin D-sufficient WT mice. Unlike the VDR-deficient or $1,25(OH)_2D_3$ -deficient mice, iNKT cells were blocked at a stage before they up-regulate CD44 expression. Analysis of precursors showed that iNKT cell precursors underwent more apoptosis. Finally, $1,25(OH)_2D_3$ feeding from day 1 after birth for 6-7 weeks failed to fully rescue iNKT cell development in D- mice. The data collectively pinpoint a critical role of $1,25(OH)_2D_3$ in iNKT cell development suggesting that early exposure to $1,25(OH)_2D_3$ is critical for normal iNKT cell development.

5.2 Results

5.2.1 Diminished response of vitamin D-deficient iNKT cells to αGalCer stimulation in vivo

To investigate the function of D- iNKT cells, WT and Cyp27B1 KO littermates were pulsed by i.p injecting with αGalCer. IL-4 and IFN-γ production in the serum was analyzed at different time points using vitamin D-sufficient WT mice (D+) as control. IFN-γ production by D+ WT mice peaked at 6 hours after injection and then disappeared by 48 hours (Figure **5-1**). The kinetics of IL-4 production was different from that of IFN-γ. Peak production of IL-4 (2h) was generated earlier than that of IFN-γ (6h). Both D- Cyp27B1 KO and D- WT mice made very low levels of both IL-4 and IFN-γ (Figure **5-1**). However, unexpectedly D- WT littermates didn't produce much IL-4 and IFN-γ either. The levels of IL-4 and IFN-γ produced by D- WT and D- Cyp27B1 KO mice were equivalent. The data showed that vitamin D-deficient mice produced extensively low amount of IL-4 and IFN-γ in response to αGalCer in vivo.

5.2.2 Equivalent frequency of IFN-γ-producing iNKT cells from vitamin D-deficient mice

To further investigate the function of iNKT cells from vitamin D-deficient mice, D+ WT, D- WT, and D- Cyp27B1 KO littermates were i.p injected with αGalCer, and IFN-γ production was assessed ex vivo by intracellular staining as described in methods. The D- WT or D- Cyp27B1 KO mice had the same percentage of IFN-γ secreting cells as

D+ WT mice (Figure **5-2**). The data demonstrated that iNKT cells from vitamin D-deficient mice were able to successfully produce IFN-γ.

5.2.3 Dramatic reduced iNKT cell numbers from vitamin D-deficient mice

Lymphocytes from the thymus, spleen and liver were isolated and analyzed for iNKT cell numbers by flow cytometry. The frequency of iNKT cells from vitamin D-deficient mice was dramatically reduced in the thymus and liver, while there were comparable percentages of iNKT cells in the spleen compared to vitamin D-sufficient WT mice (Figure **5-3)**. In the vitamin D-deficient thymus, there was only 0.08% of iNKT cells as compared to ~0.6% of vitamin D-sufficient WT (~8 fold reduction), while in the liver 1.65% to 27% (~15 fold reduction). In addition, there was no difference in iNKT cell frequency between D- WT and D- Cyp27B1 KO littermates. A similar trend was observed when absolute numbers were examined. It is notable that vitamin D-deficient mice had normal numbers of T, B, NK, and macrophages (Table **5-1**). The data showed that iNKT cell development was severely and specifically impaired in vitamin D-deficient mice. Since there was no difference in function and development of iNKT cells between D- WT and D- Cyp27B1 KO littermates, D- WT and D- Cyp27B1 KO mice will be discussed as vitamin D-deficient mice. In addition, the absence of cytokine production in vitamin D-deficient mice demonstrated that the lower frequency of iNKT cells were due to the few numbers of iNTK cells but not due to the down-regulation of TCR expression by iNKT cells in vitamin D-deficient mice.

The percentage of $CD4^+$ and DN iNKT cells were determined in the tetramer positive and TCRβ positive population of D+ WT and D- mice. Although defective development of iNKT cells was observed in D- mice, the ratio of $CD4^+$ iNKT cells to DN iNKT cells was similar suggesting that vitamin D-deficiency affected $CD4^+$ and DN iNKT cells equally (Figure **5-4**).

5.2.4 Defective maturation of vitamin D-deficient iNKT cells

In D+ WT mice, as shown in Figure **5-5** A, ~5% of iNKT cells were $NK1.1^-$ $CD44^-$ (stage 1) and ~84% $NK1.1^+CD44^+$ (stage 3). However, there was a significant increase in the percentage of stage 1 iNKT cells (18%) and correspondingly a decrease in the percentage of stage 3 iNKT cells (~74%) from D- mice. Because of the overall iNKT cell deficiency in D- mice, all three stages of iNKT cells were significantly reduced when absolute numbers of different iNKT cells were examined in comparison to D+ WT mice.

As iNKT cells mature, expression of Ly49C, CD122 and NKG2A/C/E is also up-regulated. iNKT cells from D- mice up-regulate expression of Ly49C similar to WT mice, but the D- iNKT cells failed to up-regulate expression of CD122 (Figure **5-5**B). Only 42% of iNKT cells from D- mice were $CD122^+$ as compared to 70% from D+ WT mice. In addition, a higher percentage of D- iNKT cells up-regulated NKG2A/C/E expression when compared to D+ WT iNKT cells. The data collectively suggest that development of iNKT cells in D- mice was blocked at stage 1 before they up-regulate expression of CD44.

5.2.5 Inferior selection Vβ7$^+$ iNKT cells in vitamin D-deficient mice

To decipher the mechanisms involved in vitamin D regulated iNKT cell development, the use of Vβ chains were examined. The frequency of Vβ8$^+$ and Vβ7$^+$ iNKT cells from thymus and liver were determined by staining with anti-Vβ7 or Vβ8, TCR and tetramer. As shown in Figure **5-6**A, about 56% of iNKT cells were Vβ8$^+$ in D+ WT thymus and liver. Similarly to D+ WT mice, about half of D- iNKT cells were Vβ8$^+$. The frequency of Vβ7$^+$ iNKT cells was much lower than that of Vβ8$^+$ iNKT cells in D+ WT mice (Figure 5-6B, 6.5% in thymus and 12% in the liver). By contrast, the percentage of Vβ7$^+$ iNKT cells was significantly lower in D- mice than that in D+ WT mice, more prominent in the liver (4.5% vs 12%). The results indicated that vitamin D deficiency had negative effects on the selection of Vβ7$^+$ iNKT cells.

5.2.6 Normal proliferation and increased apoptosis of iNKT cells in vitamin D-deficient mice

To further investigate the mechanisms by which vitamin D-deficiency regulates iNKT cell development, BrdU incorporation assay was performed to analyze cell-turnover of iNKT cells. In the D- liver and thymus, iNKT cells incorporated BrdU to the same extent as D+ WT iNKT cells (Figure **5-7**A) In the liver, D- mice showed 42% of BrdU$^+$ iNKT cells and D+ WT 40% BrdU$^+$ iNKT cells. The similar results were seen in the thymus. In the spleen, D- mice had a higher fraction of BrdU$^+$ iNKT cells than D+ WT mice did. The results demonstrated that impaired development of iNKT cells in the absence of vitamin D was not due to decreased cell turnover.

The apoptosis of iNKT cells in the thymus was measured by staining with annexin V. The results indicated that D- mice had significantly higher fraction of annexin V^+ iNKT cells than D+ WT mice did (47% vs 30%) (Figure **5-7**B). The data suggest that D- iNKT cells underwent cell death to a greater extent. It is reported that $CD4^{dull}CD8^{dull}$ (DP^{dull}) $tetramer^+$ cells contain the earliest iNKT cell precursors and down-regulate CD24 expression as they progress towards iNKT cells (14). To further test what populations of iNKT cells undergo apoptosis, expression of CD24 and Annexin V by $tetramer^+$ DP^{dull} cells were measured. In D- mice, $tetramer^+$ DP^{dull} cells failed to down-regulate expression of CD24 (Figure **5-8** B). 39% of $tetramer^+$ DP^{dull} cells express CD24 in D- mice as compared to 8% in D+ WT counterparts (Figure **5-8**B). Moreover, a significantly higher frequency of $tetramer^+$ DP^{dull} cells were annexin V^+ in D- mice than in D+ WT counterparts suggesting that iNKT cell precursors underwent more cell death. In addition, a significantly higher percentage of annexin V^+ cells were observed in $CD24^-$ subpopulation of $tetramer^+$ DP^{dull} cells in D- mice than D+ WT counterparts (62% vs. 15%), while $CD24^+$ subpopulation exhibited similar percentage (60% vs. 60%) (Figure **5-8**B). These results suggest that vitamin D is essential for survival of early iNKT cells ($tetramer^+$ DP^{dull} cells).

5.2.7 Requirement of early exposure to $1,25(OH)_2D_3$ for iNKT cell development

To test whether feeding D- mice $1,25(OH)_2D_3$ will rescue iNKT cell development, mothers of D- mice were fed $1,25(OH)_2D_3$ 1 day before birth and during lactation. After they were weaned, D- mice continued to be on $1,25(OH)_2D_3$ for 3-5 week and iNKT cells

were analyzed. The frequency of iNKT cells increased significantly from 0.07% to 0.11% in the thymus, 1.3% to 4.2% in the liver (Figure **5-9**). However, the percentage of iNKT cells in $1,25(OH)_2D_3$ fed D-in utero mice were still significantly lower than that of D+ WT mice. The data demonstrated that early exposure to $1,25(OH)_2D_3$ is required for iNKT cell development.

5.3 Discussion

We showed before that the VDR is required for iNKT cell development and function (197). In addition, we showed that $1,25(OH)_2D_3$ resulted in reduced iNKT cell development (Chapter 4). However, the precise requirement for $1,25(OH)_2D_3$ in iNKT cell development has not been elucidated. By feeding breeders vitamin D-deficient diet, a model of dietary vitamin D-deficiency in utero was generated. Here we have shown that in the constant absence of vitamin D, iNKT cell development was dramatically decreased with altered Vβ chain usage. Early iNKT cells in D- mice underwent more cell death. In addition, $1,25(OH)_2D_3$ feeding only partially rescued iNKT cells. These results demonstrated that early exposure to $1,25(OH)_2D_3$ is essential for iNKT cell development.

iNKT cells were able to exert innate T cell functions whereas there was barely detectable cytokines in the serum in D- mice. The discrepancy between the equivalent proportion of cytokine-producing iNKT cells and less cytokine production in the serum is probably due to the overall iNKT cell deficiency (30). Although iNKT cell development was stalled at $CD44^{low}NK1.1^-$ stage, the remaining iNKT cells were capable of making cytokines. A block of iNKT cell development at $CD44^{low}NK1.1^-$ stage has been reported

in Egr2$^{-/-}$, PLZF$^{-/-}$, RelB$^{-/-}$ and IL-15$^{-/-}$ mice (59, 67, 73-75). However, unlike D- iNKT cells, Egr2$^{-/-}$ and PLZF$^{-/-}$ iNKT cells are functionally defective. Therefore, it is less likely that vitamin D regulated iNKT cell development through Egr2 and PLZF pathway. In contrast, 1,25$(OH)_2D_3$ might function through IL-15 since IL-15 only affects iNKT cell development but not iNKT cell function (59). D- mice had much fewer iNKT cells with normal function suggesting that 1,25$(OH)_2D_3$ only affects genes important in iNKT cell development but not in function. It is demonstrated that GM-CSF-deficient mice had normal iNKT cell development, but iNKT cells in these mice are defective in cytokine secretion (62). Another possibility is that the effect of 1,25$(OH)_2D_3$ on iNKT cells is after that stage when iNKT cells deviate from conventional T cells. Resting iNKT cells have transcriptionally active IL-4 and IFN-γ loci (182). Even the CD44^{low}NK1.1^{neg} iNKT cells produce exclusively IL-4 upon TCR stimulation (6, 182, 183). It has been suggested that iNKT cells gain this effector property during thymic development at the stage when they diverge from conventional T lymphocytes (184).

D- mice had dramatically diminished numbers of iNKT cells. This is not due to decreased basal proliferation since D- mice had a similar or higher fraction of iNKT cells that were incorporating BrdU. However, a higher frequency of iNKT cells undergoing apoptosis was observed in D- mice, which was more prominent in early iNKT cell precursors. Hence early iNKT cell precursors experience more cell death in the absence of 1,25$(OH)_2D_3$, perhaps as a result of more negative selection. Moreover, D- mice showed impaired selection of Vβ7$^+$ iNKT cells. Selection of Vβ7$^+$ iNKT cells was favored over Vβ8$^+$ iNKT cells under conditions of limiting endogenous ligand concentration (212). The data again suggest that there may be more negative selection of

iNKT cells in the absence of $1,25(OH)_2D_3$. Finally, besides increased negative selection, developmental block probably contributes to reduced numbers of iNKT cells as well, as D-mice failed to convert from $CD44^{low}NK1.1^{neg}$ to $CD44^{high}NK1.1^{neg}$ stage. The positive selection of iNKT cell in D- mice seems normal as demonstrated by the antigen-presentation assay (data not shown).

However, the defective iNKT cell development in vitamin D-deficient mice couldn't be fully rescued by $1,25(OH)_2D_3$ feeding. As is discussed above, D- mice had normal basal proliferation rate. $TCR\beta^+$ thymocytes at the double-negative stage of development are shown to expand about 500-fold by day 4 (215). Each TCRβ clone divides more than three times a day. It is reported that iNKT cell from thymus are able to repopulate in lymphopenic mice (59). The remnant iNKT cell precursors should be able to fill the niche normally in the presence of vitamin D if vitamin D only regulates survival of early iNKT cells. Hence, it is very likely that the majority of precursors were permanently impaired and can not expand. Even if iNKT cell precursors were exposed to $1,25(OH)_2D_3$ later, they could not repopulate and give rise to the normal iNKT cell development whereas the minor population of iNKT cell precursors preserved expanded and filled in part of the niche.

$1,25(OH)_2D_3$, the active form of vitamin D, is a fat-soluble vitamin. $1,25(OH)_2D_3$ binds to the VDR and plays important roles in immune response. Here we have demonstrated that dietary vitamin D-deficiency resulted in a much more severely impaired iNKT cells than $1,25(OH)_2D_3$-deficiency. In addition, the phenotype of the iNKT cells were distinct from that of iNKT cells in mice with $1,25(OH)_2D_3$-deficiency.

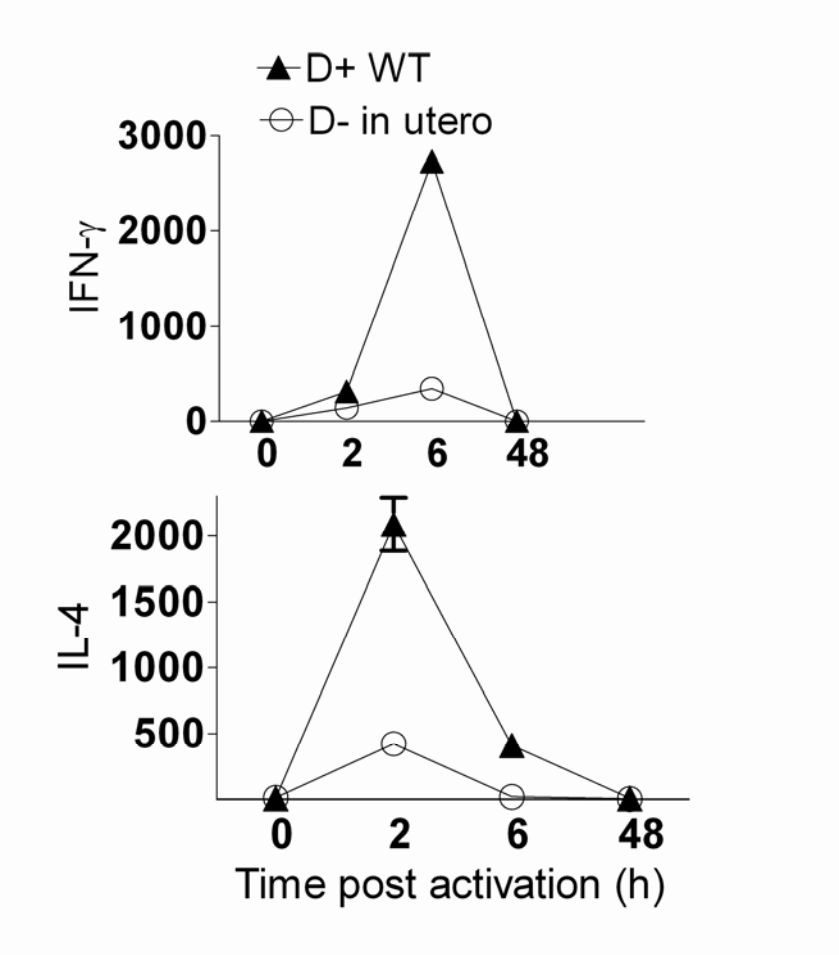

Figure **5-1**: Vitamin D-deficient iNKT mice produced little IFN-γ and IL-4 when activated with αGalCer. Serum cytokine production in vitamin D-sufficient (D+) WT and vitamin D-deficient (D-) mice induced by systemic administration of αGalCer. Levels of IFN-γ and IL-4 in the serum were determined at different times following injection (n=10 per group).

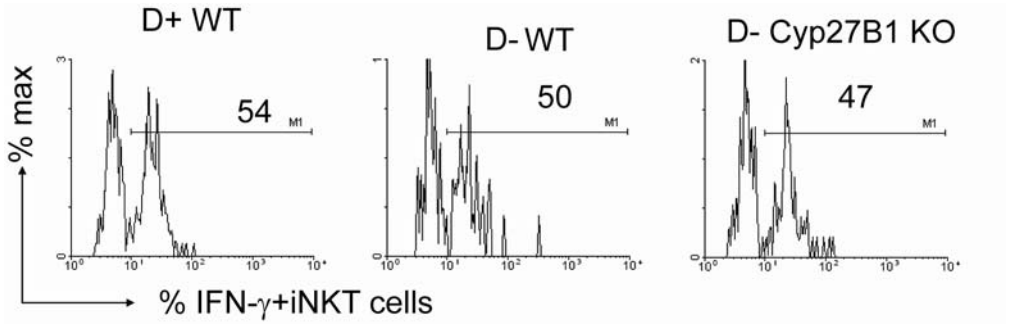

Figure **5-2**: Vitamin D-deficient iNKT cells function normally. Frequency of cytokine-producing iNKT cells in vitamin D-sufficient (D+) WT and vitamin D-deficient (D-) mice. Mononulcear cells were isolated from liver and stimulated with αGalCer for 12 hours in vitro. IFN-γ production was analyzed by intracellular cytokine staining as described in the methods. Histograms show production of IFN-γ by iNKT cells. Data from one representative of 10 mice.

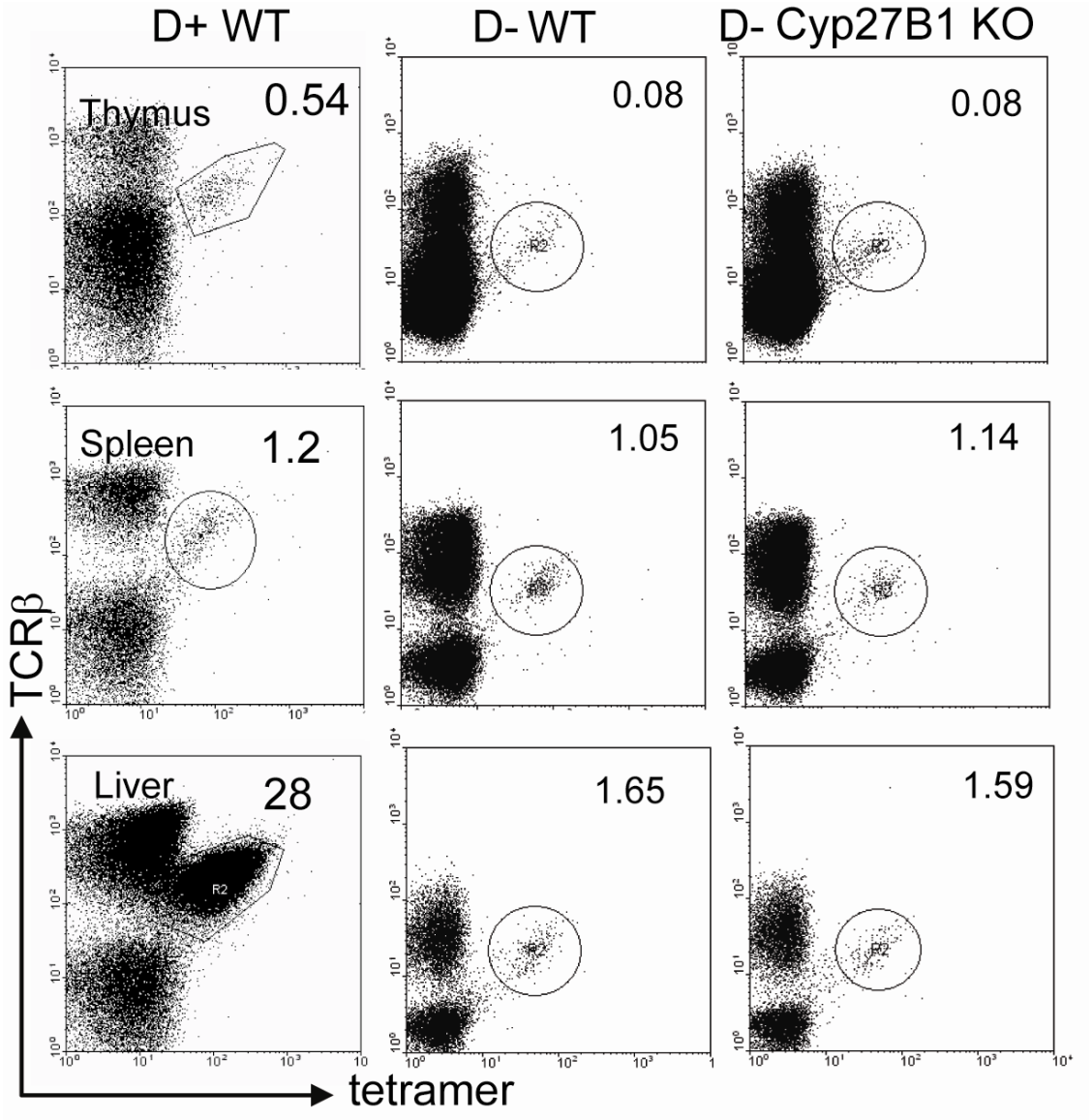

Figure **5-3**: Reduced numbers of iNKT cells in D- mice. Dot plots showing percentage of iNKT cells (TCRβ and CD1d-αGalCer double positive) in D- mice and D+ WT controls. Data show one representative of 15 mice per group. Numbers indicate percentage of iNKT cells in the circled gate.

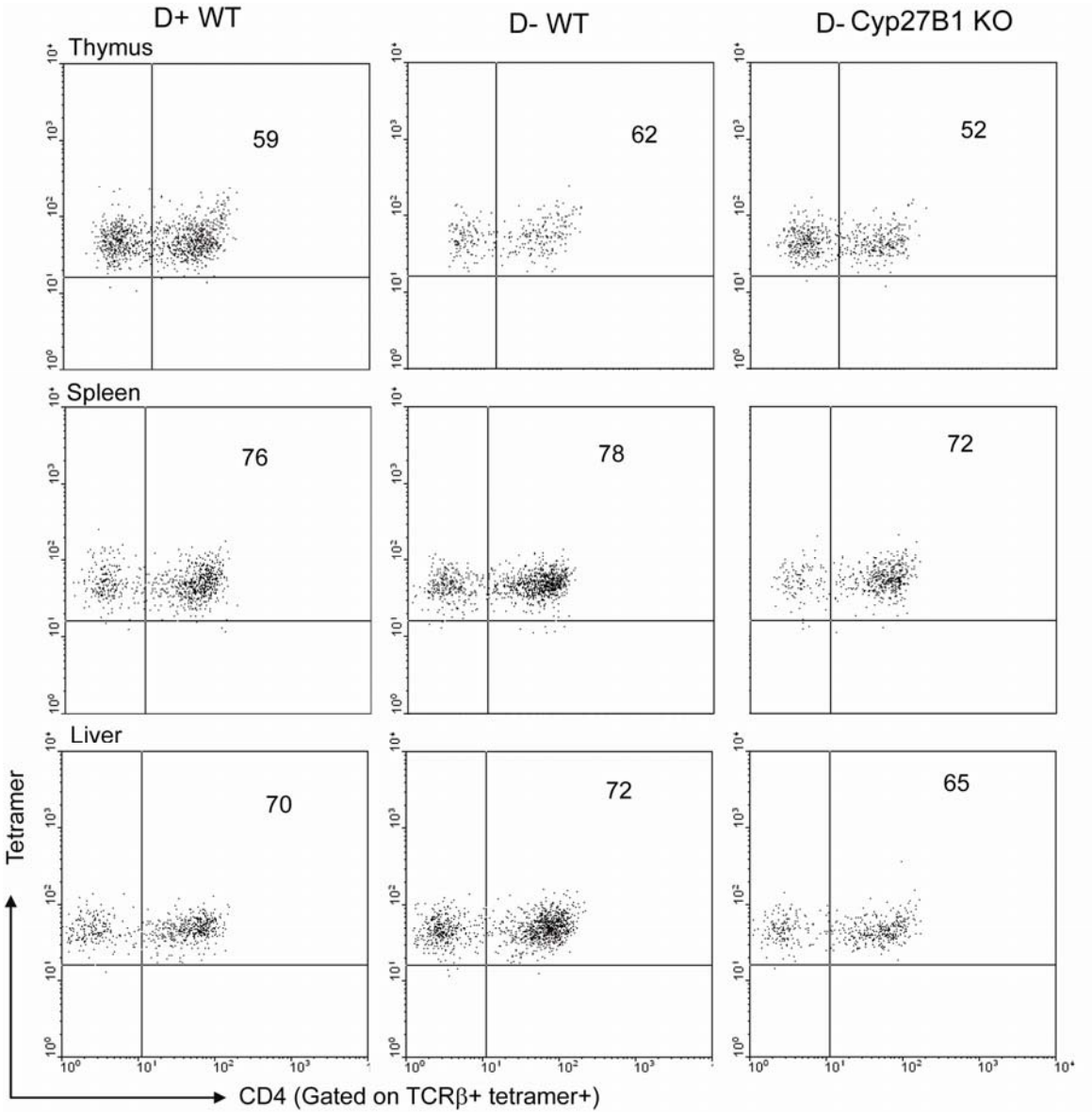

Figure **5-4**: Vitamin D affects $CD4^+$ and DN iNKT cells to the same extent. Dot plots showing expression of CD4 on TCRβ and CD1d-αGalCer tetramer double positive thymocytes. Data from one representative of 10 mice is shown.

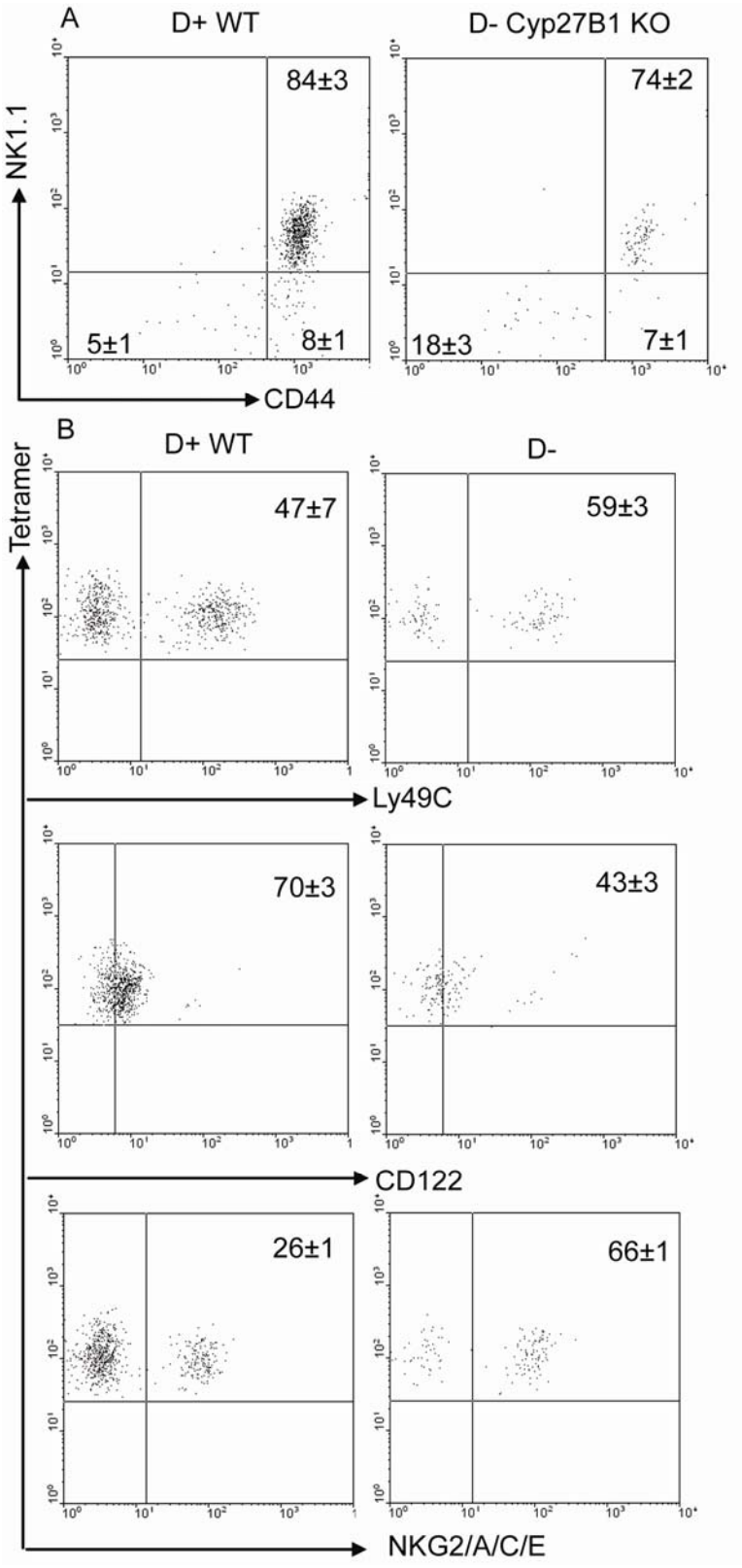
A
D+ WT
D- Cyp27B1 KO
84±3
74±2
5±1
8±1
18±3
7±1
NK1.1
CD44
B
D+ WT
D-
47±7
59±3
Tetramer
Ly49C
70±3
43±3
CD122
26±1
66±1
NKG2/A/C/E

Figure **5-5**: D- iNKT cells fail to mature fully. (A) Dot plots showing expression of CD44 and NK1.1 on TCRβ and CD1d-αGalCer tetramer double positive thymocytes. Data from one representative of 10 mice is shown. (B) Tetramer positive thymocytes were analyzed for Ly49C, CD122, and NKG2A/C/E expression. Data from one representative of 6 mice is shown. A: upper panel; B: lower panel.

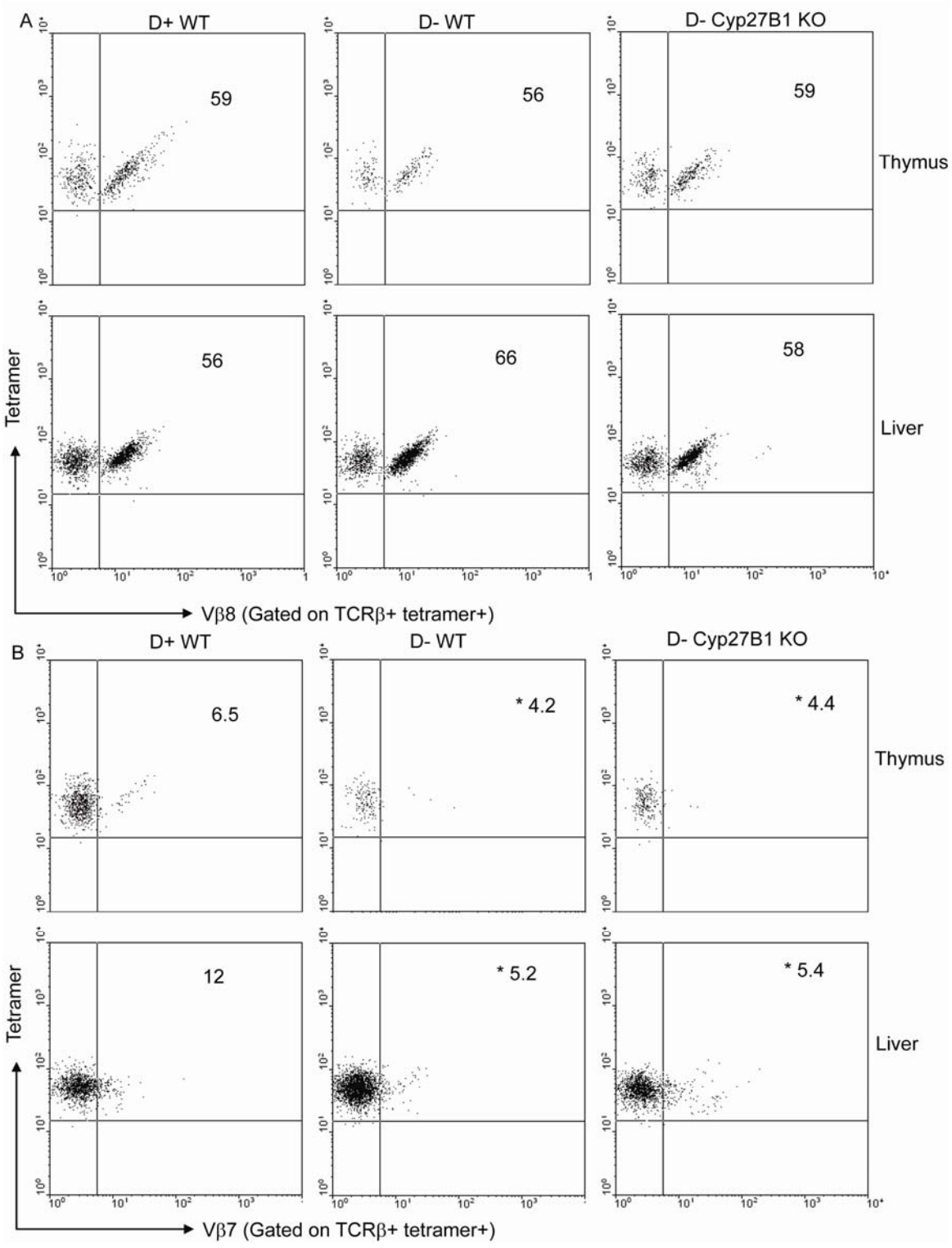

Figure **5-6**: Impaired selection of Vβ7^{+} iNKT cells in vitamin D-deficiemnt mice. (A)Dot

plots showing Vβ8 expression on TCRβ$^+$tetramer$^+$ cells. Data from one representative of 6 mice is shown. (B) TCRβ$^+$tetramer$^+$ cells were analyzed for the expression of Vβ7. Data shown is one representative of 6 mice. *, $p<0.05$

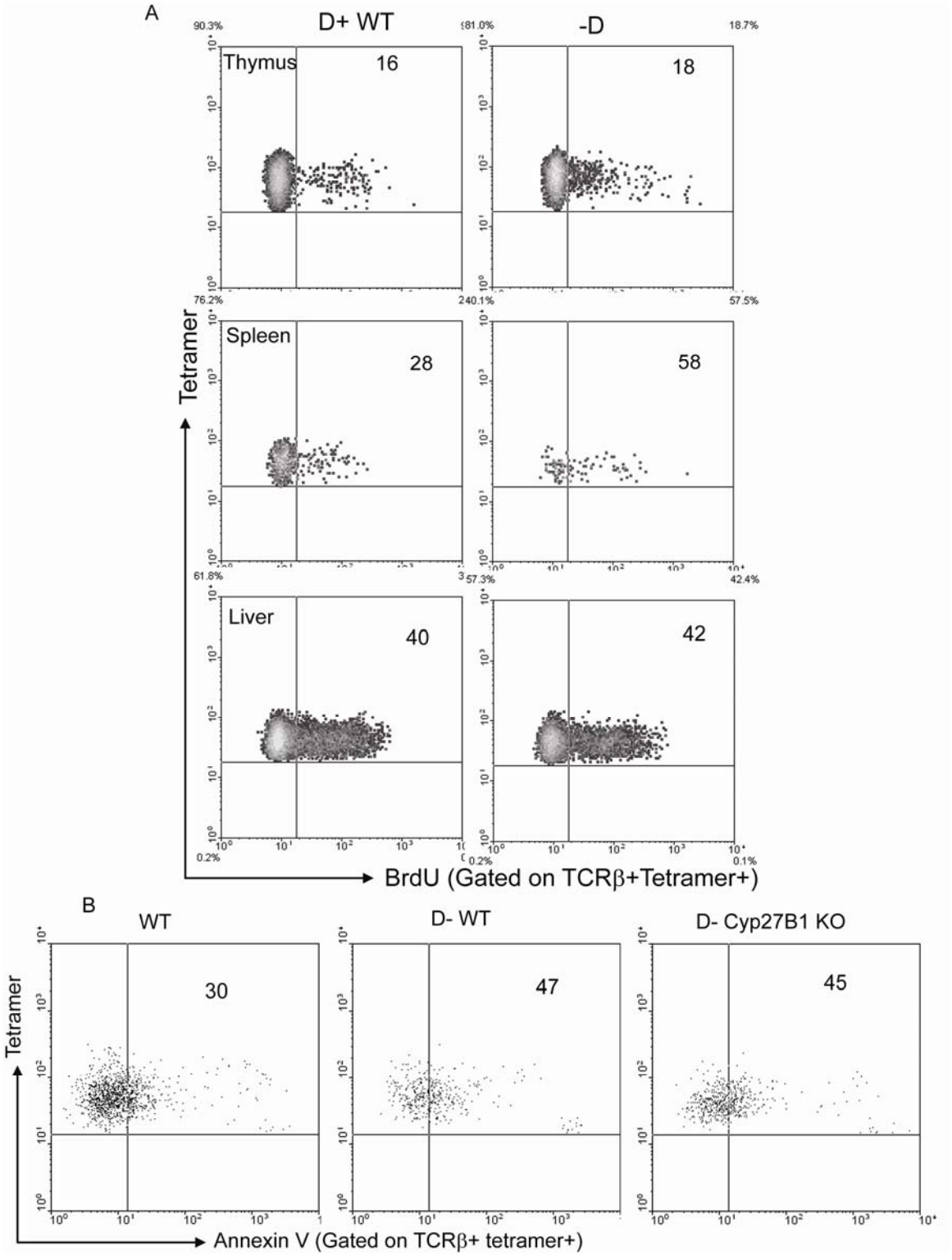

Figure 5-7: Normal basal cell proliferation but increased cell death in D- mice. (A) BrdU incorporation by iNKT cells from D+ WT and D- mice. Data from one representative of

10 mice is shown. (B) Flow cytometry of thymocytes from D+ mice and D- mice, stained with annexin V, TCRβ, and tetramer. Data shown is one representative of 10 mice.

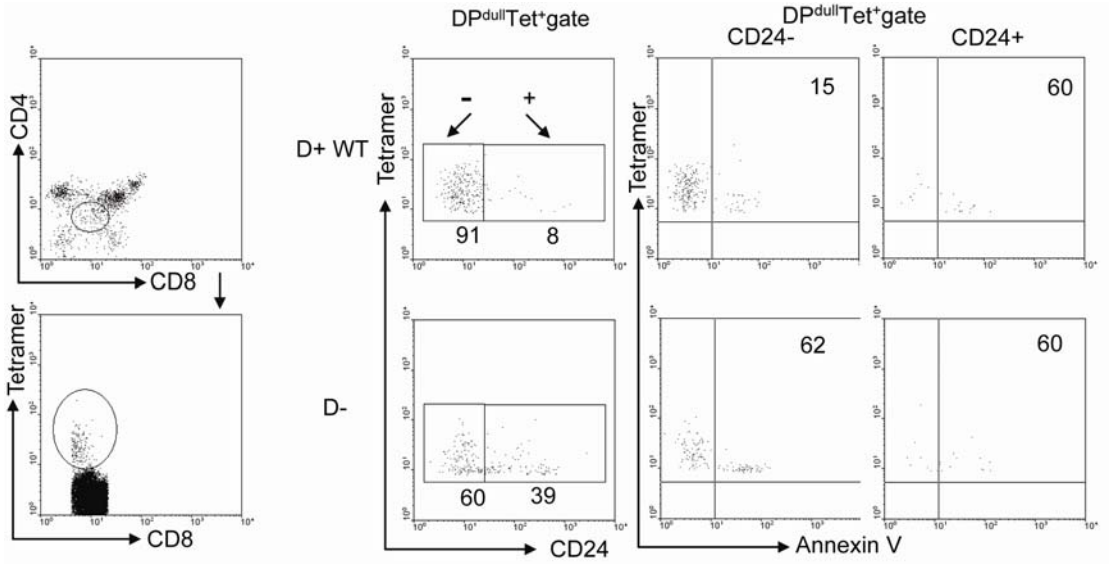

Figure **5-8**: Inreased cell death rate of iNKT cell precursors in D- mice. Flow cytometry of thymocytes from D+ WT and D- mice, stained with tetramers, annexin V, anti-CD4, anti-CD8 and anti-CD24. The small circle (far left, top) outlines the DP^{dull} gate; big circle area (far left, bottom) indicates gating on tetramer-positive cells in that DP^{dull} gate. Middle and right, numbers below outlined areas indicate percent $CD24^-$ cells (middle, left number) or $CD24^+$ cells (middle, right number); numbers in top right quadrants indicate percent tetramer-positive, annexin V–positive cells. Data represent two independent experiments with three to four mice per group per experiment.

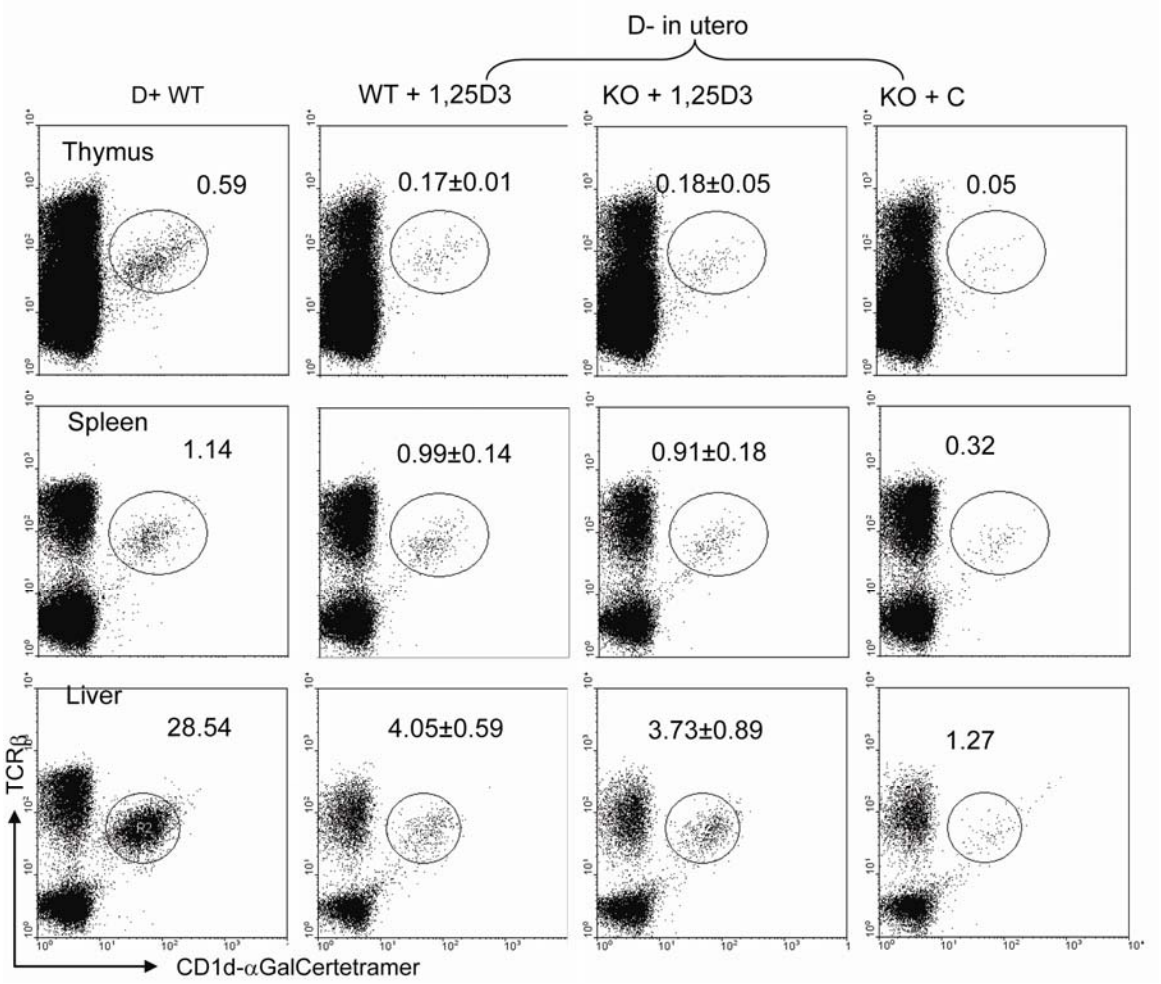

Figure **5-9**: $1,25(OH)_2D_3$ feeding partially rescues iNKT cells development in vitamin D-deficient mice. Mothers of vitamin D-deficient mice were fed 50ng/mouse/day 1 day before birth for 3 weeks. After littermates were weaned, they continued to be on the same amout of $1,25(OH)_2D_3$ for 4 week (WT+1,25D3; Cyp27B1 KO+1,25D3). Controls include D+ WT mice, D- mice fed with control diet (Cyp27B1 KO+C). Dot plots showing the percentage of $TCR\beta^+tetramer^+$ cells (means ± SEM of 8 mice).

Table **5-1**: Selective defects of iNKT cells in vitamin D-deficient mice

Cell type	Mice		Organs
	D+ WT	D-	
iNKT	1.14±0.2	**0.53±0.01**	Spleen
NKT	1.64±0.1	1.16±0.2	
T	21.68±2	25.92±1.5	
NK cells	4.98±0.5	5.33±0.7	
CD4+ T	37.39±3	35.34±4	
CD8+ T	22.89±3	25.16±2	
DP	0.36±0.04	0.44±0.05	
B cell	29.72±3	26.99±2	
Macrophage	12.08±2	13.68±2	
iNKT	0.49±0.03	***0.07±0.005***	Thymus
NKT	0.56±0.06	**0.13±0.02**	
CD4+T	27.66±3	24.86±2	
CD8+T	5.76±0.8	6.92±0.5	
DP	57.63±4	55.71±2	
iNKT	31.54±4	***1.94±0.3***	Liver
NKT	32.17±2	**18.39±3**	
NK cells	14±2	11.02±1.5	
T	27.83±3	30.02±4	

All mice were 6-8 week old and sex matched. Numbers represent percentage of different cell types. Values are the mean ± SEM of 6-8 mice per group. Values with bold letters are significantly different from D+ WT mice ($p<0.05$). Vaules with bold and italic letters ($p<0.001$). DP: CD4/CD8 double positive.

Chapter 6

Discussion and future direction

6.1 Discussion

6.1.1 Comparison of VDR-deficient and vitamin D-deficient iNKT cells

We showed here that both VDR-deficient and vitamin D-deficient mice had significantly reduced numbers of iNKT cells compared to vitamin D-sufficient WT mice. However, there is a difference in phenotype, function and mechanisms underlying the lack of iNKT cells between the VDR-deficient and vitamin D-deficient mice (Table **6-1**).

Vitamin D-deficient mice appeared to have much lower frequency of iNKT cells than the VDR KO mice did (~1.3% vs ~5% in liver). The remaining iNKT cells in vitamin D-deficient mice were stalled at $CD44^{low}NK1.1^{neg}$ stage whereas VDR-deficient iNKT cells were blocked at $CD44^{high}NK1.1^{neg}$ stage. When the ratio of $CD4^+$ to DN iNKT cells were examined, vitamin D-deficient mice had equivalent ratio compared to WT mice did showing $CD4^+$ and DN iNKT cells were affected by vitamin D-deficiency to the same extent. In contrast, the ratio of $CD4^+$ to DN iNKT cells in the VDR KO mice was higher than that in WT mice suggesting that VDR preferentially influences DN iNKT cells.

The second discrepancy was observed when iNKT cell function was compared. We showed that a comparable proportion of vitamin D-deficient iNKT cells were able to make cytokines in comparison to vitamin D-sufficient WT mice whereas a significant lower fraction of VDR-deficient iNKT cells made cytokines upon TCR stimulation. In

other words, function of vitamin D-deficient iNKT cells was normal while that of VDR-deficient was defective.

The more prominent difference between vitamin D-deficient and VDR-deficient mice was seen when the mechanisms underlying lack of iNKT cells was examined. iNKT cell development was defined by positive and negative selection. The positive selection of iNKT cells in vitamin D-deficient mice was less likely affected since antigen-presentation function of vitamin D-deficient thymoctyes was normal. However, very likely, there was more negative selection of iNKT cells in vitamin D-deficient mice as iNKT cells experienced more cell death, more pronounced in iNKT cell precursors. Other evidence for increased negative selection includes the abated usage of Vβ7 in vitamin D-deficient mice. In contrast, positive selection of iNKT cells was impaired in VDR KO mice, which was supported by the defective antigen-presentation function and results from bone marrow transplants. There was not that much evidence for altered negative selection of iNKT cells in the VDR KO mice. VDR KO mice appeared to have normal cell death. iNKT cell precursors in vitamin D-deficient mice were very likely permanently impaired and prevented from expansion. Conversely, VDR KO mice had unaltered iNKT cell precursors.

In conclusion, the requirement of vitamin D and VDR for iNKT cell development is differential. Both vitamin D and VDR are essential for iNKT cell development but they regulate iNKT cell development through different mechanisms (Figure **6-1**). In addition, only the VDR is critical for normal iNKT cell function. The data presented here identified iNKT cells as a new target of vitamin D and the VDR in immune responses. iNKT cells have been shown to play important regulatory roles in several models of

autoimmunity, infection and cancer, especially in fighting tumor growth (166, 167). Cancer-bearing mice and humans had decreased or dysfunctional NKT cells (24, 25). In a a tumor model induced by intramuscular injection of the carcinogen methylcholantrene (MCA), Jα18 KO mice had increased tumor growth rate than WT mice did when they were subcutaneously injected with tumor line derived from MCA-injected Jα18 KO mice. The tumor growth in Jα14 KO mice were inhibited when they received purified NKT cells (26). Our results shed light on the knowledge of the roles that vitamin D may play in health and potentially in diseases such as cancer. In addition, we can obtain a new treatment options for those patients with cancer.

6.1.2 Proposed model for disparity exhibited by vitamin D-deficiency and VDR-deficiency

As discussed above, function of VDR-deficient iNKT cells is inhibited, yet vitamin D-deficient iNKT cells were able to successful make cytokines upon TCR stimulation. This disparity indicates that VDR either make use of a novel, non-vitamin D ligand or it functions unliganded in iNKT cells.

Lithocholic acid (LCA) is a bile acid which is implicated in the progression of colon cancer. It is reported that it binds to the VDR with low affinity and enhances expression of the xenobiotic enzymes of the CYP3A family (216). In another report, it is shown that LCA can be absorbed into the circulation and bind to the VDR at extra-intestinal sites (217). All the data strongly suggest that LCA might be another ligand for the VDR. In addition, LCA from the diet has been shown to only exert its function in the

vitamin D-deficient model in vivo. Thus, it is less likely that the VDR carries out it function through LCA in the development of iNKT cells.

On the other hand, the model that the VDR functions unliganded is favored. The similar disparity in phenotype resulting from the VDR-deficiency and vitamin D deficiency can be found in skin/hair follicle. VDR ablation yields hair loss whereas vitamin D deficiency does not generate the same phenotype. The hair-loss phenotype can be elicited by conditional depletion of RXRα in skin and depletion of nuclear co-receptor Hr (218, 219). These findings show that the mammalian hair cycle is controlled by the VDR-RXR complex, likely recruiting Hr to inhibit a gene or a set of genes whose product(s) are prohibiting the progress of cycle from resting to growth phase.

Similarity of phenotypic disparity seen in iNKT cell development and hair loss caused by the VDR-deficiency and vitamin D-deficiency urge us to propose that the development and function of iNKT cells is driven by the unliganded VDR, recruiting a gene product of which represses the co-repressor that prevents iNKT cell development and function (Figure 6-1). In the absence of the VDR, the development and function of iNKT cells are suppressed. The gene(s) of which the product(s) curb the co- repressors in iNKT cells development needs to be identified. Our data provide strong evidence that VDR carries out its function in iNKT cell development unliganded.

6.2 Future direction

6.2.1 The mechanisms by which the VDR regulates antigen-presentation by DP thymocytes

In the VDR KO mice, the antigen-presentation was impaired partially accounting for reduced iNKT cell development. However, reduced surface expression and function of CD1d in the VDR KO thymus only partially explains the data since iNKT cells develop normally in the CD1d heterozygous mice despite reduced CD1d in the thymus and the periphery (186, 187). Therefore, decreased CD1d and function may partially explain the impaired antigen-presentation in VDR KO mice. It is reported that NPC2 protein loads and unloads self-antigen into CD1d and contributes to iNKT cell selection in thymus (220). The impaired self-antigen presentation by thymocytes and the normal exogenous antigen presentation in VDR KO mice are similar to that in NPC2 KO mice (220), suggesting that NPC2 protein might be involved in the regulation of iNKT by VDR.

To address this question, the expression of NPC2 protein will be determined by RT-PCR and western blot. Since the presentation of Galα1-2GalCer is completely dependent on endosomal function (221), WT and VDR KO thymocytes and dendritic cells from spleen will be used as stimulator for DN32.D3 hybridoma to assess the function of NPC2 in VDR KO mice. To further investigate whether NPC2 is involved in vitamin D mediated regulation of iNKT cell development, exogenous NPC2 will be added into coculture of WT and VDR KO thymocytes or splenocytes pulsed with αGalCer with DN32.D3 cells.

These studies will give us a better knowledge of expression and function of NPC2 in the VDR KO mice and may contribute to the identification of a new molecular target of the VDR.

6.2.2 Temporal requirement for $1,25(OH)_2D_3$ in iNKT cell development

We showed above that early exposure to $1,25(OH)_2D_3$ is critical for iNKT cell development. However, it is not clear the exact time points that vitamin D is required for iNKT cell development during embryonic development. To answer this question, timed pregnancies of heterozygous breeders will be fed with $1,25(OH)_2D_3$ or control diet on day 14 of embryogenesis or day 5 after birth. iNKT cell development begins early during embryonic development. By mouse embryo day 14, lymphocytes appear in the fetal liver (222). iNKT cells first appear in the thymus at day 5 after birth (12). The function, percentage and absolute numbers of iNKT cells (as described in chapter 2) from the different groups will be compared to WT littermates treated in the same way using ANOVAs.

By the end of these studies, we will have a better picture of the exact time point that $1,25(OH)_2D_3$ is required for iNKT cell development and provide useful information on adequate amount of vitamin D supplement among people of different age.

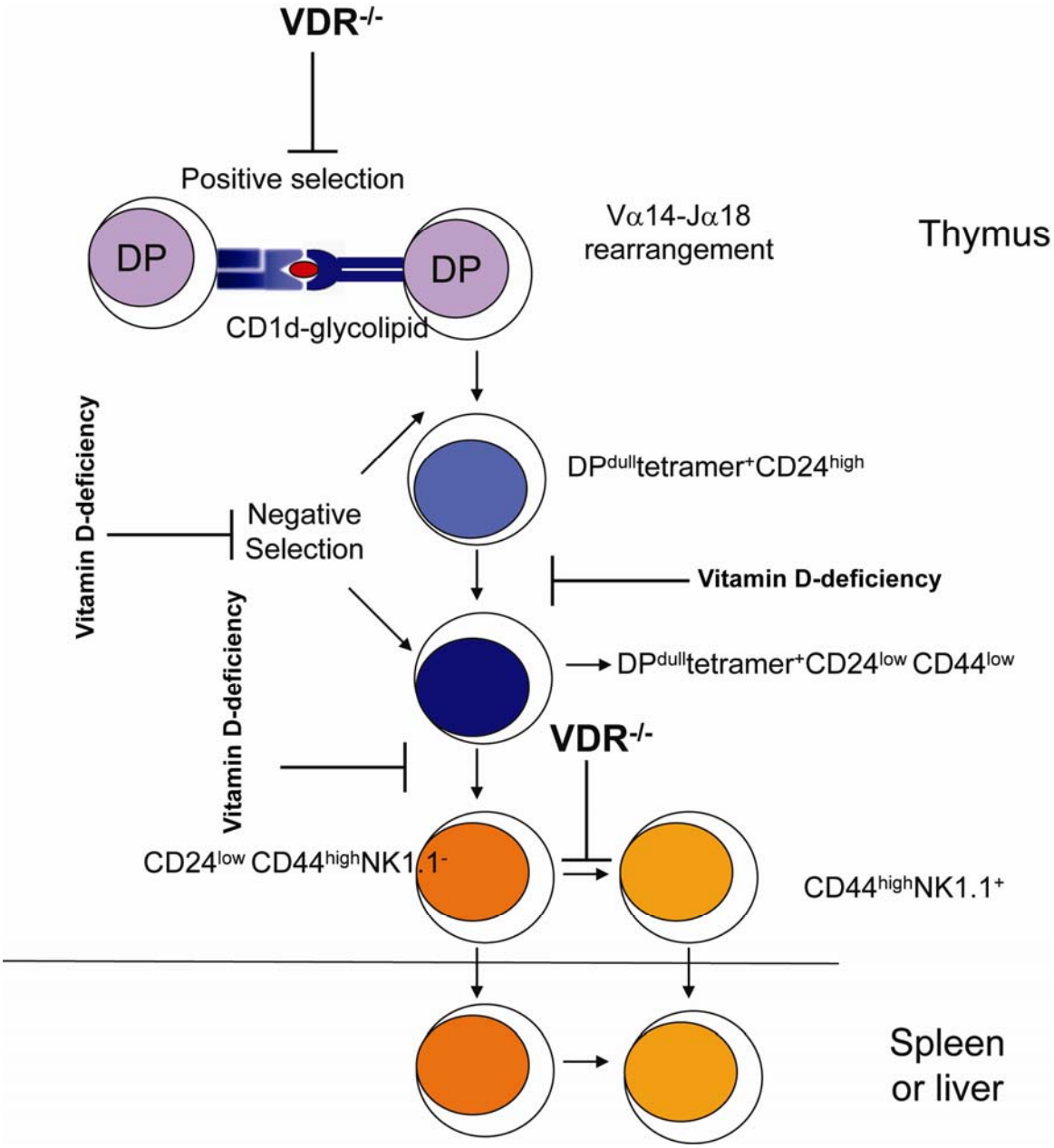

Figure **6-1**: Model of vitamin D and VDR-regulated Vα14i NKT cell development in thymus. Lack of VDR impaired positive selection of iNKT cells and transition of iNKT cells from $CD44^{high}NK1.1^{-}$ to $CD44^{high}NK1.1^{+}$ stage. The absence of vitamin D resulted in increased negative selection, permanent impaired iNKT cell precursors, and failure to up-regulate expression of CD44.

Table **6-1**: Differential role of vitamin D and VDR in iNKT cell development

	VDR-deficiency	vitamin D-deficiency
iNKT cell numbers	↓	↓ ↓
Developmental stage stalled	$CD44^{high}NK1.1^{-}$	$CD44^{low}NK1.1^{-}$
iNKT cell function	↓	Normal
Antigen-presentation	↓	Normal
Negative seletion	Less likely	very likely
iNKT precursors	normal	impaired

Comparison of VDR-deficiency and vitamin D-deficiency to vitamin D-sufficient WT mice. ↓ represents $p<0.05$. ↓↓ represents $p<0.0001$.

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Publications

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