# TRANSCRIPTIONAL ANALYSIS OF NATURAL KILLER T CELL DEVELOPMENT

**Thesis submitted by**

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# STATEMENT

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**THI XUYEN DINH**

# ABSTRACT

Type 1 NKT cells are an innate-like population of T cells that rapidly respond to both cytokine and TCR stimulation by the production of pro-inflammatory and immunoregulatory cytokines. Different from conventional αβT cells, which express a diverse repertoire of TCR sequences that are generated by random rearrangement and are positively selected by thymic epithelium expressing MHC Class I and Class II products, type 1 NKT cells express a highly restricted TCR (Vα14-Jα18 in mice, and the homologous Vα24-Jα18 chain in humans, paired with a restricted range of β chains) and are positively selected by ligating β2 Microglobulin (β2M)/CD1d and SLAM family members expressed on $CD4^+CD8^+$ (double positive – DP) cortical thymocytes. In the periphery of mice, type 1 NKT cells express a 'memory' or 'activated' like surface phenotype ($CD62L^-CD69^+CD44^{hi}$) and the great majority is either $CD4^+CD8^-$ (single positive; SP) or $CD4^-CD8^-$ (double negative; DN).

Studies on NKT cell development indicated that they are originated from the same progenitor as conventional T cells, and branched off from the mainstream lineage at DP stage. NKT cells' development is thymus dependent, they develop in fetal thymic organ culture; neonatal thymectomy on the third day of life selectively depletes them. NKT cells are not found in the peripheral tissues of mice until 1-2 weeks after birth. Many efforts have been made to understand the molecular mechanisms that govern their commitment and homeostasis. However, the very low numbers of developing NKT cells, especially DP NKT cells at Stage 0, makes it difficult to analyse transcriptional programs controlling NKT cell development.

To further dissect the events surrounding NKT cell lineage commitment and to examine transcriptional factors controlling early NKT cell ontogeny, a mouse model with increased numbers of immature type 1 NKT cells was generated on the SLAM-deficient, NOD background. These mice were found to have greatly increased numbers of $CD24^{+}NK1.1^{-}$ DP NKT cells with the characteristics of pre-selection and Stage 0 NKT cells. This provides an opportunity to compare the transcriptional profiles of these very immature $CD24^{+}NK1.1^{-}$ DP NKT cells with those of conventional T cells ($CD24^{+}NK1.1^{-}$ DP T cells), and those of more mature NKT cell subsets, including $CD24^{+}NK1.1^{-}$ $CD4^{+}$ NKT cells and $CD24^{+}NK1.1^{-}$ DN NKT cells.

Expression levels of a total of 35,556 transcripts of each biological samples of above four different cell populations were obtained by microarray analysis. Principal component analysis indicated that the four populations clearly separated, in order, across principal component 1 from $CD24^{+}NK1.1^{-}$ DP T cells, to $CD24^{+}NK1.1^{-}$ DP NKT cells, to $CD24^{+}NK1.1^{-}$ $CD4^{+}$ NKT cells, and finally to $CD24^{+}NK1.1^{-}$ DN NKT cells.

Comparison of gene expression between these populations has provided an overall transcriptional profile during TCR validation, positive selection and lineage commitment of NKT cells. These findings have further confirmed phenotypic changes during NKT cell development observed by previous studies and suggest that immature DP NKT cells are pre-selection progenitors of NKT cells. Our transcriptional regulatory network approach mapped TCR validation to the transition from DP T to DP NKT cells, while positive selection and lineage commitment were associated with the transition from DP NKT to CD4 NKT cells. This is the first time that the effects of positive and negative selection have been examined on their actual population – the immature DP NKT cells. We confirm by *in vivo* experimentation that both

positive and negative selection occur at the latter transition, separating for the first time in any T cell population the events associated with TCR validation from those associated with positive selection. NOD.*Vα14*Tg mice provide a model to study the earliest identifiable stages of NKT cell commitment and differentiation, and to help dissect factors controlling the numbers and function of this important immunoregulatory population.

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# ABBREVIATIONS

| | |
|---|---|
| α-GalCer | alpha-Galactosylceramide |
| β-ManCer | beta-Mannosylceramide |
| β2M | β2 Microglobulin |
| Ab | Antibody |
| APC | Antigen Presenting Cell/Allophycocyanin |
| ATP | Adenosine Triphosphate |
| BCG | Bacillus Calmette-Guerin |
| BrdU-5 | Bromo-2'-deoxy-uridine |
| BSA | Bovine Serum Albumin |
| CCR | CC Chemokine Receptors |
| CD1d-tet | α-GalCer loaded CD1d-tetramer |
| cDNA | complementary DNA |
| CDR3 | Complementarity Determinant Region 3 |
| CTL | Cytotoxic T Lymphocytes |
| CTLA-4 | Cytotoxic T lymphocyte Antigen 4 |
| DAVID | Database for Annotation, Visualization and Integrated Discovery |
| DC | Dendritic Cell |
| DN | Double Negative |
| DNA | Deoxyribonucleic Acid |
| DP | Double positive |

| | |
|---|---|
| DPBS | Dulbecco's Phosphate Buffered Saline |
| EAE | Experimental Autoimmune Encephalomyelitis |
| EDTA | Ethylene Diamine Tetra-Acetic Acid |
| ERK | Extracellular Signal-Regulated Kinase |
| FACS | Fluorescence Activated Cell Sorter |
| FCS | Foetal Calf Serum |
| FITC | Fluorescein Isothiocyanate |
| FTOC | Fetal Thymic Organ Culture |
| GADs | Growth Factor Receptor-Bound Protein-2-Related Adaptor Protein-2 |
| GD3 | Disialoganglioside |
| GM-CSF | Granulocyte-Macrophage Colony-Stimulating Factor |
| GSL-1 | Glycosphingolipid-1 |
| HDE | Highly differentially expressed |
| HIV-1 | Human immunodeficiency virus |
| HSV | Herpes Simplex Virus |
| Immature DP T cells | $DP^{high}$ $CD24^{high}$ $NK1.1^{-}$ $CD1d\text{-}tet^{-}TCR\beta^{+}$ cells |
| Immature DP NKT cells | DP $CD24^{high}$ $CD44^{low}$ $NK1.1^{-}$ $CD1d\text{-}tet^{+}TCR\beta^{+}$ cells |
| Immature CD4 NKT cells | $CD4^{+}CD8^{-}CD24^{high}$ $NK1.1^{-}CD1d\text{-}tet^{+}TCR\beta^{+}$ cells |
| Immature DN NKT cells | $CD4^{-}CD8^{-}$ $CD24^{high}$ $NK1.1^{-}CD1d\text{-}tet^{+}TCR\beta^{+}$ cells |
| i.p. | Intraperitoneal |
| i.t. | Intrathymic |
| i.v. | Intravenous |

| | |
|---|---|
| Id | Inhibitor of DNA-binding proteins |
| Idd | Insulin dependent diabetes |
| IDDM | Insulin-dependent diabetes mellitus |
| IFN | Interferon |
| Ig | Immunoglobulin |
| iGb3 | isoglobotrihexosyl ceramide |
| IL | Interleukin |
| iNKT cells | invariant NKT cells |
| ITAMs | Immunoreceptor tyrosine-based activation motifs |
| ITK | Inducible T-cell Kinase |
| KO | Knock-out |
| LAT | Linker for Activation of T cells |
| LPS | Lipopolysaccharide |
| mAb | monoclonal Antibody |
| MACS | Magnetic-activated cell sorting |
| MAIT | Mucosal-associated invariant T |
| MBP | Myelin basic protein |
| mCD1d | mouse CD1d protein |
| MHC | Major Histocompatibility Complex |
| mL | Mililitre |
| mM | Milimolar |
| MOG | Myelin oligodendrocyte glycoprotein |
| MRL | Murphy Roths Large |
| mRNA | Messenger RNA |

| | |
|---|---|
| MS | Multiple sclerosis |
| MTV | Mammary Tumor Virus |
| NADH | Nicotinamide Adenine Dinucleotide |
| NFAT | Nuclear factor of activated T-cells |
| NK | Natural Killer |
| NKT | Natural Killer T |
| NOD | Non-obese diabetic |
| NS | Not Significant |
| PBMCs | Peripheral blood mononuclear cells |
| PBS | Phosphate Buffered Saline |
| PCA | Principal Component Analysis |
| PCR | Polymerase Chain Reaction |
| PD-1 | Programmed Cell Death 1 |
| PE | Phycoerythrin |
| PI | Propidium iodide |
| PLCε 1 | Phospholipase C epsilon 1 |
| PLZF | Promyelocytic Leukaemia Zinc Finger |
| qPCR | Real-time quantitative PCR |
| RAG | Recombination-Activity Gene |
| RasGAP | Ras GTPase-activating protein |
| RNA | Ribonucleic Acid |
| RORγt | Retinoic acid receptor-related Orphan Receptor |
| RT-PCR | Reverse transcription PCR |
| SAP | SLAM–associated protein |

| | |
|---|---|
| SD | Standard Deviation |
| SEM | Standard Error of Mean |
| SLAM | Signaling Lymphocytic-Activation Molecule |
| SLE | Systemic Lupus Erythematosus |
| SP | Single positive |
| T1D | Type I diabetes |
| TCR | T Cell Receptor |
| Tg | Transgenic |
| Th | T helper |
| Th1 | T helper type 1 |
| Th2 | T helper type 2 |
| TLR | Toll-like receptor |
| TN | Triple Negative |
| TNF | Tumour Necrosis Factor |
| Treg | T regulatory cells |
| Vα14Tg | Vα14 -Ja18 transgenic |
| WGCNA | Weighted Gene Correlation Network Analysis |
| WT | Wild-type |

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# CHAPTER 1

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# AN INTRODUCTION TO NATURAL KILLER T CELLS

## 1.1. NATURAL KILLER T CELLS

### 1.1.1. Definition of NKT cells

Natural killer T (NKT) cells are a newly recognised member of the immune system. Research into NKT cells originated when several groups independently reported the existence of a population of T cells in mice with lower levels of αβ T cell receptor (TCR) expression, but a higher frequency of Vβ8 expression, compared to conventional T cells. These cells also exhibited a $CD4^-CD8^-$ (double negative – DN) phenotype, that is, they do not express CD4 or CD8 markers (Fowlkes *et al*., 1987; Budd *et al*., 1987a). Around that time, other groups discovered a subset of αβ-$TCR^+$ T cells that expressed NK1.1, the typical marker of the natural killer (NK) cell lineage, and were either $CD4^+CD8^-$ or DN (Syles *et al*., 1990). They were also described as a source of immunoregulatory cytokines such as interleukin 4 (IL-4), interferon γ (IFN-γ), and tumour necrosis factor (TNF) (Bendelac and Schwartz, 1991; Bendelac *et al*., 1992; Hayakawa *et al*., 1992; Arase *et al*., 1993). Initially, this new subset was referred to as $NK1.1^+$ T cells (reviewed in Bendelac *et al*., 2007).

Recently, NKT cell studies have been facilitated by the discovery of α-galactosylceramide (α-GalCer, which was originally isolated from the sea sponge *Agelas mauritianus),* and the generation of α-GalCer loaded CD1d-tetramer (also called CD1d-tet) (Kawano *et al*., 1997). The use of CD1d-tet in NKT cell identification has indicated that the name "$NK1.1^+$ T cells" was not broad enough, because in most commonly used mouse strains (except C57BL/6) and many NKT cells do not express the NK1.1 marker. Therefore, the term "NKT cells" is now widely accepted and applied to mice, humans, and other species, as a minor subset of T cells that shares some markers typical of natural killer cells, such as NK1.1 in mice, and CD161 in humans (Bendelac *et al*., 2007; Godfrey *et al*., 2010).

Over a decade of work has revealed that NKT cells are a distinct T cell subset: they originate from the same progenitors as conventional T cells but differ in terms of structure and function. Firstly, NKT cells express TCRs on their cell surface, but exhibit some markers commonly associated with natural killer cells (i.e. NK1.1 in mice or CD16, CD57, CD161 in humans). Secondly, while TCRs of conventional T cells are diverse due to the random combination of variable, joining, and diverse gene segments, TCRs of NKT cells are relatively limited. The most frequent NKT cells (called invariant (i) NKT cells or type I NKT cells) express an invariant TCR α-chain (Vα14-Jα18 in mice or Vα24-Jα18 in humans), in combination with a restricted β-chain (such as Vβ8, Vβ7, Vβ2 in mice and Vβ11 in humans). Perhaps the most striking difference is that NKT cells were shown to be reactive to both exogenous and endogenous lipid antigens presented by the non-polymorphic MHC-like molecule CD1d on cortical $CD4^+CD8^+$ (double positive - DP) thymocytes. Conversely, the bulk of mainstream αβT cells respond to short peptides in the context of highly allelic major histocompatibility complex (MHC) class I or MHC class II on antigen presenting cells (APCs), such as macrophages, B cells, and dendritic cells (Bendelac, 1995; Exley *et al*., 1997). It was noted that unlike mainstream αβT cells, NKT cells also differ in terms of immune function. While T cells have a central role in cell-mediated immunity, NKT cells play a critical role in immune regulation. They are able to rapidly release large amounts of cytokines, such as IL-4 and IFN- γ, upon stimulation. These cytokines modulate the behaviour of other cells in the immune system, such as macrophages, B cells, and T cells. Moreover, like NK cells, type I NKT cells express cytotoxic granules such as perforin and granzyme that polarise upon activation of the TCR and are able to kill target cells such as APCs and T regulatory cells ($T_{reg}$) (McCarthy *et al*., 2007; Nguyen *et al*., 2008).

Taken together, the body of work on type I NKT cells indicates that they are a very interesting subset of T cells that may bridge the innate and adaptive immune systems. Their TCR has evolved to recognise different glycolipid antigens in a conserved manner and to perform innate-like rather than adaptive functions. They can also mount specific responses to antigen with cytokine production and cytotoxic activity (Taniguchi *et al*., 2003; Bendelac *et al*., 2007).

### 1.1.2. CD1d – antigen presenting molecule of NKT cells

CD1d is a member of the MHC class I–like glycoprotein family of molecules, which plays an important role in presenting foreign and self-lipids as cognate antigens to NKT cells. CD1 molecules are encoded by the *Cd1* locus, located on chromosome 1 in humans and chromosome 3 in mice. CD1 is similar to MHC class I at both the nucleotide and amino acid level, as they have a similar intron/exon gene structure. In addition, CD1 proteins share sequence homology and overall domain structure with MHC class I molecules. They are type I integral membrane proteins comprised of a heavy chain with two extracellular domains (α1, α2) that form the antigen–binding region, and another domain (α3) that non-covalently associates with β2-microglobulin. Due to these features, CD1 molecules are also called non-classical MHC I or class I–like molecules.

There are five human *Cd1* genes that encode for the five corresponding CD1 proteins: CD1a, b, c, e and CD1d. These proteins are divided into two groups based on sequence homology in the α1 and α2 domains: group 1 (consisting of CD1a, b, c and e) and group 2 (CD1d). However, the number of CD1 isoforms expressed in any given mammal varies considerably. For example, humans express all five CD1 isoforms, rabbits lack CD1c, while mice express only the CD1d protein. The basis for such varied usage of genes within the CD1

locus across mammals is largely unknown, but presumably reflects important functional differences related to pathogen-driven evolutionary pressures.

The TCR of NKT cells engages its ligand, CD1d-lipid co-complex, with conserved germline-encoded residues in four-to-five of the six complementarity-determining regions of the combined TCR α- and β-chains (Rossjohn *et al*., 2015). Hence, phylogenetic studies of genes encoding CD1 molecules and the invariant NKTCR α-chain can reveal the origin and evolution of NKT cells (Kumar *et al*., 2017). A recent phylogenomic analysis revealed that the *Cd1* gene is an amniote innovation that evolved in the Mesozoic reptiles and was retained in the extant anapsid (green anole lizard *Anolis carolinensis*) and synapsid (Siamese crocodile *Crocodylus siamensis* and Chinese alligator *Alligator sinensis*) reptilians (Yang *et al*., 2015). *Cd1* genes diversified in mammals, wherein evolved the *Cd1d* gene that encodes the lipid agonist presenting molecule that controls the functions of NKT cells in eutherians (of placental mammals) (Boudinot *et al*., 2016, Kumar *et al*., 2017). Curiously however, the reptilian *Cd1* gene has no orthology with avian or mammalian *Cd1* genes (Yang *et al.,* 2015), suggesting that *Cd1* genes may have emerged multiple times during amniote evolution. Or alternatively, *Cd1* genes may have evolved rapidly and diverged substantially from the reptilian form within extinct synapsid and mammal-like reptiles prior to stabilization within eutherian species. The latter view is supported by the finding that egg-laying monotremes such as platypuses and echidnas do not have *Cd1* genes while a *CD1d*-like gene exists in a few metatherian (of marsupial mammals) species such as the opossum (Kumar *et al*., 2017). However, CD1 molecules seem to be conserved between species. Therefore, human NKT cells can recognise mouse CD1d and vice versa (Burdin and Kronenberg, 1999).

There are two *Cd1d* genes mice, designated *Cd1d1* and *Cd1d2* (Brigl and Brenner, 2004). These two genes are located on chromosome 3, arranged in opposite transcriptional orientation and are approximately 9 kb apart (Bradbury *et al*., 1988). The two genes are likely

the product of gene duplication, they share 95% sequence homology with each other (Zajonc, 2016; Dascher and Brenner, 2003). Mouse CD1d molecules are primarily expressed by cells of the hematopoietic lineage, including B and T cells, macrophages, and dendritic cells (Brossay *et al*., 1997; Park *et al.,* 1998). At the transcriptional level, *Cd1d1* is expressed at higher levels than *Cd1d2* in all tissues tested except the thymus, where the mRNA levels appear to be equal for both genes (Bradbury *et al*., 1988). There are the 17 amino acid differences between CD1d1 and CD1d2 proteins, in which 9 occur within the antigen-binding cleft, which includes a Cys168→Trp168 mutation. The cysteine 168 residue participates in an intradomain disulfide bond that is highly conserved in classical and nonclassical MHC I molecules (Adams and Luoma, 2013; Bradbury *et al.,* 1988; Porcelli, 1995), and is thought to be critical for the folding of MHC-I (Bjorkman *et al.,* 1987; Warburton *et al.,* 1994). The impact of this mutation on CD1d2 structure and function is unknown. Interestingly, the bovine *Cd1d* gene (Wang *et al*., 2012) and the primordial CD1-encoding gene found in chickens (Zajonc *et al.,* 2008) also contain an amino acid change at position 168. This change was previously revealed to affect the nature of the antigens presented by these molecules (Zajonc, 2016). Intriguingly, rats possess only one Cd1d gene, which, based on sequence homology, more resemble mouse CD1d1 than CD1d2, with a cysteine at position 168 (Ichimiya *et al.,* 1994).

Whereas CD1d1 has been found to play the major role in NKT cell development and function, that of CD1d2 is unclear. Chen *et al.* (1999) reported that CD1d2 is expressed on the cell surface of thymocytes in some mouse strains including CD1d1-deficient mice derived from 129 embryonic stem (ES) cells used in most gene targeting (Chen *et al.*, 1999). Studies by Sundararaj *et al*. (2018) showed that CD1d2 molecules are expressed in the thymus of some mouse strains, where they select functional type I NKT cells. However, the T cell antigen receptor repertoire and phenotype of CD1d2-selected type I NKT cells in CD1d1−/−

mice differed from CD1d1-selected type I NKT cells. High-throughput sequencing of CD1d gene transcripts from the thymus of BALB/c mice showed that the transcript ratio of CD1d1:CD1d2 was close to 1:1, which supports the possibility for a role of CD1d2 in shaping the development of iNKT cells. The crystal structure analysis of CD1d2 indicated that CD1d2 adopts an overall architecture similar to CD1d1. The Cys168Trp mutation does not collapse the antigen-binding groove, however, the CD1d2 A′-pocket was markedly restricted in size, thereby favoring the loading of lipid antigens with shorter acylchain length. (Sundararaj *et al*., 2018). Collectively, their results indicated that the two CD1d molecules present different sets of self-antigen(s) in the mouse thymus, thereby impacting the development of invariant NKT cells.

Regarding to function, CD1a, CD1b and CD1c molecules generally present self and foreign lipid antigens to polyclonal T cells expressing αβ TCRs, or sometimes to several γδ T cells. In contrast, the CD1d molecule uniquely presents self and foreign lipid antigens to CD1-dependent NKT cells (De Libero and Mori, 2005; Borg *et al*., 2007). Among microbial lipid antigens, lipids derived from *Mycobacterium tuberculosis* are restricted to presentation by CD1a, CD1b and CD1c, while CD1d tends to present those derived from other sources such as α-glucuronosyl-ceramide (from *Sphingomonas spp.)*, α-galactosyldiacylglycerol (from *Borrelia burgdorferi),* and Lipophosphoglycan (from *Leishmania donovani)*.

### 1.1.3. Lipid antigens of type I NKT cells

It has been more than two decades since type I NKT cells were identified and distinguished from conventional T cell populations based on the invariant *Vα14-Jα18* rearrangement in their T cell antigen receptor. NKT cells are now known to recognise lipid antigens presented by CD1d, a member of a third family of antigen-presenting molecules. However, the knowledge surrounding these NKT cell antigens is still limited. The first and most well-known lipid antigen that can be recognised by the TCR of type I NKT cells is α-

GalCer. α-GalCer is a glycosphingolipid, a derivative of natural agelasphins and a group of closely-related glycolipids, which only differ in the lipid portion. These compounds were originally extracted from the marine sponge *Agelas mauritanius* and their activities were first discovered in a screen of natural substances for anti-tumour activity carried out by the Kirin Pharmaceutical Company (Natori *et al*., 1994).

The most striking characteristic of α-GalCer is the α-linkage between the ceramide moiety and carbohydrate head group in its hexose sugar. Conversely, glycospingolipids from mammalian cells have β-linked glycolipids. The α-linkage is critical for its antigen activity, as challenge by the β-anomeric conformation completely abrogated or was a much less potent stimulator of type I NKT cells. Significant variations to the basic GalCer structure, via changes to the sugar and/or the stereochemistry of its linkage to either the ceramide (the sphingosine base) or the fatty acid, can still be recognised by type I NKT cells. In some cases these variants provide equal or even greater antigen potency than GalCer alone (Schmieg *et al*., 2003). For example, α glucosylceramide (where galactose is replaced by glucose), Gal(1-2)GalCer, and C-α-GalCer (a C-glycosidic form of GalCer) can engender highly differential cytokine responses, compared to α-GalCer (Pei and Kronenberg, 2012). The discovery of α-galactosylceramide and numerous related derivatives has helped to explore the range of antigen specificity among type I NKT cells, and has provided powerful agents for pharmacologic stimulation of these cells, with various functional outcomes.

Although α-GalCer and other similar compounds are useful tools for the study of type I NKT cells, they are not a natural antigen for this population, as the mammalian immune system does not typically defend against marine sponges (Sandberg and Ljunggren, 2005). Consequent studies have identified a series of microbial lipid compounds that could modulate a large fraction of type I NKT cells from mice and humans. These microbial lipid compounds

are mainly derived from three types of bacteria. Lipid antigens from *Sphingomonas* spp. and *Borrelia burgdorferi* share the common features of bearing a lipid with two acyl chains and an α-linked hexose sugar as in α-GalCer. However, in the third type of bacteria, *Helicobacter pylori*, the antigen has a very different structure that contains a cholesterol moiety derived from the eukaryotic host (Pei and Kronenberg, 2012).

The first bacterial glycolipid antigens that were obtained from *Sphingomonas* spp. were glycosphingolipid-1 (GSL-1) from *Sphingomonas paucimobilis* and glycosphingolipid-1' (GSL-1') from *Sphingomonas yanoikuyae.* They highly resemble α-GalCer in structure, with a ceramide backbone containing the α-anomeric conformation of a glycosidic bond. The main difference between *Sphingomonas* compounds (GSL-1 or GSL-1') and GalCer is that GSL-1 or GSL-1' use glucuronic acid or galacturonic acid respectively as the saccharide group, while GalCer simply uses galactose (Kinjo *et al.,* 2005; Mattner *et al.,* 2005; Sriram *et al.,* 2005; Pei and Kronenberg, 2012). GSL-1, GSL-1', and other similar compounds with more complex carbohydrates have the ability to stimulate both mouse and human type I NKT cells (both *in vivo* and *in vitro*), but with lesser potency than α-GalCer (Kinjo *et al.,* 2005; Mattner *et al.,* 2005; Sriram *et al.,* 2005).

The second type of microbial glycolipid antigens that activate most type I NKT cells are derived from *Borrelia burgdorferi*, a spirochete that causes Lyme disease, which is currently the most common vector-borne disease in the USA (Bacon *et al*., 2008). There are two abundant glycolipids present in the cell wall of *B. burgdorferi*, and they are targets of an antibody response in infected individuals (Hossain *et al*., 2001). One of these is cholesteroyl 6-O-acyl-β-D-galactopyranoside, called *B. burgdorferi* glycolipid 1 (BbGL-I), and the other is 1,2-di-O-acyl-3-O-α-D-galactopyranosyl-sn-glycerol (BbGL-II; Ben-

Menachem *et al*., 2003). BbGL-II was found to activate type I NKT cells in a CD1d-dependent manner (Kinjo *et al*., 2006).

Recently, it was indicated that a cholesterol-containing antigen from a third bacterial species, *Helicobacter pylori*, (the causative agent of many stomach ulcers) could also stimulate type I NKT cells (Chang *et al*., 2011). For example, it has been hypothesized that the antigen cholesteroyl 6-O-acyl-glucoside could stimulate type I NKT cells *in vitro* in a CD1d-dependent manner, and modulate the function of type I NKT cells *in vivo* in favour of IFN production. Cholesteroyl 6-O-acyl-glucoside protects young mice exposed to this compound from asthma (Chang *et al*., 2011), however, the underlying biochemistry of the interactions between the *H. pylori* antigen, CD1d, and the TCR is not known and requires further investigation.

In a variety of contexts, type I NKT cells exhibit CD1d-dependent stimulation in the apparent absence of microbial antigens. Therefore, it is believed that type I NKT cells are self-reactive, and there might be a single self-antigen, or a limited set of closely associated and relatively high affinity self-antigens that can stimulate the invariant TCR. Although this hypothesis remains unproven (Gapin, 2010), a large number of potential self-antigens have been proposed over the years, and all are capable of activating NKT cells in vitro and/or in vivo. They are either glycospingolipids and non-glycospingolipid antigens (Pei *et al*., 2011).

The first self-derived cellular lipid was phosphatidylinositol (PI), which was originally found by Gumperz and colleagues (2000) from an extract of tumour cells, and was presented by plate-bound mouse CD1d molecules to type I NKT cell hybridomas. Compared to its derivatives, PI has the highest antigenic potency. However, PI-binding NKT cell hybridomas did not subsequently respond to GalCer, and therefore these cells apparently do not represent the typical type I NKT cell population (Gumperz *et al.,* 2000). Another self-derived cellular

lipid, disialoganglioside (GD3), was reported to activate a subset of type I NKT cells. GD3 immunisation was required in order to detect a GD3-reactive type I NKT cell population (Wu *et al*., 2003). A number of efforts were made to identify glycospingolipid self-antigens but no particular structures were found (Paget *et al*., 2007; Salio *et al.,* 2007). However, several papers have reported the identification of factors and enzymes important for glycospingolipid biosynthesis. For example, CpG ODN, a Toll-like receptor (TLR) 9 ligand, was found to activate dendritic cells to synthesise α-linked glycospingolipids and also to secrete type I IFN, both of which are required to modulate type I NKT cells (Paget *et al*., 2007).

An intriguing finding in the identification of NKT cell self-antigens was the identification of the glycospingolipid isoglobotrihexosyl ceramide (iGb3) as a putative self-antigen required not only for NKT cell development, but also for the activation of autoreactive NKT cells in the periphery (Zhou *et al*., 2004; Mattner *et al*., 2005). There is compelling evidence against iGb3 as the main positively-selecting self-antigen because NKT cell numbers and function are normal in mice deficient for iGb3 synthase (Porubsky *et al*., 2007). While iGb3 is clearly a glycospingolipid self-antigen that activates type I NKT cells, it is highly unlikely that iGb3 is required as the major self-antigen. Data from several groups indicates that there are other glycospingolipid self-antigens (such as phosphatidylinositol, phosphatidylglycerol, and phosphatidylethanolamine) that are presented by CD1d to activate NKT cells. However, they do not have strong affinity for CD1d like other potent compounds that mimic α-GalCer (Gumperz *et al.,* 2000). Thus, synthetic α-GalCer is still an excellent lipid antigen to use to identify and study NKT cell development.

It has recently been proposed that β-linked monoglycosylceramides, such as β-glucosylceramides (β-GluCer), were natural endogenous ligands of NKT cells. Synthetic preparations of C12:0 and C24:1 β-GluCer have been shown to be strong activators of type 1 NKT cells (Ortaldo *et al*., 2004; Parekh *et al.,* 2004; Zigmond *et al*., 2007; Brennan *et al.,*

2011). However, β-GluCer is one of the most abundant glycosylceramides in all cell types, it remains very difficult to conceive the regulation of its presentation by CD1 molecules and recognition by NKT cells. In addition, because of the limitations of the analytical methods of lipids, there is a possibility that α-anomers could contaminate the synthetic preparations. In 2014, Kain and colleagues demonstrated that β-GluCer has no stimulatory properties toward NKT cells and that contamination of synthetic β-linked glycolipids with α-anomers is an important issue for above biological studies. To overcome the limitations of direct chemical methods, Kain and colleagues had used the specificity of immunological and enzymatic assays to characterize and isolate the endogenous ligands of NKT cells in the thymus and in DCs. They found that the endogenous ligands NKT cells are α-linked monoglycosylceramides, a class of glycolipids that were thought to be absent from mammalian cells given that the only two glycosylceramide synthases (glucosylceramide synthase and ceramide galactosyltransferase) were thought to be inverting glycosyltransferases. In addition, they demonstrated that α-GalCer is one of the endogenous ligands of NKT cells and that catabolic enzymes tightly control the level of α-galactosylceramide (α-GalCer) in cells and tissues.

Other potentially relevant self-antigens for type I NKT cells include lysophosphatidyl-choline (LPC) and lysosphingomyelin, which can be found at elevated levels during inflammatory responses (Fox *et al*., 2009; Lopez-Sagaseta *et al*., 2012). These two self-antigens weakly stimulate some human type I NKT cells (Fox *et al.,* 2009) but apparently fail to trigger a response by mouse type I NKT cells (Brennan *et al*., 2011; Pei *et al*., 2011; Facciotti *el al*., 2012). This raises the possibility that mouse and human self-antigen(s) for type I NKT cells might be distinct, due to differing trafficking requirements and sequence differences between mouse and human CD1d (Chen *et al*., 2007). Further, differences in the fine specificity of human and mouse type I NKT cells for the recognition of α-GalCer analogues have been reported (Wun *et al*., 2012). Taken together, it seems likely

that there is not simply one self-antigen for type I NKT cells but rather several different self-antigens with various structures might be capable of providing an agonistic signal when recognized by the type I NKT TCR.

### 1.1.4. Classification of NKT cells

Although the definition of NKT cells is still vague and broad, the classification of NKT cells has been facilitated to a certain extent by the availablity of markers such as TCRβ, CD1d-tet, and NK1.1. NKT cells are initially divided into two distinct groups: CD1d-dependent and CD1d-independent NKT cells (Bendelac *et al*., 2007, Godfrey *et al*., 2010). CD1d-dependent NKT cells are the major group. Their TCRs only recognise antigens presented in the context of CD1d, and thus, they need antigens presented by CD1d for their maturation (Godfrey and Berzins, 2007). In contrast, CD1d-independent NKT cells are a smaller group, and their TCRs recognise antigens presented on other molecules (Godfrey and Berzins, 2007). Due to the different requirements for antigen presenting molecules, the CD1d-independent NKT cells can be identified in $CD1d^{-/-}$ mice but cannot be detected in $\beta2M^{-/-}$ mouse strains (Kronenberg and Gapin, 2002).

CD1d-dependent NKT cells are further classified into two subclasses: type I and type II NKT cells (Kronenberg and Gapin, 2002). In mice, type I CD1d-dependent NKT cells are distinguished by the reactivity of their TCRs to α-GalCer loaded on CD1d tetramer. These TCRs are comprised of an invariant TCRα chain (Vα14-Jα18 in mice or Vα24-Jα18 in humans) in combination with a limited set of TCRβ chains (Vβ8.2, Vβ7 or Vβ2 in mice or Vβ11 in humans). Type I NKT cells account for approximately 80% of the total numbers of NKT cells and approximately 85% of CD1d-dependent NKT cells (Lantz *et al*., 1994; Matsuda *et al*., 2000; Park *et al*., 2001; Bendelac *et al*., 2007). Due to the use of the invariant TCRα chain, type I CD1d-dependent NKT cells are also called invariant (i) NKT cells. By

combining tetramer staining with analysis of NK1.1 expression, type I NKT cells can be classified into two smaller populations: $NK1.1^-$ and $NK1.1^+$ NKT cells. $NK1.1^-$ NKT cells are immature and can develop into mature $NK1.1^+$ NKT cells by acquiring NK1.1 on their surface (Pellicci *et al*., 2002). In mice, the larger population of type I NKT cells are $CD4^+$ and the remaining cells are $CD4^-CD8^-$. They do not simultaneously express CD4 and CD8 antigens, nor do they express only CD8 (as in humans; Lantz and Bendelac, 1994).

In humans, type I NKT cells also express an invariant TCR α chain, which is Vα24-Jα18 in combination with a limited set of TCR β chains that are biased toward the use of Vβ11. Similar with its murine counterpart, human type I NKT cells react with α-GalCer. They express CD161 (a marker, homologous and functionally equivalent to murine NK1.1) at mature stages and are $CD4^+$, DN, or $CD8^+$ (Baev *et al*., 2004; Berzins *et al*., 2005).

In contrast, type II NKT cells, also known as non-classical NKT cells, have a diverse TCRα repertoire. They do not bear a *Vα14-Jα18* rearrangement but are still CD1d-restricted and exhibit many of the same phenotypic and functional properties as invariant NKT cells. Type II NKT cells do express activated or memory markers such as NK1.1 in C57BL/6 mice, but do not recognise α-GalCer. Until now, this cell type has not been well-characterised because of the absence of a specific surface marker and agonistic antigens. Sequencing of the TCR repertoire of $V\alpha14^-$ CD1d-restricted T cell hybridomas derived from MHC class II-deficient mice has revealed that they use Vα3.2-Jα9 or Vα8 TCRα chains paired preferentially with the Vβ8 TCRβ chain (Park *et al*., 2001). Other studies have identified additional type II NKT cells that do not use Vα3.2-Jα9 or Vα8 TCRα, but still bias the use of genes from the *Vβ8* family (Behar and Cardell, 2000). This cell type recognises a range of hydrophobic antigens including sulfatide, lysophosphatidylcholine, and small aromatic molecules. Sulfatide-loaded CD1d tetramer has been produced to identify type II NKT cells,

however, this complex cannot detect all kinds of type II NKT cells (Blomqvist *et al*., 2009). Currently, the best way to study this cell subset is through the comparison of CD1d-deficient and *Jα18*-deficient mice.

In addition to above classification, recently iNKT (or type I NKT) cells have been identified into five major functionally distinct subsets, each producing a different set of cytokines. Each subset can be further characterized by the expression of distinct transcription factors that generally correlate with their cytokine response upon activation. Particularly, iNKT1 cells express T-bet$^{+}$, PLZF$^{lo}$, RORγt$^{-}$ and secrete predominantly TH1 cytokines (IFNγ); iNKT2 cells express T-bet$^{-}$, PLZF$^{hi}$, RORγt$^{-}$ and secrete TH2 cytokines (IL-4 and IL-13) (Lee *et al*., 2013); iNKT17 cells are T-bet$^{-}$, PLZF$^{int}$, RORγt$^{+}$ and secrete TH17 cytokines (IL-17); iNKT$_{FH}$ (follicular helper) express Bcl-6 and provide help to B cells in an IL-21 dependent fashion (Chang *et al*., 2012); iNKT10 cells represent a unique subset of iNKT cells that are PLZF negative but express *Nfil3* (E4BP4) and secrete IL-10 to create an immunoregulatory environment (Lynch *et al.,* 2012; 2014). Each subpopulation expresses particular cytokine receptors: iNKT1 expresses IL-12R, iNKT2 expresses IL-17RB and iNKT17 expresses IL-23R (Watarai *et al*., 2012). When stimulated with the corresponding cytokine, each subset produced only the cytokine corresponding to their subset (Watarai *et al*., 2012).

Recent studies on gene expression and epigenetic profiling largely supports the subset model of differentiation as each subtype had a distinct gene expression profile (Engel *et al*., 2016; Lee *et al*., 2016; Georgiev *et al*., 2016), yet it is likely that both pathways are interrelated. For example, the iNKT1 subset is equivalent to stage 3 iNKT cells. Furthermore, it was showed that stage 1 and stage 2 iNKT cells are composed of two populations, IL-17RB$^{+}$ cells that make up iNKT2 and iNKT17 subsets, and IL-17RB$^{-}$ cells that are progenitors of the iNKT1 subset (Watarai *et al*., 2012). Furthermore, Kronenberg and

colleagues found that the iNKT2 subset was composed of two subpopulations, in which one expressed a large amount of genes involved in cell cycle progression, indicating that it is likely a progenitor of other iNKT cells (Engel *et al*., 2016).

It is expected that all iNKT cell subsets are of thymic origin. iNKT1, iNKT2 and iNKT17 cells acquire their functional capacity in the thymus during their development (Michel *et al*., 2008; Watarai *et al*., 2012; Lee *et al*., 2013), before they migrate in the peripheral organs in a tissues specific manner (Lee *et al*., 2015). To date, $iNKT_{FH}$ and iNKT10 cells have not been described in the thymus and their origins and developmental cues remain unclear. It is possible that these two subsets develop toward these phenotypes at very low frequencies in the thymus before migration, or that they may represent distinct states of activation. It is indicated that iNKT10 and $iNKT_{FH}$ subsets arise in the periphery under the proper stimulations conditions. For example, $iNKT_{FH}$ cells are induced in the spleen of mice following injection of lipid antigens (Chang *et al*., 2012). Similarly, 'induced' iNKT10 cells that share common characteristics with the iNKT10 cells found at steady state in the adipose tissues have been described following antigen stimulation (Lynch *et al*., 2014; Sag *et al*., 2014).

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It is worth noting that the subset model is not completely set in stone. First, although each subset has a tendency to produce particular cytokines, when stimulated with a strong stimulus such as phorbol 12-myristate 13-acetate (PMA)/ionomycin or αGC, a large proportion of these cells will co-produce cytokines of other subsets, particularly IL-4 and IL-13 (Yoshimoto, 1994; Watarai *et al*., 2012), and each subset expressed some cytokines of other subsets (Engel *et al*., 2016; Georgiev *et al*., 2016). Therefore, it is interesting to speculate that upon cytokine stimulation, each subset will produce specific cytokines, yet under antigen stimulation all iNKT cells have the potential to produce all cytokines.

## 1.2. THE ROLE OF NKT CELLS IN THE IMMUNE SYSTEM

NKT cells play a crucial role in immune regulation due to their unique ability to rapidly secrete large amounts of cytokines within 90 minutes of *in vivo* TCR stimulation (Matsuda *et al.*, 2000). Once activated, the NKT cells rapidly release both Th1 and Th2 cytokines, including IL-2, IL-4, IL-10, IL-13, TNF, IFN-γ and chemokines, such as RANTES, Eotaxin, MIP-1α, and MIP-1β (Godfrey *et al*., 2004; Chang *et al*., 2005). These cytokines modulate the behaviour of other cells in both innate and adaptive immune responses (Kronenberg and Gapin, 2002). For example, IFN-γ produced by activated NKT cells promotes the activity of NK cells and macrophages as a part of innate immunity, and activates the maturation of dendritic cells (DCs) (Lang, 2009). In turn, the mature dendritic cells induce a Th1 cell response to activate adaptive immunity. In contrast, NKT cells also produce large amounts of IL-4, which controls the differentiation and development of Th2 cells, thus indirectly regulating Th1-mediated autoimmunity.

In addition to rapid cytokine production, activation of type I NKT cells through TCR stimulation also augments the bidirectional cross-talk with DCs in a CD40/CD40L and CD1d-dependent manner. This interaction promotes the maturation, activation, and the up-regulation of other co-stimulatory receptors, such as CD80 and CD86 on DCs, as well as the release of

IL-12. As a consequence of direct interaction with type I NKT cells, DCs can promote the activity of antigen-specific $CD4^+$ and $CD8^+$ T cells (Fujii *et al.*, 2003; Hermans *et al.*, 2003). Type I NKT cells can also prime the adaptive immune response through directly activating CD1d-expressing B cells (Barral *et al.*, 2008; Leadbetter *et al*., 2008). Therefore, through cytokine production and other mechanisms, NKT cells form an important link between innate and adaptive immune responses. They can enhance microbial immunity, tumour rejection, maintain self-tolerance, and suppress autoimmune diseases (Matsuda *et al*., 2008).

### 1.2.1. Role of NKT cells in autoimmune diseases

Recently, studies on various disease-prone mouse strains have demonstrated that defects in NKT cell function and numbers are correlated to autoimmune diseases. This relationship was first identified in MRL/lpr mice, a mouse model for human systemic lupus erythematosus (SLE; Chen *et al*., 2015). In MRL/lpr mice, NKT cell numbers start reducing at around 3-4 weeks of age - before the onset of the disease. When the autoimmune disease manifests, NKT cells are completely absent. The introduction of rearranged *Vα14-Ja18* gene segments into these mice delayed the decrease in NKT cell numbers and the disease onset (Mieza *et al*., 1996). In humans, the participation of NKT cells in SLE is not well understood. An increase in the αβ DN T cell proportion as well as IL-4 production after stimulation have been observed in patients with SLE, compared to healthy controls (Crispín *et al*., 2008). This cell population was not Vα24 NKT cells, but perhaps represents other pathogenic DN T cells. Therefore, the involvement of NKT cells in human SLE requires further examination.

In addition, experiments in non-obese diabetic (NOD) mice, a well-established model of type I diabetes (T1D), have shown a profound defect in NKT cell numbers and function. NOD NKT cells exhibit a dramatic reduction in IL-4 production following TCR ligation (Gombert *et al*., 1996; Baxter *et al*., 1997; Hammond *et al*., 1998). The onset of type I

diabetes was reported to be accelerated in NOD mice lacking CD1d expression for NKT cell development. Conversely, *in vivo* stimulation of NKT cells by α-GalCer, or raising NKT cell numbers either by transferring thymic type I NKT cells from nondiabetic (BALB/c x NOD) F1 mice (Baxter *et al*., 1997; Hammond *et al*., 1998), or introducing the *Vα14-Jα18* transgene (Lehuen *et al*., 1998), could suppress the development of type I diabetes in NOD mice. The protection against diabetes development is reported to be associated with the recovery of Th2 cytokine production, such as IL-4 and IL-10. However, treatment of NOD mice with Th1 cytokines (IL-12), or anti-Th2 cytokine antibodies (such as anti-IL-4), abolished the protective impact conferred by type I NKT cells (Trembleau *et al*., 1995; Hammond *et al*., 1998; Laloux *et al*., 2001).

Although it is not well documented, similar abnormalities have been found in patients with insulin-dependent diabetes mellitus (IDDM). Type I diabetic patients have been reported to have lower frequencies of DN Vα24 NKT cells in their peripheral blood than their non-diabetic twins. In addition, NKT cells from normal controls or non-diabetic siblings were found to secrete both IFN-γ and IL-4, although those from diabetic patients only produced IFN-γ (Wilson *et al*., 1998; Kukreja *et al*., 2002). These findings suggest that the decrease in NKT cell numbers results in the failure of IL-4 production, and subsequently tissue damage mediated by Th1 cells. However, a recent study using CD1d-tet has indicated that there is no difference between IDDM patients and healthy controls in NKT cell numbers and the ability to produce IL-4 (Lee *et al*., 2002b). A separate study also challenged the hypothesis that NKT cell deficiencies were associated with T1D in humans, as it indicated that NKT cell numbers were increased in the blood of T1D patients (Oikawa *et al*., 2002). Due to these discrepancies as well as the limitation in collecting samples, the involvement of NKT cells in human T1D requires further investigation.

NKT cell function has been studied in experimental autoimmune encephalomyelitis (EAE) mice, a model for multiple sclerosis (MS). EAE in mice is induced by immunising the central nervous system with myelin oligodendrocyte glycoprotein (MOG) or myelin basic protein (MBP) in a mycobacterium-containing adjuvant. In this model, NKT cells confer a Th2-biased immune response that plays a role in protection from EAE. The augmentation of NKT cells in NOD mice via the introduction of the *Vα14-Jα18* transgene has significantly suppressed MOG-induced EAE (Mars *et al*., 2002). Consistent with this, the deletion of the CD1d gene from B6 mice resulted in a more severe form of EAE compared to wild type control mice (Teige *et al*., 2004). In contrast, other groups have found no differences in disease course between $CD1d^{-/-}$ (Singh *et al*., 2001) or $J\alpha18^{-/-}$ (Furlan *et al*., 2003) mice compared to wild type littermates. There have been conflicting results for α-GalCer treatment in EAE studies. For example, the administration of α-GalCer in B10.PL and C57BL/6 mice (before immunisation with peptide from MBP or accompanied with an encephalitogenic MOG peptide, respectively) was found to reduce the severity of EAE (Jahng *et al*., 2001). On the other hand, there were reports of EAE exacerbation in B10.PL and SJL/L mice, following co-administration of α-GalCer and MBP (Singh *et al*., 2001; Furlan *et al*., 2003).

These conflicting reports raise the question as to what extent NKT cells contribute to the immune response against MS. While Jahng opposed the role of IL-4 in disease protection by α-GalCer in C57BL/6 mice (Jahng *et al*., 2001), others suggested that the protective effects of α-GalCer depend on the ability of the mouse strain to produce IL-4 upon stimulation with α-GalCer. In fact, disease suppression was seen in strains producing higher levels of IL-4 in response to α-GalCer treatment. Moreover, the protective effect of α-GalCer in EAE was not seen in IL-4- or IL-10-deficient mice (Singh *et al*., 2001), whereas disease

was suppressed in IFN-γ-deficient mice (Jahng *et al*., 2001; Pal *et al*., 2001). Although one study demonstrated that IFN-γ but not IL-4 is critical for disease protection by α-GalCer in C57BL/6 mice (Jahng *et al*., 2001), the above findings suggest that EAE protection by α-GalCer is mediated by Th2 cytokines produced by NKT cells.

There is a substantial body of evidence to support Th2-mediated protection. For example, *in vivo* injection of α-GalCer-pulsed APCs with CD86 blockade (treated with anti-B7.2 antibodies) not only polarizes NKT cells toward a Th2-like phenotype but also mediates concomitant suppression of EAE. In contrast, α-GalCer presented by anti-CD40-activated APCs induces a bias of NKT cells toward a Th1-like phenotype and exacerbated EAE (Pal *et al*., 2001). Moreover, in mice, *in vivo* administration of the altered glycolipid ligand OCH, a sphingosine-truncated analog of α-GalCer, ameliorates EAE and T1D by preferentially inducing IL-4 from NKT cells without enhancing pathogenic Th1 responses (Oki *et al*., 2004; Goff *et al*., 2004; Oki *et al*., 2005). OCH has been shown to be more effective than α-GalCer in protecting C57BL/6 mice from EAE, and it possesses some efficacy even when treatment was initiated several days after EAE induction. OCH was also effective when administered orally, which is the favored treatment route for humans. The protective effect of OCH was abrogated by neutralisation of IL-4 (Miyamoto *et al*., 2001). Taken together, this evidence indicates that NKT cells appear to work as a regulator in EAE, and targeted activation of NKT cells could lead to amelioration of EAE. These studies have provided a strong foundation for the rational design of NKT-cell-based immunotherapies for MS that induce tolerance while sparing overall immune function. Nevertheless, additional pre-clinical and clinical studies will be required to bring this goal to fruition (Van Kaer *et al.,* 2015).

### 1.2.2. Role of NKT cells in tumour surveillance

The involvement of NKT cells in the immune response against tumours has been analyzed in a range of different tumour models. *Vα14*-deficient mice have a higher incidence of methylcholanthrene-induced fibrosarcomas and earlier tumour progression than control C57BL/6 mice (Smyth *et al*., 2000; Smyth *et al*., 2001). However, this model could not rule out the effects of NK cells that are known to contribute to immunological surveillance. To confirm and extend previous observations using methylcholanthrene tumour models, the authors demonstrated that adoptive transfer of type I NKT cells into $J\alpha18^{-/-}$ mice was able to protect the mice from methylcholanthrene-induced sarcomas via direct interaction of the type I NKT TCR with CD1d molecules (Crowe *et al*., 2002).

NKT cells, perhaps, control tumour development through both direct and indirect cytotoxic activity. NKT cells could serve as cytotoxic effector cells to directly kill tumour cells and this could be the reason why *Vα14* NKT-deficient mice could not be protected against experimental liver metastasis of melanoma (Kawano *et al*., 1998). On the contrary, it is more likely that NKT cells suppress tumour development in an indirect manner through the activation of effector cells, such as $CD8^+$ T cells and NK cells (Smyth *et al*., 2000; Smyth *et al*., 2002). In particular, it has been suggested that NKT cells are activated through TCR interaction to produce IL-4, IFN-γ, and to up-regulate CD40L expression. IFN-γ and CD40L expression subsequently promote the production of IL-12 by DCs. Finally, IL-12 activates a second burst of IFN-γ production by NKT cells to stimulate NK and $CD8^+$ T cells to induce tumour killing (Smyth *et al.,* 2000; Taniguchi *et al*., 2003a).

The role of NKT cells in tumour killing has been examined recently in several mouse tumour models. A study of the 15-12RM tumour model indicated that $CD4^+$ NKT cells play an immunosuppressive role leading to tumour progression by inhibiting $CD8^+$ cytotoxic T

lymphocytes (CTL). Although the immunosuppressive mechanism remains to be determined, it could be an indirect pathway, mediated through IL-13 derived from $CD4^+$ NKT cells and other cell types (Taniguchi *et al*., 2003b). In a subsequent study, Terabe and colleagues demonstrated that type I NKT cells are not necessary for immune suppression. Interestingly, in the absence of both type I NKT cells and $T_{reg}$ cells, type II NKT cells are sufficient to suppress immunosurveillance of tumors (Terabe *et al*., 2005). A new mechanism of action was discovered a by O'Konek and colleagues, in which activation of type I NKT cells with beta-mannosylceramide (β-ManCer) induced tumour elimination in the absence of IFN-γ through a nitric oxide and TNF-α-dependent mechanism (O'Konek *et al*., 2011). Other studies of murine tumour models have indicated that two subsets of NKT cells, type I and type II, can play opposing roles and cross-regulate each other, forming an immunoregulatory axis that may influence the balance of other immune responses. Consistent with this, multiple other studies also found that type I NKT cells enhance and type II NKT cells suppress tumor immunity (Terabe and Berzofky, 2014).

NKT cells also have an impact on tumour development in humans. For example, human NKT cells exhibit cytotoxicity against several tumour cell lines, such as haematological malignancies, through effector mechanisms *with* a distinct from those of conventional T cells and NK cells (Nicol *et al*., 2000). NKT cells also display a cytotoxic activity against the U937 tumour cell line through the perforin pathway after receiving activation from α-GalCer in the presence of DCs (Takahashi *et al*., 2000). Patients with advanced cancer show a decrease in NK, T, and NKT cells compared with normal controls (Kobashi *et al*., 2001). Similar to murine type II NKT cells, CD1d-dependent type II NKT cells in humans enhance immune-suppressive functions in *MLR* mice by releasing Th2 cytokines (Baker *et al*., 2001).

### 1.2.3. Role of NKT cells in microbial infection.

In addition to their effects on autoimmune diseases and tumour surveillance, NKT cells participate in immune responses against microbial infections caused by bacterial, viral and protozoan agents. NKT cells respond to a range of infectious organisms through the recognition of microbial lipids, such as α-galactosyldiacylglycerol from *Borrelia burgdorferi* (Kinjo *et al*., 2006), lipophosphoglycan from *Leishmania donovani* (Amprey *et al*., 2004), α-glucosyldiacylglycerol from *S. pneumoniae* (Kinjo *et al*., 2011), α-glucuronosylceramide and α-galacturonosylceramide from *Sphingomonas* species (Kinjo *et al*., 2005; Sriram *et al*., 2005; Mattner *et al*., 2005), and a cholesteryl α-glucoside from *Helicobacter pylori* (Chang *et al*., 2011) presented by $CD1d^{+}$ APCs (Slauenwhite and Johnston, 2015). For example, NKT cells could form granulomas as an early immune response when the host is infected with *Mycobacterium tuberculosis*. Despite this, NKT cells are not responsible for the resistance to *Mycobacterium tuberculosis* because there is no difference between the protective immune responses against this pathogen in β2M-deficient, CD1d-deficient, and wild type mice. Studies of granuloma models indicate that NKT cells are the major cell type detected in early granulomas, and the development of a sizable, granuloma-like structure depends on the recruitment of NKT cells as *Vα14* NKT cell-deficient mice did not develop granulomas (Apostolou *et al*., 1999). Importantly, the recruitment of *Va14* NKT cells to granulomas does not require specific presentation of mycobacterial antigens by CD1d, as it can occur even when NKT cells are adoptively transferred into $CD1d^{-/-}$ mice. However, it has been demonstrated that NKT cells participate in pathogen clearance mediated by IFN-γ (Emoto *et al*., 1999; Mempel *et al*., 2002).

NKT cell stimulation by microbial ligands is not restricted to microbial lipid antigens that are recognised directly by the Vα14–Jα18 TCR. Other microbial products also activate

APCs via pattern recognition receptors (such as TLRs, NOD-like receptors, etc), leading to an increased accumulation of weak self-glycolipid antigens and the production of NKT cell-stimulating cytokines (IL-12, IL-18, and type I IFNs) (Brigl *et al*., 2003; Mattner *et al*., 2005; Paget *et al*., 2007; Salio *et al*., 2007; Nagarajan *et al*., 2007; Brigl *et al*., 2011). In that case, CD1d-presented antigens may not be required and IL-12, IL-18, or type I IFNs alone or in combination may be sufficient to drive NKT cell activation and IFN-γ production (Nagarajan *et al*., 2007; Wesley *et al*., 2008). In particular, NKT cells could indirectly induce protective immune responses toward lipopolysaccharide (LPS). It has been demonstrated that two consecutive low-dose injections of LPS are able to induce a lethal shock in mice (Dieli *et al*., 2000). In terms of a mechanism for this lethal shock syndome, it has been suggested that LPS stimulates macrophages via TLR-4 to generate IL-12, which in turn directly promotes NKT cells to produce large amounts of IFN-γ and TNF. IFN-γ continuously enhances the production of TNF, which induces hepatocyte injury (Ozmen *et al*., 1994). Although the cell type producing TNF in this step remains unclear, it could be generally concluded that the effect of the NKT cells lies in their IL-12, IFN-γ, and TNF production. Consistent with this finding, another study suggested that *Vα14*-deficient mice were resistant to this low-dose LPS shock. They also exhibit a reduction in IFN-γ and TNF secretion, but not IL-12, compared to wild type mice. The injection of recombinant IFN-γ but not IL-12 could restore LPS-induced mortality and TNF production (Ozmen *et al*., 1994). In addition, weak signalling produced by endogenous lipid ligands through the NKT-cell TCR, although not sufficient for full NKT cell activation, triggers NKT cells to release IFN-γ upon exposure to the cytokines IL-12 and IL-18 (Wang *et al*., 2012).

In contrast to the precise knowledge about the role of NKT cells in murine models of microbial infection, little is known about the role of NKT cells in human infectious diseases. Although there is a decrease in circulating NKT cells in human immunodeficiency virus (HIV-1)

patients, they do not appear to affect disease progression (van de Vliet *et al*., 2002). Another report indicated that NKT cells play an important role in controlling herpes simplex virus (HSV) (Grubor-Bauk *et al*., 2003). In fact, Vα14- and CD1d-deficient mice failed to clear HSV compared to wild type mice. Moreover, NKT cells are able to inhibit HBV replication when they are activated by α-GalCer, even though this activity is attributed to both primary cytokines (produced by NKT cells) and secondary cytokines (produced by NK cells; Kakimi *et al*., 2000).

In addition to the functions reported above, NKT cells are involved in other immune responses such as transplant rejection and allergic disorders, though the exact function of NKT cells in these instances is unclear. However, it does appear that their proinflammatory actions usually outweigh their immunosuppressive roles.

## 1.3. NKT CELL NUMBERS AND NOD MICE

### 1.3.1. NKT cell numbers

NKT cells are a minor cell population in the immune system. Their prevalence is quite different between species and between organs. In mice, NKT cell numbers vary between strains. Among the commonly used mouse strains, C57BL/6 mice have relatively high NKT cell numbers, while NOD mice exhibit a significantly lower number of NKT cells. These findings were found by both older studies using surrogate markers (Gombert *et al.,* 1996; Baxter *et al*., 1997), and more recent studies that utilise CD1d-tet for NKT cell identification (Hammond *et al*., 2001; Poulton *et al*., 2001).

In terms of tissue distribution, NKT cells are found primarily in the liver, thymus, and spleen, although the relative proportions of NKT cells vary. In mice, type I NKT cells account for 30-50% of T cells in the liver and 20-30% in the bone marrow. In the thymus, NKT cells represent 10-20% of mature ($HSA^-$) T cells and only 0.3-0.5% of total thymocytes (Eberl *et al*., 1999a; Benlagha *et al.,* 2000; Matsuda *et al*., 2000; Hammond *et al*., 2001). In other

tissues, NKT cells represent a minor proportion of T cells including spleen (3%), lymph node (0.3%), blood (4%), lung (7%) and adipose tissue (2.4%) (Eberl *et al*., 1999a; Matsuda *et al*., 2000; Huh et al., 2013).

NKT cell distribution is not well-studied in humans, as the proportion of Vα24 NKT cells is considerably lower than in mice (Bendelac *et al*., 2007). They constitute 0.1% - 0.2% of blood T cells but this proportion is highly variable among individuals (Gumperz *et al*., 2002; Lee *et al*., 2002a; Montoya *et al*., 2007). In humans, Vα24 NKT cells are not highly enriched in the liver but accumulate in the ometum, making up approximately 10% of the T cell population in that site (Lynch *et al*., 2009).

### 1.3.2. NOD mice

Due to the role of NKT cells in immune regulation, a large number of studies have focused on NOD mice, a mouse strain in which NKT cell numbers are severely reduced. Compared with eight other commonly used mouse strains, such as C57BL/6 and BALB.B6-*Cmv1*$^{r}$ mice, NOD mice have relatively lower numbers of type I NKT cells in the thymus and peripheral lymphoid organs, including the spleen and liver (Gombert *et al*., 1996; Baxter *et al*., 1997; Godfrey *et al*., 1997; Poulton *et al.,* 2001; Hammond *et al*., 2001). In addition to these numerical deficiencies, NKT cells derived from NOD mice produce relatively less IL-4, which is two to four-fold lower in plasma in response to anti-CD3 administration than in C57BL/6 mice. Similarly, there are 20-fold fewer IL-4-secreting thymic NKT cells in NOD mice than in C57BL/6 mice (Gombert *et al*., 1996; Hammond *et al*., 2001; Poulton *et al*., 2001).

The concomitant deficiencies in NKT cell numbers and function appear to contribute to the autoimmune phenotypes observed in NOD mice. These mice spontaneously develop T1D, which characterised by the destruction of pancreatic islet beta cells (Baxter *et al*., 1994).

There is a tight relationship between the spontaneous development of T1D and the severe decrease in NKT cell numbers and function in NOD mice (Makino *et al*., 1980; Baxter *et al*., 1997; Hammond *et al*., 1998). The course and the severity of T1D in these mice was able to be modulated by increasing NKT cell numbers through adoptive transfer (Hammond *et al.,* 1998; Laloux *et al*., 2001), congenesis (Jordan *et al*., 2007; Chen *et al*., 2007; Fletcher *et al*., 2008), introduction of *Vα14-Jα18* (Lehuen *et al*., 1998) or *Cd1d* transgenes (Falcone *et al*., 2004), or activation with α-GalCer (Seokmann *et al*., 2001; Sharif *et al.,* 2001).

NOD mice are also susceptible to other autoimmune diseases such as systemic lupus erythematosus (Baxter *et al*., 1994; Esteban *et al*., 2003), and hemolytic anemia (Baxter and Mandel, 1991). Notably, the injection of BCG into these mice is reported to be able to inhibit T1D but instead induces an SLE-like disease. It could be postulated that a common factor may control these two autoimmune diseases.

A genetic linkage study of a first backcross between NOD and C57BL/6 mice has revealed that the defect in NKT cell numbers in NOD mice is controlled by 2 genetic regions: *Nkt1* and *Nkt2*, which map to regions containing lupus and diabetes susceptibility genes, respectively (Esteban *et al*., 2003). The congenic replacement of these 2 regions with corresponding regions derived from C57BL/6 mice partly increased NKT cell numbers and the congenic replacement of *Nkt2* delayed the onset of type I diabetes in the NOD recipient mice (Jordan *et al*., 2007; Fletcher *et al*., 2008). However, it did not completely rescue NKT cell numbers or entirely prevent T1D (Jordan *et al*., 2007; 2011; Chung *et al*., 2005; 2008; Nichols *et al*., 2005; Pasquier *et al*., 2005; Griewank *et al*., 2007). There might be other factors that contribute to the reduced number and function of NKT cells in NOD mice, though this requires further investigation.

## 1.4. NKT CELL DEVELOPMENT

### 1.4.1. NKT cell origin

While much is known about the role of NKT cells in immune regulation, details of NKT cell origins and their developmental program are still elusive. Previously, two different models have been proposed to explain the developmental pathway of NKT cells (MacDonald, 2002). The first model, called the instructive model or mainstream model, posited that NKT cells develop from the same progenitors as conventional T cells (Bendelac *et al*., 1997), as they are a thymus-dependent T cell subset, absent from nude mice, and undetectable in mice after thymectomy (Bendelac, 1995; Taniguchi *et al*., 2000; MacDonald *et al*., 2002). In contrast, the pre-commitment model suggested that NKT cells are derived from distinct precursors (Sato *et al*., 1999). These precursors are generated very early during embryogenesis, perhaps even before the development of thymus or the formation of conventional T cells (Makino *et al*., 1996; Baur *et al*., 2001; Iwabuchi *et al*., 2001).

The most convincing argument for the pre-commitment model was reported by Iwabuchi (2001). In this research, NK1.1$^{+}$ TCR$\alpha\beta^{-}$ cells were identified in the thymus of ZAP-70$^{-/-}$ mice while neither NK1.1$^{+}$TCR$\gamma\delta^{+}$ nor NK1.1$^{+}$TCR$\alpha\beta^{+}$ cells were detected in either the thymus or the spleen. These cells did not express surface TCR$\beta$ because TCR*V$\beta$* gene segments were not rearranged. However, sorted NK1.1$^{+}$ TCR$\beta^{-}$ thymocytes in the presence of phorbol ester and ionomycin in fetal thymic organ culture (FTOC) led to the formation of some NK1.1$^{+}$ TCR$\beta^{+}$ cells and detectable *V$\alpha$14-J$\alpha$281* rearrangement. This raised the possibility that pre-NKT cells might exist in the thymus of ZAP-70-deficient mice (Iwabuchi *et al.,* 2001). In another study, two NK1.1$^{+}$TCR$\beta^{+}$ populations were identified in the spleen of *V$\beta$8.2* transgenic mice. One population expressed CD3$^{high}$ and *V$\alpha$14* mRNA, the other expressed precursor phenotypes, including CD3$^{dim}$, and no *V$\alpha$14* mRNA (but did

exhibit transcripts relevant to the immature developing T cell lineage, such as RAG-1, RAG-2 and pTα). When $CD3^{dim}$ $RAG^{+}$ $V\alpha14^{-}$ cells were purified from the bone marrow of these mice and cultured in combination with IL-15, GM-CSF, and in the presence of stroma cells, they were able to differentiate *in vitro* into mature $CD3e^{+}$ $V\alpha14^{+}$ NKT cells (Sato *et al*., 1999). In another study, $NK1.1^{+}$ thymocytes were detected in TCR/CD3-signalling-deficient mice. These cells did not exhibit *Vα14-Jα18* rearrangement or an αβTCR, even though they had undergone TCRβ VDJ rearrangement. This observation suggested that they may contain pre-NKT cells, which were blocked in the absence of CD3/TCR signaling (Baur *et al*., 2001). However, whether or not pre-NKT cells actually exist should be elucidated, because these findings do not rule out the possibility that the absence of ZAP70 and the presence of pharmacological stimulators or aberrant NKT cell precursors may result in changes in the normal pathway of thymocyte differentiation and/or abnormal TCR expression.

In sharp contrast, there is overwhelming evidence supporting the mainstream model by which mature NKT cells are derived from $CD4^{+}CD8^{+}$ (double positive - DP) thymocytes (the same progenitors as conventional T cells), because rearranged TCR α chains first appear on DP thymocytes. However, direct identification of such putative DP Vα14 NKT precursors has been controversial, because their frequency is extremely low. It is generally accepted that no cells that stain with CD1d-tet can be identified among conventional DP thymocytes expressing high levels of both CD4 and CD8. However, several groups detected rare CD1d-tet-stained cells in a DP "dull" population, which could represent post-selection Vα14 NKT progenitors that have partially down-regulated CD4 and CD8 (Benlagha *et al*., 2005; Cannons *et al*., 2006), while other groups found a detectable frequency of in-frame *Vα14-Jα18* rearrangement in purified DP thymocytes (Gapin *et al*., 2001; Dao *et al*., 2004). These

rearrangements can even be detected in DP thymocytes from $CD1d^{-/-}$ mice (Gapin *et al*., 2001), suggesting the presence of unselected DP Vα14 NKT precursors.

The most convincing evidence came from the work of Dashtsoodol and colleages, in which $CD4^+CD8^+$ thymocytes were sorted and injected into $J\alpha18^{-/-}$ (a strain that lacks type I NKT cells but possesses the CD1d ligand) and $CD1d^{-/-}$ mice. The results revealed that mature ($CD4^+$ or DN) NKT cells could be detected in the thymus, spleen and liver of $J\alpha18^{-/-}$ mice, whereas they did not exist in the thymus, spleen and liver of $CD1d^{-/-}$ mice, even though they had received DP cells intrathymically (Dashtsoodol *et al*., 2008). A similar finding was also achieved when DP thymocytes from $CD1d^{-/-}$ mice were sorted and re-injected into thymus of wild type mice that had a CD45.1-congenic region. In $CD1d^{-/-}$ mice, NKT cells cannot differentiate because of the deficiency of CD1d ligand interactions necessary for positive selection of DP thymocytes. The transfer of these cells into $CD1d^+$ recipients resulted in their differentiation into mature NKT cells (Gapin *et al*., 2001). In addition to phenotypic findings, Egawa has provided genetic evidence for type I NKT cell development by using fate mapping and mutative analysis. The results show that type I NKT cells are selected from a common pool of DP thymocytes (Egawa *et al*., 2005). Collectively, this data strongly supports the instructive model in which $CD4^+CD8^+$ thymocytes are progenitors of NKT cells.

### 1.4.2. Rearrangement of TCR gene segments.

The TCR gene rearrangement common to both the T and NKT cell lineages occurs at the $TCR^-CD4^-CD8^-$ (triple negative - TN) stage and is essentially the same as what has been described for conventional T cells. During the TN stage, the development of the common progenitors is divided into four small stages ($CD44^+CD25^-$TN -> $CD44^+CD25^+$TN -> $CD44^-CD25^+$TN -> $CD44^-CD25^-$TN), based on the expression of CD25 and CD44 markers.

The rearrangement of TCRβ and γ loci occurs first when the common precursors transit from the $CD44^{+}CD25^{+}$TN to the $CD44^{-}CD25^{+}$TN stage (reviewed in Godfrey *et al*., 1993a, Godfrey *et al*., 1993b). This transition is followed by TCRβ expression, then β-selection for a functional TCR β chain. After passing through β-selection, these common progenitors down-regulate CD25 expression to become $CD44^{-}CD25^{-}CD4^{low}CD8^{low}$ thymocytes, which is accompanied by rearrangement of TCRα gene segments. The TCRβ locus randomly combines variable (V) with diverse (D) segments, and then with one joining (J) segment. This is in contrast to the rearrangement of TCRα, which occurs between V and J segments to create a common TCRα repertoire.

In accordance with the aforementioned insights into TCRα and β rearrangement, the invariant TCRα chain of type I NKT cells is formed when *Vα14* in mice (or *Vα24* in humans) randomly combines with a *Jα18* segment. The TCRβ chains are formed by recombining a range of *Vβ* chains (mostly *Vβ8.2, Vβ7, Vβ2* in mice or *Vβ11* in humans) with *Jβ* genes. Therefore, the resulting TCRβ chains of type I NKT cells are moderately diverse, which explains why NKT cells can recognise a range of different antigens (Bendelac *et al*., 2007; Godfrey *et al*., 2010).

There are two opposing opinions regarding the method of *Vα14-Jα18* rearrangement: one is in favor of a random process and the other supports a predetermined program. However, if the pre-NKT cells implement a genetic program leading to a directed rearrangement, there would be around 50,000 type I NKT cell precursors doomed to die for every cell that achieved the correct *Vα14-Jα18* rearrangement (Asarnow *et al*., 1993; Bendelac *et al*., 1997). To date, there is no evidence supporting a directed program (Shimamura *et al*., 1997). A study on the nucleotide sequence of TCRα junctional regions (CDR3s) has shown that approximately 40% of type I NKT cells display greater nucleotide

trimming and an addition of N nucleotides, even though they still conserve the canonical amino acid length and sequence. Furthermore, analysis by RT-PCR on immature DP thymocytes has shown that *Vα14* could rearrange with a range of *Jα* gene segments, not just *Jα18* (Gapin *et al*., 2001). Collectively, these findings suggest that NKT cell precursors have been selected rather than programmed.

### 1.4.3. Positive selection of NKT cells

It is suggested that after common DP thymocytes undergo random rearrangement of the TCR *Vβ* and *Vα* gene segments and form a rearranged TCR repertoire, they quickly undergo positive selection. Unlike conventional T cells, which are selected by peptide antigens presented in the context of MHC class I or II molecules on cortical epithelial cells, NKT cells are selected by glycolipid antigens presented by CD1d on cortical DP thymocytes (Bendelac, 1995; Godfrey and Berzins, 2007). This antigen presenting activity depends on the presence of 2β-microglobulin (2βM), but is independent of MHC class II. Therefore, NKT cells are absent in 2βM KO mice but intact in MHC II-deficient mice. In contrast, NKT cell numbers are seriously affected in *Cd1d*$^{-/-}$ mice, even though *Vα14-Jα18* rearrangement is still detected in their DP thymocytes (Cole and Raulet, 2000; Chun *et al*., 2003). Furthermore, adoptive transfer of DP thymocytes into lethally-irradiated wild type mice could give rise to mature NKT cells, but could not when DP thymocytes from wild type were adoptively transferred into *Cd1d*$^{-/-}$ recipient mice (Bendelac, 1995). Collectively, these findings suggest that CD1d is required for NKT cells to be positively selected.

Due to the requirement of CD1d for positive selection, the generation of NKT cells is also affected by factors contributing to CD1d production. During the formation process, CD1d must traffic through intracellular pathways in APCs where glycolipid antigens are processed and loaded onto CD1d. Mice lacking AP-3 or prosaposin revealed impairments in

NKT cell activation and development, as AP-3 and prosaposin play important roles in the loading of glycolipids into the CD1d antigen-binding groove (Sugita *et al*., 2002). In this case, AP-3 links to a tyrosine-based motif on the cytoplasmic tail of CD1d and serves as an indicator that assists in trafficking CD1d to the endoplasmic reticulum to load lipid antigens. Prosaposin is a protein precursor of four saposins (termed A, B, C and D), which are small lysosomal glycoproteins required for the processing and hydrolysis of sphingolipids (Hiraiwa *el al*., 1997). Therefore, deficiency in either AP3 or prosaposin, or mutation in the CD1d cytoplasmic tail, leads to a blockade in NKT cell development (Chui *et al*., 2002; Lawton *et al*., 2005).

While conventional CD4 and CD8 T cells are positively selected by peptide antigens in the context of MHC class I or II on cortical epithelial cells, NKT cells are positively selected by lipid antigens presented by CD1d on cortical DP thymocytes. The cortical DP thymocytes are the major cell population that exhibit CD1d in thymus, while cortical epithelial cells do not seem to express CD1d. According to Bendelac and colleagues (1995), they are immature cortical DP thymocytes rather than a professional antigen-presenting cell, such as a B cell or a mature T cell (Bendelac *et al.*, 1995). NKT cells could not be generated in lethally irradiated $\beta 2M^{+/+}$ mice reconstituted with $\beta 2M^{-/-}$ fetal liver cells, though they were able to be generated in reciprocal chimeras ($\beta 2M^{-/-}$ mice constituted with $\beta 2M^{+/+}$ fetal liver cells) (Bendelac, 1995; Cole and Raulet, 2000; Gapin *et al*., 2001). This indicates that the generation of NKT cells requires the presence of bone marrow-derived cells. The proximal Lck promoter was used to restrict transgenic expression of mouse or human CD1d on DP thymocytes, and subsequently demonstrated that CD1d-expressing DP thymocytes are sufficient to promote positive selection of V$\alpha$14 NKT cells (Wei *et al*., 2005; Zimmer *et al*., 2006).

As mentioned above, the random rearrangement of TCRα and β produces an unselected, varied TCR repertoire, which will later determine the avidity for peptide or lipid antigens on APCs. The DP thymocytes expressing TCRαβ with appropriate avidity for self-peptide antigens are positively selected upon interaction with MHC class I or II molecules expressed on thymic epithelial cells (Godfrey and Zlotnik, 1993a). The cells that successfully pass through thymic selection subsequently undergo maturation to become conventional $CD4^+CD8^-$ or $CD4^-CD8^+$ (single positive) T cells. In contrast, the DP thymocytes whose TCR has appropriate avidity for glycolipid antigens presented in the context of CD1d on cortical DP thymocytes are positively selected as NKT cells (Bendelac, 1995; Godfrey and Berzins, 2007). DP thymocytes whose TCR have insufficient or overly strong avidity for endogenous antigens (either peptide or glycolipid antigens) are deleted by neglect or negative selection, respectively (Pellicci *et al*., 2003; Chun *et al*., 2003; Schumann *et al*., 2005; Godfrey and Berzins, 2007).

However, precisely how iTCR-CD1d interaction could induce the positive selection of type I NKT cells remains an enigma. Several findings indicated that NKT cells could be selected by the specificity of their invariant TCR repertoire for CD1d. The TCRα amino acid chains of type I NKT cells are typically invariant, their TCRα DNA junction regions are variable as a result of trimming, followed by N-region additions. For example, the analysis of nucleotide sequence data has revealed that the complementarity-determining region 3 (CDR3) of 40% NKT cells are trimmed and contain added N nucleotides, even though they still conserve the canonical amino acid length and sequence (Lantz *et al*., 1994; Ronet *et al*., 2001).

TCRβ chains could also partly contribute to positive selection of NKT cells (Ohteki and MacDonald, 1996; Dao *et al*., 2004). In fact, the most prominent β chain families that were found to be associated with type I NKT cells were Vβ8.2, Vβ7, Vβ8.3, Vβ2 and Vβ8.1

in mice, and Vβ11 in humans (Bendelac *et al*., 1997). With this observation arises the question of whether this biased usage of Vβ chains is a result of TCR repertoire selection or the inability of the invariant TCRα chain to pair other Vβ chains. A study on *Vα14-Jα18* TCRα transgenesis has shown that an invariant TCRα chain could pair indiscriminately with many Vβ families (Wei *et al*., 2006), which rules out the Vβ pairing issue. Furthermore, differences within the Vβ domains (such as Vβ8.2 and Vβ7) of NKT TCR-CD1d complexes resulted in altered TCRβ-CD1d-mediated contacts, while altered recognition was mediated by the invariant alpha chain (Pellicci *et al.*, 2009). Mallevaey and colleagues demonstrated that specific sequences within the CDR2β and CDR3β loops are responsible for shaping the type I NKT cell repertoire *in vivo*, and do so by constraining type I NKT TCR interaction with antigen(s)/CD1d complexes (Mallevaey *et al*., 2009). Moreover, transgenesis of *Vβ8.2* and *Vβ8.1* TCRβ chains resulted in decreased NKT cell numbers (Takahama *et al*., 1991). The transgenesis of *Vα14-Jα18* gene segments into B6 mice increased the affinity for CD1d in T cells bearing CD8. As a result, this led to a selective depletion of 50-90% $CD8^+$ T cells bearing Vβ8 or Vβ7 chains, which are most commonly expressed by mouse Vα14 NKT cells (Bendelac *et al*., 1996). Two recent studies also provide further evidence corroborating the notion that the restricted TCRβ repertoire is shaped by positive selection (Pellicci *et al*., 2009; Mallevaey *et al*., 2009). Altogether, these results suggest a role for Vβ regions in contributing CD1d specificity for positive selection (Bendelac *et al*., 1997).

It is well demonstrated in conventional T cell positive selection that the interactions between the newly formed TCRs and endogenous ligands induce a series of intracellular signalling cascades, which will direct the DP thymocytes to positive or negative selection (Starr *et al*., 2003). Upon engagement of the TCRs by antigens presented by MHC molecules, the Src family kinases Lck and Fyn are activated and phosphorylate immunoreceptor tyrosine-

based activation motifs (ITAMs) on CD3 (Kane *et al*., 2□□□□. Phosphorylated ITAMs promote the recruitment and subsequent activation of another tyrosine kinase, ZAP-70, which, in turn, phosphorylates the tyrosine residues on the adapter molecule Linker for Activation of T cells (LAT) (Samelson, 2002). The phosphorylation of LAT results in recruitment of several components of the TCR signal transduction pathway, including Growth Factor Receptor-Bound Protein-2-Related Adaptor Protein-2 (GADs), SH2 Domain-Containing Leukocyte Protein-76 (SLP-76), Phospholipase C gamma 1 (PLCγ 1) and Growth factor receptor-bound protein 2 (GRB2) (Wange, 2000; Koretzky *et al*., 2006). In the presence of a moderate-avidity TCR ligand, partial LAT phosphorylation recruits PLCγ 1 and GADs/SLP-76/IL2 Inducible T-cell Kinase (ITK), resulting in the activation of the Ras pathway and calcium signalling. The Ras pathway stimulates sustained, low-level Extracellular Signal-Regulated Kinase (ERK) activation, while the calcium flux activates Nuclear Factor of Activated T-cells (NFAT) (Brownlie and Zamoyska*,* 2013). These signals ultimately lead to thymocyte survival, cessation of TCR gene recombination, antigen receptor tuning, cell migration into a new thymic environment, and lineage commitment. In contrast, a high-avidity TCR interaction results in complete phosphorylation of LAT, which, in turn, recruits GRB2/Son of Sevenless homolog 1 (SOS1). This results in strong, transient activation of ERK, coupled with p38 and c-Jun N-terminal Kinase (JNK) activation, which leads to negative selection or programmed cell death (Starr *et al*., 2003).

### 1.4.4. Negative selection of NKT cells

While positive selection of NKT cells is gradually being clarified, the question of whether type I NKT cells are subject to negative selection remains controversial. The most compelling evidence indicates that the early introduction of α-GalCer, both *in vivo* and *in vitro*, could specifically ablate NKT cell development. However, it has been shown that the

introduction is unable to do so if the challenge is delayed until after NKT cells have differentiated (Pellicci *et al*., 2003). In addition, a study using FTOC has shown that NKT cells could not be generated in the thymic lobes if cultivated in the presence of α-GalCer, while normal development was observed in cultures which employed β-GalCer or DMSO (Chun *et al*., 2003; Pellicci *et al*., 2003). However, treatment of mice with α-GalCer via intraperitoneal injection from days 3 to 14 after birth results in depletion of α-GalCer-loaded CD1d-tet$^{+}$ cells, with no significant effect on any other T lymphocyte populations (Pellicci *et al*., 2003). Moreover, addition of this antigen to FTOC or *in vivo* administration after the development of type I NKT cells does not affect the absolute number of cells. In these studies, the presence of α-GalCer, a potent antigen, mediated negative selection of type I NKT cells. Unfortunately, α-GalCer is not a mammalian self-antigen for the semi-invariant TCR, thus these studies do not address the question of whether type I NKT cells can be negatively selected under physiological conditions.

More recent studies have examined the effects of ligand-expressing cells by altering ligand density. In these experiments, overexpression of CD1d (derived from both mice and humans) in transgenic mice impairs NKT cell development and leads to reduced NKT cell numbers (Schumann *et al.*, 2005). This is consistent with the possibility that the high levels of CD1d result in significant negative selection of NKT cells.

In addition, by creating mice expressing a transgenic TCR-β chain that pairs with the canonical Vα14-Jα18 TCR-α chain and confers high affinity for self-lipid/CD1d complexes, Bedel *et al*. (2014) indicated that effective functional maturation of invariant natural killer T cells is constrained by negative selection and T-cell antigen receptor affinity. Analysis with PBS57-loaded CD1 tetramers revealed a large reduction in, but not complete loss of, the proportion and total number of iNKT cells in the thymus, spleen, and liver of the 2A3-D Tg

mice compared with the wildtype mice. While the impairment occurs at the transition from stage 1 to stage 2 of NKT cell development the remained iNKT cells that avoid negative selection in these mice express natural sequence variants of the canonical TCR-α and decreased affinity for self/CD1d. These results directly demonstrates that the TCR signaling by agonist self-ligands is the trigger of the iNKT cell differentiation pathway. It also emphasizes that only a limited range of TCR affinity for self is actually compatible with iNKT cell development versus death by neglect or negative selection (Bedel *et al.,* 2014). Collectively, these findings support the hypothesis that the increased affinity or avidity of antigen presented intrathymically can induce negative selection of NKT cells.

The specific types of APCs that could mediate negative selection of NKT cells remain a source of controversy. By transferring CD1d-overexpressing DCs into irradiated $RAG^{-/-}$ mice reconstituted with wildtype bone-marrow cells, Chun and colleagues demonstrated that bone marrow-derived DCs, but not thymic epithelial cells, might be the main cell population mediating negative selection of Vα14NKT cells (Chun *et al*., 2003). Another study on human CD1d transgenic mice has revealed that both thymocytes and APCs (such as DCs, macrophages and B cells) may independently induce negative selection. However, professional APCs appear to be more efficient in negatively selecting type I NKT cells than thymocytes (Schumann *et al*., 2005).

### 1.4.5. NKT cell lineage commitment and maturation

Following positive selection, nascent NKT cells progress down a pathway of lineage commitment and maturation that is marked by the sequential acquisition of specific patterns of surface marker expression. Currently, it is believed that this differentiation pathway can be subdivided into at least four sequential stages based upon the expression of a variety of phenotypic markers, such as the heat stable antigen (HSA or CD24), CD44 and NK1.1. The

most immature NKT cells are defined as $CD24^{high}CD44^{low}NK1.1^{-}$ (Stage 0), followed by $CD24^{low}CD44^{low}NK1.1^{-}$ (Stage 1), $CD24^{low}CD44^{high}NK1.1^{-}$ (Stage 2), and finally $CD24^{low}$ $CD44^{high}$ $NK1.1^{+}$ (Stage 3) (Benlagha *et al*., 2002; Gadue and Stein, 2002; Pellicci *et al.*, 2002; Benlagha *et al*., 2005; Bendelac *et al*., 2007; MacDonald *et al*., 2007). Stage 0 NKT cells exit the DP pool as $CD4^{+}$ cells and are characterised by high expression of heat stable antigen, a phenotype consistent with developmental immaturity. This $CD4^{+}$ $CD24^{high}$ subset has a $CD44^{low}$ $NK1.1^{-}$ phenotype indicative of its immature status. Interestingly, $CD4^{+}$ $CD24^{high}$ Vα14 NKT cells are small in size and present in very low numbers (approximately 1 in every million thymocytes), (Godfrey and Berzins, 2007) consistent with the observation that they do not undergo proliferation. Although these cells are non-cycling, they already display a marked bias for expression of Vβ8, indicating that Vβ repertoire selection by CD1d precedes cell expansion in this lineage. Furthermore, most $CD4^{+}$ $CD24^{high}$ Vα14 NKT cells express the early activation marker CD69, suggesting that they may represent cells that have recently undergone positive selection (Bendelac *et al*., 2007; MacDonald *et al*., 2007).

Conversely, intrathymic type I NKT cells in Stage 1 are characterised by the decreased expression of CD24 and CD69, although they still remain $CD44^{low}$ and do not express NK1.1. They are rapidly cycling, as determined by their large size and ability to incorporate 5-bromo-2-deoxyuridine (BrdU) in short-term *in vivo* labelling studies (Berzins *et al*., 2006). This suggests that the post-selection expansion of lineage-committed type I NKT cells occurs at this stage. In addition, transition from Stage 0 to Stage 1 marks another unique phase of NKT cell maturation, where a proportion of cells lose CD4 expression and give rise to a subset of NKT cells that are DN. Using intrathymic cell transfers of purified Vα14 NKT cell subsets, several groups have indicated that DN Vα14 NKT cells are in fact derived from immature $CD4^{+}$ (Stage 2 or 3) precursors (Benlagha *et al.*, 2002; Gadue and Stein, 2002; Pellicci *et al*.,

2002). However, the mechanism responsible for down-regulation of CD4 expression during this maturation step remains unclear.

Stage 2 of Vα14 NKT cell development is defined by up-regulation of CD44, and the cells thus acquire a phenotype similar to memory or activated conventional T cells. $CD4^+$ $CD24^{low}$ $CD44^{high}$ Vα14 NKT cells do not yet express NK1.1 or other NK-associated markers such as the Ly-49 family of inhibitory receptors, but continue to undergo rapid proliferation. A majority of the $CD24^{low}CD44^{high}NK1.1^-$ cells emigrate to peripheral tissues, where they stop proliferating and rapidly express NK1.1 and other NK lineage markers such as NKG2D, Ly49A, C/I, and G2, as well as markers of T cell activation such as CD69 and CD122 (Matsuda *et al.*, 2006). Interestingly, a small fraction of $CD44^{high}$ and $NK1.1^+$ cells stay in the thymus and accumulate there over the life of the mouse as terminally mature long-lived resident cells (Berzins *et al.,* 2006).

The final stage (Stage 3) is distinguished by the acquisition of NK1.1 and Ly-49 inhibitory receptors. Microarray analysis has indicated concomittant expression of multiple NK-related markers at this stage (Matsuda *et al.*, 2006). Stage 3 cells also differ from their precursors as they have both $CD4^+$ and $CD4^-CD8^-$ phenotypes. The $CD4^-CD8^-$ NKT cells account for 30-40% of CD1d-tet-staining cells in adult mouse thymi (MacDonald and Mycko, 2007). DN Vα14 NKT cells appear to be mature cells since they can express NK cell markers such as NK1.1 and Ly-49 family members. Significantly, Stage 3 Vα14 NKT cells cease to cycle, consistent with entering a terminal stage of differentiation.

The classic ‘stage 0-1-2-3’ model has been largely enriched by the discovery of alternative NKT1, NKT2 and NKT17 sublineages (Lee *et al*., 2013; Constantinides and Bendelac, 2016; Jameson *et al*., 2016). However, the new findings also challenge the conventional classification of iNKT cells, in which $CD44^{lo}$ $NK1.1^-$ (stage 1) cells upregulate

CD44 (stage 2) before finally expressing NK1.1 (stage 3) (Benlagha *et al.,* 2002; Berzins *et al*., 2006; Godfrey and Berzins, 2007). In fact, iIL17RB$^{+}$ NK1.1$^{-}$ cells do not give rise to NK1.1$^{+}$ cells (Wararai *et al*., 2012) while NKT2 cells are not precursors of NKT1 cells, and that NK1.1$^{-}$ iNKT cells are heterogeneous, containing terminally differentiated IL-4 producers (hCD2$^{+}$ NKT2 cells), NKT17 cells, as well as the progenitors for NKT1 cells (Lee et al., 2013; Constantinides and Bendelac, 2016; Jameson *et al.,* 2016).

Recent advances have elucidated, in great detail, the TCR signaling pathway and the transcriptional network governing NKT lineage development (Lee *et al*., 2013; Constantinides and Bendelac, 2016; Jameson *et al*., 2016; Georgiev *et al*., 2016). It has been suggested that IL-17RB, a key component of the receptor for IL-25, was selectively expressed in NKT2 and NKT17 precursors but not in NKT1 precursors (Watarai *et al*., 2012). Strikingly, mice lacking IL-17RB lacked both NKT2 and NKT17, but not NKT1 thymocytes, suggesting that commitment to these sublineages required signaling through this receptor (Watarai *et al*., 2012). As IL-17RB is expressed as early at stage 1 cells, the commitment to NKT1 vs NKT2 or 17 sublineages may occur very early. Because ~50% of stage 1 and 75% of stage 2 cells are IL-17RB$^{+}$ in the B6 background, the implication of these findings is that there are more thymic precursors to the NKT2 and NKT17 lineage than to the NKT1 lineage, which is in apparent conflict with the overall predominance of mature NKT1 cells in B6 mice both in thymus and in periphery. One possibility is that the NKT2 and NKT17 cells are short-lived compared with NKT1 cells, which exhibit IL-15-driven self-renewing properties (Matsuda *et al.*, 2002).

In addition, studies of mice lacking T-bet, GATA3 and RORγt, the signature transcription factors of NKT sublineages have shed partial light on the mechanisms regulating the NKT sublineage decisions. T-bet-deficient (Townsend *et al.,* 2004) and IL-15-deficient mice (Matsuda *et al.,* 2002) exhibited a block in the development of NKT1 thymocytes at a

IL-2Rβ$^{-}$ NK1.1$^{-}$ and IL-2Rβ$^{low}$ NK1.1$^{low}$ stage 2, respectively. Interestingly, they also exhibited a relative increase in NKT2/NKT17 cells (Watarai *et al*., 2012; Townsend *et al.,* 2004). These findings warrant a modification of the early model of NKT cell development proposed by Godfrey and colleagues, and raise the question of the branch point at which NKT1, NKT2 and NKT17 cells emerge.

## 1.5. TRANSCRIPTIONAL REGULATION IN NKT CELL DEVELOPMENT

The major questions in any developmental system, as well as in NKT cell ontogeny, are: 1) what are the factors determining cell fate? 2) how do extrinsic factors influence cell fate decisions? and 3), how do cell fate decisions determine cellular function? In the context of NKT cell development, there might be special factors at play, as this lineage shows a combination of features from multiple cell types (Th1, Th2 and NK cells), and arises from an unconventional selection event. In order for type I NKT cells to develop and function properly, signals emanating from TCR ligation and the signaling lymphocytic-activation molecule (SLAM) receptor interactions must culminate in the coordinated regulation of genes that allow for the specification and maturation of this lineage. A number of studies, including some quite recent, have provided valuable insights into the transcriptional mechanisms regulating type I NKT cell development, such as *Vα14-Jα18* rearrangement, selection, expansion, maturation and acquisition of effector function.

### 1.5.1. *Vα14-Jα18 rearrangement and factors controlling DP thymocyte lifespan*

The rearrangement of *Vα14-Jα18* gene segments is controlled by a large range of factors. Firstly, this process occurs in order from proximal to distal, and as a result, the combination of *Vα14* with *Jα18* is secondary (Hu *et al*., 2010). Therefore, in the process of NKT cell development, DP precursors need a relatively long lifespan to achieve *Vα14-Jα18* recombination. Mice lacking several transcription or transition factors such as retinoic acid

receptor-related orphan receptor (RORγt), and anti-apoptotic protein Bcl-xL, possess DP precursors with a shortened lifespan, consequently leading to an absence of type I NKT cells (Guo *et al.*, 2002). In this case, RORγt acts as a transcription factor that induces the expression of Bcl-xL, thus prolonging the survival of DP thymocytes (Sun *et al.,* 2000), allowing sufficient time for rearrangement of the more distal *Vα-Jα* gene segments required for *Vα14-Jα18* recombination (Bezbradicae *et al*., 2005; Gadola *et al*., 2006).

In addition to these factors, c-Myb - a transcription factor expressed in progenitors of all hematopoietic lineages, hematopoietic stem cells, and necessary for definitive hematopoiesis – has been shown to influence NKT cell development (Mucenski *et al*., 1991). The expression of c-Myb is highest in DN and DP thymocytes, and then decreases after positive selection. c-Myb deficiency strongly impacts early T cell development (especially the transition from DN to DP) (Allen *et al*., 1999), the survival of DP thymocytes, and the differentiation of $CD4^+$ single positive thymocytes (Bender *et al*., 2004). The presence of c-Myb is also critical for type I NKT cell ontogeny, during which c-Myb primes $CD4^+CD8^+$ immature thymocytes for selection into the type I NKT lineage. In *c-Myb*-deficient mice, both *Vα14-Jα18* rearrangement and type I NKT cells are absent. Quantitative RT-PCR analysis of the expression of survival factors in sorted DP thymocytes from *c-Myb*-deficient mice showed that Bcl-xL expression is 50% lower in *c-Myb*$^{-/-}$ mice compared to wild type controls, while the expression of other survival factors (such as RORγt, Bcl2 and Mcl-1) is almost intact (Hu *et al*., 2010). The effects of c-Myb may be independent of RORγt and upstream of Bcl-xL, because the retrovirus-mediated addition of Bcl-xL has been shown to restore the *Vα14-Jα18* rearrangement, while the introduction of both Bcl-xL and RORγt transgene into *c-Myb*$^{-/-}$ *Cd4*-Cre mice is not able to correct the deficiency in NKT cell numbers (Hu *et al*., 2010). This complete block in NKT cell development may be a consequence of a defect in CD1d and

SLAM expression because CD1d and SLAM expression is reduced in *c-Myb*$^{-/-}$ mice. In fact, *Jα18*-deficient thymocytes can support the differentiation of CD1d-deficient type I NKT cells in mixed-bone marrow chimeras, while *c-Myb*$^{-/-}$ *Cd4*-Cre thymocytes could not. Therefore, it is possible that the deficiency in c-Myb leads to alterations in Bcl-xL, CD1d, and SLAM expression, and thus defects in NKT cell development.

Another signaling molecule that acts very early during NKT cell ontogeny is the Src tyrosine kinase Fyn. Results from Gadue's work indicated that NKT cells are severely deficient in Fyn-mutant mice, especially immature NK1.1$^{-}$ NKT cells, while T or NK cell development is largely normal (Gadue *et al*., 1999). Interestingly, the introduction of transgenic *Vα14-Jα18* rearrangement completely rescued NKT cell development, suggesting that Fyn is required before α-chain rearrangement, rather than for positive selection or NKT cell expansion in the periphery. The studies in RORγt$^{-/-}$ mice demonstrated that Fyn deficiency reduces the period of time for TCR rearrangement, and consequently impairs NKT-associated TCR generation (Gadue *et al*., 1999).

The E protein family of basic helix-loop-helix transcription factors is comprised of HEB, E12, E47 (E2A), and E2-2. Homodimers or heterodimers of HEB members bind to DNA at E-box sites and control the expression of genes essential for lineage development. The role of E proteins in conventional T cell development has been studied extensively. Both E2A and HEB promote TCR gene rearrangement and control thymocyte survival, proliferation and positive selection (Bain *et al*., 1997; Barndt *et al.*, 1999; Barndt *et al*., 2000; Wojciechowski *et al.*, 2007). However, D'Cruz and colleagues (2010) have recently reported that specific E proteins also play a role in type I NKT cell development. In this work, deletion of HEB in DP thymocytes led to extreme paucity of type I NKT cells at the earliest stage (CD24$^{high}$ CD44$^{-}$NK1.1$^{-}$), but had minimal impact on the development of conventional CD4$^{+}$

or $CD8^+$ T cells. In contrast, deletion of E2A has no distinguishable effects on type I NKT cell ontogeny. Interestingly, loss of expression of both HEB and E2A impairs T cell development, but does not have further effects on type I NKT cell ontogeny, compared to the absence of HEB only. The authors concluded that there is a unique requirement for HEB in the development of type I NKT cells (D'Cruz *et al*., 2010). Deficiency of HEB results in reduced levels of RORγt and Bcl-$X_L$ mRNA, poor survival of DP thymocytes, and impairments in generation of distal *Jα* gene rearrangements, including *Vα14-Jα18*. Directed expression of Bcl-$X_L$ or a rearranged Vα14 TCR rescues the type I NKT cell phenotype and function, providing further evidence that HEB regulates type I NKT cell development by driving the expression of genes stimulating thymocyte survival, thereby allowing for distal TCRα rearrangements (D'Cruz *et al*., 2010; D'Cruz *et al*., 2014).

### 1.5.2. *Transcriptional factors that regulate NKT cell ontogeny at the DP stage*

In addition to the strict requirement for signals from invariant TCR-CD1d interaction, positive selection of NKT cells requires the homotypic interactions between two members of the SLAM family (Griewank *et al*., 2007). SLAM family molecules are encoded by the *Slam* locus. There are 6 members in the SLAM family, namely SLAMF1, SLAMF3, SLAMF4, SLAMF5, SLAMF6, and SLAMF7. They are mostly expressed by cells of hemopoietic origin and play a role as TCR-dependent or independent regulators of adhesion and cellular activation (Engel *et al*., 2003). During positive selection of NKT cells, the expression of SLAM on thymocytes induces the formation of SLAM-receptor-initiated signaling, which is mediated by SLAM–associated protein (SAP), an adaptor that recruits the Src kinase Fyn. SAP acts as a stimulator that phosphorylates SLAM receptors by binding to their cytoplasmic tails. SAP also binds to the tyrosine kinase Fyn and enhances kinase activity (Simarro *et al*.,

2004). These complexes subsequently act as a docking site for a set of signalling molecules in NKT cell development.

There is clear evidence for the relationship between SLAM expression and NKT cell development. Mice lacking SLAM or SAP exhibit lower NKT cell numbers, although conventional T cells are intact (Chung *et al*., 2005; Chung *et al*., 2008). In the absence of SAP and Fyn, NKT cell development is arrested at the $CD24^{high}$ CD1d-$tet^{high}$ $CD69^{+}$ stage, which results in the reduction of mature type I NKT cells by 85% (Griewank *et al*., 2007). In humans, mutations in SAP cause an X-linked immunodeficiency (XLP), which is characterised by a deficiency in Vα24 NKT cells. In the same study by Griewank and colleagues (2007), a genetic condition was engineered so that each partner cell in the DP-DP interaction was lacking only one SLAM receptor, either SLAMF1 (CD150) or SLAMF6 (Ly108). The results indicated that NKT precursors lacking one of the two SLAM receptors exhibited only modest defects (Griewank *et al*., 2007). However, in mixed bone marrow chimeras designed to abrogate both SLAMF1 and SLAMF6 signalling during CD1d ligand recognition in the thymus, a stronger developmental arrest was observed. This scenario proved that SLAMF1 and SLAMF6 exerted essential and intrinsic (but partly redundant) effects through homophilic self-association across the cell-cell synapse. The Vα14-Jα18 TCR recognition of CD1d guided the DP-DP interaction and concurrently permitted other signals such as SLAM-SLAM engagement (Griewank *et al*., 2007).

Previously, Jordan and colleagues (2007) reported that SLAMF1 expression on DP thymocytes from NOD mice is far lower than that of DP thymocytes from C57BL/6 mice. The authors have postulated that this low expression is partly responsible for the decreased NKT cell numbers observed in the NOD genetic background (Jordan *et al*., 2007). A genetic linkage study in a first backcross from C57BL/6 to NOD.*Nkrp1*$^{b}$ mice indicated that the *Slam* locus maps to distal chromosome 1, in the same region as the NOD mouse lupus

susceptibility gene *Babs3/Bana3* (Esteban *et al.,* 2003; Jordan *et al*., 2007). This genetic region together with the chromosome 2 genetic region *Nkt2* (containing diabetes susceptibility loci), controls NKT cell numbers in NOD mice (Esteban *et al*., 2003; Jordan *et al*., 2007; Fletcher *et al.,* 2008). Introgression of the C57BL/6-derived *Slamf1* allele by both congenics and transgenesis corrected the defect of retarded SLAM expression in NOD mice and resulted in increased NKT cell numbers in both the thymus and the periphery (Jordan *et al*., 2007; Jordan *et al*., 2011).

However, the mechanism by which the SLAM receptor-SAP signals coordinate with those emanating from the TCR to support type I NKT cell development remains enigmatic. In T cells, stimulation of the SLAM-SAP-Fyn pathway leads to recruitment of the SH2 domain–containing inositol phosphatase (SHIP), the Dok1/2 adaptor proteins, and the Ras GTPase-activating protein (RasGAP) (Latour *et al*., 2001; Wang *et al*., 2004). It was possible that in the absence of SAP, Dok1/2 binds to RasGAP and consequently inhibits Ras-MAPK activation (Latour *et al*., 2001; Wang *et al*., 2004), which might result in impairment of type I NKT cell development because of a failure to negatively regulate Ras signaling. However, when the Ras-MAPK pathway was inhibited (using dominant-negative *Ras* and *Mek-1* transgene), NK1.1$^{+}$ T cell development seemed unperturbed (Alberola-Ila *et al*., 1996). As these studies were performed prior to the availability of CD1d-tet, it would be worthwhile re-examining these models using this more specific reagent.

The SLAM-SAP-Fyn complex also binds to NFκB via protein kinase θ (PKCθ) and the Bcl10 adaptor protein (Cannons *et al*., 2004). In the absence of SAP or Fyn, T cells have impaired IκBα degradation, reduced NFκB nuclear translocation in response to TCR activation, and fail to recruit PKCθ and Bcl10 to the immunological synapse. This suggests that this pathway may interact directly with TCR signaling cascades (Cannons *et al*., 2004). PKCθ or Bcl-10 knockout mice show a severe reduction in type I NKT cell numbers in the

thymus and spleen, respectively (Schmitdt-Supprian *et al*., 2004; Stanic *et al*., 2004b). Taken together, PKCθ, Bcl-10, and NFκB seem to critically modulate type I NKT cell ontogeny, however, the developmental stage(s) at which these molecules are required have not yet been characterised (Sivakumar *et al*., 2003; Stanic *et al*., 2004b).

The nuclear factor NFκB is likely implicated in *Vα14* NKT cell development by another important mechanism: Mice deficient in NFκB, or those expressing a dominant negative IκBα transgene, showed severely reduced type I NKT cell populations. Importantly, the few remaining *Vα14* NKT lineage cells in the NFκB-deficient mice did not exhibit mature NK markers, such as NK1.1 and Ly-49 inhibitory receptors, and displayed a predominantly $CD4^+$ $CD44^{low}$ phenotype (Stanic *et al*., 2004a). A proportion of these $CD4^+$ $NK1.1^-$ $CD44^{low}$ Vα14 NKT cells also expressed high levels of CD24, suggesting that they were impaired at Stage 1. This block at the immature Vα14 NKT cell stage could be completely rescued by overexpression of a Bcl-$X_L$ transgene, suggesting that the other function of NFκB in early Vα14 NKT cell development is to promote survival (Stanic *et al*., 2004a).

The Runt related transcription factor 1 (Runx1) is also a critical regulator of type I NKT cell ontogeny at the DP stage. In the absence of Runx1, type I NKT cell development is impaired at the earliest detectable type I NKT committed subset, but *Vα14-Jα18* rearrangement is still identified in DP cells lacking Runx1 (Egawa *et al*., 2005). This indicates that Runx1 activity is required for different aspects of type I NKT development, perhaps during positive selection or for expansion of post-selection precursors.

The Myelocytomatosis oncogene (c-Myc) transcription factor is also known to play important roles in regulating cell proliferation, survival and apoptosis of the T cell lineage. c-Myc is expressed at high levels in immature DN thymocytes but reduces as cells enter the DP stage (Huang *et al.,* 2008). Targetted deletion of *c-Myc* during early T cell development

results in profound defects in pre-TCR-mediated proliferation, consequently leading to a severe impairment of T cell development (Dose *et al*., 2006). *c-Myc* deficiency (by the *Cd4*-Cre system) in DP thymocytes selectively blocks NKT cell development without effects on conventional and natural regulatory T cells. For example, in the absence of c-Myc, type I NKT cells appear to be arrested at the $CD24^{low}CD44^{-}$ stage because of a failure to undergo lineage expansion (Mycko *et al*., 2009), or a later stage of intrathymic proliferation that precedes memory acquisition (Dose *et al*., 2009). Both studies show that the type I NKT cell developmental block is not due to impaired survival, as transgenic expression of Bcl-2 does not compensate for this defect. Although the reason for the discrepancy between the two reports remains unclear, they both demonstrate that c-Myc is indispensible for acquisition of a mature $CD44^{+}NK1.1^{+}$ phenotype (Mycko *et al*., 2009).

### *1.5.3. Transcriptional factors regulating lineage commitment, maturation and homeostasis*

**GATA-3**

GATA-3 is a C2C2 type zinc finger transcription factor that is expressed on NK, T, and type I NKT cells (Kim *et al*., 2006). Regarding the T-cell lineage, GATA-3 functions at several levels, including differentiation of common lymphoid progenitors into early triple negative (TN) cells, progression through β-selection, and during the development of $CD4^{+}$ T cells (Pai *et al.,* 2003; Hernandez-Hoyos *et al*., 2003). GATA-3 is also important for the differentiation of $CD4^{+}$ T cells into Th2 cytokine-secreting cells (Pai *et al*., 2003; Hernandez-Hoyos *et al*., 2003). GATA-3 operates upstream, downstream, or synergistically (Wang *et al*., 2008; Ho *et al*., 2009) with Zinc finger transcription factor (ThPOK) in driving the CD4 lineage. Lack of GATA-3 at the DP stage results in a cell-intrinsic block in the generation of $CD4^{+}$ type I NKT cells (Kim *et al*., 2006). This defect is evident in mice from two weeks of age, and involves both $NK1.1^{-}$ and $NK1.1^{+}$ progenitors. Interestingly, in the event of GATA-3 loss extra-thymic

NKT cells undergo increased apoptosis, leading to diminished numbers of splenic and more notably hepatic type I NKT cells (Kim *et al*., 2006). Peripheral type I NKT cells also fail to express CD69 and do not secrete Th1- or Th2-type cytokines in response to α-GalCer (Kim *et al*., 2006). When proximal TCR signals are bypassed using PMA and ionomycin, CD69 expression and IFN-γ production are restored; however, this challenge fails to compensate for the loss of IL-4 and IL-13 secretion. Several important characteristics regarding GATA-3 in type I NKT cell development have been emphasized by this work. Firstly, GATA-3 is important for the formation of the $CD4^+$ type I NKT cell lineage and secretion of Th2-type cytokines. Secondly, GATA-3 is crucial for type I NKT cell survival, especially in the liver. However, whether this mechanism is mediated by altered modulation of genes controlling hepatic type I NKT cell apoptosis, such as CXC chemokine receptor 6 (CXCR6) (Germanov *et al*., 2008) remains unclear. Thirdly, given the observation that PMA and ionomycin restore TCR-induced up-regulation of CD69 and IFN-γ secretion, this suggests that GATA-3 seems to function downstream of the TCR to modulate type I NKT cell activation.

**ThPOK**

ThPOK, a member of the BTB-POZ family of zinc finger transcription factors, is required for the generation of CD4 NKT cells. Although mice that possess spontaneous mutations in *Zbtb7b* (encoding ThPOK) have normal frequencies and numbers of thymic and peripheral NKT cells, their mature NKT cells completely lack CD4 expression and express CD8 on large numbers of NKT cells (Dave *et al*., 1998; He *et al*., 2005). Additionally, ThPOK-deficient mice exhibit defects in the production of IL-4, and, to a lesser extent, IFN-γ (Dave *et al*., 1998; He *et al*., 2005). It has been suggested that GATA-3 may play a role upstream of ThPOK in $CD4^+$ T cell lineage commitment (Germain, 2002). Therefore, it is possible that

GATA-3 and ThPOK might function in a similar linear pathway in type I NKT cells to permit development of the $CD4^+$ subset and subsequently acquisition of specific effector functions.

**PLZF**

Promyelocytic leukaemia zinc finger (PLZF) transcriptional factor is a key regulator of type I NKT cell development and function. *Plzf* $^{-/-}$ mice or animals bearing a natural mutation in *Zbtb16* (the gene encoding PLZF), show a severe reduction in type I NKT cell numbers in the thymus, but no changes are observed in the development of conventional T cells (Kovalovsky *et al*., 2008; Savage *et al*., 2008). Thymic NKT cells of PLZF-deficient mice do not acquire the typical "activated" phenotypic characteristics, while peripheral NKT cells fail to co-secrete Th1 and Th2 cytokines upon primary stimulation. However, PLZF-deficient type I NKT cells are able to produce IL-4 and IFN-γ upon secondary activation (Kovalovsky *et al*., 2008; Savage *et al*., 2008). Transgenic expression of PLZF in T cells leads to the acquisition of memory-like characteristics in peripheral $CD4^+$ and $CD8^+$ T cells, such as increased CD44 and reduced CD62L expression. However, these cells show no acquisition of NK or NKT cell-associated markers, such as NK1.1, DX5, NKG2D and 2B4 (Savage *et al*., 2008; Raberger *et al.,* 2008; Kovalovsky *et al.,* 2010). PLZF transgenic T cells also express altered patterns of cytokine production and enhanced proliferation compared to non-transgenic cells (Kovalovsky *et al.,* 2010) that are independent of SAP- and Fyn-mediated signals (Kovalovsky *et al.,* 2010). PLZF is expressed in the most immature $CD24^{high}CD44^-$ type I NKT cells, while the remaining rare CD1d-$tet^+$ cells in the thymi of SAP- or Fyn-deficient mice express normal levels of PLZF (Kovalovsky *et al*., 2008; Savage *et al*., 2008), indicating that PLZF might function early in ontogeny after lineage commitment ((Kovalovsky *et al*., 2008), and play a role downstream of the SLAM receptor-SAP-Fyn signaling axis to direct the development of type I NKT cells (Das *et al.,* 2010).

**T-bet**

T-bet is a transcription factor that was originally revealed to be a regulator of Th1 lineage specification which also played a critical role in the final maturation stages of type I NKT cells (Townsend *et al*., 2004; Matsuda *et al*., 2006). Vα14 NKT cells deficient in T-bet are selectively blocked at a relatively late stage of development, between Stage 2 ($CD44^{high}$ $NK1.1^{-}$) and Stage 3 ($CD44^{high}$ $NK1.1^{+}$). Thymic *T-bet*$^{-/-}$ type I NKT cells do not express NK1.1 or other NK-related markers such as members of the Ly-49 inhibitory receptor family. They also lack CD122, a component of the IL-15 receptor. Consequently, type I NKT cells lacking T-bet fail to proliferate in response to IL-15 (Townsend *et al*., 2004). In addition, T-bet-deficient cells do not acquire characteristics of type I NKT cell effector functions, such as the ability to produce IFN-γ in response to TCR stimulation, or exhibition of cytolytic activity (Townsend *et al*., 2004; Matsuda *et al*., 2006; Matsuda *et al*., 2007). Consistent with these developmental and functional defects, T-bet expression increases from Stage 1 to 3 of type I NKT cell maturation and directly regulates the stimulation of genes associated with mature type I NKT cell functions, such a perforin, FasL, and IFNγ (Matsuda *et al*., 2006). T-bet knockout results in a similar developmental impairment in NK cells (Townsend *et al*., 2004), which indicates that T-bet controls multiple genes involved in migration, survival, and effector function of Vα14 NKT cells (Matsuda *et al*., 2000), and that its role in Vα14 NKT cell development may be relatively complex.

**CXCR6 and Inhibitor of DNA-binding proteins (Id)**

CXCR6 has been shown to play a critical role in homeostasis and activation of NKT cells by mediating the accumulation of NKT cells in the liver (Germanov *et al*., 2008). Cells lacking CXCR6 show depleted NKT cells in the liver and lungs, but alternatively accumulate NKT cells in the bone marrow (Geissmann *et al*., 2005; Germanov *et al*., 2008). Similarly,

NKT cell survival and homeostasis are also under the regulation of Id2, the inhibitor of DNA-binding protein. Id2 deficiency results in a drastic and selective reduction of NKT cells in the liver, while normal numbers remain in the thymus and spleen. Studies into the mechanisms responsible for the reduced number of hepatic cells have revealed no defects in homing to the liver. In contrast, Id2-deficient cells showed greater levels of apoptosis. Additionally, Id2-deficient cells have lower expression of CXCR6, which, as was mentioned above, is implicated in the recruitment of hepatic type I NKT cells (Geissmann *et al*., 2005; Germanov *et al*., 2008; Monticelli *et al*., 2009). Mice lacking Id2 showed more severely reduced type I NKT cell numbers in their livers compared to CXCR6-deficient individuals. Hepatic Id2-deficient type I NKT cells exhibited reduced expression of Bcl-2 and Bcl-$X_{L,}$ while genetic deletion of the pro-apoptotic molecule Bim in Id2-deficient cells rescued type I NKT cell numbers. This suggests that Id2 modulates type I NKT cell homeostasis in the liver by modulating E protein activity to maintain a favorable balance between pro- and anti-apoptotic signals.

Despite the progress made over the last decade, many questions remain regarding the molecular mechanisms controlling type I NKT cell development and maturation. For example, what is the functional relationship between these crucial transcription factors? Also, what governs the activity of these and other transcriptional regulators that function later in type I NKT cell development or in the homeostasis of type I NKT cells? Answering these questions could lead to a greater understanding of the transcriptional networks regulating type I NKT cell development.

Recently, the identification of an increasing number of αβT cell subsets deriving from a common DP precursor has prompted studies investigating genetic factors of these groups. Related studies have indicated a number of factors are involved, and these have been characterised as “master regulators” of different T cell lineages (Szabo *et al*., 2000; Hori *et*

*al*., 2003; He *et al*., 2005; Ivanov *et al.*, 2006). The concept of a master regulator is derived from empirical evidence implicating the necessity and sufficiency of a gene in controlling a cellular phenotype. However, it should be emphasised that in this context, determination of the sufficiency is most often an examination of whether the gene has a dominant impact on lineage choice of the immediate progenitor, or changes the fate of the lineage. Generally, the dominance of master regulators is typically only evaluated in the transcriptional milieu in which they are active. One transcription factor may be necessary for gene expression, but it may not be truly sufficient. It can only seem sufficient under precisely limited conditions, when all the other needed transcription factors are already present. There might be antagonistic or cooperative effects of multiple transcription factors, called a "transcription factor network" playing a pivotal role in establishment of lineage diversities from stem cells (Sieweke and Graf, 1998; Orkin, 2000; reviewed in Rothenberg, 2007).

In post-selection T cell ontogeny, ThPOK, Foxp3, T-bet, and RORγt are considered master regulators by the criteria mentioned above. They respectively drive CD4 (He *et al*., 2005; Sun *et al.*, 2005), regulatory (Hori *et al*., 2003; Khattri *et al*., 2003; Fontenot *et al*., 2003), Th1 (Szabo *et al*., 2000), and Th17 (Ivanov *et al*., 2006) T cell phenotypes. However, a closer examination of these systems has revealed additional complexities. For example, studies of Foxp3 regulation and activity by Rudensky and colleagues have indicated that Foxp3 is not the primary factor in $T_{reg}$ lineage development (Fontenot *et al*., 2005; Gavin *et al.,* 2007). Likewise, there is continued debate regarding the primacy of ThPOK in CD4 lineage commitment, with evidence that Gata-3 operates upstream, downstream, or synergistically with ThPOK in driving the CD4 lineage (Wang *et al*., 2008; Ho *et al*., 2009). Thus, it is clear that developmental choices are not a linear progression but rather a network of interrelated processes, which synergise toward a stable program. Nonetheless, accumulating evidence supports the idea that a limited number of unique transcriptional

regulators are largely responsible for the differentiation and characteristics of a given lineage, perhaps occupying critical "nodes" in the network of interactions or representing the output of complex molecular decision circuits (Rothenberg, 2007; Laslo *et al*., 2008). The behaviour of most complex systems can be modelled by the activity of many critical components (nodes) that interact with each other through pairwise interactions (termed edges) forming a network. Therefore, the compelling question in NKT cell ontogeny is "is the unique NKT cell transcriptional milieu organized in the pattern of a distinct lineage?" or "is the NKT cell program governed by a signature transcription factor(s)?"

While transcriptional factors driving conventional T cell development have been well studied, those of NKT cell ontogeny are still enigmatic. Since the time NKT cells were first described, many efforts have been made to understand the molecular mechanisms that govern their homeostasis and function. Transcriptional analyses have previously been made to compare gene expression between NKT cells with NK cells, naïve conventional $CD4^+$ T helper, and regulatory T cells (Niemeyer *et al*., 2007), or to shed light on the transcriptional programs regulating type I NKT cell development (Cohen *et al*., 2013). However, due to the lack of DP NKT cells (the earliest NKT cell progenitors), these authors can only compare $CD44^-NK1.1^-$ (Stage 1) with $CD44^+NK1.1^-$ (Stage 2) and $CD44^+NK1.1^+$ (Stage 3) NKT cells.

More recent, many attempts were made to determine the original signals that determine which and to what extent a given signaling pathway and/or transcription factor expression is required for the commitment to various iNKT cell subsets. The 'choice' to become iNKT1/ iNKT2/iNKT17 cells or CD4/DN NKT cells appears to be set in the thymus during development, however, it remains unclear whether each 'fate' is permanently set or can be further modulated. Therefore, understanding the mechanisms of iNKT cell subset differentiation is a prerequisite to the manipulation of the iNKT cell response for therapeutic purposes. By combining single-cell RNA-Seq, 'micro-scaled' RNA-Seq and H3K27ac CHIP-

Seq assays, Engel and colleagues comprehensively elucidated the differentiation and function of thymic iNKT1 (T-bet$^{+}$PLZF$^{lo}$RORγt$^{–}$); NKT2 (PLZF$^{hi}$RORγt$^{–}$T-bet$^{–}$) and NKT17 (RORγt$^{+}$ T-bet$^{–}$PLZF$^{int}$) cell subsets. Their findings demonstrated that these subsets were substantially different in their expression of not only transcriptional factors and cytokine but also molecules involved in homing, inhibitory receptors that presumably help to modulate autoreactivity, and also effector molecules that provide host defense and the regulation of inflammation (Engel *et al*., 2016).

To provide new insights into iNKT cell function and development based on the iNKT1/2/17 classification, Georgiev and colleagues investigated the transcriptomes of iNKT1, 2 and 17 cells from thymus of BALB/c and C57BL/6 mice. The results confirmed that a subdivision into iNKT1, 2 and 17 cells is suitable to characterize iNKT cells independent of the strain but also revealed candidate genes that may explain strain dependent variations in iNKT subset composition reported earlier by Lee *et al*. (2013). Many candidate genes that may affect iNKT cell development, migration or function were identified. They showed that *Fcer1g* is involved in generation of iNKT1 cells and that *SerpinB1* modulates frequency of iNKT17 cells. The results provided comprehensive support for the iNKT1/2/17-concept. The in depth characterization of iNKT1, 2 and 17 thymocytes reveals that each subset is not composed of a uniform population of cells because IL-4$^{+}$ iNKT2 cells are clearly distinct from IL-4$^{–}$ iNKT2 cells and a substantial proportion of iNKT17 cells express IL-4 along with IL-17 (Georgiev *et al*., 2016). However, it remains to be determined whether IL-4$^{+}$IL-17$^{+}$ iNKT cells represent a transitory population differentiating into IL-17$^{+}$ cells. Their current view would be in line with a hypothesis that each iNKT subset contains a population of terminally matured cells (CD4$^{–}$ iNKT1, IL-4$^{+}$ iNKT2, IL-17$^{+}$ iNKT17) but also a population of cells not yet finally differentiated (CD4$^{+}$ iNKT1 cells, IL-4$^{–}$ iNKT2, IL-4$^{+}$ IL-17$^{+}$ iNKT17) (Georgiev *et al*., 2016).

To address these issues, Lee *et al*. (2016) also performed RNAseq analysis of iNKT subsets, including NKTp, NKT1, NKT2 and NKT17 cells. Importantly, they found only NKT1 cells, but not NKT2 and NKT17 cells, shared a transcriptional program with NK (Cohen *et al.,* 2013), activated CD8 T and intraepithelial γδ-T cells. They also identified that NKTp signature genes were shared amongst differentiating or proliferating hematopoietic cells including developing thymocytes, which were associated with an upstream regulator Myc protein (Lee *et al*., 2016). Using previously published data sets, these authors measured the transcriptional similarity of iNKT subsets to those of analogous γδ-T cells, ILC and Th cells. Signature genes of NKT1 cells were defined, and found to be highly shared with ILC1 and Th1 cells, indicating profound similarity between the transcriptional programs of all IFN-γ - producing cells (Lee *et al*., 2016). NKT2 cells were most similar to thymic $CD24^{low}$ $V\delta6^{+}$ γδ-T cells, both of which expressed high levels of PLZF, followed by ILC2. NKT17 cells were similar to thymic $CD24^{low}$ $V\gamma2^{+}$ γδ-T cells and ILC3 cells. Although Th2 and Th17 cells shared a small core of effector signature genes with the analogous subsets of ILC, γδ-T and iNKT cells, their overall transcriptional profiles were more distinct (Lee *et al*., 2016).

There was also a majority of work been done to study transcriptional regulation of NKT cells, however, these work mainly queried common factors or reaffirmed the roles of specific factors, which were referred from conventional T cell development, or mainly performed on relatively mature or mature NKT cells (i.e. at Stage 3). The early development of NKT cells especially stage 0 remains unclear. One reason is the very low numbers of developing NKT cells, especially DP NKT cells, makes it difficult to analyse transcriptional programs controlling NKT cell development. Therefore, to examine transcriptional factors controlling early NKT cell ontogeny, especially during the DP stage, transgenic expression of rearranged *Vα14-Jα18* segment has been used to increase NKT cell numbers at different stages. This has paved the way for transcriptional analysis of NKT cell development at its early stages by microarray.

## CONCLUSION

Like many other cell lineages, NKT cells play an important role in bridging innate and adaptive immunity. They have various effects on microbial infection, allergy, cancer, and autoimmune diseases. In this work, transgenic expression of rearranged *Vα14-Jα18* segment has increased numbers of $CD4^+CD8^+$ (DP) NKT cells in both NOD and C57BL/6 mice. This provides an opportunity to compare the transcriptional profiles of the very immature $CD24^+NK1.1^-$ DP NKT cells with those of conventional T cells ($CD24^+NK1.1^-$ DP T cells), and those of more mature NKT cell subsets, which include $CD24^+NK1.1^-$ $CD4^+$ NKT cells and $CD24^+NK1.1^-$ DN NKT cells. Via microarray analysis, expression levels of a total of 35,556 transcripts for each biological sample of four different cell populations were obtained. Principal component analysis (PCA), which was conducted on all transcripts under investigation, indicated that the four populations clearly separated, in order, across principal component 1 (PC1) from $CD24^+NK1.1^-$ DP T cells (P1), to $CD24^+NK1.1^-$ DP NKT cells (P2), to $CD24^+NK1.1^-$ $CD4^+$ NKT cells (P3), and finally to $CD24^+NK1.1^-$ DN NKT cells (P4). Comparison of gene expression between the populations (in this order) has revealed highly differentially expressed genes at transitions. Here, the aim has been to extend this work to understand the change in expression of transcriptional factors during NKT cell ontogeny. Gene co-expression network analyses were also undertaken to assess the relationship between transcripts during mouse NKT cell development.

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# CHAPTER 2

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# GENERAL MATERIALS AND METHODS

## 2.1. ANIMALS FOR STUDIES

Non-obese diabetic (NOD) and C57BL/6J (B6), congenic, transgenic and mutant mice were maintained under specific pathogenic-free conditions at the Immunogenetics Research Facility of James Cook University (Douglas, QLD, Australia). Mice of both sexes were used at 6-12 weeks of age. In a single experiment, experimental mice were age and sex - matched with controls. All mouse experiments were reviewed and approved by the Animal Ethics Committee at James Cook University (A1753, A2142 and A2162).

As this study focuses on NKT cell development, the natural killer (NK) cell gene complex had previously been introduced into all NOD mouse strains to permit the use of the NK1.1 marker for characterising NKT cell maturation. This complex (called *Nkrp1*$^{b}$) was originally derived from chromosome 6 of B6 mice; the proximal boundary lay between *D6mit323* and *D6mit105* and the distal boundary lay between *D6mit135* and *D6mit259*. There was no evidence that the *Nkrp1*$^{b}$ congenic segment altered the number or function of NKT cells in the NOD.*Nkrp1*$^{b}$ congenic line (Matsuda *et al.*, 2001; Hammond *et al*., 2001; Poulton *et al*., 2001; Matsuki *et al*., 2003; Rogner *et al*., 2001), therefore, NOD.*Nkrp1*$^{b}$ mice were used as wild type controls for all experiments on a NOD background.

### 2.1.1. Inbred mouse strains

A number of mouse strains used in this research were originally purchased from the Jackson laboratory (Jackson lab., Maine, USA) and Animal Resource Centre (Canning Vale, WA), or kindly provided from previous studies. They were inbred in the Immunogenetics Research Facility at Douglas campus, James Cook University, Australia.

**Table 2.1. Inbred mouse strains used in this study**

| Mouse strains | Description | Source |
|---|---|---|
| NOD/Lt | Wild type NOD strain | The Jackson lab. |
| NOD.*Nkrp1*$^{b}$ | Congenic for NK1.1 marker from B6 background | The Jackson lab. |
| NOD.*Nkrp1*$^{b}$.*Cd1d*$^{-/-}$ | Congenic for NK1.1 marker from B6 and mutant for *Cd1d*. | Dr. Margaret Jordan |
| C57BL/6J | Wild type B6 strain | The Jackson lab. |
| B6.*Ccr2*$^{-/-}$ | B6 with *Ccr2* knock out | The Jackson lab. |
| B6.*Ccr7*$^{-/-}$ | B6 with *Ccr7* knock out | The Jackson lab. |
| B6.*Nkg7*$^{-/-}$ | B6 with *Nkg7* knock out | Australian Phenomics Facility |
| B6.*Cd1d*$^{-/-}$ | B6 with *Cd1d* knock out | The Jackson lab. |

### 2.1.2. Production of *Vα14-Jα18* transgenic (*Va14*Tg) mouse strains

NOD and B6 *Vα14-Jα18* transgenic mice were generated by Dr. Margaret Jordan using the previously published construct, kindly provided by Prof. Albert Bendelac (Lantz and Bendelac, 1994). Briefly, the pre-rearranged *Vα14-Jα18* TCR α chain complementary DNA (cDNA) was PCR-amplified from the CD1-specific T cell hybridoma DN32.D3 (Lantz and Bedelac, 1994). Then, *Vα14-Jα18* cDNA was inserted into the *Sal*I site of a plasmid containing the minimal CD4 promoter, enhancer and the intronic silencer (Sawada *et al*., 1994; Matsuda *et al*., 2001). The linearized (via *NotI*) construct was purified by agarose gel electrophoresis, agarase treated, re-purified and injected directly into NOD/Lt embryonic pronuclei at the Walter and Elisa Hall microinjection unit (Melbourne, Australia). The manipulated embryos were placed in the reproductive tracts of pseudo-pregnant NOD/Lt recipient female mice. Transgenic mice were screened by polymerase chain reaction (PCR) and crossed onto the NOD.*Nkrp1*$^{b}$ line to permit the use of the NK1.1 developmental marker.

**Table 2.2. *Vα14* transgenic mouse strains**

| Mouse strains | Description | Source |
|---|---|---|
| NOD.*Nkrp1*$^{b}$.*Vα14*Tg (line 1, 2, 3, 4) | Transgenic for *Vα14-Jα18* segment. | Generated by Dr. Margaret Jordan using a construct gifted by Prof. A. Bendelac |
| B6.*Vα14*Tg (line 2, 5) | | |

### 2.1.3. In-house established mouse strains

Three mouse strains (Table 2.3) were established in-house by crossing inbred mouse strains carrying different genes of interest. In short, mice from two different strains were crossed to generate heterozygous progeny. Resultant heterozygous mice were then intercrossed and the subsequent generations were genotyped to identify founder mice for the next generation. If the genes or genetic regions of interest were congenic (such as *Nkrp1*$^{b}$), homozygous congenic founders were selected to establish the congenic line. If the genes of interest were targeted deletion mutations, mutant mice were selected for the next generation. Intercrosses were set up until mice with the desired combination of genes were produced.

**Table 2.3. In-house established mouse strains**

| Strain | Description | Source |
|---|---|---|
| NOD.*Nkrp1*$^{b}$.*Cd1d*$^{-/-}$.*Vα14*Tg | A strain resulting from intercrossing *Cd1d*$^{-/-}$ from NOD.*Nkrp1*$^{b}$.*Cd1d*$^{-/-}$ to NOD.*Nkrp1*$^{b}$.*Vα14*Tg | Generated in this study |
| B6.*Cd1d*$^{-/-}$*Vα14*Tg. | A strain resulting from intercrossing *Cd1d*$^{-/-}$ from B6.*Cd1d*$^{-/-}$ to B6.*Vα14*Tg | Generated in this study |
| B6.*Ccr7*$^{-/-}$.*Vα14*Tg | A strain resulting from intercrossing *Ccr7*$^{-/-}$ from B6.*Ccr7*$^{-/-}$ to B6.*Vα14*Tg | Generated in this study |

For the remainder of this thesis, the strain designation "NOD" refers to NOD.*Nkrp1*$^{b}$ mice. For example, "NOD.*Vα14*Tg" refers to NOD.*Nkrp1*$^{b}$.*Vα14*Tg mice, NOD.*Cd1d*$^{-/-}$ refers to NOD.*Nkrp1*$^{b}$.*Cd1d*$^{-/-}$ mice, and so on.

## 2.2. GENOTYPING

Pups from intercrosses were genotyped to set up new combinations of target genes. The appearance or absence of a gene was checked by PCR, or by flow cytometry, depending on the availability of suitable antibodies.

### 2.2.1. Genotyping by PCR

Genomic DNA was extracted from tail tips or ear punches using a CAS-1810 X-tractor Gene (Corbett Robotics) liquid handling platform. Tail tips (11 mm from top) or ear punches were collected into 1.5 ml Eppendoft tubes, then digested overnight in 420 µL Tissue digest buffer (100mM TrisHCl pH 8, 10 mM EDTA, 100 mM NaCl, 0.5% SDS, 50 mM DTT, 100 mM Proteinase K) at 56$^{o}$C, 30 rpm in a VORTEMP 56EVC (Labnet, Hialeah, FL, USA) or overnight, in a 56$^{o}$C incubator (MIR-262, Sanyo, Japan). Samples were mixed by vortexing and then centrifuged to separate out fur and/or bones. Two hundred and twenty µL from each digested sample was loaded in a 96-deep well plate, before adding 440 µL Lysis buffer (consisting of 5.25 M Guanidine thiocyanate, 10 mM Tris HCl pH 6.5, 20 mM EDTA, 4% Triton-X, 64.8 mM DTT) was added to each sample. Samples were then loaded on a glass filter (GF/B) polypropylene microplate (Whatman International, Clifton, USA) and vacuumed to capture the DNA on the filter. Samples were washed once by adding 200 µL Lysing buffer then vacuumed to remove unbound fractions. Samples were washed twice in Propanol wash buffer and once in 100% ethanol. DNA was then eluted in 100 µL Elution buffer (10 mM Tris-HCl pH 8.5, 0.5 mM EDTA). DNA concentration was measured using a NanoDrop ND1000 spectrophotometer (Bio-Rad Laboratories).

Genotyping of the crosses between mouse strains carrying congenic regions from B6 (*Nkrp1*$^{b}$) were performed by using primers chosen from the simple sequence length polymorphism library, Whitehead Institute (Cambridge, MA; Dietrich 1996), or primers

designed in-house on the basis of PCR product length polymorphism between C57BL/6 and NOD/Lt strains (Table 2.4). Genotyping of knock-out genes was based on the protocols provided.

With regards to the transgenic mouse strains (*Vα14*Tg), genotyping followed in-house established primers and PCR protocols. DNA from subsequent generations and controls (including homozygous, heterozygous, and negative DNA controls) was measured and diluted to the same concentration (usually at 7.5 ng/μL). PCR reactions and electrophoresis were performed at the same conditions between samples and controls. The results were assessed based on the intensity of the electrophoretic bands compared to controls. The samples from homozygous mice possibly have stronger bands than samples from heterozygous mice. Mice with the brightest transgenic bands were chosen as founders for the next generation. Confirmation of homozygosis was performed by outercrossing to wild type mice. Male and female homozygous transgenic mice were used to generate the homozygous transgenic mouse strains.

**Table 2.4. Primer details**

| Targeted gene or region | Primer (from 5'-3') | Expected results |
|---|---|---|
| ***Nkrp1*$^b$** | ***D6mit135***<br>**Primer 1:** CCTAACAGTTCAATTTGTCAGCC<br>**Primer 2:** CCAGCCCCCAATTTGATATA | B6 allele = 140 bp<br>NOD allele = 130 bp |
| ***Ccr7-/-*** | **WT reverse:** GGTGATCAAGGCCTCCACT<br>**Mutant reverse:** AGACTGCCTTGGGAAAAGCG<br>**Common:** TAAGGGCATCTTTGGCATCT | Mutant = ~ 110 bp<br>Het = ~ 110 bp and 280 bp<br>WT = 280 bp |
| ***Vα14Tg*** | **Forward primer:** TGTAGGCTCAGATTCCCAACC<br>**Reverse primer:** GAGGATGGAGCTTGGGAGTCAGG | WT = no band<br>Het. or Hom. = 1000 bp |

### 2.2.2. Confirmation of genotype of target mutant *Cd1d* mice by FACS

The presence or absence of a gene's products can be identified using a fluorescent conjugated antibody. For example, in this work, knock-outs of *Cd1d* genes from mice resulted in the absence of the CD1d marker on the surface of their lymphocytes, which can be confirmed by flow cytometric analysis, using a fluorescent conjugated anti-mouse CD1d antibody. Briefly, 30µL blood samples were collected by retro-orbital bleeding. Red blood cells were lysed using Red blood cell lysing buffer (Sigma-Aldrich). Peripheral blood mononuclear cells (PBMCs) were collected by centrifugation at 300g, then labeled with PE-conjugated anti-mouse CD1d antibody (eBioscience). Results were assessed by fluorescent intensity of CD1d compared to controls (*Cd1d* knock out and wild type blood samples; Figure 2.1.).

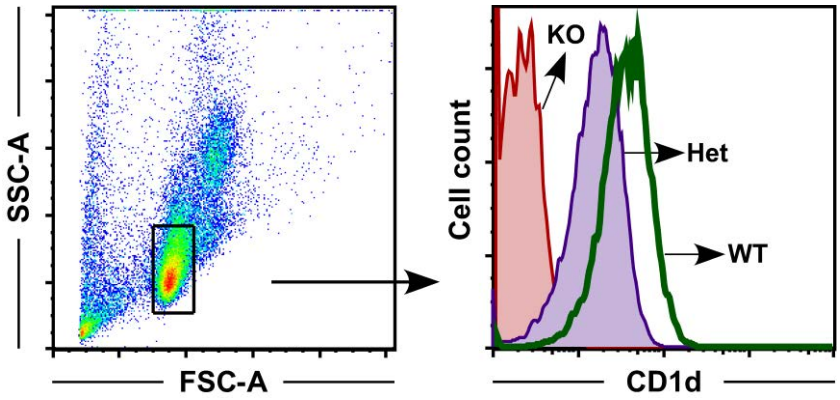


**Figure 2.1. Genotyping *Cd1d* knockout mice by FACS from blood**. Density plot (left panel) presents PBMCs by forward scatter area and side scatter area. The histogram (right panel) shows the fluorescent intensity of CD1d expression on lymphocytes. KO stands for *Cd1d* knockout; Het stands for *Cd1d* heterozygous; and WT stands for *Cd1d* wild type.

## 2.3. FLOW CYTOMETRIC CELL ANALYSES

### 2.3.1. Organ harvesting and single cell suspension preparation

Experimental mice were sacrificed by $CO_2$ asphyxiation. Thymi and spleens were collected into 10 ml tubes containing 3 ml ice-cold MACS buffer (DPBS containing 2 mM EDTA (Amresco, Solon, Ohio, USA), 10% (v/v) Bovine Serum (Invitrogen, Melbourne,

Australia), and 0.02% (w/v) Sodium Azide; Jordan *et al*., 2011). Livers were perfused with 5-10 ml chilled PBS via the portal vein to flush red blood cells out of the liver tissues; they were then harvested into marked 50 ml conical tubes containing 5 ml 1x PBS. Single cell suspensions were prepared and counted using a cell counter (Beckman Counter); total cell numbers per organ were calculated based on the volume of the single cell suspension and the number of cells counted per milliliter.

Single cell suspensions from thymus samples were prepared by gently disrupting the thymus between two frosted microscope slides in MACS buffer. Cell suspensions were collected and passed through 40µm meshes. The thymic cells were washed by spinning at 300 g for 8 min at 4$^{o}$C and re-suspended in 1 ml MACS buffer for flow cytometric cell analyses.

Single cell suspensions from spleens were prepared by flushing with 3 ml MACS buffer using a 3 ml syringe and a 25 gauge needle, and then gently disrupting them between two frosted microscope slides. Cell suspensions were collected and passed through 40µm meshes. The cells were centrifuged at 300 g for 8 min at 4$^{o}$C before being treated with 2 ml Red Blood Cell Lysing buffer (Sigma Aldrich, Castle Hill, NSW, Australia), at room temperature for 5 min. After adding 2 ml MACS buffer to stop the reaction, cells were washed and resuspended in 1 ml MACS buffer.

Individual livers were cut into small pieces and gently pressed through an 180µm wire stainless steel mesh. The cells were collected by spinning at 500 g for 5 min at 4$^{o}$C and washed twice with 25 ml chilled PBS. Parenchymal cells were removed over a 33.75% Percoll gradient (GE Healthcare, NSW, Australia). Liver lymphocytes were collected then washed twice with 1x PBS following by spinning at 300g for 8 min at 4$^{o}$C. Cells were treated with 3 ml Red Blood Cell Lysing buffer at room temperature for 5 min. After adding 25 ml MACS buffer to stop the reaction, cells were washed by spinning at 300 g for 8 min at 4$^{o}$C and re-suspended in 1 ml MACS buffer for flow cytometric cell analyses.

### 2.3.2. Surface staining and flow cytometric cell analyses

For flow cytometric cell analyses, 3-5 million cells were surface labeled with various combinations of the following antibodies:

**Table 2.5. List of antibodies used in staining mouse cells for flow cytometric cell analyses**

| Antibody | Fluorescence conjugated | Host | Isotype | Reactivity | Clone | Company |
|---|---|---|---|---|---|---|
| anti-CD16/32 | Unconjugated | Rat | IgG2a, λ | Mouse | 93 | eBioscience |
| anti-TCRβ | APC or FITC | Hamster | IgG2, λ1 | Mouse | H57-597 | BD Bioscience |
| | PerCP Cy5.5 | Hamster | IgG | Mouse | H57-597 | eBioscience |
| anti-CD4 | FITC, V500, BV510 or BV650 | Rat | IgG2b, κ | Mouse | RM4-5 | BD Bioscience |
| anti-CD8 | APC-Cy7, FITC or V450 | Rat | IgG2a, κ | Mouse | 53-6.7 | BD Bioscience |
| anti-CD44 | V450 or FITC | Rat | IgG2b, κ | Mouse | IM7 | BD Bioscience |
| anti-NK1.1 | APC, PE-Cy7 or PerCP Cy5.5 | Mouse | IgG2a, κ | Mouse | PK136 | BD Bioscience |
| | PerCP Cy5.5 | Mouse | IgG2a, κ | Mouse | PK136 | Biolegend |
| anti-CD24 | FITC or BV605 | Rat | IgG2b, κ | Mouse | M1/69 | BD Bioscience |
| anti TCR Vβ8.1, 8.2 | FITC | Mouse | IgG2a, κ | Mouse | MR5-2 | BD Bioscience |
| anti TCR Vβ8.3 | FITC | Hamster | IgG3, λ1 | Mouse | 1B3.3 | BD Bioscience |
| anti TCR Vβ7 | FITC | Rat | IgG2b, κ | Mouse | TR310 | BD Bioscience |
| anti TCR Vβ2 | FITC | Rat | IgG2a, κ | Mouse | B20.6 | BD Bioscience |
| anti-CD1d | PE | Rat | IgG2b, k | Mouse | 1B1 | eBioscience |
| anti-CD150 | PE, APC or PE-Cy7 | Rat | IgG2a, λ | Mouse | TC15-12F12.2 | Biolegend |
| anti-CD5 | V450 | Rat | IgG2a, λ | Mouse | 53-7.3 | BD Bioscience |
| anti-CD25 | APC | Rat | IgG1, λ | Mouse | PC61.5 | eBioscience |
| anti-CD53 | Alexa Fluor 647 | Rat | IgM, k | Mouse | OX-79 | BD Bioscience |
| anti-ICOS (CD278) | PE | Rat | IgG2b, κ | Mouse | 7E.17G9 | eBioscience |

Alpha galactosylceramide (α-GalCer) loaded CD1d-tetramers (CD1d-tet) were produced by Mr. Marcin Ciula in Prof. Dale Godfrey's laboratory (University of Melbourne). Firstly, mouse CD1d protein (mCD1d) was made in a baculovirus expression system as described previously (Matsuda *et al*., 2000). Purified mCD1d protein was then biotinylated with BirA enzyme and loaded with α-GalCer. Consequently, α-GalCer loaded mCD1d was tetramerized by adding neutravidin -PE, APC, or BV421. This protocol was originally provided by Prof. Mitchell Kronenberg's laboratory (La Jolla Institute for Allergy and Immunology, San Diego, CA).

For surface staining, antibodies were diluted in MACS buffer. Cells were pre-incubated with unconjugated anti-CD16/32 (clone 93, eBiosciences, San Diego, CA, USA) for 15 min at 4$^{o}$C to prevent Fc receptor binding of labelled antibodies, before the addition of surface staining antibody cocktails. Lymphocytes were identified by forward and side scatter profiles. A forward scatter-area against forward scatter-height gate was used to exclude doublets from analysis. Dead cells were excluded from analysis by propidium iodide staining. Where possible, an empty fluorescent channel was used to exclude auto-fluorescent cells. Data were acquired on a BD LSRII Fortessa cytometer (BD Bioscience, San Jose, California, USA) and analysed using FlowJo software (Tree Star, Inc., Ashland, OR).

### 2.3.3. Cell sorting

Single cell suspensions from thymi of 6 week old female NOD.*Vα14*Tg line 1 mice were stained on ice for 25 min with specific antibodies, such as anti-TCR*β* (H57-597), CD1d-tetramer, anti-CD4 (RM4-5), anti-CD8 (53-6.7), anti-CD24 (M1/69) and anti-NK1.1 (PK 136). Cells were sorted on a BD FACSAria II. Cell doublets were excluded using 3 comparisons: forward scatter-area to forward scatter-height, forward scatter-height to forward

scatter-width, and side scatter-height to side scatter-width. Dead cells were excluded from analysis by propidium iodide staining. All samples were analysed after sorting to confirm viability and cell purities were over 97%.

## 2.4. FUNCTIONAL ASSAYS

### 2.4.1. *In vivo* treatment with α-GalCer

Alpha-GalCer (Enzo Life Science, NY, USA) was reconstituted in PBS containing 0.5% Tween-20 (Sigma-Aldrich) to the concentration of 200 mg/ml by sonicating for 2 hours at 37$^{o}$C. Six-week old mice were intravenously injected with 4 μg of α-GalCer in 200 μl PBS1X or control vehicle (200 μl PBS1X containing 0.05% Tween-20). Mice were bled four hourly following injection; sera were collected and immediately stored in a - 80$^{o}$C freezer for cytokine bead assays to assess NKT cell function. In different experiments, 6-week old mice were injected with α-GalCer (4 μg in 200 μl PBS for an intravenous injection or 2 μg in 10 μl PBS for an intrathymic injection) or control vehicle. Injected mice were culled 40 hours later to harvest lymphoid organs (thymus, spleen and liver) for flow cytometric cell analyses.

### 2.4.2. *In vitro* NKT cell culture

Single-cell suspensions obtained from whole thymus were labeled with anti-TCR$\beta$ (H57-597), CD1d-tetramer, anti-CD4 (RM4-5), anti-CD8 (53-6.7), anti-CD24 (M1/69) and anti-NK1.1 (PK 136). Immature CD4 NKT cells and immature DN NKT cells were isolated by FACS on an BD Aria II (as described previously in section 2.3.3). Isolated NKT cells were resuspended and plated at 1 x $10^4$/200μl in RPMI 1640 supplemented with 10% (v/v) FCS (Invitrogen Life Technologies, Mulgrave, Victoria, Australia), 2mM L-glutamine, 100U/ml penicillin, 100μg/ml streptomycin sulfate, and 50 μM 2-ME (Invitrogen Life Technologies, Mulgrave, Victoria, Australia). Culture wells were coated with 100 μl anti CD3e

(eBioscience, Campus Vienna Biocenter 2, 1030 Vienna, Austria) overnight at the concentration 10μg/ml PBS. Cells were cultured in the presence or absence of 100U/ml murine rIL-2 and 100U/ml murine rIL-15 (R&D system, Inc., Minneapolis, MN, USA) for 70h at 37$^{o}$C, 5% $CO_2$ and humidity in an incubator (MCO-20AIC, Sanyo, Japan).

### 2.4.3. Cytokine bead assays

Quantitative detection of cytokines from mouse sera and cell culture supernatants was performed by cytometric bead array using Bender Medsystems mouse Th1/Th2 10plex (Bender Medsystems, Vienna, Austria). Samples were analysed following the manufacturer's protocol. The detection limits were 10.9 pg/ml for GM-CSF, 6.5 pg/ml for IFN-γ, 15.7 pg/ml for IL-1α, 8.8 pg/ml for IL-2, 0.7 pg/ml for IL-4, 4.0 pg/ml for IL-5, 22 pg/ml for IL-6, 5.5 pg/ml for IL-10, 2.4 pg/ml for IL-17 and 2.1 pg/ml for TNF-α. Data were acquired on a BD LSRII Fortessa flow cytometer (BD Bioscience, San Jose, California, USA) and analysed with FlowCytomisTM Pro version 3.0 (eBioscience, Campus Vienna Biocenter 2, Vienna, Austria).

### 2.4.4. Diabetes incidence and insulitis assessment

NOD wild type control mice and NKT cell transgenic mice (NOD.*Vα14*Tg line 1) (both males and females) were checked weekly for diabetes from 10 weeks of age by random blood glucose measurements. Mice were considered diabetic if measurements of blood glucose were > 12.5 mg/L on two consecutive readings. Mice with blood glucose measurements < 12.5 mg/L were followed until at least 32 weeks of age. At this point, they were considered protected.

### 2.4.5. *In vivo* BrdU incorporation assays

NOD.*Va14*Tg mice received 3 intraperitoneal (i.p.) injections of 1mg (in 100 µl PBS) BrdU (Sigma-Aldrich; B5002) at 12 hour intervals for a total of 3 mg per mouse at the indicated time point before harvest. The mice were culled and thymocytes were isolated. One million cells from each sample were distributed into the wells of a 96 well round bottomed microplate (Greiner). Cells were pre-incubated with unconjugated anti-CD16/32 (provided with FITC BrdU flow kit; BD Bioscience, Pharmingen) for 15 min on ice to prevent Fc receptor binding of labelled antibodies. The cells were then surface stained with antibodies in a U bottom well plate. The surface stained cells were fixed, permeabilised and intracellularly stained with anti-BrdU FITC following the manufacturer's protocol. Cells were washed twice and resuspended in MACS buffer. Five hundred thousand cells were acquired on a BD LSRII Fortessa flow cytometer at the flow rate around 400 events per second.

## 2.5. MICROARRAY – GENE EXPRESSION ANALYSES

### 2.5.1. RNA preparation

Sorted immature thymic T and NKT cell subsets (7 biological replicates / subset) were individually homogenized in the RLT buffer of an RNeasy kit (Qiagen, Venlo, Limburg, Netherlands). Homogenates were passed through Qiashedder columns (Qiagen, Limburg, Netherlands) and RNA was extracted using the standard RNeasy protocol (RNeasy; Qiagen, Limburg, Netherlands). The RNA yields were quantified spectrophotometrically on a Nanodrop ND-1000 using the convention that 1 absorbance unit at 260 nm is equal to 40 µg/ml RNA. Absorbance of sample at 260 and 280 nm was checked and aliquots electrophoresed for determination of sample concentration and purity. This technique was carried out by Dr. Margaret Jordan and Ms. Letitia Smith (Comparative Genomic Centre, James Cook University, Australia).

### 2.5.2. Microarray analysis

100ng of RNA from each sample were labelled and hybridized. The expression microarray hybridizations were performed using the WT Expression kit (Life Technologies, CA, USA), WT Terminal Labelling and Controls Kit (Affymetrix, CA, USA) and Affymetrix Mouse Gene_1.0ST arrays, which contained 770,317 probe sets representing an estimated 28,853 mouse genes. The probed arrays were washed and stained using the GeneChip Hybridization Wash and Stain Kit (Affymetrix, CA, USA), and then scanned using the GeneChip Scanner 3000. Images (.dat files) were processed using GeneChip Command Console (Affymetrix, CA, USA) and the CEL files were imported into Partek Genomics Suite 6.6 (Partek SG, Singapore) using an RMA summarisation algorithm for further analysis. A statistical significance threshold was set by a Mann-Whitney U test and a U-statistic of 0 (i.e. no overlap in signals between two subsets); subsequently the highly differentially expressed gene list was refined. This technique was carried out with assistance from Dr. Margaret Jordan and Ms. Letitia Smith (Comparative Genomic Centre, James Cook University, Australia).

### 2.5.3. Gene Co-expression Network

A gene co-expression network was generated using the Affymetrix Mouse Gene_1.0ST array analyses of thymocyte subsets – a total of 28 microarrays. The Affymetrix CEL files were normalised using RMA background subtraction in Bioconductor and batch effects were removed using the nonparametric CombatR algorithm (Johnson *et al.,* 2007). Variability of transcripts across all arrays was ranked by standard deviation (Hahne *et al*., 2008) and the 1,929 most variable were used for network construction. Application of the WGCNA algorithm in R (Langfelder and Horvath, 2008) generated a weighted gene co-expression network of 1,929 nodes (transcripts) and 10,626 edges (representing significant

correlations at a $p<0.02$) assigned to 12 significantly co-expressed modules. This technique was carried out with assistance from Prof. Alan Baxter, Dr. Margaret Jordan (Comparative Genomic Centre, James Cook University, Australia) and Dr Dragana Stanley (Central Queensland University, School of Medical and Applied Sciences, Rockhampton, Queensland, Australia).

### 2.5.4. Gene Ontology Analysis

Gene lists were generally split into those upregulated and those downregulated before being submitted to The Database for Annotation, Visualization and Integrated Discovery (DAVID) Bioinformatics Resources v6.7 (Huang *et al*., 2007; 2009) for gene ontology analysis of functional annotation clustering against Clusters of Orthologous Groups (COG) Analysis Ontology, Sp Pir Keywords (SP-PIR), Gene Ontology Biological Process (GO-BP), Molecular Function (GO-MF), Cellular Compartment (GO-CC), UniProt Sequence annotation (UP_SEQ_FEATURE), Online Mendelian Inheritance in Man (OMIM_DISEASE), Biological Biochemical Image Database (BBID), BioCarta Pathways (BIOCARTA), and KEGG. This technique was carried out with assistance from Prof. Alan Baxter, Dr. Margaret Jordan (Comparative Genomic Centre, James Cook University, Australia).

## 2.6. REAL-TIME QUANTITATIVE PCR

Real-time quantitative PCR (qPCR) was conducted with assistance from Dr. Morgane Moreau to verify microarray data on gene expression comparison between immature thymic T and NKT cell subsets. Briefly, primers were designed and titrated template standards were processed in parallel with unknown controls. qPCR were performed on independent sample sets of RNA from FACS sorted immature thymic T and NKT cell subsets. PCR mixes were set up using a CAS1200 liquid handling platform (Corbett Robotics, Brisbane) and all qPCR were carried out on the Rotorgene 3000 or Rotorgene 6000 (Corbett, Sydney, Australia). Each

25 µl (15 µl) reaction contained 12.5 µl (7.5 µl) Platinum Syber Green qPCR Supermix, 0.1 mM (0.08 mM) each primer, and 5 µl (2.5 µl) cDNA. PCR condition included denaturation 50$^{o}$C, 2 min, 95$^{o}$C, 2 min, then 40 cycles (95$^{o}$C, 30 sec; 50-56$^{o}$C (primer dependent annealing) 30 sec; 72$^{o}$C, 30 sec; 78$^{o}$C, 30 sec). Data were acquired at both 72$^{o}$C and 78$^{o}$C.

## 2.7. STATISTICAL ANALYSES

Qualitative data were compared by Fisher's exact test or contingency table (Chi squared) analysis. Quantitative data were analysed using GraphPad Prism software versions 6.0 for Mac and Windows (La Jolla, CA, USA). In flow cytometry experiments, data were analysed using BD FACSDiva software (BD Bioscience, San Jose, California, USA) or FlowJo software (Tree Star, Inc., Ashland, OR). Data were then exported into .csv or Microsoft Excel files and copied into GraphPad for further analyses. Depending on the sample size, appropriate statistical test were performed.

The statistical significance threshold of the microarray study comparing FACS sorted thymic DP$^{high}$ CD24$^{high}$ NK1.1$^{-}$CD1d-tet$^{+}$TCRβ$^{+}$ cells (immature DP NKT cells) and DP$^{high}$ CD24$^{high}$ NK1.1$^{-}$ CD1d-tet$^{-}$TCRβ$^{+}$ cells (immature DP conventional T cells) from individual NOD.*Vα14*Tg mice was set at a Mann-Whitney U statistic of 0 (i.e. $p < 0.001$, n=7; equating to no overlap between groups). We have previously published empiric validations of microarray expression analyses of similar design by using congenic intervals to differentiate "on target" from "off target" differential expression (Jordan *et al*., 2007, Fletcher *et al*., 2008). A comparison group of CD4$^{+}$CD8$^{-}$CD24$^{high}$ NK1.1$^{-}$CD1d-tet$^{+}$TCRβ$^{+}$ cells (immature CD4$^{+}$ NKT cells) and CD4$^{-}$CD8$^{-}$CD24$^{high}$ NK1.1$^{-}$CD1d-tet$^{+}$TCRβ$^{int}$ cells (immature DN NKT cells) sorted from the same thymi were also analysed, but required some pooling of samples to obtain adequate amounts of RNA. Uncorrected significance values are provided for a comparison of transcripts of these cells with those from immature DP NKT cells.

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# PART A

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# NKT CELL TRANSGENIC MICE

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# CHAPTER 3

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# CHARACTERISATION OF TYPE I NKT CELLS FROM TRANSGENIC MICE

## 3.1. INTRODUCTION

Type I NKT cells are the most prevalent NKT cell subpopulation in mice and humans. In mice, they account for more than 85% of $NK1.1^+$ T cells (Lantz *et al*., 1994; Matsuda *et al*., 2000). Type I NKT cells are characterised by the expression of an invariant TCR α chain, which is Vα14-Jα18 in mice or Vα24-Jα18 in humans (Lantz *et al*., 1994), in combination with a limited set of TCR β chains (mostly Vβ8, Vβ7, Vβ2 in mice and Vβ11 in humans; Fowlkes *et al*., 1987; Budd *et al*., 1987b; Takahama *et al*., 1991; Hayakawa *et al*., 1992; Arase *et al*., 1992; Porcelli *et al*., 1993; Park *et al*., 2001). This cell type has evolved to recognise glycolipid antigens presented by the non-classical major histocompatibility complex class I – like molecule CD1d (Bendelac, 1995). Upon stimulation, they are able to rapidly produce large amounts of cytokines, which modulate the behavior of other cells in the immune system, including dendritic cells, T cells, B cells and natural killer cells. Through this activity, they form an important link between innate and adaptive immune responses and have various biological effects on infection, allergy, asthma, cancer and autoimmunity (Berzins *et al*., 2011).

The definition and classification of type I NKT cells has been made possible through the discovery by Kawano and colleagues (1997) that the NKT-associated invariant TCR α chain, in both mice and humans, shows a uniform reactivity to the marine sponge derived glycolipid alpha-galactosylceramide (α-GalCer) presented by CD1d (Kawano *et al*., 1997), and the development of α-GalCer-loaded CD1d tetramers 3 years later. Prior to these innovations, the co-expression of $NK1.1^+$ and TCRβ as a surrogate phenotype could not track type I NKT cells as a whole, because some mouse strains, such as NOD/Lt and BALB/c, do not express the NK1.1 marker (Giorda *et al*., 1992; Matsuda *et al*., 2000). Furthermore, large numbers of type I NKT cells do not express NK1.1 at certain stages during their development.

It is now clear that type I NKT cells can be found among mature $CD24^{low}CD44^{high}$ thymocytes (Lantz *et al*., 1994). In mice, they express either CD4 at intermediate levels or else express neither CD4 nor CD8 (double negative – DN). Type I NKT cells can be further subdivided on the basis of expression of NK1.1. While $NK1.1^+$ type I NKT cells seem to be mature, thymic $NK1.1^-$ populations likely represent a pool of immature type I NKT cells. In contrast, peripheral $NK1.1^-$ type I NKT cells are composed of immature cells that have emigrated from thymus and mature type I NKT cells, which have down-regulated the NK1.1 marker following antigenic stimulation (Chen *et al*., 1997; Chen and Paul, 1998). In humans, NKT cells are either $CD4^+$ or DN or $CD8^+$, with or without the expression of CD56.

The frequency of type I NKT cells varies consistently between organs. In mice, they account for about 0.5% of total thymocytes, but have a higher frequency in peripheral lymphoid tissues (around 1% of spleen cells and 30-40% of liver cells; Benlagha *et al*., 2000; Matsuda *et al*., 2000; Hammond *et al*., 2001). In humans, the proportion of type I NKT cells is not well studied, due to the limitation of sample access. However, it is now known that they constitute 0.008-1.176% of blood T cells in the periphery (Marsh *et al*., 2009), 0.001-0.01 of total thymocytes (Baev *et al*., 2004; Berzins *et al*., 2005) and 1% of liver lymphocytes (Benlagha *et al*., 2000; Matsuda *et al*., 2000).

Studies in the 1990's and 2000's have revealed that type I NKT cell numbers are strain dependent. Both proportions and absolute numbers of type I NKT cells vary between mouse strains. Compared with eight other commonly used mouse strains, such as C57BL/6 and BALB.B6-*Cmv1*$^r$ mice, non-obese diabetic (NOD) mice have relatively lower numbers of type I NKT cells in their thymi and peripheral lymphoid organs, including their spleens and livers (Gombert *et al*., 1996; Baxter *et al*., 1997; Godfrey *et al*., 1997; Poulton *et al*., 2001; Hammond *et al*., 2001). In addition to the numerical deficiencies, NKT cells derived from NOD mice produce relatively less IL-4; 2 to 4-fold lower IL-4 plasma levels in response to anti-CD3

administration and 20-fold fewer IL-4 secreting thymic NKT cells than C57BL/6 mice (Gombert *et al*., 1996; Hammond *et al*., 2001; Poulton *et al*., 2001).

The concomitant deficiencies in NKT cell numbers and function have contributed to the autoimmune phenotypes seen in NOD mice. These mice spontaneously develop type I diabetes (T1D), characterised by the destruction of pancreatic islet beta cells (Baxter *et al*., 1994). They are also susceptible to other autoimmune diseases, such as systemic lupus erythematosus (SLE; Esteban *et al*., 2003), and hemolytic anemia (Baxter *et al*., 1991). There is a tight relationship between the spontaneous development of type I diabetes and the severe decrease in NKT cell numbers and function in NOD mice (Makino *et al*., 1980; Baxter *et al*., 1997; Hammond *et al*., 1998); the course and the severity of type I diabetes in these mice could be moderated by increasing NKT cell numbers through adoptive transfer (Hammond *et al*., 1998; Laloux *et al*., 2001), congenesis (Jordan *et al*., 2007; Chen *et al*., 2007; Fletcher *et al*., 2008), transgene of *Vα14-Jα18* (Lehuen *et al*., 1998) or CD1d (Falcone *et al*., 2004), or activating them with α-GalCer (Seokmann *et al*., 2001; Sharif *et al*., 2001).

Many endeavours have sought to identify the original factors leading to the reduction of NKT cell numbers and function in NOD mice. A genetic linkage study of a first backcross between NOD and C57BL/6 mice has revealed that the defect in NKT cell numbers in NOD mice is controlled by at least two loci: *Nkt1* and *Nkt2*, which map to regions containing lupus and diabetic susceptibility genes, respectively (Esteban *et al*., 2003). The congenic replacement of these two regions, with corresponding regions derived from C57BL/6 mice, partly increased NKT cell numbers and delayed the onset of type I diabetes in NOD recipients (Jordan *et al*., 2007; Fletcher *et al*., 2008). However, it did not completely rescue NKT cell numbers nor NOD mice from type I diabetes (Chung *et al*., 2005; 2008; Nichols *et al*., 2005; Pasquier *et al*., 2005; Griewank *et al*., 2007; Jordan *et al*., 2007; 2011).

In this chapter, type I NKT cell transgenic mice were generated to increase NKT cell numbers on both NOD and B6 backgrounds. We were able to provide sufficient numbers of immature NKT cells to dissect transcriptional changes associated with NKT cell selection and lineage commitment.

The following hypothesis was tested: "**Expression of *Vα14-Jα18* on a CD4 minimal transgenic construct on the NOD strain background will produce functional NKT cells and increase NKT cell numbers in early developmental stages, including NKT cells at stage 0**".

Specifically, the aims of this chapter were:

1. To investigate the effect of *Vα14-Jα18* expression on type I NKT cell numbers;

2. To assess the phenotype of type I NKT cells generated by *Vα14-Jα18* transgenic mice;

3. To assess TCR Vβ usage of type I NKT cells generated by *Vα14-Jα18* transgenic mice;

4. To assess the function of type I NKT cells generated by *Vα14-Jα18* transgenic mice.

## 3.2. RESULTS

### 3.2.1. Production of *Vα14-Jα18* transgenic mice

*Vα14-Jα18* transgenic (*Vα14*Tg) mice have been previously developed to assist functional and developmental studies of type 1 NKT cells in B6 mice (Bendelac *et al*., 1994; Griewank *et al*., 2007). Here, we used their validated transgenic construct containing a type I NKT-associated *Vα14-Jα18* TCR α chain complementary DNA (cDNA) under the regulatory control of a minimal CD4 promoter, enhancer and intronic silencer (Griewank *et al*., 2007; Figure 3.1) to produce transgenic mice on both B6 and NOD backgrounds. Firstly, the pre-rearranged *Vα14-Jα18* TCR α chain cDNA was PCR-amplified from the CD1 specific T cell hybridoma DN32.D3 (Lantz *et al*., 1994). *Vα14-Jα18* cDNA was then ligated into the *Sal*I site of a plasmid containing the minimal CD4 promoter, enhancer and the intronic silencer (Sawada *et al*., 1994; Matsuda *et al*., 2001; Fig. 3.1 A).

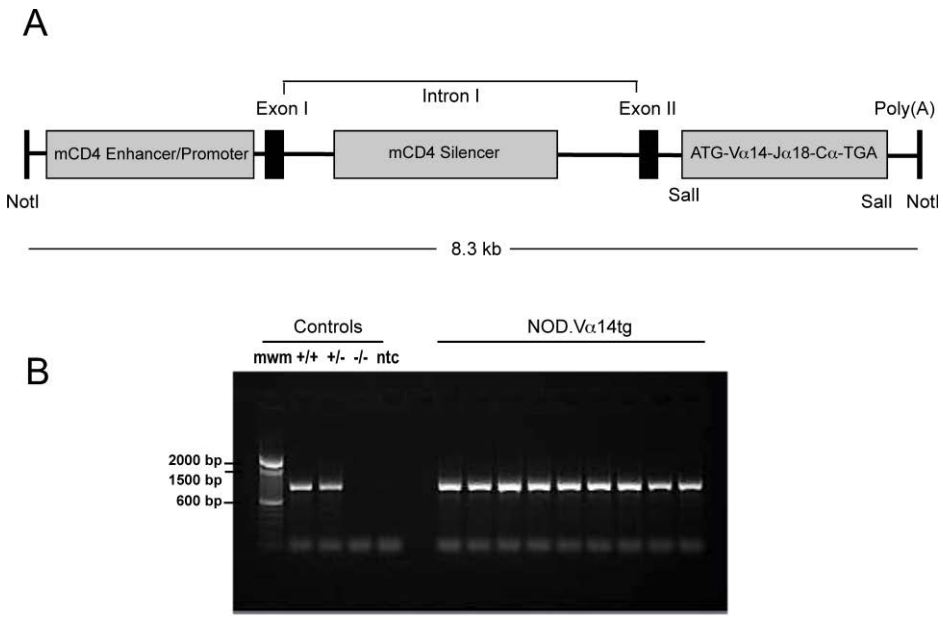


**Figure 3.1. Generation of NOD.*Vα14*Tg mice**. **A**. A diagram of the transgenic construct, which consists of a CD4 minigene (including a minimal CD4 promoter/enhancer and the CD4 intronic silencer), 5' of *Vα14-Jα18* TCR α chain cDNA and a poly-A tail (Griewank *et al*., 2007). **B**. Genotyping gel picture. Pups were genotyped using construct-specific primers. mwm = molecular weight marker; +/+ = homozygote for *Vα14*Tg; +/- = heterozygote for *Vα14*Tg; -/- = wild type (WT); ntc = no template control.

The construct was prepared by digestion of 10-15μg pDNA with *Not*1 and undergone gel electrophoresis to separate the transgene from the backbone. It was then agarase-treated and dialysed before being stored at -20C in injection buffer at a concentration of 2μg/ml until ready to inject. The purified, linearized constructs were directly injected into B6 and NOD/Lt embryonic pronuclei at the Transgenic Production Facility of the Walter and Eliza Hall Institute; and the manipulated embryos mice were placed in the reproductive tracts of pseudo-pregnant B6 and NOD/Lt recipient females. The resultant mice were then genotyped by a PCR spanning the CD4 to TCR sequence to identify transgene incorporation and germ-line transmission.

Seven different transgenic founders were identified on the B6 background and seven on the NOD background. We ultimately created four different homozygous NOD transgenic lines (lines 1, 2, 3, 4) and two B6 transgenic lines (lines 2 and 5). The other founding lines either did not obtain germ-line transmission, were poor breeders, or else had malocclusion and were therefore culled. All *Vα14*Tg NOD lines have been crossed onto the NOD.*Nkrp1*$^{b}$

line to permit the use of the NK1.1 developmental marker. However, in the text we will use the abbreviation NOD.*Vα14*Tg instead of NOD.*Nkrp1*$^{b}$.*Vα14*Tg.

### 3.2.2. NKT cells in *Vα14-Jα18* transgenic mice

To investigate the effect of *Vα14-Jα18* transgenic expression on type I NKT cell development, lymphoid organs were collected from 6 - 8 weeks old *Vα14*Tg, and wild type control mice, to make single cell suspensions. Total cells were counted and 5 million cells from each organ were stained with antibodies, and then analysed on a flow cytometer (FACS Fortessa from BD Bioscience). Lymphocytes were gated by side scatter area and forward scatter area to exclude red blood cells and debris. Single lymphocytes were then selected by using forward scatter height against forward scatter area before an exclusion of dead cells by using PI and an empty channel (if possible) to exclude autofluorescent cells. Thymic, hepatic and splenic NKT cells were identified using fluorochrome conjugated anti-mouse TCRβ antibody and α-GalCer loaded CD1d-tetramers. NKT cells from *Vα14*Tg NOD and B6 mice were compared with NOD and B6 wild type controls, respectively.

Flow cytometric cell analyses revealed that transgenic expression of *Vα14-Jα18* had increased NKT cell numbers on a NOD background. However, the proportions and absolute numbers of NKT cells differed between the different transgenic lines.

In the thymi, there were significant increases in both proportion and absolute numbers of NKT cells in all *Vα14*Tg NOD lines ($p$<0.0001, Mann-Whitney U test; Fig. 3.2). Of total thymocytes, NKT cells constituted 6.6% in line 1; 8.0% in line 3, and 0.23% in lines 2 and 4 compared with 0.09% in NOD WT mice. In terms of absolute numbers, transgenic lines 1 and 3 had a 60-90 fold increases in thymic NKT cell numbers, while lines 2 and 4 had 3-fold increases. There were no differences in thymus size between NOD WT control and *Vα14*Tg mice, except in line 3, which had a significant reduction in total thymocyte numbers (p=0.0115; Fig 3.2).

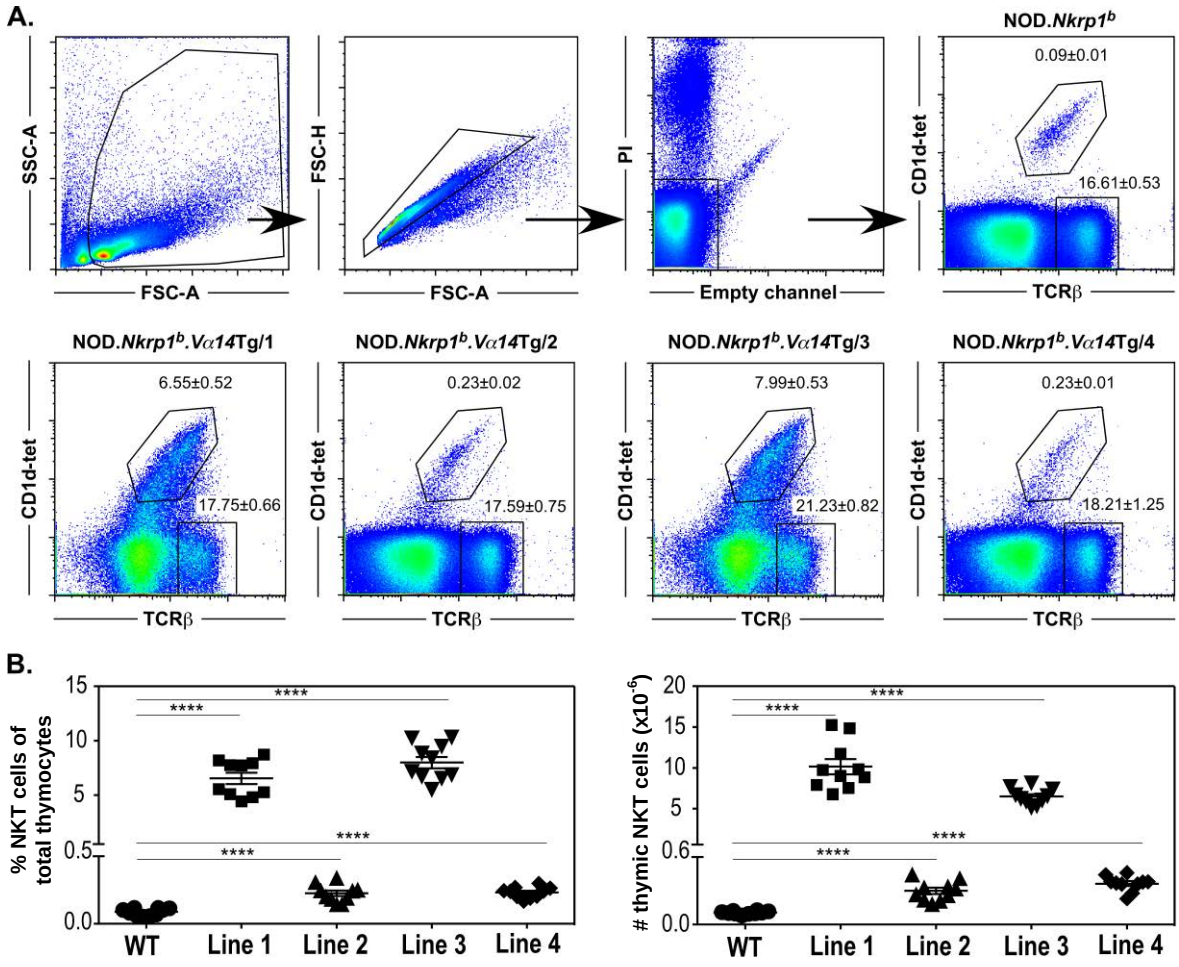


**Figure 3.2. Characterisation of NKT cells in the thymi of NOD.*Vα14*Tg lines**. **A**. Representative FACS plots showing NKT cell gating strategy and the frequencies of NKT cells (TCRβ$^{int}$CD1d-tet$^{+}$) among total cells from thymi of NOD WT and four NOD.*Vα14*Tg lines (1, 2, 3 and 4). **B**. Dot plots show the frequencies (left panel) and absolute numbers (right panel) of NKT cells among total cells from thymus of NOD WT mice compared to four NOD.*Vα14*Tg lines. Data are means (±SEM) from 5 males and 5 females for each group. Data illustrated are combined from two independent experiments. Mann-Whitney U test, **** p<0.0001.

To assess the effect of *Vα14* transgene on T cell development, thymic T cells were gated (Fig. 3.2). There were no significant differences in the proportions of thymic T cells between NOD WT and *Vα14*Tg mice, except a considerable increase in T cell proportions in line 3 (Fig. 3.3; $p<0.001$). With regard to the absolute numbers, there were significantly greater numbers of T cells in lines 1 and 4, while similar T cell numbers were seen in lines 2 and 3 compared with NOD WT mice (Fig. 3.3).

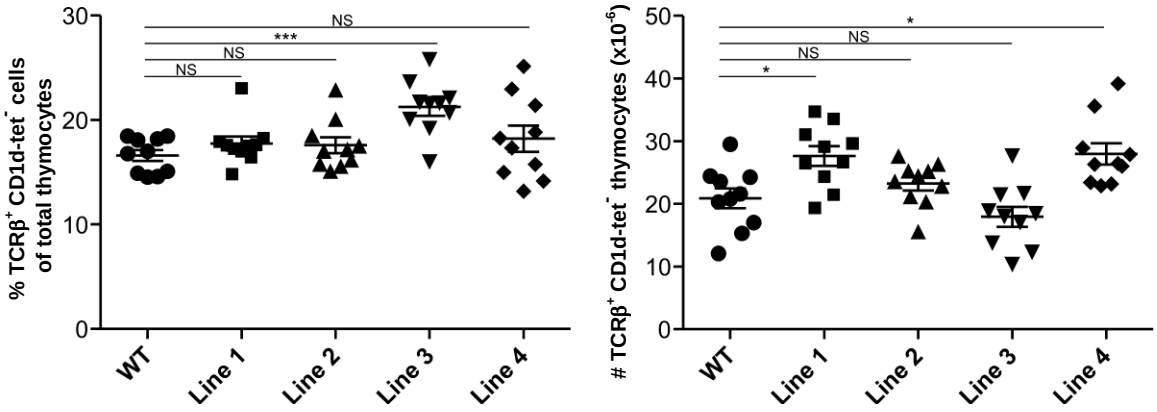


**Figure 3.3. Frequencies and absolute numbers of thymic T cells** from NOD WT mice compared to four NOD.*Vα14*Tg lines. Thymic T cells were identified as TCRβ$^{+}$ CD1d-tet$^{-}$ from total thymocytes (Figure 3.2). Data are means (±SEM) from 5 males and 5 females for each group. Data illustrated are combined from two independent experiments. Mann-Whitney U test, * p<0.05, *** p<0.001.

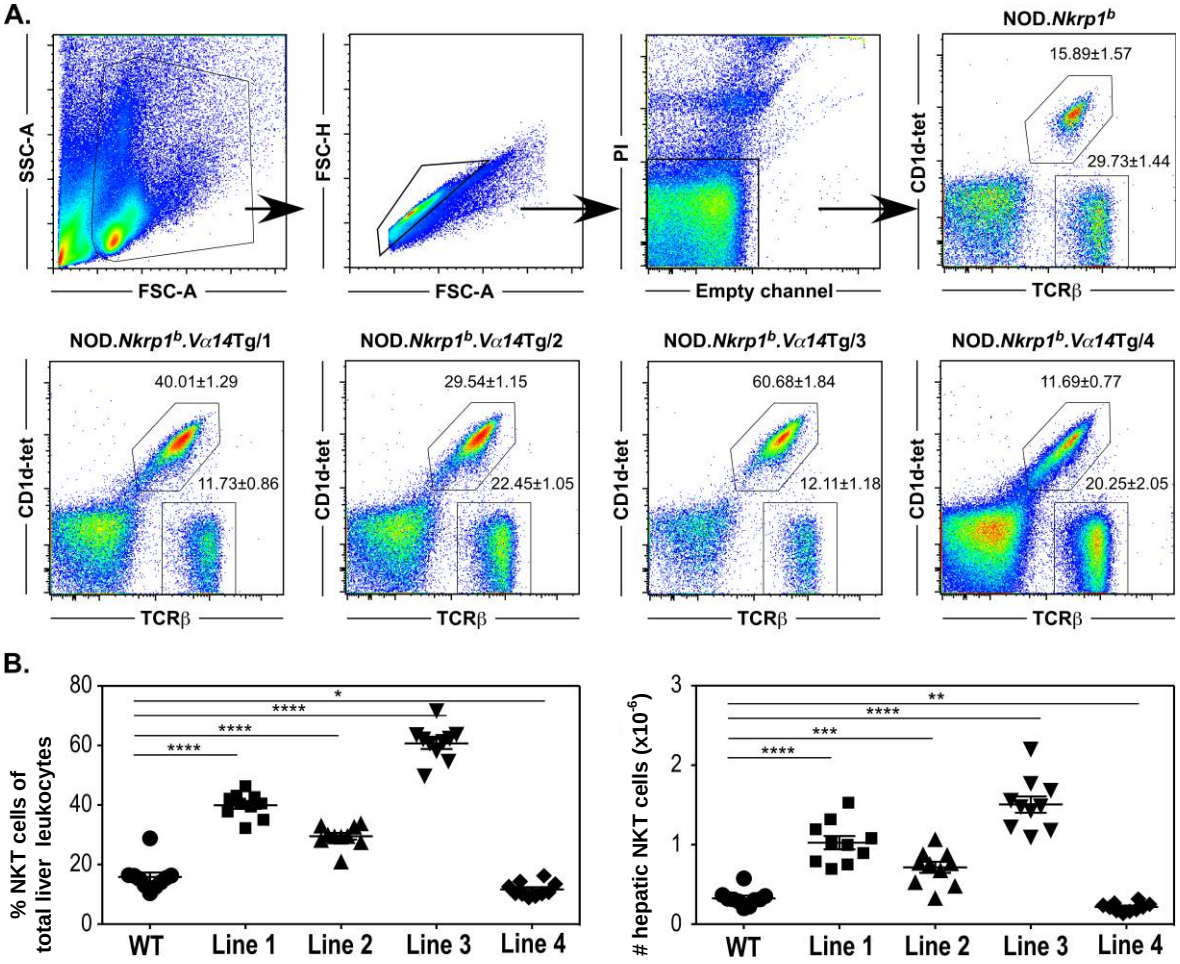


**Figure 3.4. Characterisation of NKT cells in the livers of NOD.*Vα14*Tg mice**. **A**. Representative FACS plots showing NKT cell gating strategy and the frequencies of NKT cells (TCRβ$^{int}$CD1d-tet$^{+}$) among total cells from livers of NOD WT and four NOD.*Vα14*Tg lines (1 to 4). **B**. Dot plot graphs show the frequencies (left panel) and absolute numbers (right panel) of NKT cells among total cells from livers of NOD WT mice compared to four NOD.*Vα14*Tg lines. Data are means (±SEM) from 5 males and 5 females for each group. Data illustrated are combined from two independent experiments. Mann-Whitney U test, * p<0.05, ** p<0.01, *** p<0.001, **** p< 0.0001.

*Vα14*Tg expression also increased hepatic NKT cell numbers in *Vα14*Tg lines 1, 2 and 3. The numbers of hepatic NKT cells were raised ~4-fold in lines 1 and 3, and by 50% in line 2. In sharp contrast, *Vα14*Tg line 4 had considerably fewer NKT cells, in both proportion and absolute numbers, than NOD WT mice (Fig. 3.4). There were no differences in total lymphocyte numbers in the livers. Thus, the increase in hepatic NKT cells may explain a slight reduction in proportions of conventional T cells in the liver of all NOD.*Vα14*Tg lines (Fig. 3.5).

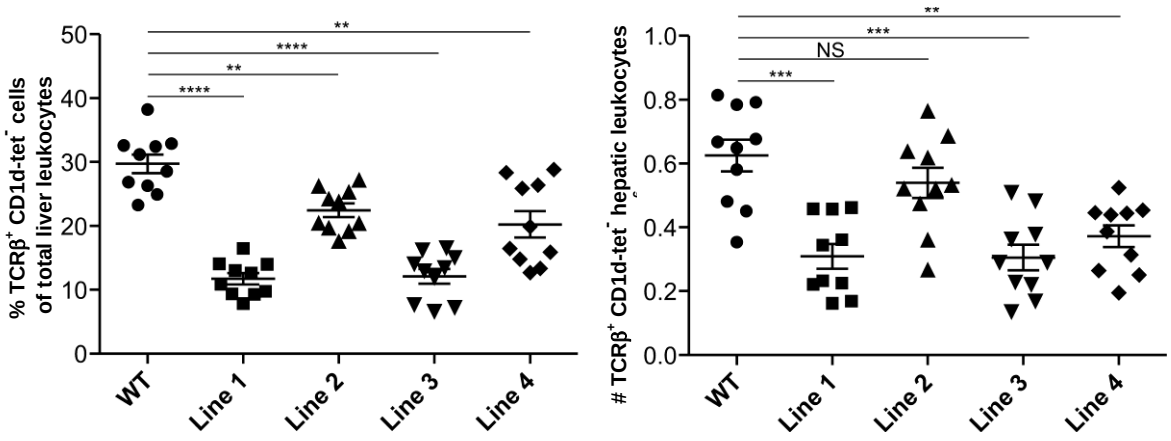


**Figure 3.5. Frequencies and absolute numbers of hepatic T cells from NOD WT mice compared to the four NOD.*Vα14*Tg lines**. Hepatic T cells were identified as TCRβ$^+$ CD1d-tet$^-$ from total hepatic leukocytes (Figure 3.4). Data are means (±SEM) from 5 males and 5 females for each group. Data illustrated are combined from two independent experiments. Mann-Whitney U test, ** p<0.01, *** p<0.001, **** p< 0.0001.

In the spleens, there were significant increases in the percentage of NKT cells in the four *Vα14*Tg lines (Fig. 3.6). While in WT controls, only 0.56% of total spleen cells were NKT cells, the proportion of NKT cells in spleens of *Vα14*Tg line 1 was 3.8%, that of line 2 was 0.80%, line 3 was 5.1% and line 4 was 0.88%. In terms of absolute numbers, lines 1 and 3 had ~7-fold increases in splenic NKT cell numbers, whereas lines 2 and 4 had similar numbers to the non-transgenic parental strain.

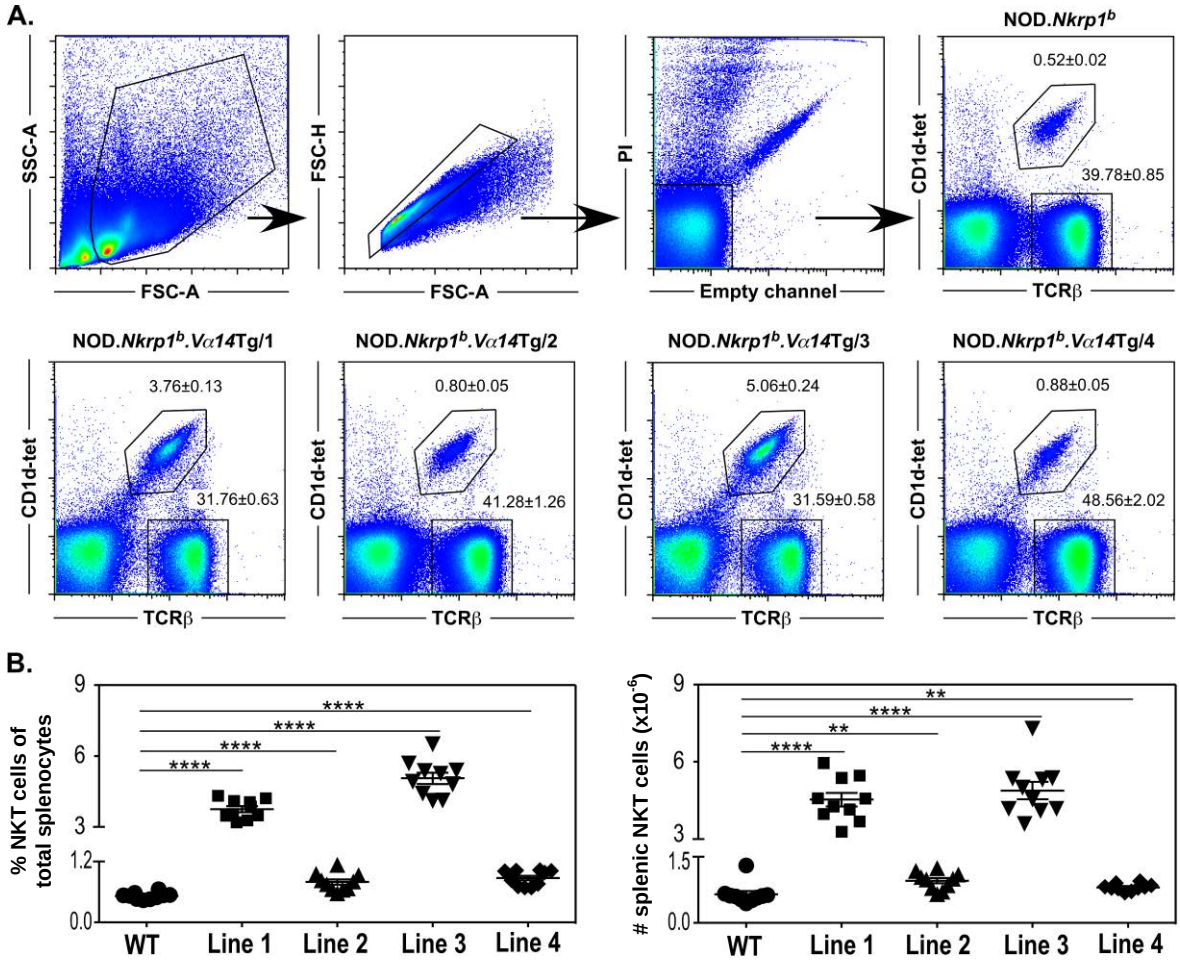


**Figure 3.6. Characterisation of NKT cells in the spleens of NOD.*Vα14*Tg mice**. **A**. Representative FACS plots showing NKT cell gating strategy and the frequencies of NKT cells ($TCR\beta^{int}CD1d\text{-}tet^{+}$) among total cells from spleens of NOD WT and four NOD.*Vα14*Tg lines (line 1, 2, 3, 4). **B**. Dot plot graphs show the frequencies (left panel) and absolute numbers (right panel) of NKT cells among total splenocytes of NOD WT compared to four NOD.*Vα14*Tg lines. Data are means (±SEM) from 5 males and 5 females for each group. Data illustrated are combined from two independent experiments. Mann-Whitney U test, ** p<0.01, **** p< 0.0001.

The introduction of *Vα14*Tg also affected T cell production in the spleens of NOD.*Vα14*Tg mice (Fig. 3.7). There were considerable decreases in proportions of conventional T cells in lines 1 and 3 compared to the NOD WT controls; line 2 was unchanged, while splenic T cell proportions in line 4 were slightly greater. With regards to the absolute numbers of conventional T cells, there were no significant differences between WT controls and *Vα14*Tg lines, except for line 3, in which they were markedly decreased ($p$=0.0001; Mann-Whitney U test; Fig. 3.7).

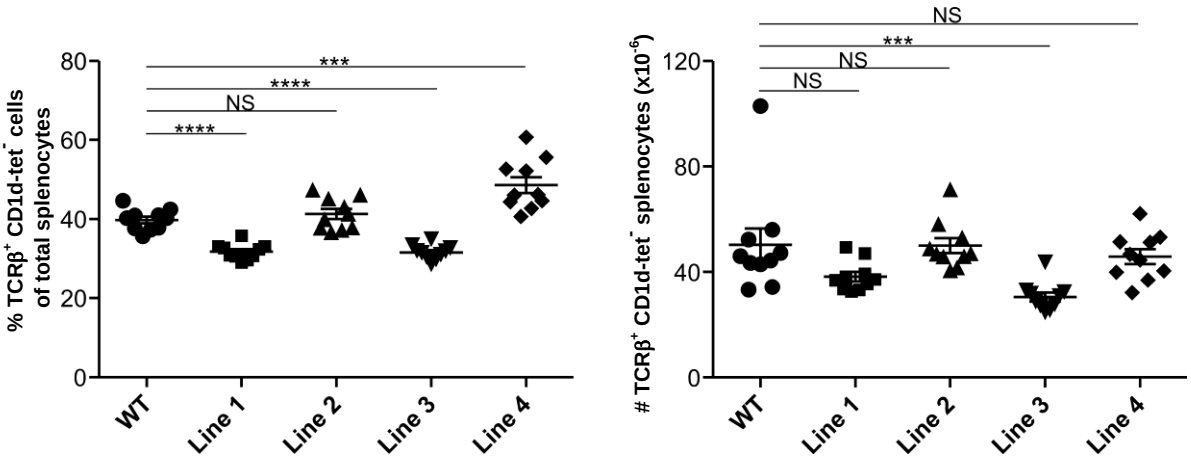


**Figure 3.7. Frequencies and absolute numbers of splenic T cells from** NOD WT mice compared to the four NOD.*Vα14*Tg lines. Splenic T cells were identified as TCRβ$^+$ CD1d-tet$^-$ from total splenocytes (Figure 3.6). Data are means (±SEM) from 5 males and 5 females for each group. Data illustrated are combined from two independent experiments. Mann-Whitney U test, *** p<0.001, **** p< 0.0001.

The effects of the *Vα14* transgene on B6 mice was examined in comparison with B6 WT control mice. There was a three-fold reduction in thymus size of both B6.*Vα14*Tg line 2 and line 5 mice compared to B6 WT mice (Fig. 3.8). No significant differences were seen in total cell counts from the livers and spleens between line 2 and B6 WT control mice. In contrast, total cell counts from the livers of B6.*Vα14*Tg line 5 mice were slightly increased ($p<0.05$), and those of spleens from line 5 were decreased compared to B6 WT control mice ($p<0.05$; Fig. 3.8).

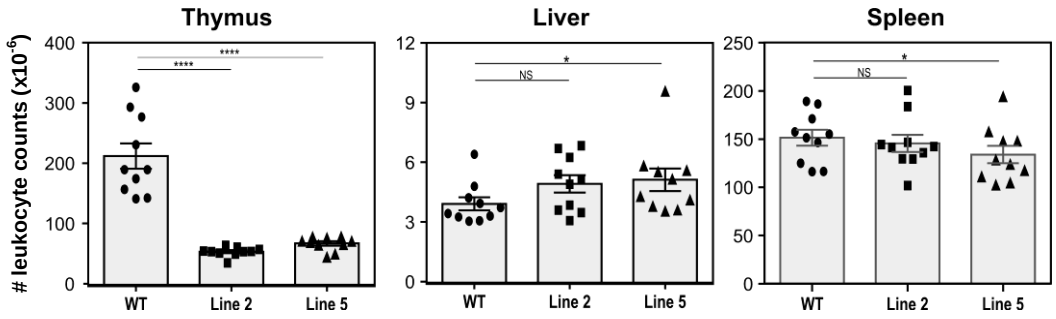


**Figure 3.8. Total leukocyte counts of thymi, spleens and livers from B6 WT and B6.*Vα14*Tg lines 2 and 5 mice**. Data are means (±SEM) from 5 males and 5 females for each group. Data illustrated are combined from two independent experiments

NKT cell analyses revealed that the B6 *Vα14-Jα18* transgenic mice also exhibited an augmented frequency of thymic type I NKT cells identified as TCRβ$^{int}$ CD1d-tet$^{+}$ (Fig. 3.9). This was reflected in 35- and 46-fold increases in both proportions and absolute numbers of thymic NKT cells in B6.*Vα14*Tg lines 2 and 5, respectively. The frequencies and absolute numbers of NKT cells in the livers of B6.*Vα14*Tg lines 2 and 5 were increased by 75%, whereas those of splenic NKT cells were increased more than 6-fold in line 2, and 9-fold in line 5. B6.*Vα14*Tg line 5 had significantly more NKT cells in their thymi and spleens compared to line 2, but they had similar NKT cell proportions and numbers in their livers (Fig. 3.9).

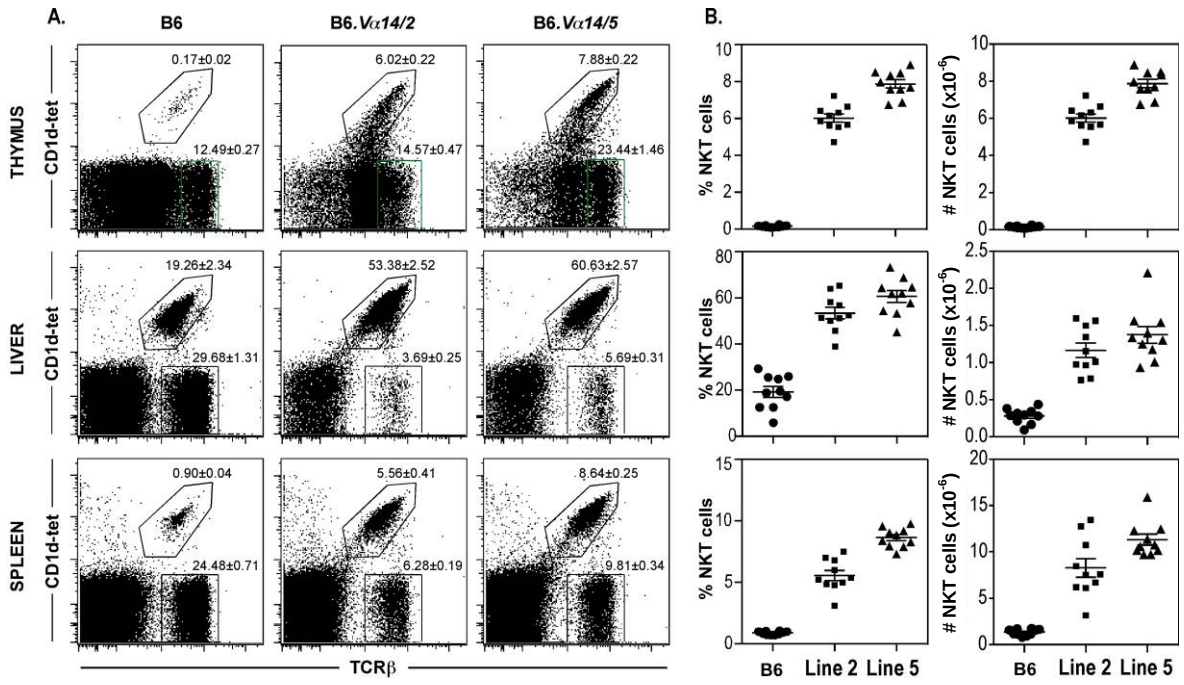


**Figure 3.9. Characterisation of NKT cells from B6.*Vα14*Tg mice**. **A**. Representative FACS plots showing the frequencies of NKT cells among total cells from thymi (top row), livers (middle row) and spleens (lower row) of B6, B6.*Vα14*Tg line 2 and 5 mice. **B.** Dot plot graphs show the frequencies (left panel) and absolute numbers (right panel) of NKT cells in thymi (top row), livers (middle row) and spleens (lower row) from the two B6.*Vα14*Tg mouse lines compared to B6 mice. Data are means (±SEM) from 5 males and 5 females for each group. Data are combined from two different experiments.

In contrast, there were decreased frequencies and absolute numbers of conventional T cells in all thymi, livers and spleens of B6.*Vα14*Tg lines 2 and 5, except for a slight increase in the frequency of mature thymic T cells in line 5 (Fig. 3.10).

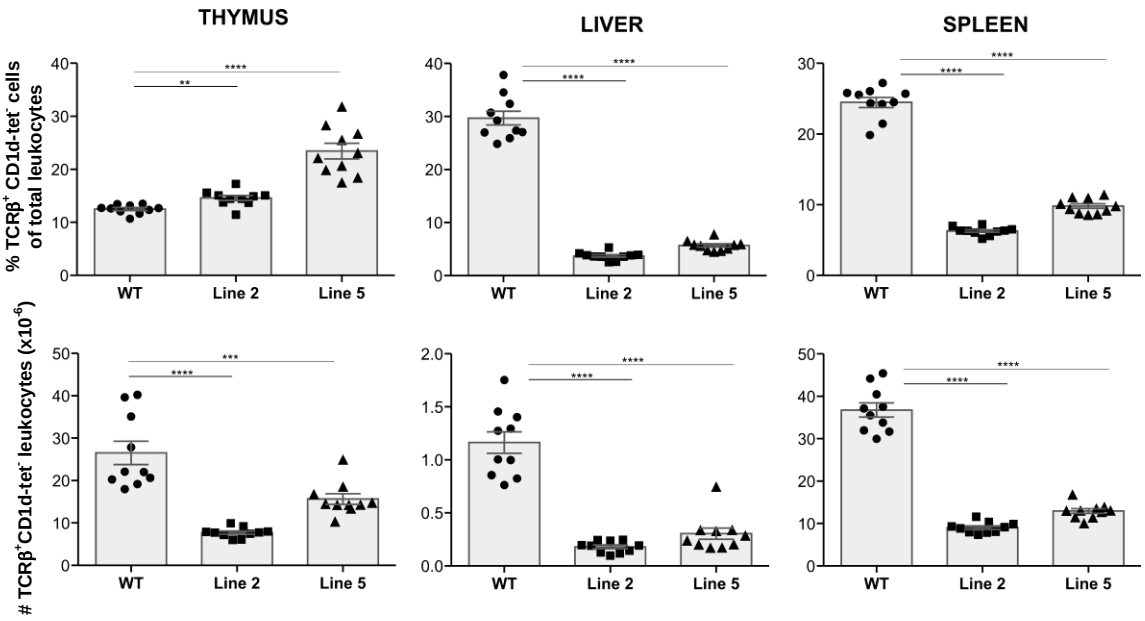


**Figure 3.10. Frequencies and absolute numbers of thymic, hepatic and splenic T cells of the two B6.*Vα14*Tg lines compared to B6 WT mice**. Data are means (±SEM) from 5 males and 5 females for each group. Data are combined from two independent experiments

Taken together, these data indicated that the introduction of a rearranged *Vα14-Jα18* transgene significantly increased NKT cell numbers in NOD and B6 mice.

### 3.2.3. Characterisation of NKT cell subsets from *Vα14*Tg mice

#### 3.2.3.1. NKT cell subsets from NOD.*Vα14*Tg mice

To investigate whether the introduction of the *Vα14* transgene could increase NKT cell numbers at different developmental stages and to analyse NKT cell phenotypes in greater detail, TCRβ$^{int}$CD1d-tet$^{+}$ cells were labelled with stage-specific markers (CD4, CD8, CD24, CD44 and NK1.1) and subjected to flow cytometry. Using these markers, NKT cells were divided into subsets based on the cell surface expression of CD4 and CD8, CD24 and CD44, or CD44 and NK1.1 molecules.

Flow cytometric cell analyses revealed that both the $CD4^+$ and $CD4^-CD8^-$ (double negative - DN) populations of NKT cells could be identified in the thymi (Fig. 3.11 and 3.12), livers (Fig. 3.13) and spleens (Fig. 3.14) of WT mice and mice from the NOD.*Vα14*Tg lines. In the thymi of NOD.*Vα14*Tg mice, these two major subsets totaled 30-60% of NKT cells with a DN:CD4 ratio of 3:2, compared to 95% and a 3:4 ratio in WT mice (Fig. 3.11 A).

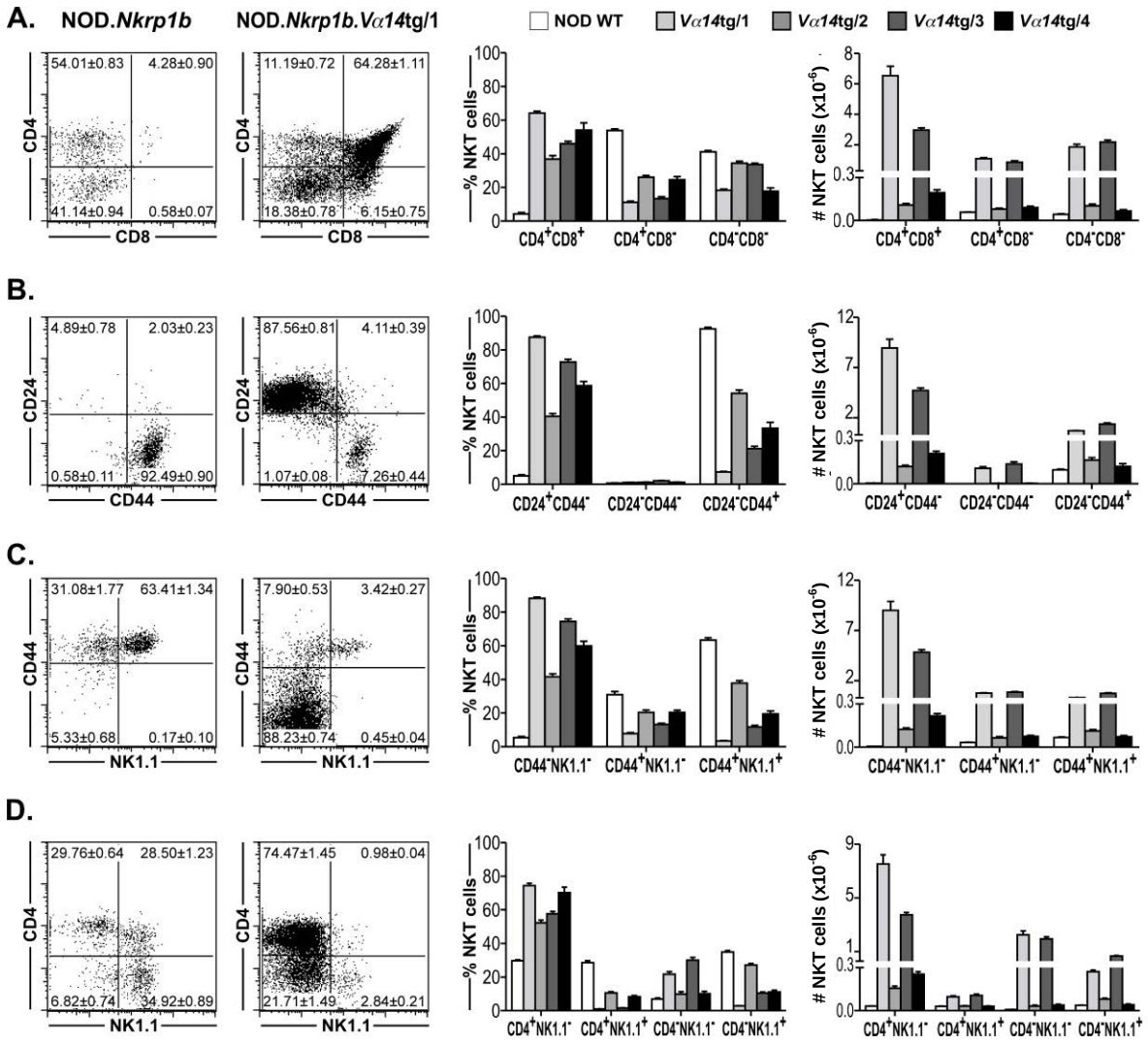


**Figure 3.11. NKT cell subsets from thymi of NOD.*Vα14*Tg mice**. **A.** CD4/CD8 NKT cell subsets. **B.** CD24/CD44 NKT cell subsets. **C.** CD44/NK1.1 NKT cell subsets. **D.** CD4/NK1.1 NKT cell subsets. For all figures, the representative FACS plots show the mean frequencies (±SEM) of NKT cell subsets in total NKT cells from thymi of NOD WT and NOD.*Vα14*Tg line 1 mice. Bar graphs show the mean frequencies and absolute numbers of thymic NKT cell subsets between NOD WT (clear bars) and four NOD.*Vα14*Tg mouse lines (filled bars). n = 10 for each group. Data are combined from two independent experiments. The NKT gates applied are those illustrated in Figure 3.2.

Within the NKT cell population of NOD WT mice, ~92% of thymic NKT cells expressed the relatively mature $CD24^{low}$ $CD44^{high}$ phenotype, and <5% expressed the less mature $CD24^{high}$ $CD44^{low}$ phenotype (Fig. 3.11 B). By contrast, within the transgenic lines, the larger the number of NKT cells present, the lower the proportion of mature $CD24^{low}$ $CD44^{high}$ NKT cells present. For example, line 1, which had the highest numbers of thymic NKT cells ($1.0 \times 10^7$ cells/thymus; Fig. 3.2), also had the lowest proportion of $CD24^{low}$ $CD44^{high}$ NKT cells (about 7.2% of total NKT cells; Fig. 3.11 B). A semi-log fit of thymic NKT cell numbers against proportion of $CD24^{low}$ $CD44^{high}$ cells in a combined data set, incorporating five male and five female mice of each strain (WT and the 4 transgenic lines), showed a very strong inverse correlation ($r^2 = 0.94$; df=48; n=50; data not shown).

Thymic NKT cells were further analysed for the expression of CD44, CD4 and NK1.1, which can be used to classify NKT cells into developmental stages such as immature $CD44^{low}NK1.1^-$, semi-mature $CD44^{high}NK1.1^-$, and mature $CD44^{high}NK1.1^+$ (Fig. 3.11 C and D). A similar trend was seen with the proportion of NK1.1 expressing NKT cells. The proportions of mature $NK1.1^+$ NKT cells were reduced in transgenic lines with more NKT cells ($r^2 = 0.74$; data not shown). For example, in line 1, only ~3.4% of thymic NKT cells expressed NK1.1, compared to >60% in WT mice (Fig. 3.11 C).

One of the most striking characteristics of the thymic NKT cell population in NOD.*Vα14*Tg mice was the presence, in very large numbers, of the $CD4^+CD8^+$ (double positive - DP) subset (Fig. 3.12 A). This population constituted less than 5% of all thymic NKT cells in WT NOD mice (estimated at less than $5/10^6$ thymocytes), but between 37 and 64% of thymic NKT cells in the various lines of NOD.*Vα14*Tg mice. The abnormal presence of large numbers of DP NKT cells encouraged further analysis of the $CD4^+CD8^-$, $CD4^+CD8^+$, $CD4^-CD8^-$ and $CD4^-CD8^+$ NKT cell subsets for the expression of CD24, CD44 and NK1.1 (Fig. 3.12).

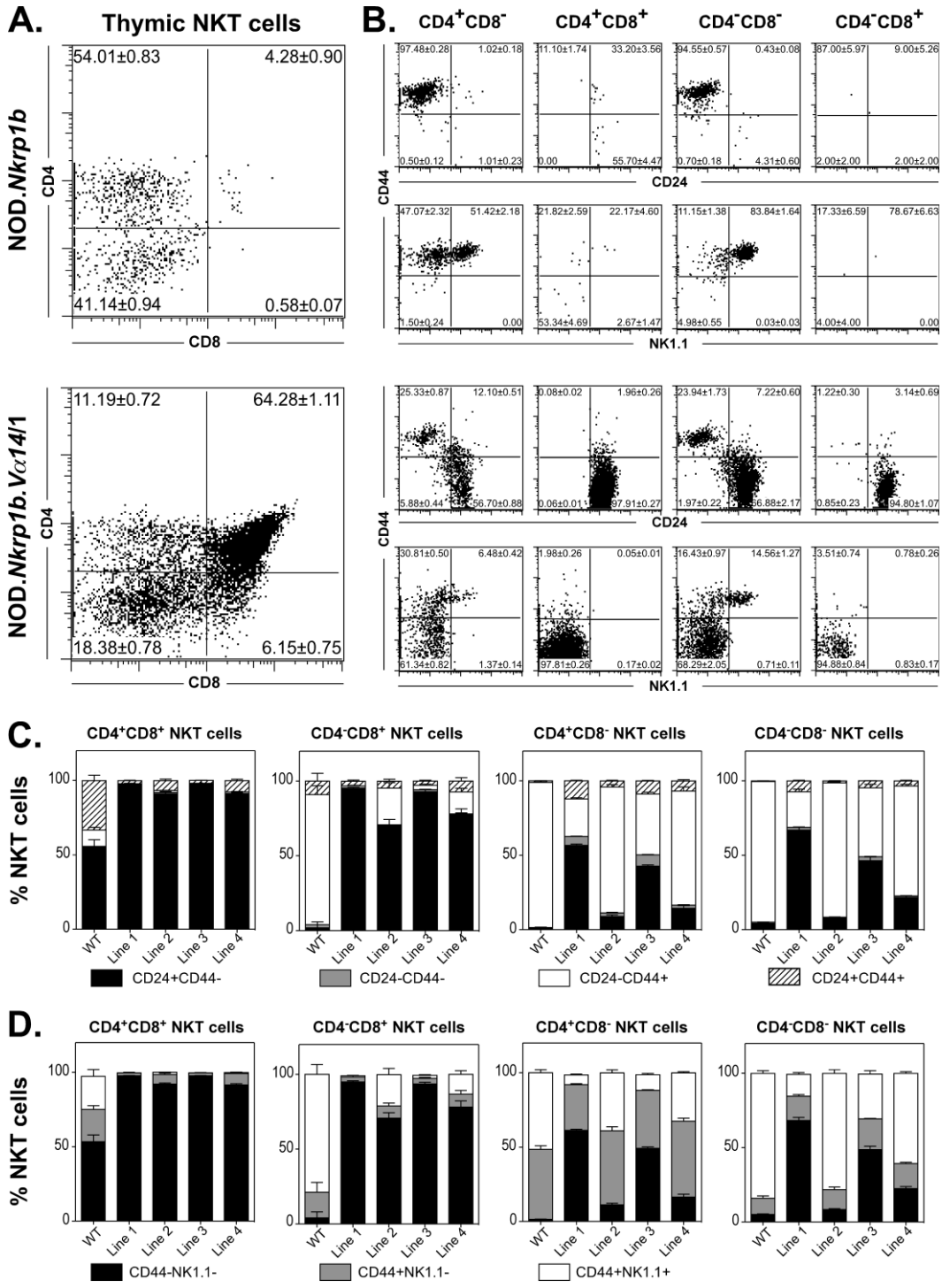


**Figure 3.12. Flow cytometric comparison of thymic CD4/CD8 NKT cell subsets in NOD WT and NOD.*Vα14*Tg line 1 mice**. **A**. Representative FACS plots show the mean frequencies (±SEM) of CD4/CD8 NKT cell subsets of total NKT cells from thymi of NOD WT and NOD.*Vα14*Tg/1 mice. **B**. Representative FACS plots show profiles of the expression of CD24, CD44 and NK1.1 in the four different thymic CD4/CD8 NKT cell subsets of NOD WT (upper rows) and NOD.*Vα14*Tg/1 mice (lower rows). **C**. Graphs show CD24/CD44 expression of the 4 different CD4/CD8 NKT cell subsets. **D**. Graphs show CD44/NK1.1 expression of the 4 different CD4/CD8 NKT cell subsets. Data are presented for ten mice/group combined from two independent experiments. The NKT gates applied are illustrated in Figure 3.2.

As shown in Figure 3.12 B, more than 97% of DP NKT cells expressed a $CD24^{high}$ $CD44^{low}$ $NK1.1^{-}$ phenotype, consistent with being the earliest identifiable NKT cells (Fig. 3.12 C and D). Interestingly, the $CD4^{low}CD8^{+}$ NKT cell subset (lying within the $CD4^{-}CD8^{+}$ quadrant gate in our analysis of NOD.*Vα14*Tg mice) also expressed a relatively immature phenotype: $CD24^{high}$ $CD44^{low}$ $NK1.1^{-}$. Comparison with Figure 2 of Gapin and colleagues' (2001) paper suggested that these cells were the $DP^{dull}$ population, previously identified as post-selection NKT cells. Conversely, 23-25% of the $CD4^{+}$ and DN NKT cells had the more mature phenotype $CD24^{low}CD44^{high}$ (Fig. 3.12). Taken together, the above data indicated that the transgenic expression of *Vα14-Jα18* enhanced the production of NKT cells in the thymi of NOD.*Vα14*Tg mice. However, this enhancement is biased toward the production of immature NKT cells at different development stages, especially the generation of DP NKT cells at stage 0 (Fig. 3.12).

In contrast to the thymi, virtually all (>99%) NKT cells in the livers and spleens of NOD.*Vα14*Tg mice were either $CD4^{+}$ or DN, although the proportions of these major subsets differed from those of WT NOD mice. In the livers, the DN:CD4 ratio was 1:2 in WT mice, but ranged from 1.5:1 to 5:1 in NOD.*Vα14*Tg mice (Fig. 3.13 A). Similarly, in the spleens, the DN:CD4 ratio was 1:4 in WT mice, but varied from 1:2 to 2:1 in NOD.*Vα14*Tg lines (Fig. 3.14 A).

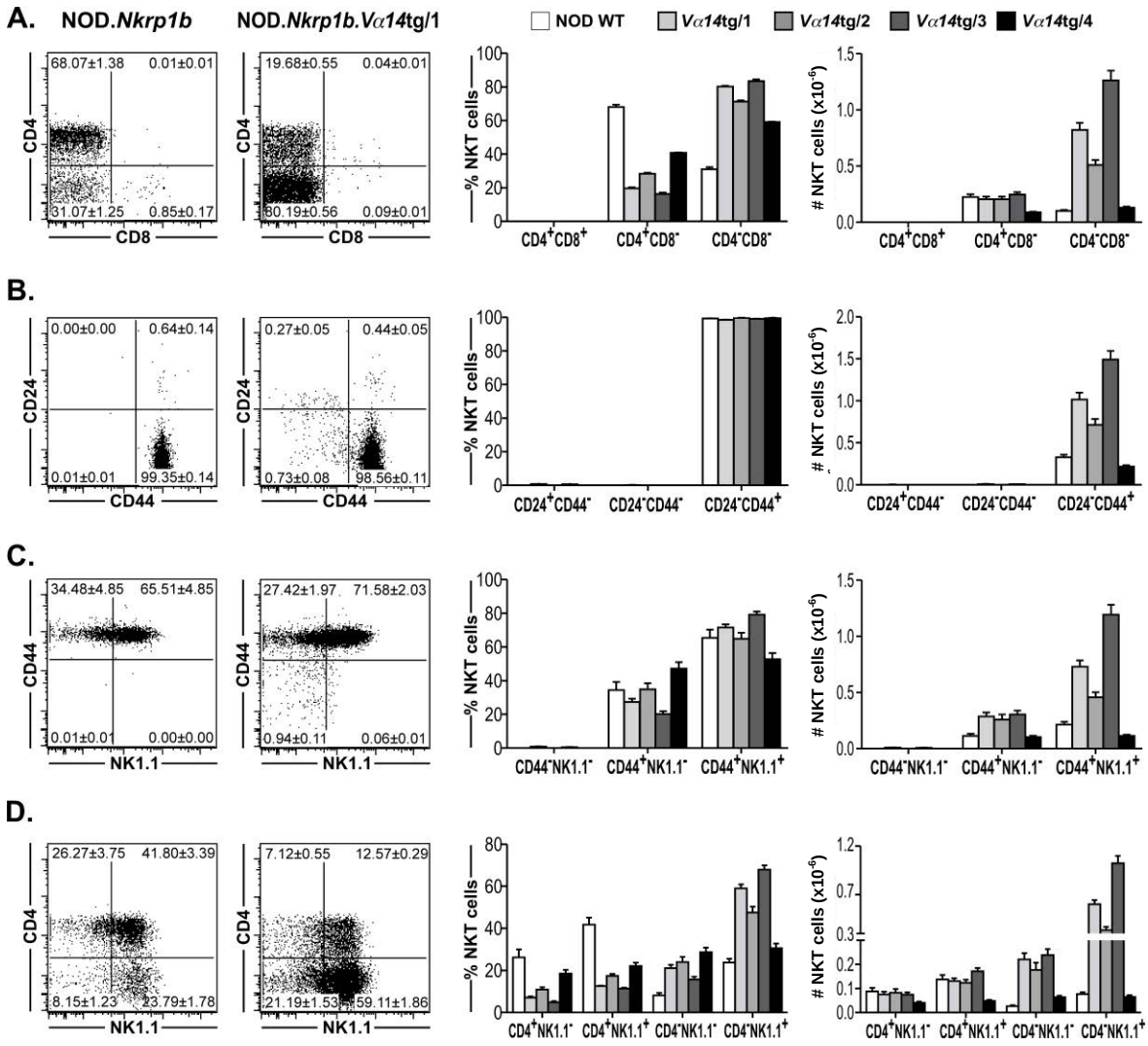


**Figure 3.13. NKT cell subsets in the livers of NOD.*Vα14*Tg mice**. **A.** CD4/CD8 NKT cell subsets. **B.** CD24/CD44 NKT cell subsets. **C.** CD44/NK1.1 NKT cell subsets. **D.** CD4/NK1.1 NKT cell subsets. For all figures, representative FACS plots (left panel) show the mean frequencies (±SEM) of NKT cell subsets in total hepatic NKT cells of NOD WT and NOD.*Vα14*Tg/1 mice. Bar graphs (right panel) show the mean frequencies and absolute numbers of hepatic NKT cell subsets between NOD WT (clear bars) and the four NOD.*Vα14*Tg mouse lines (filled bars). n = 10 for each group. Data are combined from two independent experiments. The NKT gates applied are illustrated in Figure 3.4.

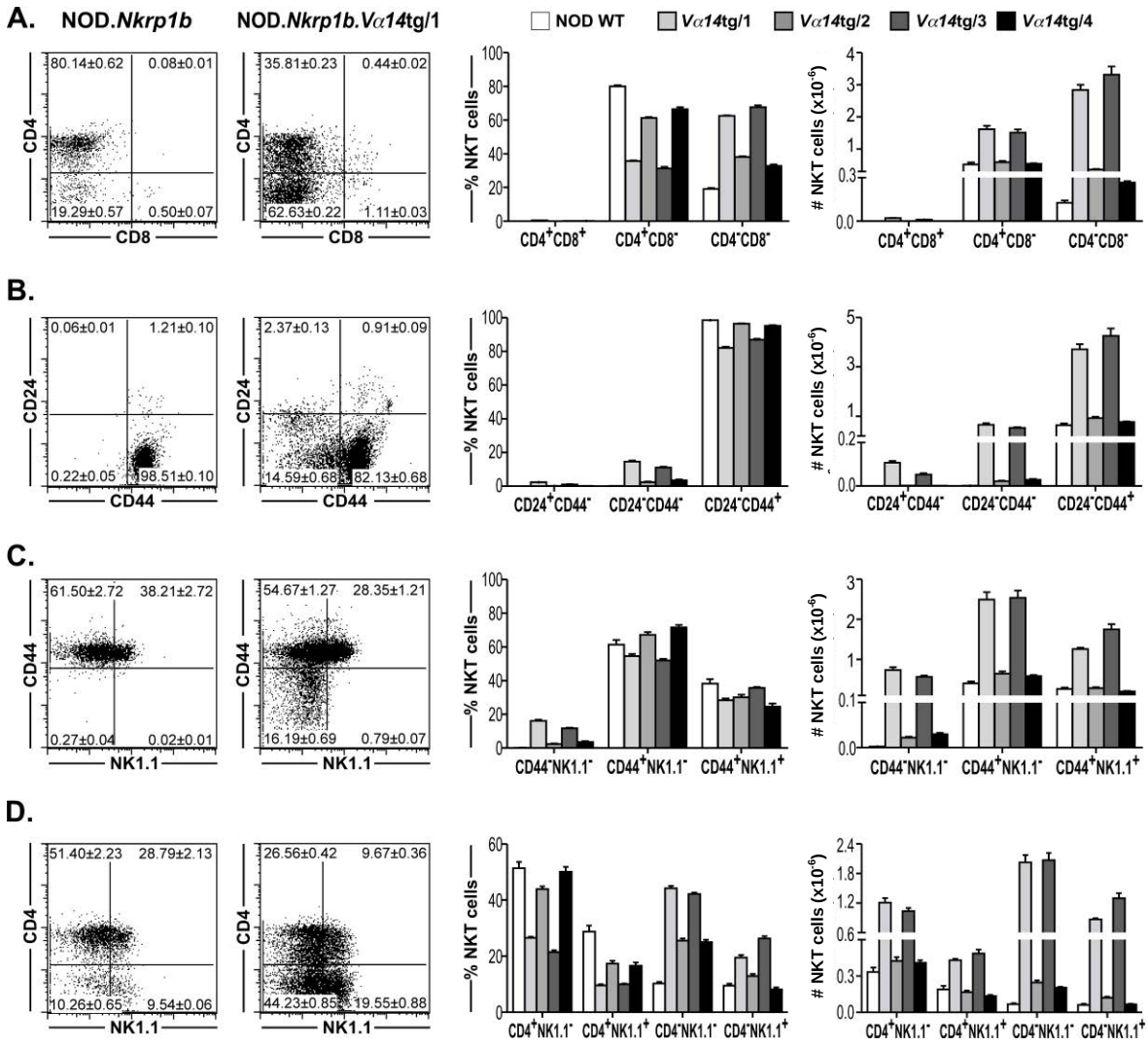


**Figure 3.14. NKT cell subsets from the spleens of NOD.*Vα14*Tg mice**. **A.** CD4/CD8 NKT cell subsets. **B.** CD24/CD44 NKT cell subsets. **C.** CD44/NK1.1 NKT cell subsets. **D.** CD4/NK1.1 NKT cell subsets. For all figures, the representative FACS plots (left panels) show the mean frequencies (±SEM) of NKT cell subsets in total splenic NKT cells of NOD WT and NOD.*Vα14*Tg/1 mice. Bar graphs (right panels) show the mean frequencies and absolute numbers of splenic NKT cell subsets between NOD WT (clear bars) and the four NOD.*Vα14*Tg mouse lines (filled bars). n = 10 for each group. Data are combined from two dependent experiments. The NKT gates applied are illustrated in Figure 3.6.

Additionally, NKT cells from both livers and spleens of NOD.*Vα14*Tg mice expressed a relatively mature $CD24^{low}$ $CD44^{high}$ phenotype. These $CD24^{low}$ $CD44^{high}$ NKT cells comprised more than 98% of hepatic leukocytes and over 82% of splenocytes. This phenotype was relatively similar to that of NOD WT controls (Fig. 3.13 B and 3.14 B). Peripheral NKT cells were also examined for the expression of CD44 and NK1.1. The proportions of NKT

cells with $CD44^-NK1.1^-$, $CD44^+NK1.1^-$ or $CD44^+NK1.1^+$ phenotypes in NOD.*Vα14*Tg mice were similar to those of NOD WT mice (Fig. 3.13 C and 3.14 C).

In terms of absolute numbers, NOD.*Vα14*Tg mice produced more mature NKT cells in their peripheries than NOD WT control mice, except for the slightly lower number of hepatic $CD24^-CD44^+$ NKT cells in line 4 (Fig 3.13 B). For example, the absolute numbers of hepatic $CD24^-CD44^+$ NKT cells were twofold higher in line 2, threefold higher in line 1 and more than 4-fold higher in line 3 compared to those of NOD WT mice (Fig. 3.13 B). In the spleens, the absolute numbers of $CD24^-CD44^+$ NKT cells were relatively similar between NOD.*Vα14*Tg line 2 and line 4 mice and NOD WT mice, but it was doubled in NOD.*Vα14*Tg lines 1 and 3 (Fig. 3.14 B).

Taken together, these data suggest that NOD.*Vα14*Tg mice produced increased numbers of immature NKT cells in their thymi and mature NKT cells in their peripheries.

**3.2.3.2. NKT cell subsets from B6.*Vα14*Tg mice**

Similar to what had been observed in NOD.*Vα14*Tg mice, both CD4 and DN NKT cells were identified in the thymi, spleens and livers of B6.*Vα14*Tg mice. In the thymi of B6.*Vα14*Tg mice, the proportion of these two subsets were 45% of total NKT cells in line 2, and 67% in line 5, compared to 97% in B6 WT mice (Fig. 3.15 A). The DN:CD4 ratio was 2:3 in WT, whereas it was 4:1 in B6.*Vα14*Tg line 2 and 1.2:1 in line 5. DP NKT cells were also seen in the thymi of B6.*Vα14*Tg, but to a lower extent than in those of NOD.*Vα14*Tg mice. They accounted for 49% of total thymic NKT cells in line 2, and 29% in line 5, relative to between 37% and 64% on the NOD background. In terms of absolute numbers, B6.*Vα14*Tg mice produced significantly lower numbers of DP NKT cells than did NOD.*Vα14*Tg lines 1 and 3. However, in comparison with B6 WT mice, B6.*Vα14*Tg mice produced much higher numbers of $CD4^+$, DN NKT and DP NKT cells.

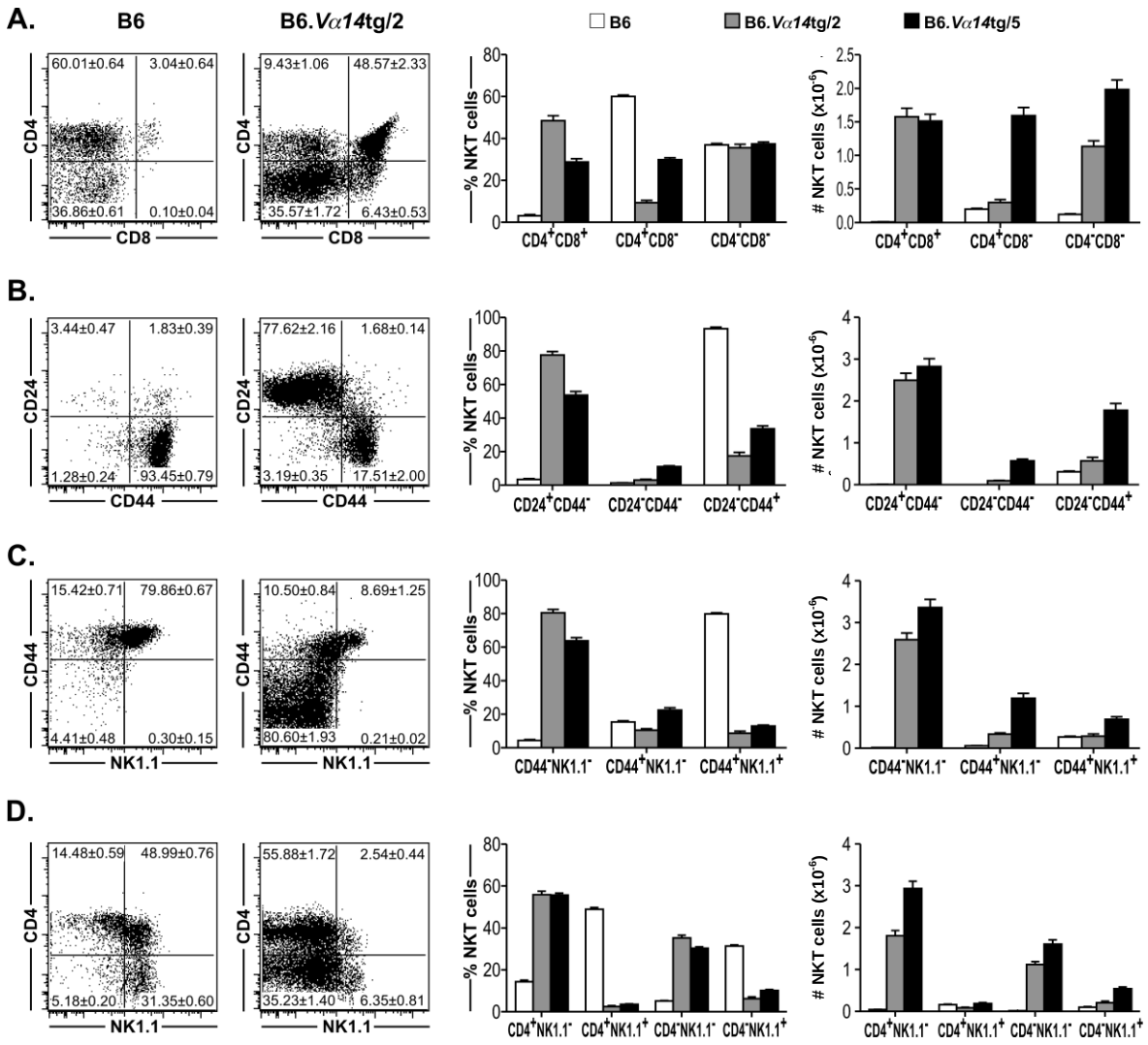


**Figure 3.15. NKT cell subsets in the thymi of B6.*Vα14*Tg mice**. **A.** CD4/CD8 NKT cell subsets. **B.** CD24/CD44 NKT cell subsets. **C.** CD44/NK1.1 NKT cell subsets. **D.** CD4/NK1.1 NKT cell subsets. For all figures, representative FACS plots (left panels) show the mean frequencies (±SEM) of NKT cell subsets in total thymic NKT cells of B6 and B6.*Vα14*Tg/2 mice. Bar graphs (right panels) show the mean frequencies and absolute numbers of thymic NKT cell subsets between B6 (clear bars) and B6.*Vα14*Tg line 2 and 5 mice (filled bars). n = 10 for each group. Data are combined from two independent experiments. The NKT gates applied are illustrated in Figure 3.9.

Additionally, ~94% of thymic NKT cells from B6 WT mice expressed the mature phenotype with $CD24^{low}$ $CD44^{high}$ and less than 4% expressing the immature $CD24^{high}$ $CD44^{low}$ phenotype (Fig. 3.15 B). In sharp contrast, 18% of thymic NKT cells in B6.*Vα14*Tg line 2 and 34% in line 5 expressed the mature $CD24^{low}$ $CD44^{high}$ phenotype, whereas the immature $CD24^{high}$ $CD44^{low}$ compartment comprised 78% in line 2, and 54% in line 5 (Fig. 3.15 B).

The same pattern was observed when the expression of CD44 and NK1.1 by thymic NKT cells was examined (Fig. 3.15 C). In B6 WT mice, 80% of thymic NKT cells showed the mature $CD44^{high}NK1.1^{+}$ phenotype, 15% were $CD44^{high}NK1.1^{-}$ and only 4.4% expressed the $CD44^{low}NK1.1^{-}$ phenotype. In comparison, the mature $CD44^{high}NK1.1^{+}$ NKT cells constituted only 8.7% and 13% of total thymic NKT cells in B6.*Vα14*Tg lines 2 and 5, respectively, and the $CD44^{high}$ $NK1.1^{-}$ subset made up 11% of total thymic NKT cells in line 2, and 22% in line 5. Most of the thymic NKT cells in B6 transgenic mice were the relatively immature $CD44^{low}NK1.1^{-}$ subset. They accounted for 81% in line 2 and 64% in line 5. With regards to absolute numbers, although the transgenic expression of *Vα14-Jα18* considerably increased mature $CD44^{high}NK1.1^{+}$ and $CD44^{high}NK1.1^{-}$ NKT cells, the increase in total thymic NKT cell numbers was largely contributed by the expansion of the immature $CD44^{low}NK1.1^{-}$ NKT cell subset (Fig. 3.15 C).

In contrast to the thymi, virtually all (>99%) NKT cells in the livers and spleens of B6.*Vα14*Tg mice were either $CD4^{+}$ or DN, although the proportions of these major subsets differed from those of WT B6 mice (Fig. 3.16 A; 3.17 A). In the livers, the DN:CD4 ratio was 1:3 in WT mice, but was 3:1 in both B6.*Vα14*Tg lines. With regard to the absolute numbers, there was a slight increase in the numbers of $CD4^{+}$ NKT cells, whereas those of the DN NKT cells were more than 10-fold higher (Fig. 3.16 A). Similarly, in the spleens, the DN:CD4 ratio was 1:5.6 in WT mice but was 5:2 and 3:2 in B6.*Vα14*Tg lines 2 and 5, respectively (Fig. 3.17 A). These data are consistent with a previous observation by Lehuen *et al*. (1998), in which they found that the $NK1.1^{+}$ T cells were mainly of the DN phenotype.

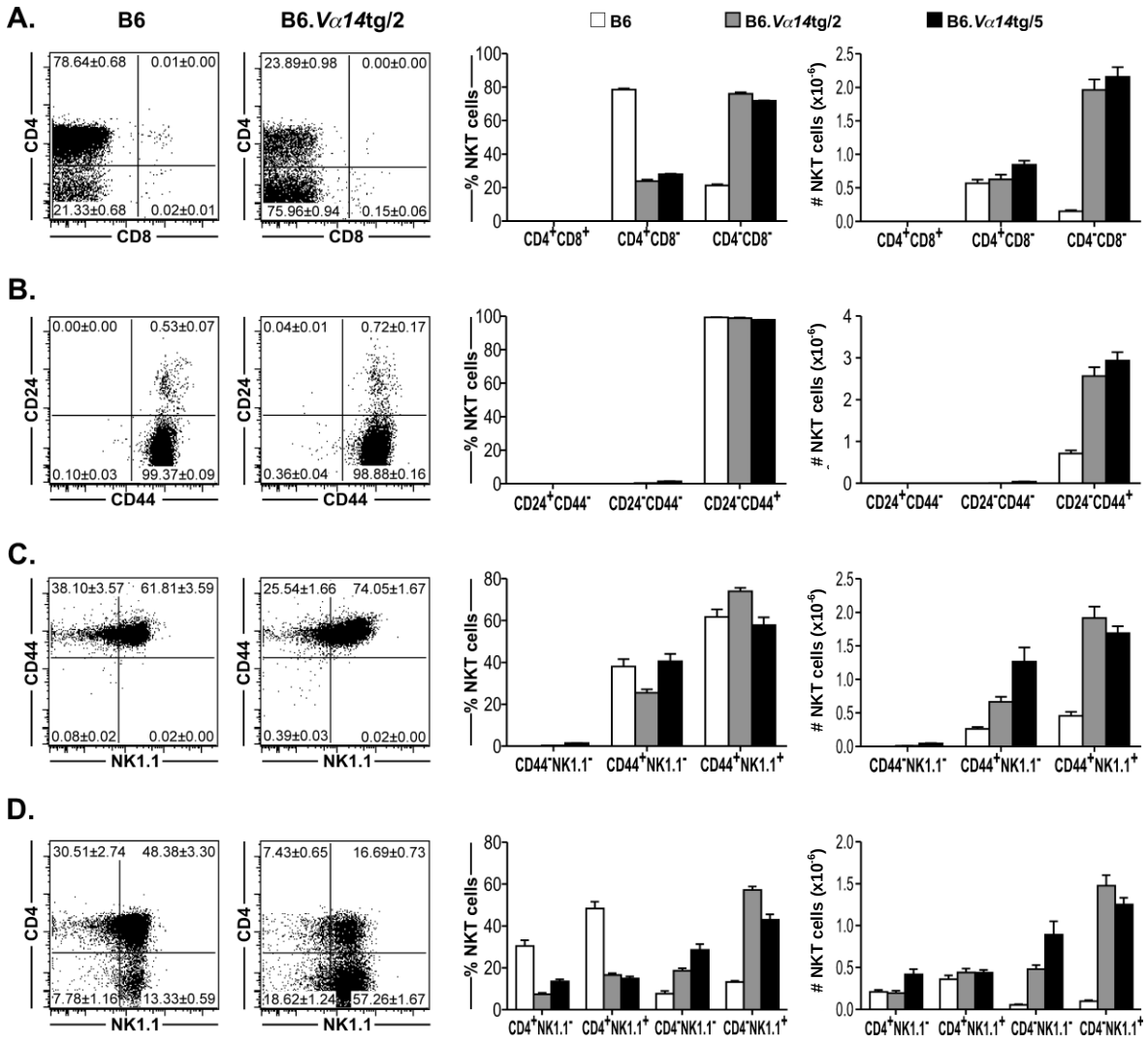


**Figure 3.16. NKT cell subsets in the livers of B6.*Vα14*Tg mice**. **A.** CD4/CD8 NKT cell subsets. **B.** CD24/CD44 NKT cell subsets. **C.** CD44/NK1.1 NKT cell subsets. **D.** CD4/NK1.1 NKT cell subsets. For all figures, representative FACS plots (left panels) show the mean frequencies (±SEM) of NKT cell subsets in total NKT cells from livers of B6 and B6.*Vα14*Tg/2 mice. Bar graphs (right panels) show the mean frequencies and absolute numbers of hepatic NKT cell subsets between B6 (clear bars) and B6.*Vα14*Tg line 2 and 5 mice (filled bars). n = 10 for each group. Data are combined from two independent experiments. The NKT gates applied are illustrated in Figure 3.9.

Similar to what was seen in the peripheral organs of NOD.*Vα14*Tg mice, more than 98% of hepatic NKT cells (Fig. 3.16 B) and more than 80% of splenic NKT cells (Fig. 3.17 B) of B6.*Vα14*Tg mice expressed the mature $CD24^{low}CD44^{high}$ phenotype. Of those, the majority expressed the NK1.1 marker (Fig. 3.16 C; 3.17 C). In terms of absolute numbers, both B6.*Vα14*Tg line 2 and line 5 mice produced considerably more $CD24^{low}CD44^{high}$ NKT

cells compared to B6 WT mice. For example, the numbers of hepatic $CD24^{low}CD44^{high}$ NKT cells in B6.*Vα14*Tg lines 2 and 5 were more than three times higher than those of B6 mice. Similarly, in the spleens, there were four times more $CD24^{low}CD44^{high}$ NKT cells in line 2 and five times more in line 5 compared to B6 WT mice (Fig. 3.16 B; 3.17 B).

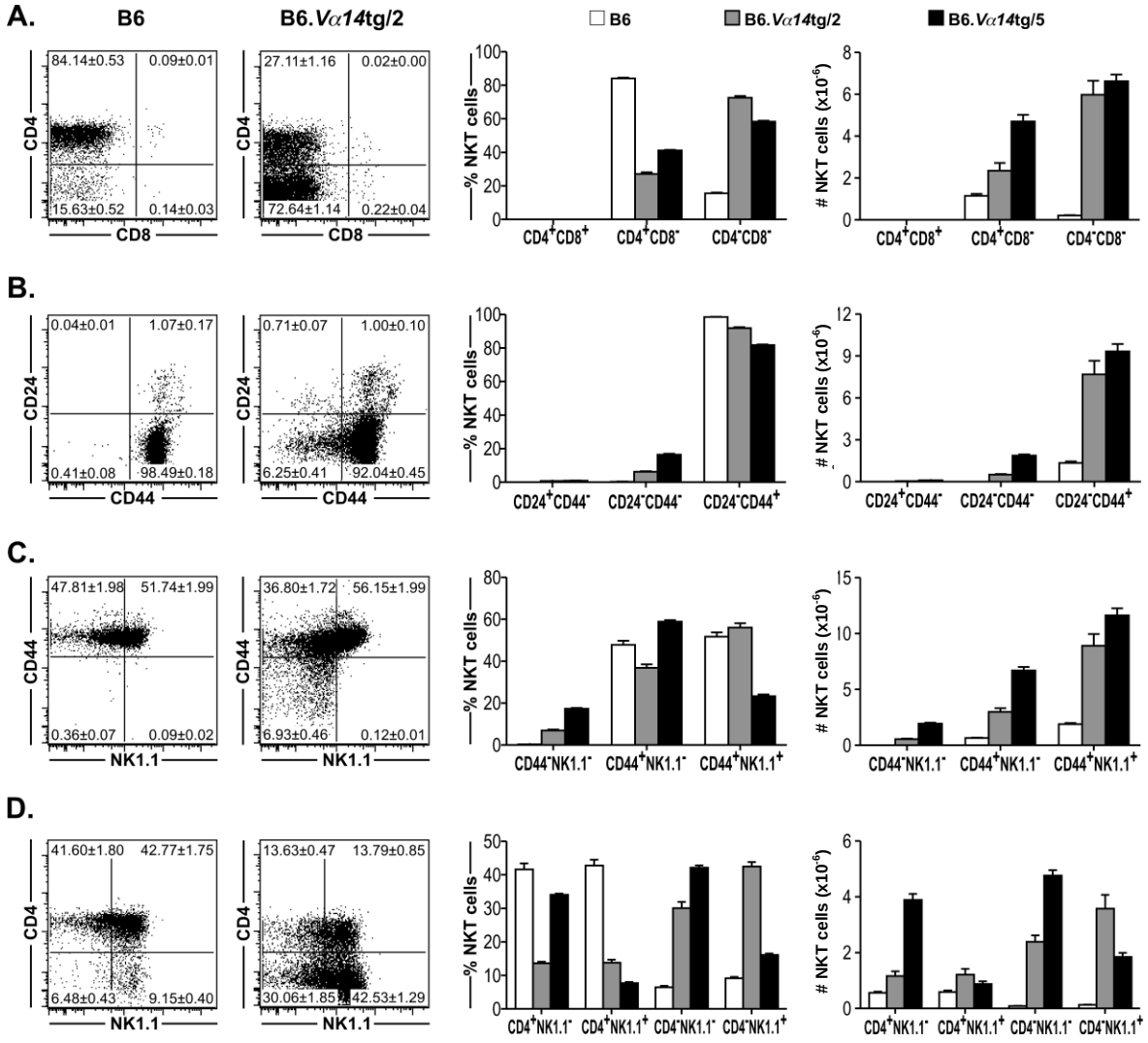


**Figure 3.17. NKT cell subsets in the spleens of B6.*Vα14*Tg mice**. **A:** CD4/CD8 NKT cell subsets. **B:** CD24/CD44 NKT cell subsets. **C:** CD44/NK1.1 NKT cell subsets. **D:** CD4/NK1.1 NKT cell subsets. For all figures, representative FACS plots (left panel) show the mean frequencies (±SEM) of NKT cell subsets in total NKT cells from spleens of B6 and B6.*Vα14*Tg/2 mice. Bar graphs (right panel) show the mean frequencies and absolute numbers of splenic NKT cell subsets between B6 (clear bars) and B6.*Vα14*Tg lines 2 and 5 mice (filled bars). n = 10 for each group. Data are combined from two dependent experiments. The NKT gates applied are illustrated in Figure 3.9.

As presented earlier, there were a large number of very immature NKT cells ($DP^{high}$ $CD24^{high}$ $CD44^{low}$ $NK1.1^{-}$) in the thymi of both NOD and B6 *Vα14* transgenic mice. However, their absence in the periphery and the finding that the vast majority of peripheral NKT cells were mature suggested that the transgenic expression of *Vα14-Jα18* had successfully resulted in an increase in production of mature NKT cells. The thymic $DP^{high}$ $CD24^{high}$ $CD44^{low}$ $NK1.1^{-}$ NKT cells likely represent a very immature - possibly pre-selection - population of NKT cells.

### 3.2.4. Vβ usage of NKT cells in *Vα14*Tg mice

We further examined if NKT cells generated by *Vα14*Tg mice exhibited typical biological features of NKT cells. TCR Vβ usage of NKT cells from NOD.*Vα14*Tg line 1 mice was compared with that of NOD WT mice. In NOD WT mice, thymic and splenic NKT cells were gated using CD3ε and CD1d-$tet^{+}$. The proportions of these NKT cells using TCR Vβ2, Vβ7, Vβ8.1-8.2 and Vβ8.3 were identified via histogram plots (Fig. 3.18) and represented by bar graphs (Fig. 3.19). In NOD.*Vα14*Tg line 1 mice, TCR Vβ usage was examined in five different thymic cell populations, including immature DP T cells, DP $CD44^{low}NK1.1^{-}$ NKT cells, CD4 $CD44^{low}NK1.1^{-}$ NKT cells, DN $CD44^{low}NK1.1^{-}$ NKT cells and mature $CD44^{+}$ NKT cells (Fig. 3.18 C). TCR Vβ2, Vβ7, Vβ8.1-8.2 and Vβ8.3 usage was identified using histogram plots (Fig. 3.18 C) and represented via bar graphs (Fig. 3.19). The TCR Vβ usage of splenic NKT cells in NOD.*Vα14*Tg line 1 mice was identified in a similar manner to that of spleens of NOD WT mice.

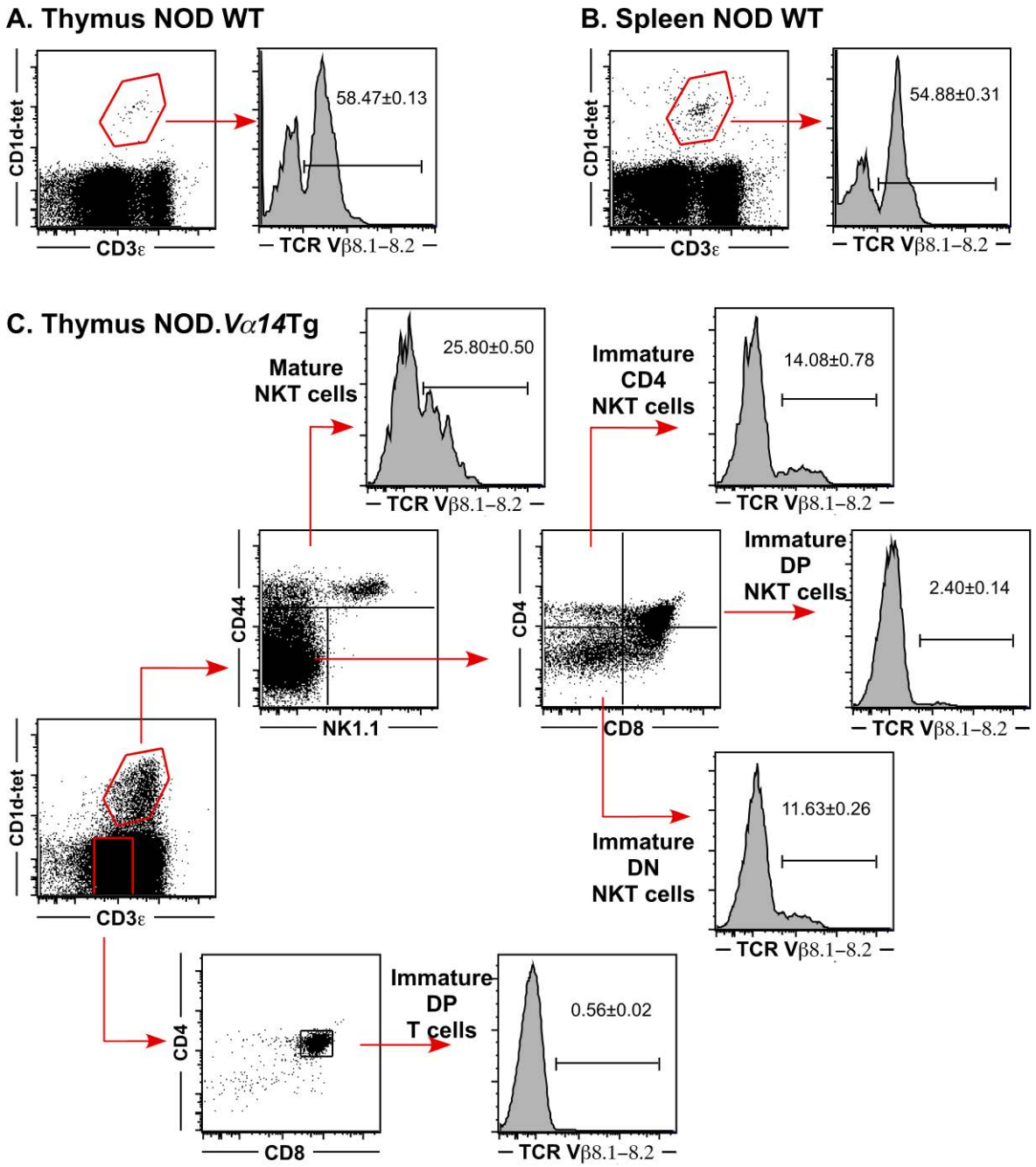


**Figure 3.18. Gating strategy for TCR Vβ usage of NKT cells from NOD WT and NOD.*Vα14*Tg/1 mice. A.** Representative FACS plots show the gating strategy for TCR Vβ8.1-8.2 analysis of thymic NKT cells from NOD WT mice. **B.** Representative FACS plots show the gating strategy for TCR Vβ8.1-8.2 analysis of splenic NKT cells from NOD WT mice. **C.** Representative FACS plots show the gating strategy for TCR Vβ8.1-8.2 analysis of thymic NKT cells from NOD.*Vα14*Tg/1 mice (n = 6 for NOD WT mice and n=10 for NOD.*Vα14*Tg/1 mice).

Flow cytometric analyses indicated that TCR Vβ usage was variable between cell populations and between NOD WT and NOD.*Vα14*Tg/1 mice. In wild type mice, almost all

thymic and splenic NKT cells used TCR Vβ8.1-8.2, Vβ8.3, Vβ7 or Vβ2; 58% of thymic NKT cells used TCR Vβ8.1-8.2, 9.2% used TCR Vβ8.3, 19% used TCR Vβ7 and 13% used TCR Vβ2. The same pattern was seen with NKT cells from the spleens of NOD WT mice, with a total of 83% of splenic NKT cells presenting this bias (Fig. 3.19 A).

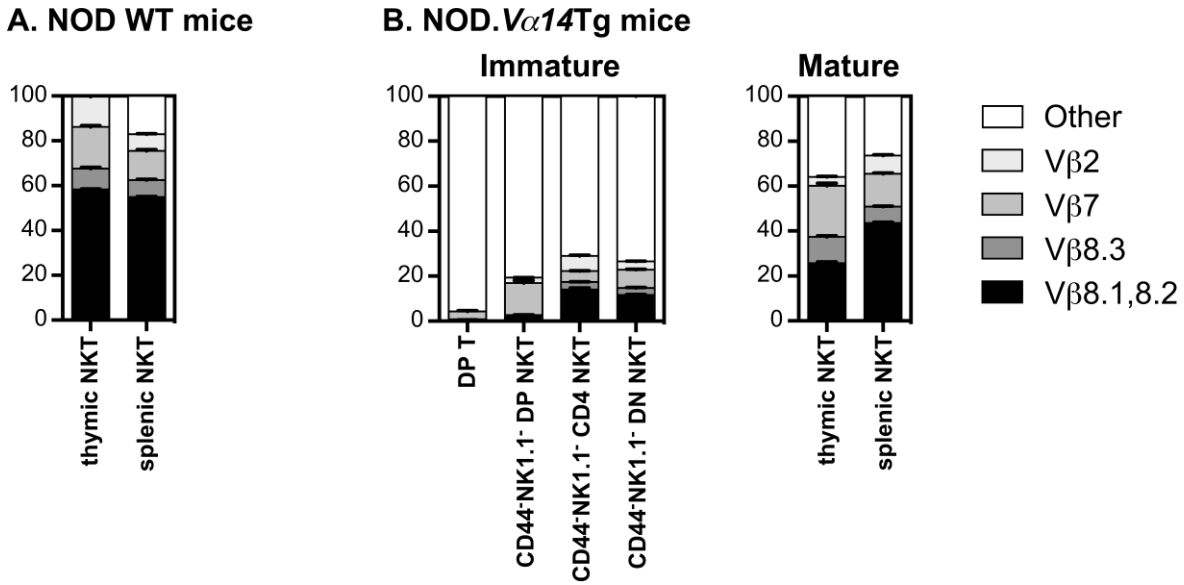


**Figure 3.19. TCR Vβ usage in NOD.*Vα14*Tg/1 mice**. **A.** Bar graph representatives of the mean frequencies of TCR Vβ usage of NKT cells in thymus and spleen of NOD WT mice. **B.** Bar graphs show the mean frequencies of TCR Vβ usage of immature and mature thymic and splenic cells from NOD.*Vα14*Tg/1 mice (n = 6 for NOD WT mice and n=10 for NOD.*Vα14*Tg/1 mice).

NKT cells generated by NOD.*Vα14*Tg mice were also biased toward the use of TCR Vβ8.1-8.2, Vβ8.3, Vβ7 and Vβ2, as were the NKT cells from NOD WT mice, but to a lesser extent in immature thymic NKT cells. A total of 19% of immature DP NKT cells, 29% of immature CD4 NKT cells and 27% of DN NKT cells presented the TCR Vβ8.1-8.2, Vβ8.3, Vβ7 and Vβ2 chains. In contrast, 64% of mature thymic $CD44^+$ NKT cells and 74% of splenic NKT cells from NOD.*Vα14*Tg mice used TCR Vβ8.1-8.2, Vβ8.3, Vβ7 and Vβ2 (Fig. 3.19 B). Although the results are showing that NKT cells generated by NOD.*Vα14*Tg/1 mice exhibited the same characteristic of TCR Vβ usage as NKT cells from

WT mice, 70-80% of $CD44^-$ NKT cells and ~38% of $CD44^+$ NKT cells in the thymus of NOD.Vα14Tg mice use TCR Vβ chains other than Vβ8, Vβ7 or Vβ2, exceeding by far the frequency detected in the thymus of WT NOD mice (~0%). These findings suggest that the choice of TCRβ chain affects the probability that a α-GalCer/mCD1d tetramer-binding thymocyte will transition from a $CD44^-$ to a $CD44^+$ phenotype.

### 3.2.5. Generation of functional NKT cells in *Vα14*Tg mice

In order to confirm that NOD.*Vα14*Tg mice generated increased numbers of functionally mature NKT cells, six-week old NOD WT and NOD.*Vα14*Tg/1 mice were intravenously injected with 4μg α-GalCer and bled for cytokine analyses by bead array four hours later. The following cytokines were examined: GM-CSF, IFN-γ, IL-1α, IL-2, IL-4, IL-5, IL-6, IL-10, IL-17 and TNF-α (Fig. 3.20).

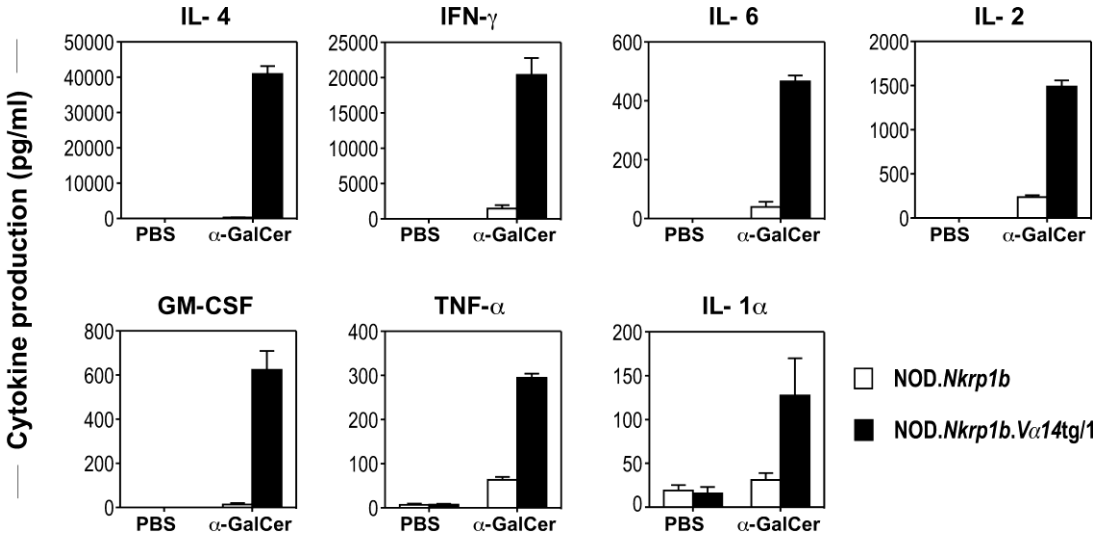


**Figure 3.20. *Vα14* transgenic expression increased cytokine production in NOD.*Vα14*Tg mice**. Bar graphs show the mean concentration (±SEM) of IL-4, IFN-γ, IL-6, IL-2, GM-CSF, TNF-α and IL-1 in serum of NOD WT mice (clear bars) and NOD.*Vα14*Tg line 1 mice (filled bars), 4 hours after challenge with α-GalCer (n = 6 for control and injected NOD WT mice, n = 10 for control and injected NOD.*Vα14*Tg line 1 mice).

Without stimulation, both NOD WT and NOD.*Vα14*Tg/1 mice produced detectable levels of TNF and IL-1α, but not of the other cytokines assayed. However, in response to α-

GalCer injection, NOD.*Vα14*Tg line 1 mice produced large amounts (broadly in the ng range) of IL-4, IFN-γ, IL-2, GM-CSF, IL-6, TNF-α and IL-1α compared with NOD WT mice (Fig. 3.20). However, IL-5, IL-10, and IL-17 could not be detected in any samples. Among detected cytokines, IL-4 and IFN-γ were produced in the largest amounts. NOD WT mice secreted three fold greater IFN-γ than IL-4; whereas an opposite profile was seen in NOD.*Vα14*Tg/1 mice with two times higher the amount of IL-4. NOD.*Vα14*Tg/1 mice released 120-fold more IL-4 and 20-fold greater IFN-γ in response to α-GalCer stimulation than NOD WT controls. These results are consistent with the substantial increases in NKT cell numbers observed in the NOD transgenic mice and suggested a major expansion of functional NKT cells in NOD.*Vα14*Tg*/1* mice.

To test whether transgenic expression of *Vα14-Jα18* also produced functionally mature NKT cells on a B6 background, the same experiment was conducted to compare cytokine production between B6 WT and B6.*Vα14*Tg line 2 and line 5 mice. Consistent with the findings in NOD mice, sera from both un-injected WT and transgenic B6 mice contained detectable levels of TNF-α and IL-1α, but not of the other cytokines assayed. In contrast, B6.*Vα14*Tg mice produced large amounts (also in the ng range) of IL-4, IFN-γ, IL-2, GM-CSF, IL-6, TNF IL-5 and IL-1α in response to α-GalCer compared to B6 WT control mice (Fig. 3.21). IL-10 and IL-17 were not detected in any samples.

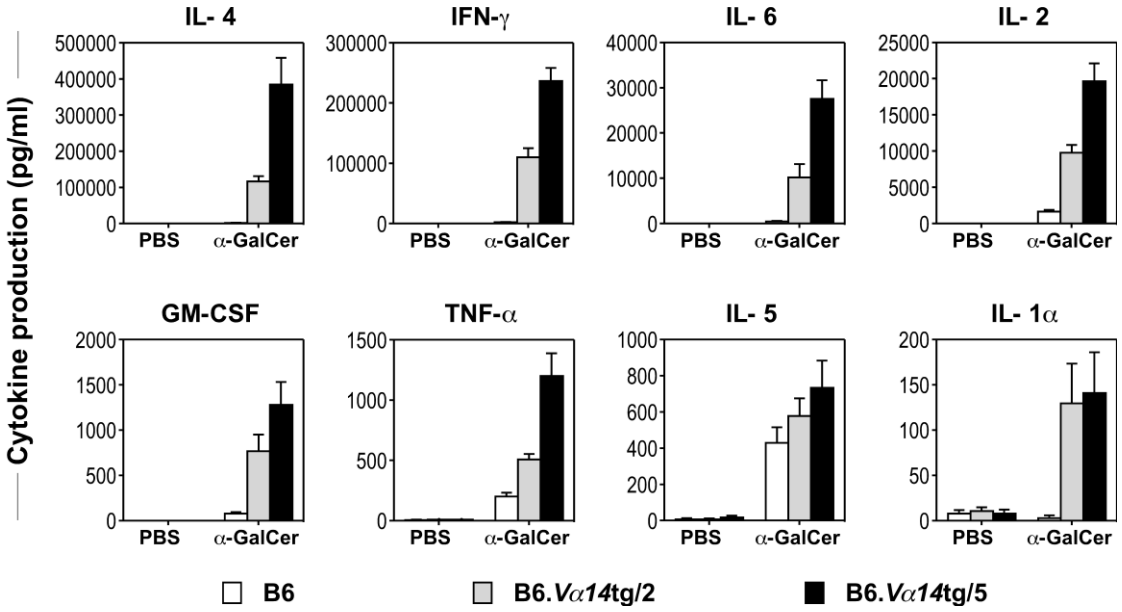


**Figure 3.21. *Vα14* transgenic expression increased cytokine production in B6.*Vα14*Tg mice**. Bar graphs show the mean concentration (±SEM) of IL-4, IFN-γ, IL-6, IL-2, GM-CSF, TNF-α, IL-5 and IL-1α in serum of B6 WT (clear bars), B6.*Vα14*Tg line 2 (grey filled bars) and line 5 mice (black filled bars) 4 hours after challenge with α-GalCer. (n = 10 for B6 WT control and injected B6.*Vα14*Tg line 2 groups, n = 9 for injected B6.*Vα14*Tg line 5 group).

In agreement with a previous study by Bendelac *et al*. (1996), our data showed that upon α-GalCer stimulation, B6.*Vα14*Tg mice produced markedly higher amounts of IL-4 and IFN-$\gamma$ than did B6 WT mice (Fig. 3.21): B6.*Vα14*Tg line 2 mice released at least 50 times more IL-4 and IFN-$\gamma$ than B6 WT controls injected with the same amount of α-GalCer, while line 5 secreted at least 115 times more of each cytokine than the control mice. In comparison between the two transgenic lines, B6.*Vα14*Tg line 5 produced 2.5 times more IL-4 and 2.3 times higher IFN-$\gamma$ than did line 2 (Fig. 3.21).

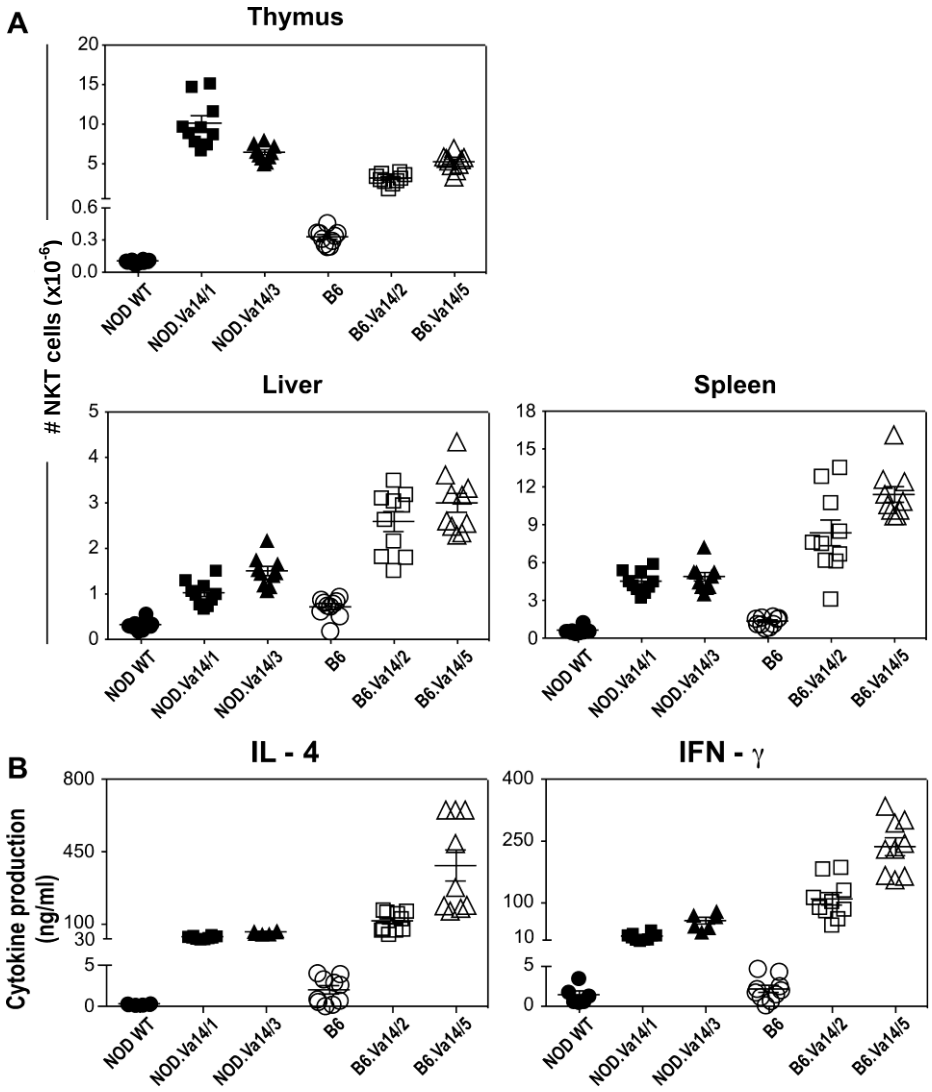


**Figure 3.22. The correspondence between NKT cell numbers and cytokine production in WT and *Vα14*Tg mice injected with α-GalCer. A**. Absolute numbers of thymic, hepatic and splenic NKT cells from NOD and B6, WT and *Vα14*Tg mice. **B**. Mean concentration of IL-4 and IFN-γ in serum of NOD and B6 mice, WT and *Vα14*Tg, 4 hours after challenge with α-GalCer.

Although cytokine production on the NOD and B6 backgrounds were tested on two different days, the sera were collected and immediately kept at -80°C and then were analysed in parallel. Comparison of the two backgrounds revealed that B6 transgenic mice produced significantly more cytokines than NOD transgenic mice. For example, B6.*Vα14*Tg line 2 produced six times more IL-2, 23 times more IL-6, five times more IFN-γ and three times more IL-4 than did NOD.*Vα14*Tg line 1. When the numbers of NKT cells were compared to the amount of cytokines released by WT and *Vα14* transgenic mouse lines (NOD.*Vα14*Tg

lines 1 and 3, B6.*Vα14*Tg lines 2 and 5), the increase in IL-4 and IFN-γ production ran parallel to the numerical increase in mature NKT cells in the peripheral organs (Fig. 3.20, 3.21 and 3.22). These results are consistent with greater numbers of functionally mature NKT cells being produced by B6.*Vα14*Tg mice than by NOD.*Vα14*Tg mice.

### 3.2.6. Incidence of spontaneous diabetes in NOD.*Vα14*Tg line 1 mice

It is well demonstrated that NOD mice spontaneously develop diabetes at early ages and that their reduction in NKT cell numbers and function contributed to this autoimmune phenotype (Baxter *et al*., 1994). Increases in NKT cell numbers by adoptive transfer (Hammond *et al*., 1998; Laloux *et al*., 2001), Vα14 TCR transgene expression (Lehuen *et al*., 1998) and stimulation of NKT cells in NOD mice by α-GalCer (Singh *et al*., 2001; Hong *et al*., 2001) delayed the disease onset and the severity of insulitis. As presented in early sections of this chapter, NOD.*Vα14*Tg mice showed the increased proportion and absolute numbers of NKT cells. They also produced large amounts of cytokine, such as IL-4 and IF-γ, after *in vivo* stimulation with α-GalCer. Therefore, we further examined the function of NKT cells produced by NOD.*Vα14*Tg mice in protecting these mice from spontaneous development of diabetes. NOD wild type control mice and *Vα14* transgenic mice (NOD.*Vα14*Tg line 1; both males and females) were checked weekly for diabetes from 10 weeks of age by random blood glucose measurements (Fig. 3.23). Mice were considered diabetic if measurements of blood glucose were >12.5 mg/L on two consecutive readings. Mice with blood glucose measurements <12.5 mg/L were followed until at least 32 weeks of age. At this point, they were considered protected.

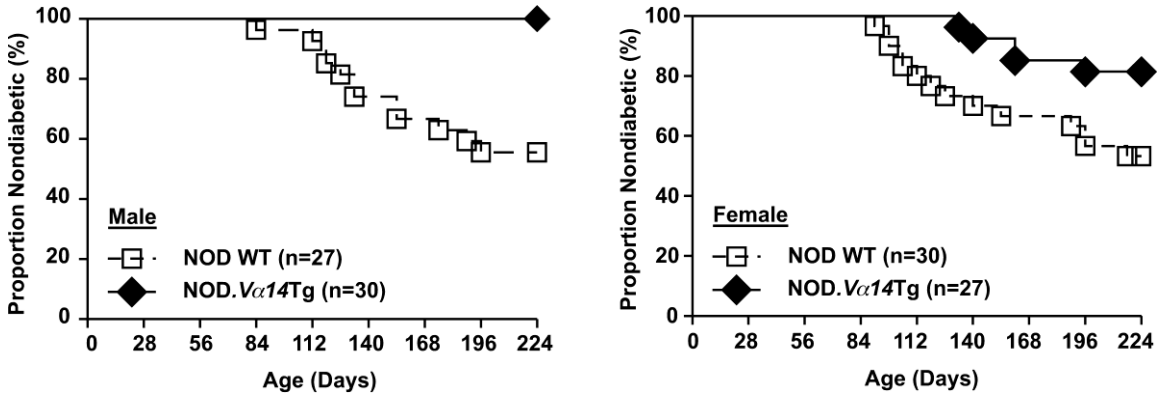


**Figure 3.23. Diabetes incidence of the NOD.*Vα14*Tg line 1 mice.** Life-table analysis of diabetes in cohorts of male and female NOD WT and NOD.*Vα14*Tg mice, which were checked weekly for glucose blood levels from 10-32 weeks of age

Our data (Fig. 3.23) showed that by 32 weeks of age, male NOD.*Vα14*Tg mice had not developed diabetes, while 12/27 (44%) male NOD WT mice did so ($p<0.0001$, Fisher's exact test). By the same age, 5/27 (19%) female NOD.*Vα14*Tg mice had spontaneously developed diabetes, compared to 14/30 (47%) female NOD WT control mice ($p<0.05$, Fisher's exact test; Fig. 3.23). Although 5 out of 27 female NOD.*Vα14*Tg mice developed diabetes, this occurred at a later stage (19 weeks old) than either male or female NOD WT mice. These data, which are consistent with those of Lehuen 1998, show that raising NKT cell numbers by expression of a *Vα14-Jα18* transgene reduced the incidence of diabetes in NOD.*Vα14*Tg mice compared to NOD WT controls (Fig. 3.23).

## 3.3. DISCUSSION

In the present study, the transgenic expression of a *Vα14-Jα18* TCR α chain resulted in overproduction of type I NKT cells on both NOD and B6 genetic backgrounds. This augmentation was seen in the thymi, spleens and livers of the transgenic mice.

Flow cytometric analysis of NKT cell subsets using stage-specific markers indicated that there was a major expansion of TCRβ$^{+}$ CD1d-tet$^{+}$ DP$^{high}$ CD24$^{high}$ CD44$^{low}$ and NK1.1$^{-}$ (immature DP NKT) cells in the thymi of both NOD and B6 *Vα14* transgenic mice. In contrast, this cell population was relatively rare in the thymi of WT mice, estimated at less than 5/10$^{6}$ thymocytes. They were therefore normally excluded from previous analyses. Gapin *et al*. (2001) applied a magnetic bead enrichment strategy to examine the earliest NKT cell progenitors. After the enrichment process, the authors identified a DP$^{dull}$ population, which accounted for around 25% of enriched CD1d-tetramer$^{+}$ thymocytes of 9-day old mice. This cell population was reported to decrease with age and was almost undetectable in the thymi of adult mice. Benlagha *et al*. (2005) applied a similar CD1d tetramer-based enrichment process and identified DP$^{low}$ CD24$^{high}$ CD44$^{low}$ NK1.1$^{-}$ NKT cells from MACS-enriched CD1d tet$^{+}$ thymocytes of 8-day to 5-week old mice. In comparison with the findings of Gapin *et al*. (2001) and Benlagha *et al*. (2005), the immature thymic DP$^{high}$ NKT cells from our NOD and B6 *Vα14* transgenic mice are likely to precede the DP$^{dull}$ and DP$^{low}$ stages of development and represent a very immature - possibly pre-selection - population of NKT cells.

In earlier studies, Bendelac *et al*. (1996) and Lehuen *et al*. (1998) produced *Vα14*Tg mice on B6 and NOD backgrounds, respectively, using a TCR α shuttle vector containing the *Vα11* endogenous promoter and the Ig enhancer. In their studies, *Vα14*Tg mice also produced an increase in NKT cell numbers; however, neither study reported the presence of immature DP NKT cells in the thymi of their transgenic mice. One possibility is that they used α/β

$TCR^+$ and $NK1.1^+$ markers to identify the NKT cells, inadvertently missing the relatively immature NKT cells from their analyses. Alternatively, their transgenic system may not have increased numbers of immature DP NKT cells. Here, the use of TCRβ and CD1d-tet, along with stage-specific markers, identified large numbers of very immature thymic DP NKT cells expressing a $CD24^{high}$ $CD44^{low}$ and $NK1.1^-$ phenotype. This therefore represents the first mouse model that produced large numbers of immature DP NKT cells.

Although both NOD and B6 V$\alpha$14 transgenic mice produced immature thymic DP NKT cells, numbers were higher in NOD.*Vα14*Tg mice. In contrast, the peripheral NKT cells in both strains were mature, either $CD4^+$ or DN and expressed the $CD24^{low}CD44^{high}$ mature phenotype. They accounted for more than 80% of total NKT cells in the spleens and 95% of the livers of both NOD and B6 *Vα14*Tg mice. In terms of absolute numbers, *Vα14* transgenic mice produced >two-fold increase in mature $CD24^{low}$ $CD44^{high}$ NKT cells in the periphery than did WT mice. Comparing between the two transgenic mouse strains, B6.*Vα14*Tg mice produced ~2-fold more mature NKT cells in both spleens and livers than did NOD.*Vα14*Tg mice. For example, B6.*Vα14*Tg mice produced 7.7 million $CD24^{low}CD44^{high}$ NKT cells per spleen in line 2 and 9.3 million cells in line 5, whereas NOD.*Vα14*Tg mice has only 3.7 million cells per spleen in line 1, and 4.2 million cells in line 3.

The presence of larger numbers of immature NKT cells in the thymi of NOD.*Vα14*Tg mice, but lower numbers of mature NKT cells in the periphery compared to B6.*Vα14*Tg mice suggested that many immature NKT cells failed to leave the thymus or that there was a deficiency in NKT cell development in the thymi of NOD.*Vα14*Tg compared B6.*Vα14*Tg. Mingueneau *et al.* (2012) identified a selective defect in Erk1/2 activation in response to TCR engagement in NOD versus B6 thymocytes and T cells. When transgenes drive premature TCR expression in thymocytes, this difference appears to modulate the cellular response to

pre-TCR engagement, resulting in an expanded DP population in NOD mice. Because of a selective defect in the Erk1/2 signaling module, the same Tg TCRs wired to NOD signalosomes are perceived like pre-TCRs, rather than γδ-TCRs, leading to amplification and abundant differentiation into DPs. Competition for selecting ligands may be altered when the DP population is expanded. As a consequence, thymi from NOD TCR Tg mice show higher numbers of DP thymocytes than their B6 counterparts, and relatively inefficient positive selection, promoting compensatory rearrangements of endogenous TCR-α loci, which rescue some measure of positive selection (Mingueneau *et al.,* 2012). The novel perspective available in this thesis is that the DP population contains both the cells vying for selection, and the cells presenting the ligand for selection, into the NKT cell lineage.

Supporting these hypotheses, the positive selection of invariant NKT cells, which is also Ras-Mapk-Egr dependent (Hu *et al*., 2011), is impaired in NOD mice that have few thymic iNKT cells (Gombert *et al*., 1996). Because iNKT cells express an invariant TCR, no repertoire compensation could counterbalance the Erk1/2 defect, potentially explaining their decreased numbers in NOD mice.

In support of this marked increase in mature NKT cells in the periphery of the *Vα14* transgenic mouse studies, NKT cells from both NOD and B6 *Vα14* transgenic mice rapidly released massive amount of cytokines, such as IL-4 and IFN-γ, shortly after an intravenous injection of α-GalCer. These data are consistent with previous studies in mice showing that NKT cells contain pre-formed mRNA for these cytokines (Matsuda *et al*., 2003); they are able to produce both IFN-γ and IL-4 immediately after α-GalCer injection (Matsuda *et al*., 2000; 2003; Stetson *et al*., 2003).

In addition to the evidence of massive increases in cytokine production, the fewer numbers of NOD.*Vα14*Tg mice developing diabetes compared to NOD mice supported the

hypothesis that the increased NKT cell numbers prevented NOD.*Vα14*Tg mice from spontaneous development of type I diabetes. However, it is raising another possibility that the *Vα14* transgene limited the TCR repertoire diversity of conventional T cells, which may contributed to the delayed onset of the disease in these mice. The data in this chapter indicated that splenic T cells of NOD.*Vα14*Tg line 1 mice were not affected by the introduction of a *Vα14-Jα281* rearranged TCR transgene, the numbers of thymic T cells even slightly increased but the numbers of hepatic T cells significantly decreased in NOD.*Vα14*Tg line 1 mice compared to NOD wild type mice.The fixation of one TCR chain through the preferential expression of the rearranged Vα14-Jα281 transgene has been shown to blunt or even prevent the development of immunopathological disease (Doherty *et al.,* 1994; Mars *et al*., 2002).

Previous studies indicate that there are strain-dependent differences in NKT cell numbers between commonly used mouse strains; however, NOD mice particularly exhibited a severe reduction in NKT cell numbers and function compared to other strains (Baxter *et al*., 1997; Godfrey *et al*., 1997; Poulton *et al*., 2001). This natural reduction in NKT cell numbers in NOD mice has been associated with their susceptibility to several autoimmune diseases, including type 1 diabetes, lupus and experimental autoimmune encephalomyelitis (EAE), and that increasing NKT cell numbers by adoptive transfer (Baxter *et al*., 1997; Godfrey *et al*., 1997), transgenic expression of *Vα14-Jα18* TCR α chain (Lehuen *et al*., 1998 and our own data) or stimulating them with the superantigen-like ligand, α-GalCer, all inhibit the onset of type I diabetes, transgenic expression of the *Vα14-Jα18* TCR α chain in NOD mice dramatically reduced the incidence and severity of neurological signs in EAE – a mouse model for multiple sclerosis (Mars *et al*., 2002), and NKT cell numbers are decreased in lupus-prone NOD mice (Baxter *et al*., 1994; Horsfall *et al*., 1998; Hawke *et al*., 2003).

Several genetic studies of mice congenic between the NOD strain and various diabetes-resistant inbred strains have identified insulin dependent diabetes (*Idd*) susceptibility loci. In addition, a genetic linkage analysis of the genetic control of NKT cell numbers was undertaken in a first backcross from C56BL/6 to NOD.*Nkrp1b* mice. Here two major regions were identified exhibiting significant linkage to NKT cell numbers (Esteban *et al*., 2003). The first region (*Nkt1*) mapping to distal chromosome 1, was in the same region as lupus susceptibility gene *Babs2/Babs3*. The second region (*Nkt2*) was on chromosome 2, mapped in the same region as *Idd13* (Jordan *et al*., 2007; Fletcher *et al*., 2008). NOD mice congenic for these two regions derived from B6 showed significant increase NKT cell numbers (Rocha-Campos *et al*., 2006; Jordan *et al*., 2007; Chen *et al*., 2007; Fletcher *et al*., 2008; Jordan *et al*., 2011). The *Nkt1* region harbours SLAM family members, *Slamf1* and *Slamf6* (Jordan *et al.,* 2007; which have been confirmed as NKT-control genes through congenic and transgenic manipulation (Jordan *et al*., 2007; Jordan *et al*., 2011), while *Pxmp4* has been implicated as the control gene for *Nkt2* (Fletcher *et al*., 2008). In addition, Zekavat *et al*. (2010) found that *Cd93*, an autoimmune susceptibility gene residing within the *Idd13* locus, also plays a role in regulating absolute numbers of $CD4^+$ NKT cells. Congenesis for a further region, on chromosome 4, harbouring the *Idd9* gene has also been shown to increase NKT cell numbers, but in this case the increase in NKT cell numbers was only slightly (Matsuki *et al.*, 2003).

An additional explanation for the higher numbers of DP NKT cells in NOD.*Vα14*Tg mice is that DP thymocytes on the NOD background expressed lower levels of SLAMF1 (CD150) expression than did DP thymocytes on B6 mice (Jordan *et al*., 2007; Jordan *et al*., 2011). In our own observations, DP NKT cells from NOD.*Vα14*Tg mice expressed lower levels of SLAMF1 than did DP NKT from B6.*Vα14*Tg mice. The lower expression of SLAMF1 on the NOD background may contribute to the higher accumulation of DP NKT

cells in the thymi of NOD.*Vα14*Tg mice because SLAMF1 expression on both DP NKT cells and DP T cells is required for successful NKT cell development (Godfrey *et al*., 2010).

Evidence supporting the relationship between SLAM expression and NKT cell ontogeny includes: 1) Mice lacking SLAM or SLAM adaptor protein (SAP) - a necessary factor for SLAM signaling, are deficient in NKT cells, although conventional T cells are intact (Chung *et al*., 2005; 2008); 2) In the absence of SAP and Fyn, NKT cell development is arrested at the $CD24^{hi}$ $Tetramer^{hi}$ $CD69^{+}$ stage, which resulted in the reduction of 85% of mature type I NKT cells (Griewank *et al*., 2007); 3) Mutations in SAP cause an X-linked lymphoproliferative (XLP) immunodeficiency in humans, which is characterized by deficiency in Vα24 NKT cells; and 4) A genetic linkage study of a first backcross between NOD and B6 mice indicated that the SLAM locus was mapped to the same region as the SLE susceptibility locus (*Nkt1*). This genetic region, along with the genetic region *Nkt2* (containing diabetic susceptibility locus), control NKT cell numbers in NOD mice (Jordan *et al*., 2007).

In addition to the abnormally increased numbers of DP NKT cells in the manipulated *Vα14* transgenic mice, it was reported in this chapter that the *Vα14* transgene also promotes DN NKT development to a greater extent than CD4 NKT development. The DN:CD4 ratio of thymic NKT cells in NOD.*Vα14*Tg mice was 3:2, compared to a 3:4 ratio in WT mice (Fig. 3.11 A). In the livers, the DN:CD4 ratio was 1:2 in WT mice, but ranged from 1.5:1 to 5:1 in NOD.*Vα14*Tg mice (Fig. 3.13 A). In the spleens, the DN:CD4 ratio was 1:4 in WT mice, but varied from 1:2 to 2:1 in NOD.*Vα14*Tg lines (Fig. 3.14 A). Similar patterns were seen in all thymi, spleens and livers of B6.*Vα14*Tg lines (Fig. 3.16 A; Fig. 3.17 A). These data are consistent with a previous observation by Lehuen *et al*. (1998), in which they found that the $NK1.1^{+}$ T cells were mainly of the DN phenotype.

It is well demonstrated that the T cell development begins with the migration into the thymus of bone marrow-derived lymphoid progenitors and by the differentiation of $CD4^-CD8^-$ double negative (DN) thymocytes (Godfrey and Zlotnik, 1993). These common progenitors develop through sequential stages, including DN1 ($CD25^-CD44^+$), DN2 ($CD25^+CD44^+$), DN3 ($CD25^+CD44^-$), and DN4 ($CD25^-CD44^-$). At the DN3 stage, random rearrangement of TCRβ chain (VDJ) gene segments occurs and subsequently the beta selection takes place for a functional TCRβ chain (Godfrey and Zlotnik, 1993). The polypeptide TCRβ chain then pairs with an invariant pre-TCRα and signals the thymocyte to undergo further differentiation. At this point, the DN progenitor also up-regulates CD4 and CD8 to become double positive (DP) and initiates rearrangement at the TCRα gene locus. If a productive TCRα gene rearrangement occurs, the α chain can pair with the already expressed TCRβ chain and be expressed on the surface. All subsequent selection events are based on the antigen-binding site formed by this heterodimer. It is currently demonstrated that thymocytes bearing a TCR with high affinity for self-MHC-peptide complexes are deleted from the repertoire, whereas those with a low affinity are positively selected. If the TCR has negligible affinity for self-MHC, the thymocyte undergoes death by neglect while the cells that successfully pass through thymic selection subsequently undergo maturation to conventional $CD4^+CD8^-$ or $CD4^-CD8^+$ single positive T cells (Godfrey and Zlotnik, 1993; Michie and Zuniga-Pflucker, 2002; Starr *et al.,* 2003). A key feature of these selective events and a hallmark of T cell development are the ordered and sequential rearrangement and expression of the TCRβ and TCRα chains to ensure the production of a clonally expressed repertoire with a minimum of energy expenditure.

However, this temporal control is lost in TCR transgenics because TCR transgenic model systems both TCRα and -β chains are expressed early at the CD4CD8 double negative (DN) stage (Terrence *et al*., 2000; Lacorazza *et al*., 2001; Wolfer *et al*., 2002). The early expression of TCRα has been suggested to affect β-selection even in the presence of the pre-TCR because TCRβ has a higher affinity for TCRα than it does for pre-Tα (Trop *et al*., 2000). Although the αβTCR heterodimer can mediate β-selection if expressed at the DN stage, it is highly inefficient (Borowski *el al*., 2004). In addition, early expression may affect αβ/γδ lineage commitment, which results in a large population of mature DN $TCR^+$ cells both in the thymus and the periphery (Bruno *et al*., 1996; Fritsch *et al*., 1998; Terrence *et al*., 2000; Lacorazza *et al*., 2001). In the thymus, these cells represent a terminally differentiated population without the ability to seed the DP compartment (Lacorazza *et al*., 2001) while in the periphery, they display properties consistent with γδ-lineage cell (Terrence *et al*., 2000). These lineage-misdirected cells are not observed in wild-type mice or mice that express a transgenic TCRβ chain.

In this work the premature expression of transgenic *Vα14*TCR promotes DN T cell development (Baldwin *et al.,* 2005; McDonald *et al,.* 2014). The DN:CD4 inversion seen in both NOD and B6 *Vα14* transgenic mice might be due to TCR engagement in developing NKT cells occurring earlier, on average, in Vα14 transgenic mice compared to controls. The results could be postulated that, in the normal thymus, NKT cells might bifurcate into DN and CD4 subsets due to a difference in the "age" of the DP cell when it is positively selected into the NKT lineage.

In summary, data from this chapter confirm:

1. *Vα14-Jα18* transgenic expression increased NKT cell numbers in both NOD and B6 mice.

2. NOD and B6 *Vα14* transgenic mice produce far more of the most immature NKT subset in the thymus, while the increase in the periphery is due mainly to an increase in mature NKT cells.
3. The NKT cells generated by *Vα14*Tg NOD mice also express the strong bias toward the use of the Vβ8.1−8.2, Vβ8.3, Vβ7 and Vβ2 as seen in WT NKT cells.
4. Mature NKT cells from *Vα14* transgenic mice produced large amounts of cytokines typical of NKT cells.
5. NOD. *Vα14*Tg line 1 mice showed significant protection from diabetes compared to NOD WT mice.

The generation of immature DP NKT cells in *Vα14* transgenic mice provides an opportunity to study the earliest identifiable stages of NKT cell development, and will help dissect factors controlling the numbers and function of this important immune regulatory population.

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# PART B

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# TRANSCRIPTIONAL ANALYSIS OF NKT CELL DEVELOPMENT

## I. INTRODUCTION

Natural killer T (NKT) cells are recently recognised members of the immune system. They express both the T cell receptor (TCR) and several markers typical of natural killer (NK) cells on their cell surfaces (Godfrey *et al*., 2010). NKT cells differ from conventional T cells in several ways. While conventional T cells exhibit a diverse TCR repertoire, the TCRs of NKT cells are relatively limited. The best-studied and most frequent NKT cells express an invariant TCR α chain and a restricted set of TCR β chains. In addition, the TCR of NKT cells recognises glycolipid antigens presented by the CD1d molecule rather than peptide antigens presented on major histocompatibility (MHC) class I or II (Bendelac, 1995; Kronenberg, 2005).

NKT cells are well known for their role in immune-regulation. Upon stimulation, they rapidly secrete large amounts of cytokines (Matsuda *et al*., 2000) that modulate the behavior of other cells of the immune system, including T cells, B cells, macrophages, dendritic and natural killer cells. Through this activity, they form an important link between innate and adaptive immune responses (Kronenberg and Gapin, 2002) and modulate immune responses in the context of autoimmunity, cancer and microbial infection (Matsuda *et al*., 2008; Slauenwhite and Johnston, 2015).

Recently, there has been overwhelming research supporting the mainstream model of NKT cell development, in which NKT cells are thought to derive from the same progenitors as conventional T cells. Consistent with this model, NKT cells are not found in the peripheral tissues of mice until 1-2 weeks after birth; their development is thymus dependent. NKT cells develop in fetal thymic organ culture (Bendelac *et al*., 1994; Pellicci *et al*., 2002), while neonatal thymectomy on the third day after birth, selectively depletes them (Hammond *et al*., 1998).

The NKT cell lineage shares the early developmental progression with conventional T cells, which begins with the migration into the thymus of bone marrow-derived lymphoid progenitors and is followed by the differentiation of lineage $(Lin)^-$, $CD4^-CD8^-$ double negative (DN) thymocytes (Godfrey and Zlotnik, 1993). These common progenitors develop through sequential stages, including DN1 ($CD25^-CD44^+$), DN2 ($CD25^+CD44^+$), DN3 ($CD25^+CD44^-$), and DN4 ($CD25^-CD44^-$). At the DN3 stage, random rearrangement of TCR β chain (VDJ) gene segments occurs and subsequently the beta selection takes place for a functional TCR β chain (Godfrey and Zlotnik, 1993). The cells that survive the beta selection process mature to the DN4 stage and then become $CD4^+CD8^+$ double positive (DP) thymocyes. During the DP stage, rearrangement of the TCR α chain (VJ) gene segments occurs forming the unselected TCRαβ repertoire (MacDonald *et al*., 2001). The DP thymocytes expressing TCRαβ with appropriate avidity for self-peptide antigens are positively selected upon interaction with MHC class I or II molecules expressed on thymic epithelial cells (Godfrey and Zlotnik, 1993). The cells that successfully pass through thymic selection subsequently undergo maturation to conventional $CD4^+CD8^-$ or $CD4^-CD8^+$ single positive T cells.

In contrast, the DP thymocytes whose TCR has appropriate affinity for glycolipid antigens presented in the context of CD1d on cortical DP thymocytes are positively selected as NKT cells. These NKT cells will engage in maturation processes and up-regulate the expression of the activation markers, CD44 and NK1.1 (Bendelac, 1995; Godfrey and Berzins, 2007). DP thymocytes whose TCR have insufficient affinity or overly strong affinity for endogenous antigens (either peptide or glycolipid antigens) are deleted by neglect or negative selection, respectively (Pellicci *et al*., 2003; Chun *et al*., 2003; Schumann *et al*., 2005; Godfrey and Berzins, 2007).

In an attempt to identify the earliest CD1d-tet$^{+}$ thymic NKT cells, Gapin *et al.* (2001) stained mouse thymocytes with CD1d-tet-phycoerythrin (PE), and enriched positive cells with anti-PE magnetic beads. Of the NKT cells obtained from thymi of nine-day old mice, 25% were "DP$^{dull}$", a population whose frequency declined with age, was not seen in the periphery at any time and was absent in *Cd1d*$^{-/-}$ mice (Gapin *et al*., 2001). This population probably represented early post-selection NKT cells because, in conventional T cell development, TCR ligation of DP$^{high}$ thymocytes triggers CD4 and CD8 down-regulation (Swat *et al*., 1992; Page *et al*., 1993). In contrast, quantitative polymerase chain reaction (PCR) analysis found equivalent proportions of Vα14-Jα18 encoded transcripts from sorted DP$^{high}$ thymocytes in wild type (WT) and *Cd1d*$^{-/-}$ mice, consistent with the DP$^{hi}$ phenotype containing pre-selection NKT cells (Gapin *et al*., 2001). There were also Vα14-Jα18 encoded transcripts in the DN4 thymocytes from B6 WT and *Cd1d*$^{-/-}$ mice. These cells were reported to have the potential to generate mature NKT cells both *in vivo* and *in vitro* (Dashtsoodol *et al*., 2008). Further evidence of the DP$^{high}$ to DP$^{dull}$ transition representing positive selection was obtained by CDR3 spectratyping. In WT mice, only a single peak could be detected in the DP$^{dull}$ population, which corresponded to the canonical *Vα14-Jα18* rearrangement. This contrasted with the multiple rearrangements detected in DP$^{high}$ thymocytes (Gapin *et al*., 2001).

Benlagha *et al.* (2005) applied a similar tetramer-based enrichment strategy and identified CD24$^{high}$ NKT cells in the thymi of newborn mice. The average proportion of the CD24$^{high}$ NKT cells decreased with age, from 70% of tet-enriched cells at day 3 to 57% at day 4, 13% at day 5 and almost undetectable at 5 weeks of age. Nearly all (90%) of the CD24$^{high}$ NKT cells found in 3-day old mice were CD4$^{+}$ CD44$^{low}$ NK1.1$^{-}$, while very few cells were DP$^{low}$ CD44$^{low}$ NK1.1$^{-}$. However, in contrast with the reduction of tet-enriched CD24$^{high}$ NKT cells, their DP$^{low}$ CD24$^{high}$ NKT cell subset increased with age, from very few cells at day 3 to 10% at day 8, 68% at day 14 and 73% at 5 weeks of age. As they were CD24$^{high}$ CD44$^{low}$

NK1.1$^-$, and the DP$^{dull}$ cells identified by Gapin *et al.* (2001) were CD24$^{low}$, these DP$^{low}$ CD24$^{high}$ NK1.1$^-$ NKT cells are likely to precede the DP$^{dull}$ population discovered by Gapin *et al*. (2001). Furthermore, as the CD24$^{high}$ NKT cells already exhibited the same Vβ8 bias characteristic of mature NKT cells and are non-dividing, they are likely to be post-selection, making them the earliest population of post-selection NKT cells identified to date. Conversely, the tet-enriched CD24$^{low}$ NKT cells contained two subsets (CD44$^{low}$ and CD44$^{high}$), both of which were rapidly proliferating. On the basis of the changing proportions with aging from 2 to 6 weeks, Benlagha *et al.* (2005) proposed that the CD44$^{low}$ population precedes the CD44$^{high}$ population in maturation. Therefore, the postulated developmental pathway of NKT cells in mice is: DP CD24$^{high}$ CD44$^{low}$ NK1.1$^-$ → selection → DP$^{low}$ CD24$^{high}$ CD44$^{low}$ NK1.1$^-$ → DP$^{low}$ CD24$^{low}$ CD44$^{low}$ NK1.1$^-$ → DN or CD4$^+$ CD24$^{low}$ CD44$^{low}$ NK1.1$^-$ → DN or CD4$^+$ CD24$^{low}$ CD44$^{high}$ NK1.1$^-$ → DN or CD4$^+$ CD24$^{low}$ CD44$^{high}$ NK1.1$^+$ (Benlagha *et al*., 2005; Godfrey *et al*., 2010).

As presented in Chapter 3, the presence of large numbers of DP$^{high}$ CD24$^{high}$ CD44$^{low}$ NK1.1$^-$, CD1d-tet$^+$TCRβ$^+$ cells in the thymus and their absence in the periphery of *Vα14*Tg mice, suggested that these cells likely represent a very immature - possibly pre-selection - population of NKT cells. To test this hypothesis, thymocytes from NOD.*Va14*Tg mice were subjected to Fluorescence-activated cell sorting (FACS) to isolate four subsets: DP$^{high}$ CD24$^{high}$ NK1.1$^-$ CD1d-tet$^-$ TCRβ$^+$ (immature DP conventional T) cells, DP$^{high}$ CD24$^{high}$ CD44$^{low}$ NK1.1$^-$ CD1d-tet$^+$ TCRβ$^+$ (immature DP NKT) cells, CD4$^+$CD8$^-$CD24$^{high}$ NK1.1$^-$ CD1d-tet$^+$ TCRβ$^+$ (immature CD4 NKT) cells and CD4$^-$CD8$^-$ CD24$^{high}$ NK1.1$^-$CD1d-tet$^+$ TCRβ$^+$ (immature DN NKT) cells for transcriptional analysis by microarray. This provided us with an opportunity to compare the transcriptional profiles of very immature DP NKT cells with those of more mature NKT cell subsets in order to identify the transcriptional transitions associated with TCR validation, positive selection and lineage commitment.

Generally, the aims of part B were:

1. To profile gene expression of the four populations: immature DP T cells, immature DP NKT cells, immature CD4 NKT cells and immature DN NKT cells by microarray.

2. To provide data for the transcriptional changes at the early stages of NKT cell development and events surrounding thymic selection and lineage commitment of NKT cells.

3. To functionally validate gene expression changes during NKT cell development.

## II. OVERVIEW OF RESULTS

Immature DP conventional T cells (P1), immature DP NKT cells (P2), immature CD4 NKT cells (P3) and immature DN NKT cells (P4) were sorted from the thymi of NOD.*Va14*Tg mice (Fig. B.1). Cell samples were kept cool during the sorting processes and then immediately stored in RLT buffer at -80$^{o}$C for RNA isolation. RNA preparation, labeling and scanning were conducted by Dr. Margaret Jordan and Ms. Letitia Smith (Comparative Genomic Centre, James Cook University, Australia), as described in Chapter 2: Materials and Methods. The expression microarray hybridizations were performed using a WT Expression kit (Life Technologies, CA, USA), WT Terminal Labelling and Controls Kit (Affymetrix, CA, USA) and Affymetrix Mouse Gene_1.0ST arrays, which contained 35,556 transcripts representing an estimated 28,853 mouse genes. The probed arrays were washed and stained using the GeneChip Hybridization Wash and Stain Kit (Affymetrix, CA, USA), and then scanned using a GeneChip Scanner 3000. Images (.dat files) were processed using GeneChip Command Console (Affymetrix, CA, USA) and the CEL files were imported into Partek Genomics Suite 6.6 (Partek SG, Singapore) using an RMA summarisation algorithm for further analysis.

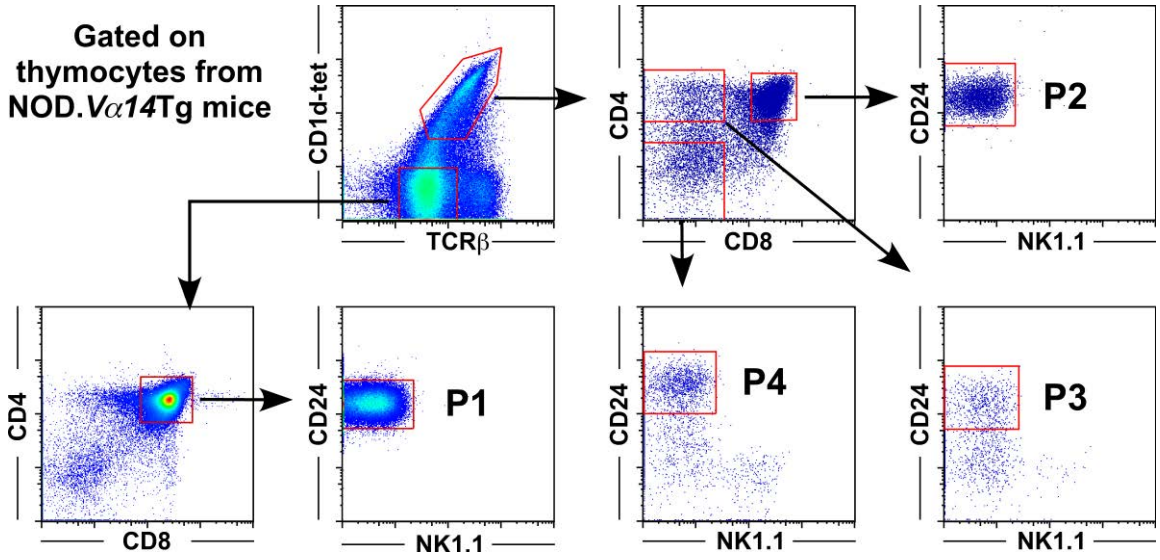


**Figure B.1. Gating strategy for sorting of populations by FACS**. Population (**P**) **1** represents for $DP^{high}$ $CD24^{high}$ $NK1.1^{-}$ CD1d-$tet^{-}$ $TCR\beta^{+}$ cells or immature DP T cells, **P2** represents $DP^{high}$ $CD24^{high}$ $CD44^{low}$ $NK1.1^{-}$ CD1d-$tet^{+}$ $TCR\beta^{+}$ cells or immature DP NKT cells, **P3** represents $CD4^{+}CD8^{-}CD24^{high}$ $NK1.1^{-}$CD1d-$tet^{+}$ $TCR\beta^{+}$ cells or immature CD4 NKT cells, and **P4** represents $CD4^{-}CD8^{-}$ $CD24^{high}$ $NK1.1^{-}$CD1d-$tet^{+}$ $TCR\beta^{+}$ cells or immature DN NKT cells.

Microarray data were analysed by Dr. Margaret Jordan using Partek Genomics Suite 6.6 software. In our experience of microarray expression analyses of mouse thymocytes, the use of seven replicates and a significance threshold of a Mann-Whitney U statistic of zero (i.e. no overlap between groups) provides robust and reliable identification of differentially expressed transcripts (Jordan *et al*., 2007, Fletcher *et al*., 2008; Jordan *et al*., 2011). After data processing, expression levels of a total of 35,556 transcripts for each biological sample of four different cell populations (seven biological samples from each cell population) were obtained.

Principal component analysis (PCA) was conducted on all transcripts under investigation to determine expression trends within the data set. Figure B.2 illustrates the scores of seven biological samples from each of the four different cell populations on the first three principal components. The PCA revealed that the seven samples from each cell population clustered together, with the PC1 accounting for 29% of total variance, the PC2 accounting for 19% of variance and the PC3 accounting for 10% of variance. The PCA indicated that the four populations clearly separated in order across PC1, from immature DP

T cells (P1) to immature DP NKT cells (P2) then to immature CD4 NKT cells (P3) and to immature DN NKT cells (P4).

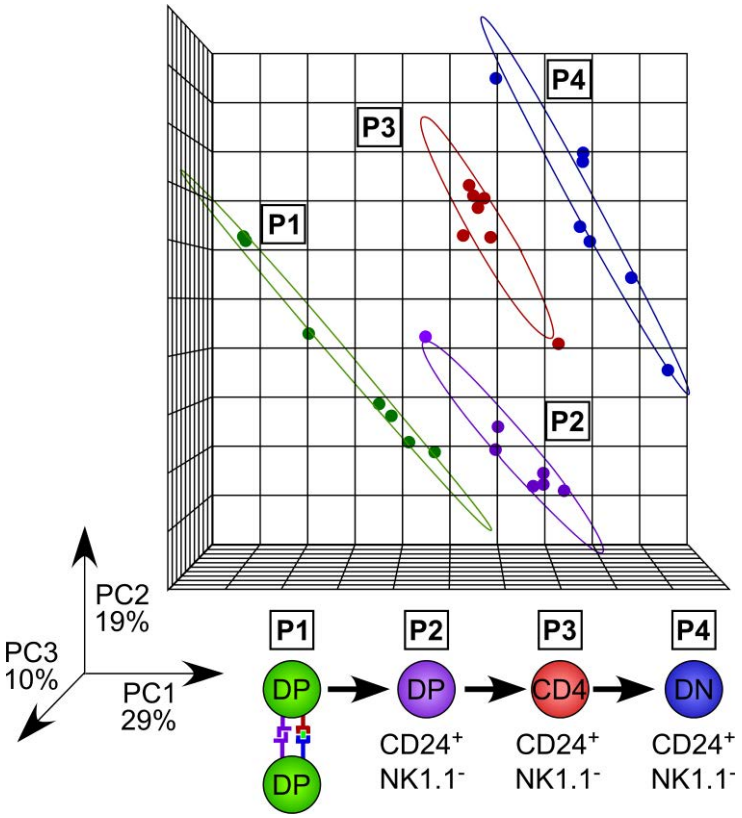


**Figure B.2. Principal component analysis of 35,556 transcripts of 7 biological replicates from 4 different cell populations during NKT cell development**. The green circles correspond to gene expression of immature DP T cell samples (**P1**); the purple circles correspond to gene expression of immature DP NKT cell samples (**P2**); red circles correspond to gene expression of immature CD4 NKT cell samples (**P3**); and blue circles correspond to gene expression of immature DN NKT cell samples (**P4**). The three largest PC explain >58% of variation; samples from each of the four cell types cluster together into groups, and the groups are distributed separately across PC1 (X axis), which explains >29% of variation. A graphic illustrating the proposed NKT cell maturation pathway is provided for comparison. Data were generated with assistance from Dr. Margaret Jordan and Prof. Alan Baxter.

This transcriptional progression of NKT cell subsets, therefore, mirrors the postulated developmental progression described by Godfrey's group based on their and other's work (Coquet *et al*., 2008; Godfrey *et al*., 2010). Of particular interest is the possibility that our analysis of DP NKT cells might identify the transcriptional events associated with Control Point 1 of NKT cell development, which Godfrey and Berzins (2007) attributed to selection.

A transcriptional regulatory network was constructed by Dr. Dragana Stanley (School of Medical and Applied Sciences, Central Queensland University) using the Weighted Gene Correlation Network Analysis (WGCNA) algorithm in R (Langfelder *et al*., 2008), representing the expression data from 35,556 transcripts from each of the 28 samples (7 replicates x 4 populations). The resulting network contained 1,929 nodes (transcripts) in twelve modules (co-expressed transcript clusters) with 10,626 edges (pair-wise significant correlations; Fig. B.3).

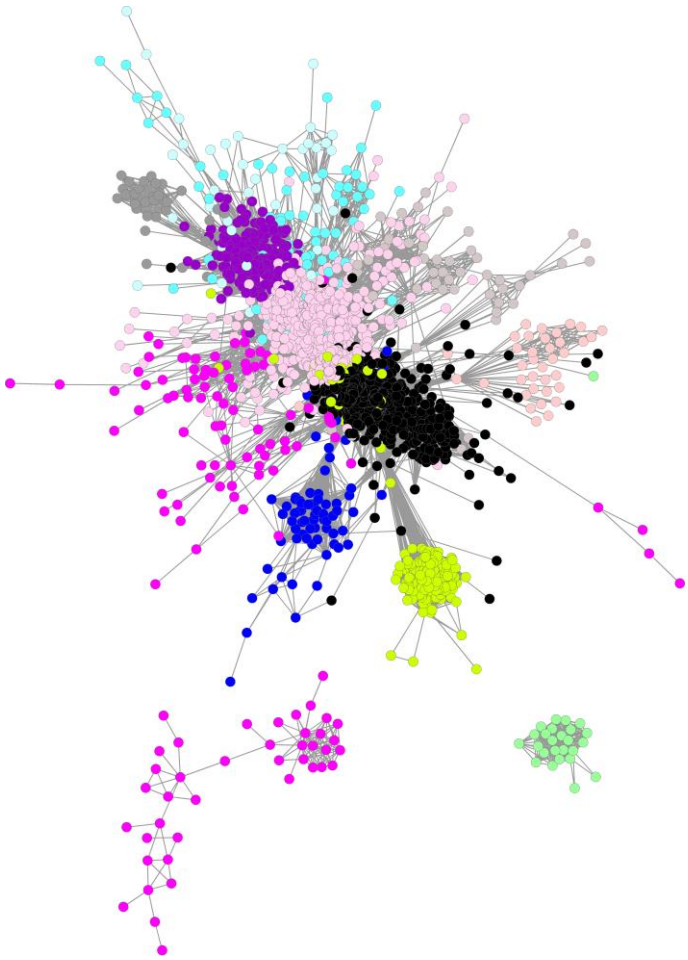

**Figure B.3. Weighted Gene Co-expression Network Analysis**. Undirected, weighted transcriptional regulatory network generated from thymic NKT cell subsets P1-P4 (n=7/population), consisting of 1,929 transcripts (nodes) and 10,626 pair-wise correlations (edges) across twelve co-regulated clusters (modules), named: Black (587 nodes), Cyan (71), GreenYellow (177), Grey (32), LightCyan (42), LightGreen (26), Magenta (127), MidnightBlue (68), Pink (525), Purple (188), Salmon (37) and Tan (49). Data were generated with assistance from Dr. Dragana Stanley, Dr. Margaret Jordan and Prof. Alan Baxter.

The network was then analysed as three progressive transitions: 1) DP T cells → DP NKT cells; 2) DP NKT cells → CD4 NKT cells; 3) CD4 NKT cells → DN NKT cells. For each transition, uncorrected Student's t-Test p values of pair-wise comparisons of each transcript were mapped onto the nodes within the network and visualised using a heat map in Cytoscape 3 (Shannon *et al*., 2003). Highly differentially expressed (HDE) transcripts were defined as significant after Bonferroni p value correction ($p<1.5 \times 10^{-6}$).

The next three chapters present findings from transcriptional analyses across each transition to provide transcriptional evidence of an early developmental program for NKT cells.

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# CHAPTER 4

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# TRANSITION 1 -

# ALLELIC EXCLUSION AND TCR VALIDATION

## 4.1. INTRODUCTION

Mainstream αβ T cell development encompasses two consecutive phases of thymic selection: β selection and repertoire selection. β selection occurs during the $CD4^-CD8^-$ double negative (DN) stage of thymocyte development, following rearrangement of the T cell receptor (TCR) β genes (Levelt and Eichmann, 1995). The expression of successfully recombined TCRβ chains with a surrogate α chain generates signals essential for survival, proliferation and maturation to the $CD4^+CD8^+$ double positive stage (Saint-Ruf *et al*., 1994; Wiest *et al*., 1994; Baur *et al*., 2001). The second phase of selection, termed repertoire selection or positive selection, takes place among DP cells following TCRα gene rearrangement. Development of thymocytes through the positive selection checkpoint requires the rearrangement and expression of a suitable TCRα chain that can pair with the already-expressed β chain to make a TCR that is selectable. That is, it must have sufficient avidity for self MHC-peptide interaction to induce signals required for differentiation, but not too strong so as to induce cell death.

During thymic selection processes, allelic exclusion takes place to ensure most thymocytes express only a single specificity of T cell receptor, which is encoded by one TCRα allele and one TCRβ allele. Allelic exclusion of TCR genes is regulated differently between α and β chains (Rybakin *et al*., 2014). For the TCRβ chain, a successfully completed in-frame rearrangement of one locus effectively inhibits the rearrangement of the second locus. This inhibition is achieved at the DNA level as a result of signalling originated from a successfully formed dimer of the β chain with pre-Tα. In contrast, both TCRα alleles are rearranged, often multiple times, until the positive selection stage, when a single α chain in conjunction with a fixed β chain allows the thymocyte to differentiate and the protein product

of the alternative α locus to be internalised, ubiquitinated and degraded (Borgulya *et al*., 1992). A majority of immature thymocytes express more than one α chain on the cell surface, while after positive selection only one chain is expressed on the vast majority of conventional T cells (Gascoigne and Alam, 1999).

Allelic exclusion of antigen receptor chains is a fundamental mechanism of immunological self-tolerance. Incomplete allelic exclusion results in dual TCR expression, which can allow developing autoreactive αβ T lymphocytes to escape clonal deletion and cause autoimmunity in some systems (Sarukhan *et al*., 1998; Auger *et al*., 2012).

NKT cells represent a minor subset of T lymphocytes expressing αβTCR as well as markers typical of the NK cell lineage (MacDonald, 1995; Bendelac *et al*., 1997). The repertoire of αβTCR of invariant NKT cells is dominated by an invariant TCR α chain (Vα4-Jα18 in mice or Vα24-Jα18 in humans) and a limited choice of TCR β chain (mostly Vβ8, Vβ7 and Vβ2). Although NKT cells share the common progenitors with conventional T cells; they branch off from the mainstream lineage at the DP stage. The developmental pathway of NKT cells is less well understood compared to conventional T cells. NKT cell development is impaired in mice bearing a variety of genetic defects, which are partly equivalent to the requirements for mainstream αβ T cells. The requirement for *Cd1d* (Chen *et al*., 1997; Mendiratta *et al*., 1997), and TCR *Vα14* and *Jα18* genes (Cui *et al*., 1997) for positive selection of NKT cells is analogous to the roles of homologous genes in the repertoire selection of mainstream αβ T cells. The dependence on molecules associated with CD3 complex signalling (CD3ζ, Lck; Baur *et al*., 2001) and pre-TCR α chain (Eberl *et al*., 1999) are consistent either with a requirement of positive selection in NKT cell development or with a pre-TCR dependent checkpoint, similar to β selection of mainstream αβ T cells.

As suggested by the flow cytometric analysis in Chapter 3 and the principal component analysis of the four populations analysed by microarray, the immature DP NKT cells are likely the pre-selection population of NKT cells. Therefore, in this chapter, gene expression comparison will be performed between immature DP T cells and immature DP NKT cells. The following hypothesis was tested: "**The thymic immature DP NKT cells from *Vα14* transgenic mice represent a pre-selection population of NKT cells or NKT cell progenitors at stage 0**".

Specifically, the aims of this chapter were:

1. To profile gene expression changes across Transition 1, between immature DP T cells and immature DP NKT cells.

2. To identify significant changes in gene expression related to the differentiation of DP NKT cells.

3. To functionally validate the observed changes in the expression of genes across development from immature DP T cells to immature DP NKT cells

## 4.2. RESULTS

### 4.2.1. Weighted gene co-expression network analysis of DP T cells and DP NKT cells across Transition 1

Uncorrected Student's t-Test p values of pair-wise comparisons of each transcript across Transition 1 were mapped onto the nodes within the network (described in Figure B.3) and then visualised using a heat map in Cytoscape 3 (Shannon *et al*., 2003; Fig. 4.1). After applying the Bonferroni corrected significance threshold ($p<1.5 \times 10^{-6}$), 311 highly significantly differentially expressed (HDE) transcripts were defined across the transition between DP T cells and DP NKT cells. Of these, 116 were members of the module named "Pink" (of 525 nodes) and 48 were in the "Purple" module (of 188 nodes; illustrated in Figure B.3; $p<0.0001$ $\chi^2$ contingency).

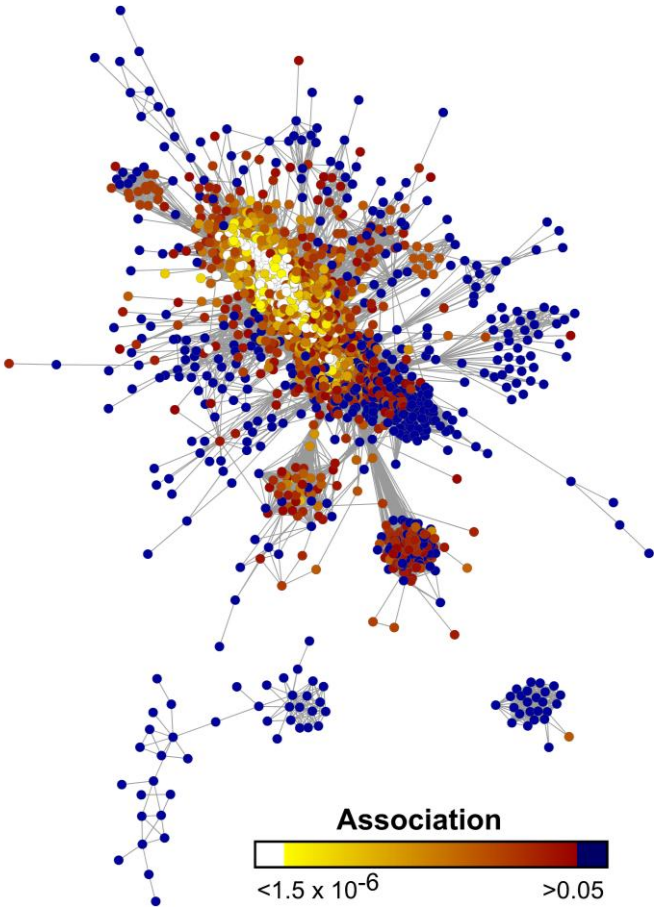


**Figure 4.1. Weighted gene co-expression network analysis across Transition 1**. Heat map encoding of Students' t-Test p values generated by pair-wise comparisons of transcript abundance across Transition 1 (between DP T cells and DP NKT cells) mapped onto the transcriptional regulatory network illustrated in Figure B.3. Data were generated with assistance from Dr. Dragana Stanley, Dr. Margaret Jordan and Prof. Alan Baxter.

Gene Ontology analysis was performed on the transcripts within the Pink module in the Database for Annotation, Visualization and Integrated Discovery (DAVID) Bioinformatics Resources v6.7 (Huang *et al*., 2009). The top ranked Category (SP_PIR_KEYWORDS) obtained for the transcripts in the Pink module was Oxidative Phosphorylation with a Bonferroni corrected p value of $<4.1 \times 10^{-6}$. Within this category, there were genes encoding several components of the mitochondrial electron transport chain, such as NADH Dehydrogenase (*Nd1*, *Nd2, Nd4l, Nd5*), Cytochrome C Oxidase (*Cox1*, *Cox2*, *Cox3*) and ATP Synthase 6 (*Atp6*), all of which were down-regulated across Transition 1. This finding is suggestive of the Warburg phenomenon (Fig. 4.2), as the down regulation of

NADH Dehydrogenase, Cytochrome C Oxidase and ATP Synthase 6 suggests the intracellular deviation of small carbon chains, away from ATP production to fatty acid synthesis, implying increased cell proliferation (Warburg, 1956).

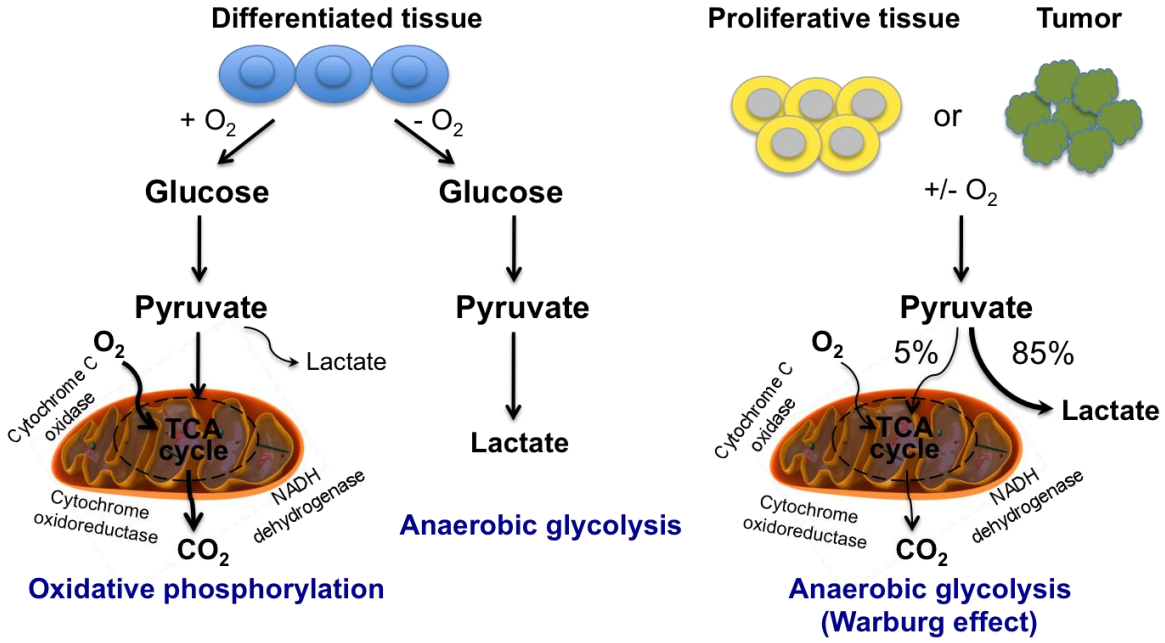


**Figure 4.2. Warburg phenomenon** (concept adapted from Vander *et al*. 2009)

### 4.2.2. Transcriptional analysis of DP T cells and DP NKT cells across Transition 1

In support of the hypothesis that increased proliferation occurs across Transition 1, a transcriptional comparison between immature DP T cells and immature DP NKT cells was performed. The expression levels of all 35,556 transcripts across Transition 1 were compared pairwise using the Mann-Whitney U test; and those with a U statistic of zero (i.e. no overlap between groups) were shortlisted for ranking by t-test (i.e. transcripts for which the means of the groups were separated by largest multiples of SEM were prioritised). Across this transition, 5,565 transcripts generated a Mann-Whitney U score of zero, and of these, 1,195 were highly differentially expressed (HDE; $p < 1.4 \times 10^{-6}$, by t-Test). This HDE gene list was split into transcripts up-regulated (624 transcripts), and those down-regulated (570 transcripts), before being submitted to DAVID for functional annotation clustering.

### 4.2.2.1. Increased expression of genes related to cell cycle and cell division

Gene Ontology analysis was performed on 624 up-regulated, HDE transcripts in the DAVID Bioinformatics Resources v6.7 (Huang *et al*., 2009). The top up-regulated annotation cluster (Enrichment Score 14.7) was dominated by genes associated with the cell cycle and cell division and contained the annotation categories DNA Metabolic Process (GO:0006259) and DNA Replication (GO0006260) with Bonferroni corrected p values between $10^{-10}$ and $10^{-14}$, respectively (Table 4.1).

**Table 4.1. Annotation clusters 1 and 2 (ranked by enrichment score) from GO analysis of 624 up-regulated HDE genes across Transition 1 using DAVID Bioinformatics Resources** (data were generated with assistance from Dr. Margaret Jordan and Prof. Alan Baxter).

| **Annotation Cluster 1** | **Enrichment Score: 14.67** | | | | |
|---|---|---|---|---|---|
| **Category** | **Term** | **Count** | **p value** | **Fold Enrichment** | **Bonferroni** |
| GOTERM_BP_FAT | GO:0006259~DNA metabolic process | 49 | 3.35E-17 | 4.194963362 | 5.28E-14 |
| SP_PIR_KEYWORDS | dna replication | 24 | 4.86E-17 | 10.26706601 | 1.59E-14 |
| GOTERM_BP_FAT | GO:0006260~DNA replication | 28 | 9.33E-15 | 6.639396901 | 1.47E-11 |
| KEGG_PATHWAY | mmu03030:DNA replication | 15 | 1.39E-12 | 12.94285714 | 1.76E-10 |
| **Annotation Cluster 2** | **Enrichment Score: 8.67** | | | | |
| **Category** | **Term** | **Count** | **p value** | **Fold Enrichment** | **Bonferroni** |
| GOTERM_BP_FAT | GO:0007049~cell cycle | 58 | 4.04E-16 | 3.421377313 | 7.00E-13 |
| SP_PIR_KEYWORDS | cell cycle | 49 | 5.26E-16 | 3.986048652 | 1.82E-13 |
| SP_PIR_KEYWORDS | cell division | 29 | 5.16E-10 | 4.119192337 | 1.69E-07 |
| GOTERM_BP_FAT | GO:0000279~M phase | 30 | 1.21E-09 | 3.820753391 | 1.91E-06 |
| GOTERM_BP_FAT | GO:0022403~cell cycle phase | 32 | 2.33E-09 | 3.516335641 | 3.68E-06 |
| GOTERM_BP_FAT | GO:0022402~cell cycle process | 35 | 3.75E-09 | 3.209886542 | 5.91E-06 |
| GOTERM_BP_FAT | GO:0051301~cell division | 29 | 4.44E-09 | 3.719682453 | 7.00E-06 |
| GOTERM_BP_FAT | GO:0000278~mitotic cell cycle | 24 | 3.02E-07 | 3.545158064 | 4.77E-04 |
| GOTERM_BP_FAT | GO:0007067~mitosis | 21 | 3.12E-07 | 3.98363814 | 4.91E-04 |
| GOTERM_BP_FAT | GO:0000280~nuclear division | 21 | 3.12E-07 | 3.98363814 | 4.91E-04 |
| GOTERM_BP_FAT | GO:0000087~M phase of mitotic cell cycle | 21 | 4.37E-07 | 3.901501272 | 6.89E-04 |
| SP_PIR_KEYWORDS | mitosis | 20 | 5.10E-07 | 4.062852007 | 1.67E-04 |
| GOTERM_BP_FAT | GO:0048285~organelle fission | 21 | 5.59E-07 | 3.842087547 | 8.81E-04 |

The top ranked category (GOTERM_BP_FAT: GO:0006259) with a Bonferroni corrected $p < 5.3 \times 10^{-14}$ contained 49 transcripts (of all up-regulated HDE genes) associated with DNA metabolic process, such as DNA Primase (*Prim1, Prim2*), Polymerase (*Pold2, Pold3*) and Replication factor C (*Rfc2, Rfc3, Rfc4, Rfc5*).

The microarray data revealed that genes involved in DNA replication: *Pole 2* (encoding Polymerase 1 epsilon 2), *Prim1* (encoding DNA Primase 1), *Hells* (encoding Lymphoid-Specific Helicase) and *Rpa2* (encoding Replication Protein A2) were significantly up-regulated (between 1.7 and 2.6 fold) in immature DP NKT cells compared to immature DP T cells (T test $p<0.001$; Mann-Whitney U statistic =0; Fig. 4.3 A). Up-regulation of *Rpa2* expression was confirmed by qPCR on an independent sample set (Fig. 4.3 B).

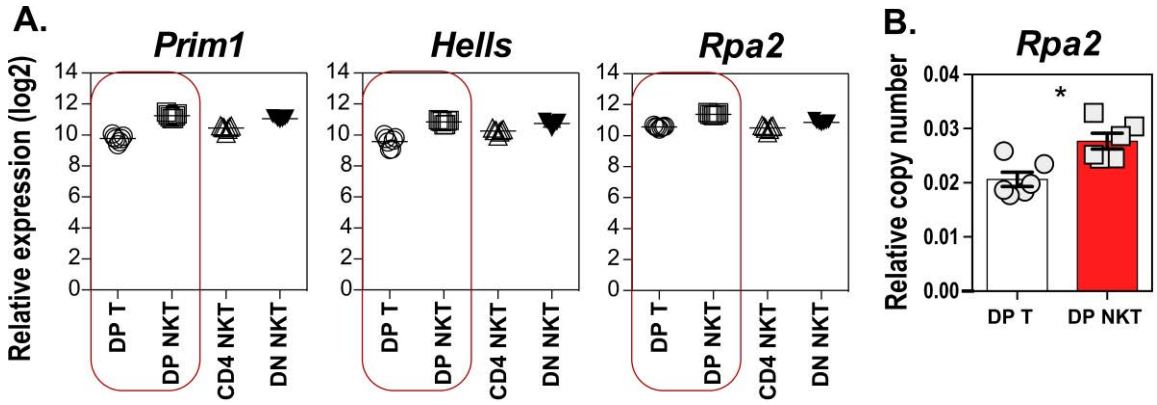


**Figure 4.3. Expression profiles of genes related to DNA replication. A**. Gene expression of *Prim1, Hells* and *Rpa2* as determined by microarray (data were generated with assistance from Dr. Margaret Jordan and Prof. Alan Baxter). **B**. *Rpa2* expression was confirmed by qPCR. n = 6 per cell population; Mann-Whitney test, * $p<0.05$. Data were generated with assistance from Dr. Morgane Moreau.

The second most up-regulated annotation cluster (Enrichment score: 8.7; Table 4.1) contained the annotation categories Cell Cycle (GO:0007049; representing 58 transcripts of a total 624 up-regulated HDE genes; Bonferroni corrected $p< 7 \times 10^{-13}$) and Cell Division (Bonferroni corrected $p< 1.8 \times 10^{-13}$; Table 4.1). Several functional families of genes related to cell cycle and cell division dominated the gene list, including members of the E2F signalling pathway (*E2f1, E2f2, E2f3*), Cyclin E (*Ccne1, Ccne2*), Cell division cycle (*Cdc6, Cdc7, Cdc45*) and Centromere protein (*Cenph, Cenpi, Cenpp*).

The E2F family of transcription factors play an essential role in regulating cell cycle progression (Dimova *et al*., 2005; Stevaux and Dyson, 2002; Iaquinta and Lees, 2007; Fig. 4.4). E2F1 and E2F2 are downstream of the pre-TCR and TCR signalling pathways

(DeRyckere and DeGregori, 2005), and they regulate the expression of a relatively large set of genes that are induced in the $G_1$ to S phase transition of the cell cycle. In contrast, E2F4 and E2F5 appear to function as transcriptional repressors in combination with the p130 protein in $G_0$ and early $G_1$ (Sears and Nevins, 2002; Fig 4.4).

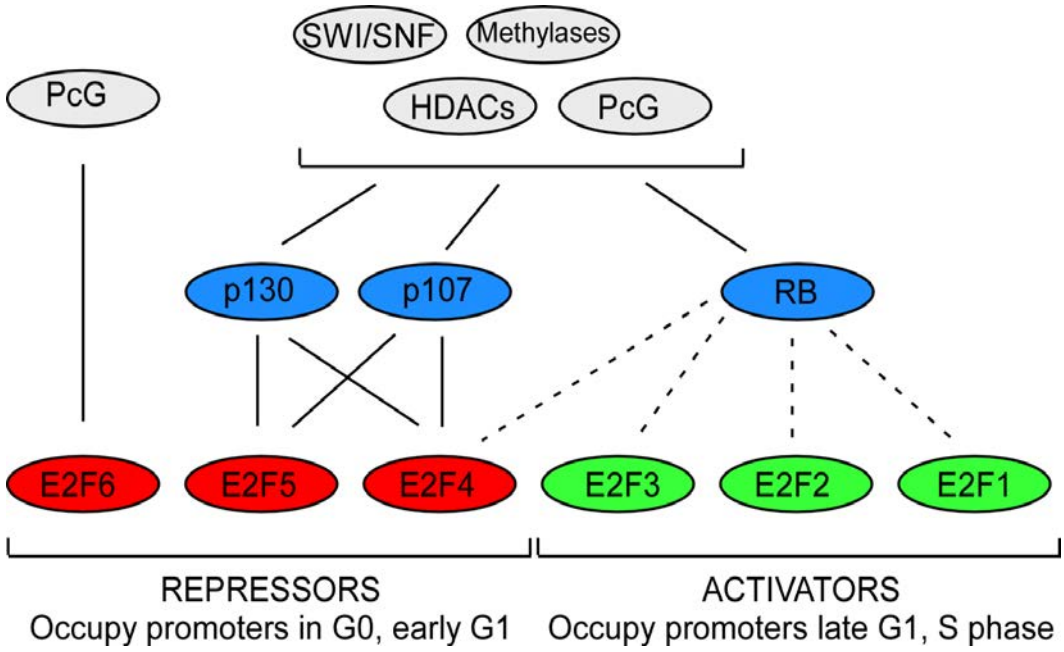


**Figure 4.4. E2F signalling pathway** (concept adapted from Stevaux and Dyson, 2002).

Differential expression analysis of immature DP T cells and immature DP NKT cells, covering Transition 1, revealed that gene expression of *E2f1*, *E2f2* and *E2f3* was significantly increased by 2.0, 1.3 and 2.3 times, respectively (T test $p<0.001$; Mann-Whitney U test =0; Fig. 4.5 A). In contrast, *E2f4* and *E2f5* expression was unchanged (data not shown). *E2f* signalling components, including *Rb-1*, *Cdc6* and *Ccne1* were also up-regulated. The increased expression of genes associated with E2F signalling was confirmed by qPCR (Fig. 4.5 B), and provided further evidence to support an increase in proliferation by immature DP NKT cells compared to immature DP T cells.

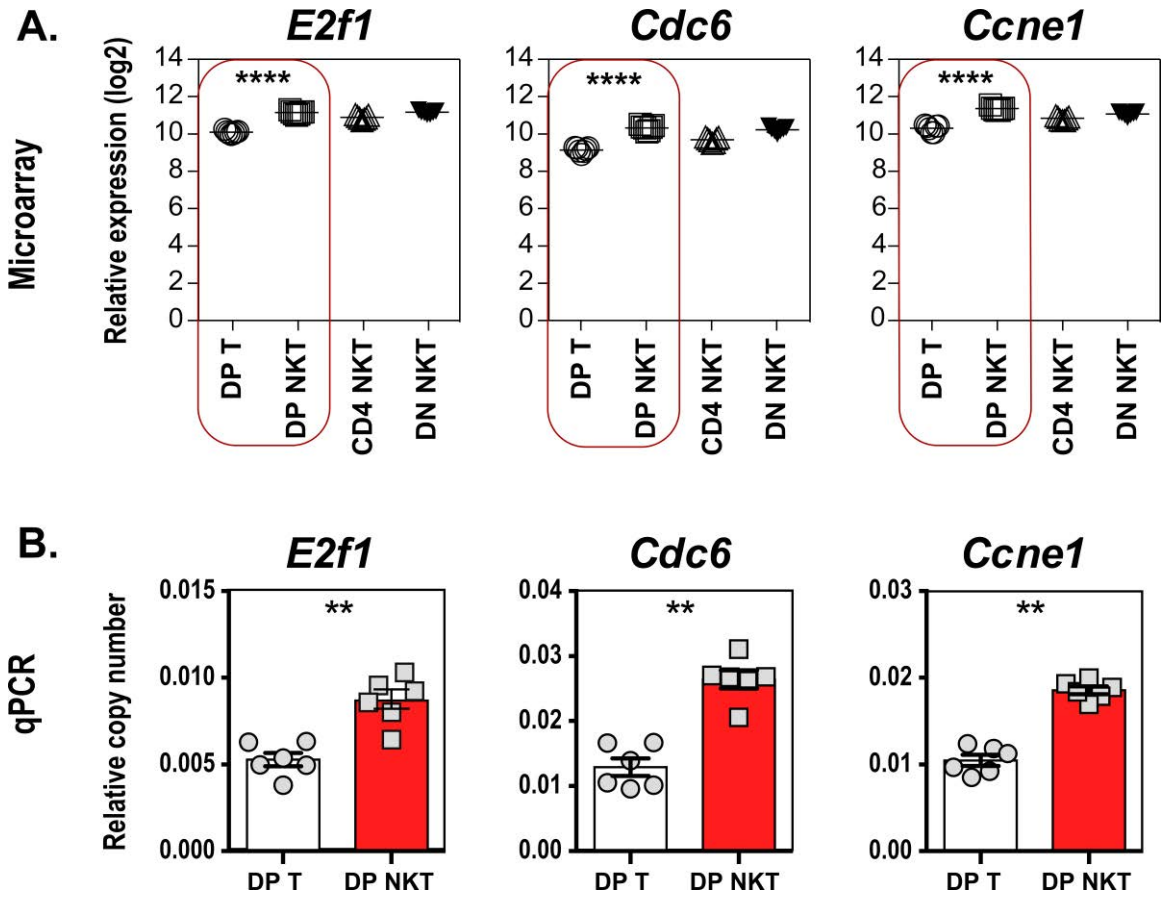


**Figure 4.5. Expression profiles of genes related to the E2F signalling pathway. A.** Gene expression of *E2f1, Cdc6* and *Ccne1* as determined by microarray (data were generated with assistance from Dr. Margaret Jordan and Prof. Alan Baxter)**. B.** *E2f1, Cdc6* and *Ccne1* expression was validated by qPCR across Transition 1. n = 6 per cell population. Data were generated with assistance from Dr. Morgane Moreau. Mann-Whitney test, ** $p<0.01$, **** $p<0.0001$.

The expression of Centromere protein encoding genes was also up-regulated across Transition 1. *Cenph* (Centromere protein H), *Cenpi* (Centromere protein I), *Cenpk* (Centromere protein K) and *Cenpm* (Centromere protein M) were all 2.2 to 3.4-fold up-regulated by immature DP NKT cells compared to immature DP T cells (T test $p<0.001$; Mann-Whitney U statistic =0; Fig. 4.6 A). To confirm our microarray data, gene expression of *Cenpm* was validated by qPCR (Fig. 4.6 B).

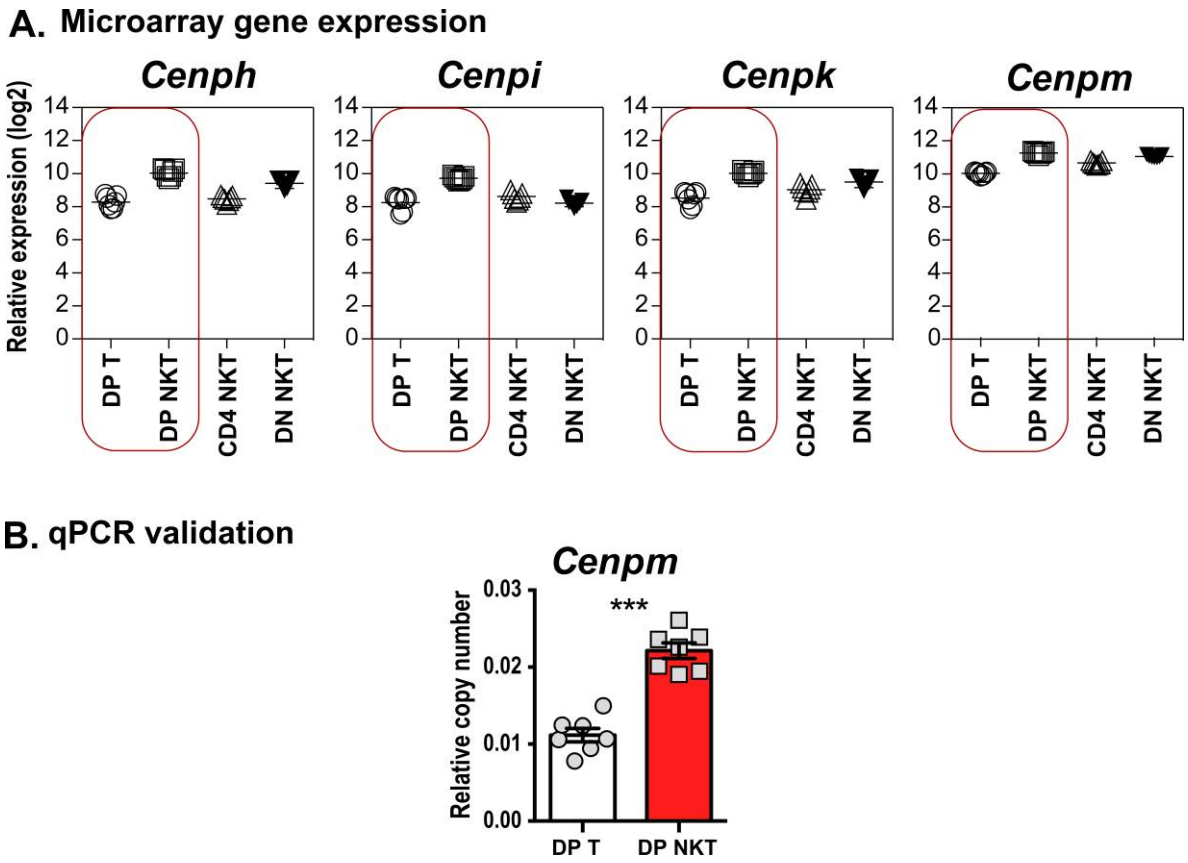


**Figure 4.6. Expression profiles of genes encoding Centromere proteins. A**. Gene expression of *Cenph, Cenpi, Cenpk* and *Cenpm* as determined by microarray (data generation were generated with the assistance from Dr. Margaret Jordan and Prof. Alan Baxter). **B**. qPCR validation of *Cenpm.* n = 7 per cell population. Data were generated with assistance from Dr. Morgane Moreau. Mann-Whitney test, *** p<0.001.

In total, we found 18 genes encoding Centromere proteins in our microarray data set. All 18 showed significantly increased expression in immature DP NKT cells compared to immature DP T cells (T test p<0.001; Table 4.2), of which, 15 genes had a Mann-Whitney U statistic =0, while 3 genes had a U statistic >0 (Table 4.2).

**Table 4.2: Expression profile of centromere protein-encoded genes at Transition 1.**

| Gene symbol | U Statistic | T test p-value | Fold Change (DP NKT cell/ DP T cell) |
|---|---|---|---|
| *Cenpa* | 0 | 1.56E-07 | 1.87384 |
| *Cenpb* | 2 | 0.0005753 | 1.18468 |
| *Cenpc1* | 0 | 0.0001395 | 1.80325 |
| *Cenpe* | 0 | 1.17E-05 | 2.37934 |
| *Cenpf* | 0 | 6.16E-05 | 2.28946 |
| *Cenph* | 0 | 2.33E-07 | 3.36125 |
| *Cenpi* | 0 | 8.86E-07 | 3.00947 |
| *Cenpj* | 1 | 0.0138735 | 1.23093 |
| *Cenpk* | 0 | 8.99E-07 | 2.84247 |
| *Cenpl* | 0 | 2.23E-06 | 2.02518 |
| *Cenpm* | 0 | 7.16E-11 | 2.26051 |
| *Cenpn* | 0 | 7.76E-09 | 2.00455 |
| *Cenpo* | 0 | 4.85E-07 | 1.59497 |
| *Cenpp* | 0 | 1.64E-07 | 2.90862 |
| *Cenpq* | 2 | 0.000535 | 1.46906 |
| *Cenpt* | 0 | 3.27E-05 | 1.22957 |
| *Cenpv* | 0 | 7.16E-09 | 1.66326 |
| *Cenpw* | 0 | 1.08E-05 | 1.8043 |

The down-regulation of genes related to the electron transport chain, increased expression of DNA replication genes, activation of the E2F signalling pathway and the up-regulation of genes encoding centromere proteins all suggested that there was an increase in proliferation in the immature DP NKT cells compared to immature DP T cells.

### 4.2.2.2. Down-regulation of genes related to TCR signalling

Gene Ontology analysis of 570 down-regulated, HDE transcripts using DAVID Bioinformatics Resources v6.7 (Huang *et al*., 2009) showed that the top down-regulated annotation cluster (Enrichment Score 7.3) contained the annotation categories Lymphocyte Activation (GO:0046649), Leukocyte Differentiation (GO:0002521) and T Cell Activation with Bonferroni corrected p values between $10^{-6}$ and $10^{-4}$. Remarkably, genes encoding almost all of the proximal components of the TCR signalling cascade were significantly down-regulated (Mann-Whitney U statistic =0): *Lck* ($p<6.6 \times 10^{-8}$; t-Test), *Fyn* ($1.4 \times 10^{-7}$), *Zap70* ($5.4 \times 10^{-5}$), *Vav1* ($1.3 \times 10^{-6}$), *Plcg1* ($1.2 \times 10^{-10}$; Fig. 4.7).

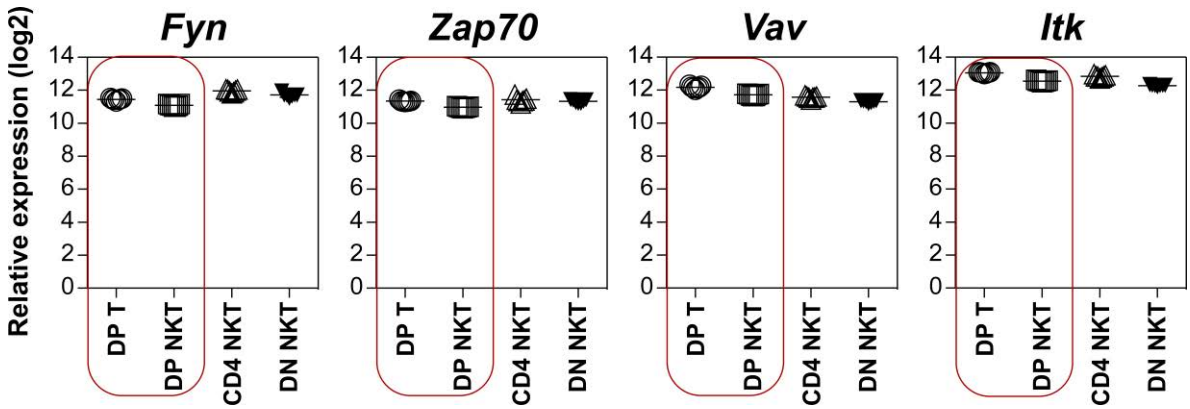


**Figure 4.7. Down-regulation of transcriptional factors related to TCR signalling across Transition 1**. Gene expression profiles of *Fyn, Zap70, Vav* and *Itk* as determined by microarray (data were generated with assistance from Dr. Margaret Jordan and Prof. Alan Baxter).

Although not previously reported, the effects of this transition would be akin to other mechanisms of TCR tuning, such as down-regulation of the TCR and CD4/CD8 co-receptors, up-regulation of accessory molecules such as CD5, CD2 and CD28, and phosphorylation of SHP-1 (Grossman and Paul, 2015), suggesting that the TCR had been ligated and successfully signalled, constituting validation of a functionally rearranged (in this case transgenic) receptor. Taken together, the transcriptional evidence supported the hypothesis that the increase in proliferation across Transition 1 was due to TCR signalling.

#### 4.2.2.3. Widespread down-regulation of TCR *Vα* genes

If the proliferation observed across Transition 1 was due to successful TCR signalling and validation, then allelic exclusion of endogenous alpha chain loci should have occurred. Consistent with this, TCR alpha chains dominated the most significantly differentially expressed transcripts, constituting 10 of the top 20 most significant. In particular, *Tcra-V22.1* (TCR Vα22-1), *Trav12-2* (TCR Va12-2), *Trav9-n1* (TCR Vα9-n1), *Trav9-4* (TCR Vα9-4), *Trav8d-1* (TCR Vα8d-1), *Trav7d-4* (TCR Vα7d-4), *Trav7d-3* (TCR Vα7d-3), and *Trav6-3* (TCR Vα6-3) all showed 1.8 to 5.8 fold down-regulation, with T test $p<10^{-8}$ and Mann

Whitney U statistic =0 (Fig. 4.8). The down-regulation of *Trav7d-3* gene by DP NKT cells was also confirmed by qPCR in comparison with DP T cells (Fig. 4.9).

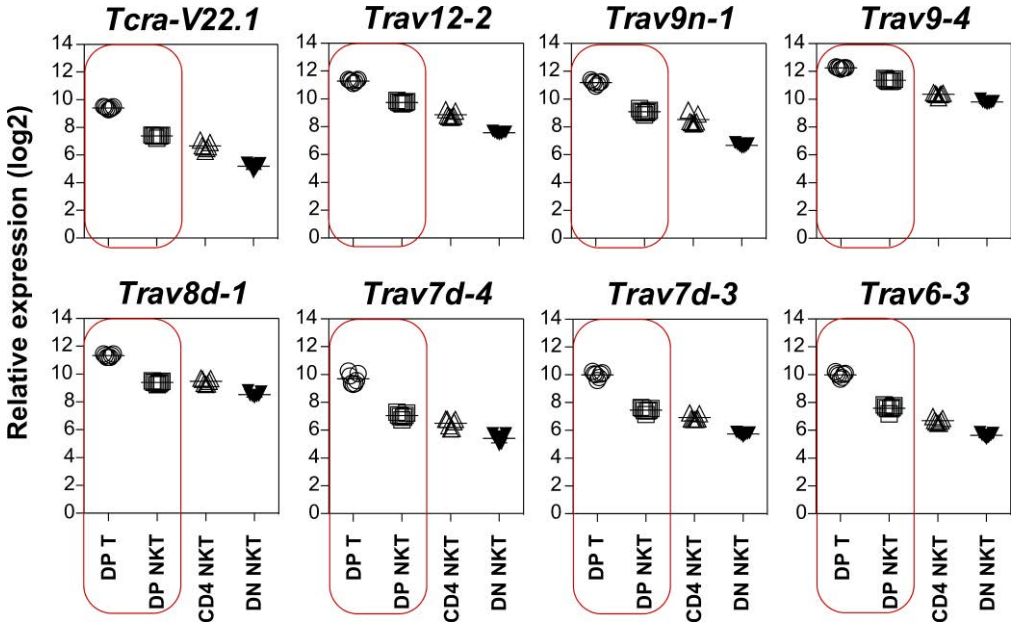


**Figure 4.8. Down-regulated expression of genes encoded TCR V alpha chains across Transition 1.** Gene expression profiles of *Tcra-V22.1*, *Trav12-2*, *Trav9-n1*, *Trav9-4*, *Trav8d-1*, *Trav7d-4*, *Trav7d-3*, and *Trav6-3* (data were generated with assistance from Dr. Margaret Jordan and Prof. Alan Baxter).

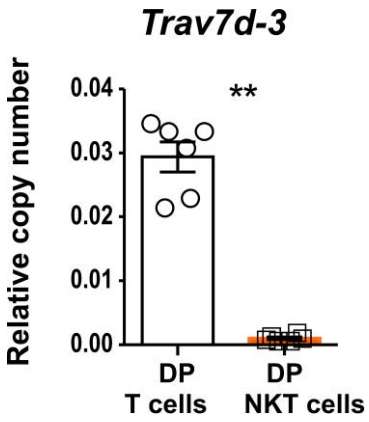


**Figure 4.9 *TCR-Vα7d-3* expression was validated by qPCR between DP T cells and DP NKT cells.** n = 6 per cell population. Data were generated with assistance from Dr. Morgane Moreau. Mann-Whitney test, ** p<0.01.

Forty-nine of a total of 60 transcripts, representing 31 different TCR Vα chains, showed significant down-regulated expression in immature DP NKT cells compared to immature DP T cells (Fig. 4.10). The loss of expression of the vast majority of non-NKT cell

associated Vα chains is consistent with successful validation of the transgene-encoded *Vα14* TCR initiating the mechanism for allelic exclusion of competing TCR.

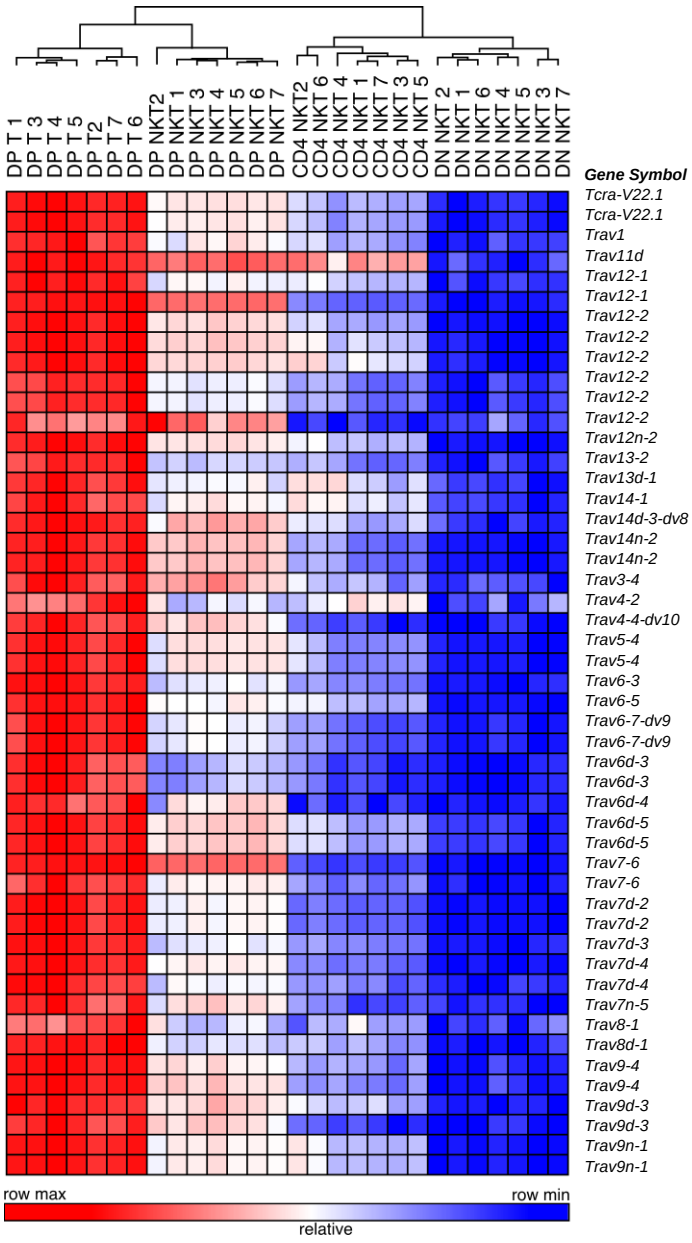


**Figure 4.10. Widespread down regulation of TCR V alpha genes across Transition 1.** Rows correspond to individual transcripts and columns correspond to tested populations. *Trav11d* encodes the NKT cell-associated TCR Vα14. Data were generated with assistance from Dr. Margaret Jordan and Prof. Alan Baxter.

In summary, the combination of activation, proliferation, TCR tuning and allelic exclusion provide compelling evidence that TCR validation occurred at the DP stage, across Transition 1.

### 4.2.3. Functional analysis of TCR validation by immature DP NKT cells

#### 4.2.3.1. Immature DP NKT cells express increased proliferation *in vivo*

Transcriptional analyses in sections 4.2.1 and 4.2.2 revealed that immature DP NKT cells proliferate more than immature DP T cells. In order to validate this finding, *in vivo* 5-bromodeoxyuridine (BrdU) incorporation was performed to compare their proliferative rates. NOD.*Vα14*Tg mice received three intraperitoneal (*i.p.*) injections of 1mg BrdU at 12-hourly intervals and thymi were collected for analysis by flow cytometry 36 hours after BrdU administration. Single cells from thymi of both control and BrdU injected mice were initially stained with surface antibodies then intracellularly stained with fluorescence-conjugated anti-BrdU antibody. The gating strategy applied was the same as that used for sorting cells for the microarray experiment (Fig. 4.11).

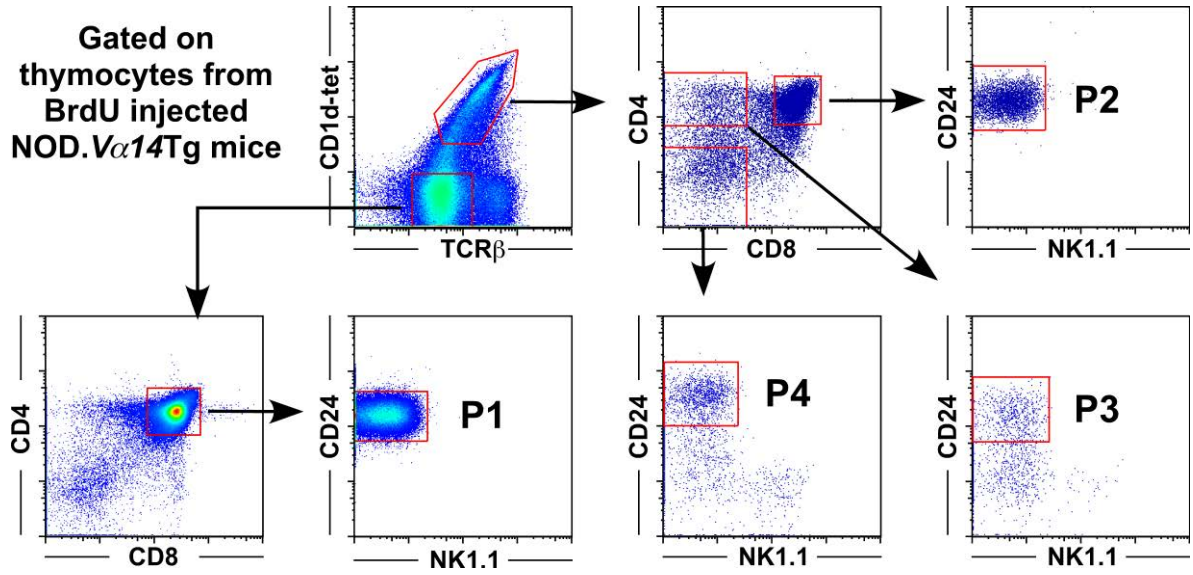


**Figure 4.11. Representative FACS plots showing gating strategy for immature DP T cells (P1), immature DP NKT cells (P2), immature $CD4^+$ (P3) and DN NKT cells (P4) from thymi of BrdU injected NOD.*Vα14*Tg mice.**

Following gating, the proportions of $BrdU^+$ cells from immature DP T cells, immature DP NKT cells, immature $CD4^+$ NKT cells and immature DN NKT cells from the thymi of NOD.*Vα14*Tg mice were identified via histogram plots as presented in Figure 4.12.

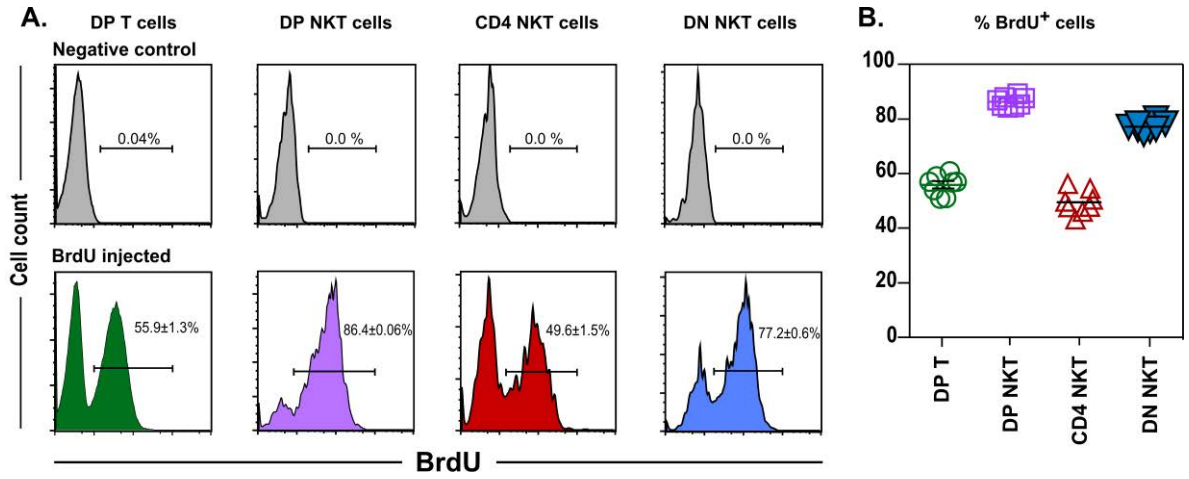


**Figure 4.12. Proportions of BrdU positive cells among DP T cells, DP NKT cells, CD4 and DN NKT cells.**
**A.** Representative histograms showing proportions of $BrdU^+$ cells in total DP T cells, DP NKT cells, CD4 and DN NKT cells between control and BrdU injected mice. **B.** Proportions of $BrdU^+$ cells of each subset in BrdU injected mice.

The flow cytometric cell analyses revealed that immature DP NKT cells had significantly higher proportions of $BrdU^+$ cells compared to immature DP T cells. The average proportion of $BrdU^+$ cells rose from 56% of total immature DP T cells to 86% of total immature DP NKT cells (p=0.0002; Mann-Whitney U test). This finding was consistent with a higher frequency of proliferative cells in the immature DP NKT population compared to DP T cells. In contrast, the proportions of $BrdU^+$ cells decreased in immature CD4 and DN NKT cells with 51% of immature CD4 NKT cells bearing $BrdU^+$ and 77% of immature DN NKT cells (Fig. 4.12). Taken together, these data support the hypothesis that immature DP NKT cells have a higher proliferative rate than immature DP T cells.

### 4.2.3.2. Validation of allelic exclusion of TCR Vα chain

Allelic exclusion is a mechanism whereby a product of a single allele of T cell receptors is selected for expression. This process is a fundamental mechanism of immunological self-tolerance. Incomplete allelic exclusion leads to dual T cell receptor expression, which can allow developing autoreactive αβ T lymphocytes to escape clonal

deletion (Morris and Allen, 2009; Kekalainen *et al*., 2010). Allelic exclusion at the TCRβ locus is more stringent (primarily at the gene level not the protein level) than that at the TCR α locus. Both alleles of TCR α genes continue to rearrange until a positively-selectable heterodimer is formed with the previously formed β chain (Borgulya *et al*., 1992). Therefore, immature thymocytes frequently express dual α chain on the cell surface, whereas almost all mature thymocytes express a single αβ combination (Alam *et al*., 1995a; Boyd *et al*., 1998).

To further confirm the finding that allelic exclusion of NKT cells occurs at Transition 1, immature and mature NKT cells were examined for the expression of TCR Vα2, Vα3.2 and Vα8.3 by flow cytometry. In particular, thymic, splenic and hepatic cells from NOD.*Vα14*Tg mice were stained with TCRβ and CD1d-tet to identify NKT cells; CD4, CD8, CD24 and NK1.1 markers were used to identify NKT cell subsets. As illustrated in Figures 4.13 and 4.14, immature DP NKT cells, immature CD4 NKT cells and immature DN NKT cells were gated from the thymus of NOD.*Vα14*Tg mice, while mature NKT cells were gated from the thymi, spleens and livers. These NKT cell populations (those already expressing the TCR Vα14-Jα18 chain) were then tested for the co-expression of TCR Vα2, Vα3.2 and Vα8.3 chains. For this experiment, immature thymic DP T cells, mature thymic, splenic and hepatic T cells were used as controls (Fig. 4.13 and 4.14).

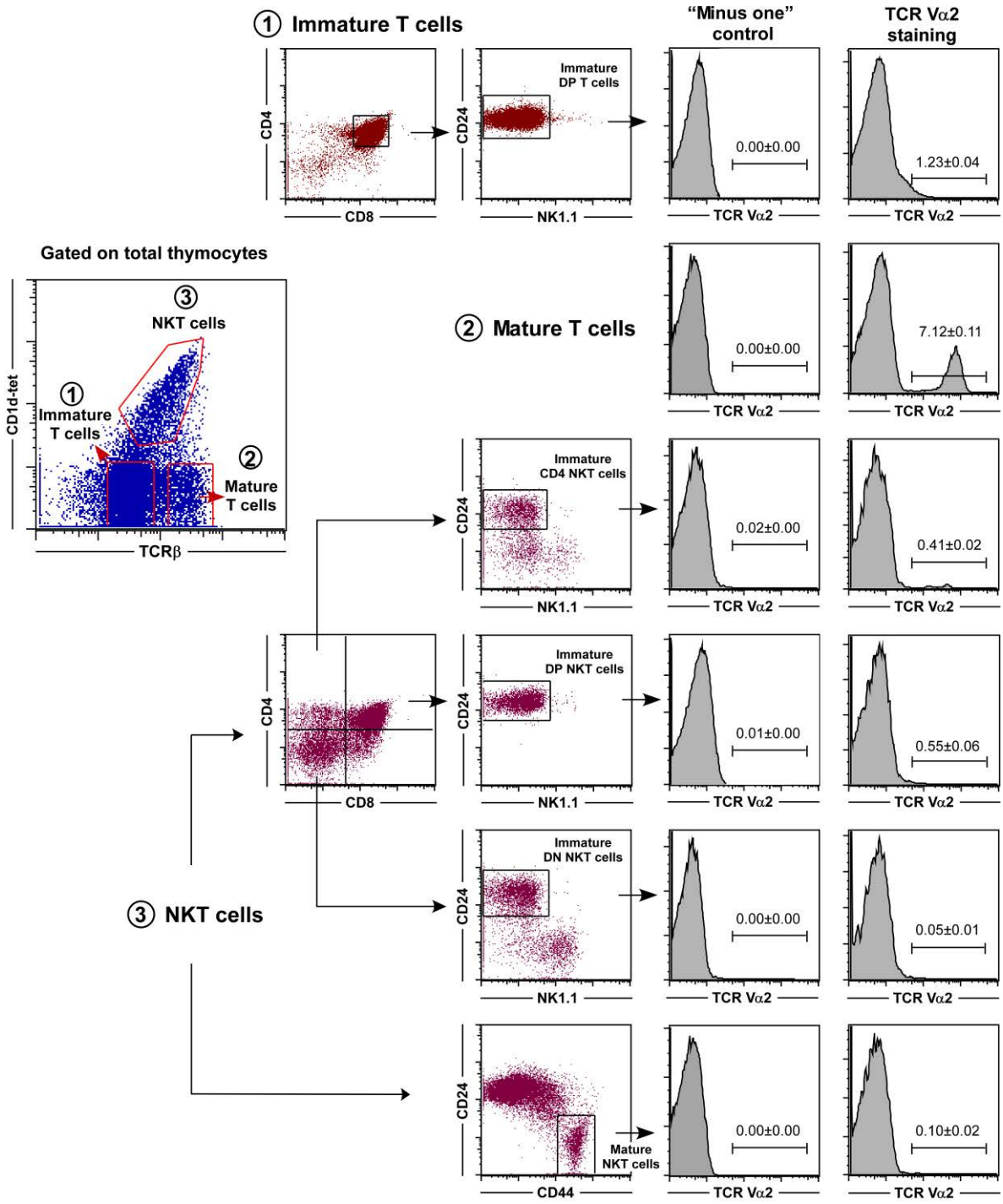


**Figure 4.13. Representative FACS plots showing gating strategy for Vα2$^+$ thymic cell populations.** Thymocytes were stained with stage-specific antibodies. Immature DP T cells, immature DP NKT cells, immature CD4 NKT cells, immature DN NKT cells and mature NKT cells were gated from the thymocytes of NOD.*Vα14*Tg mice. The proportions of Vα2$^+$ cells from these 5 cell populations were identified by histogram plots (right). The lower gate boundary for Vα2$^+$ cells was set based on "minus one" fluorescence controls (left histogram plots). The same strategy was applied to identify the proportions of Vα3.2$^+$ and Vα8.3$^+$ cells.

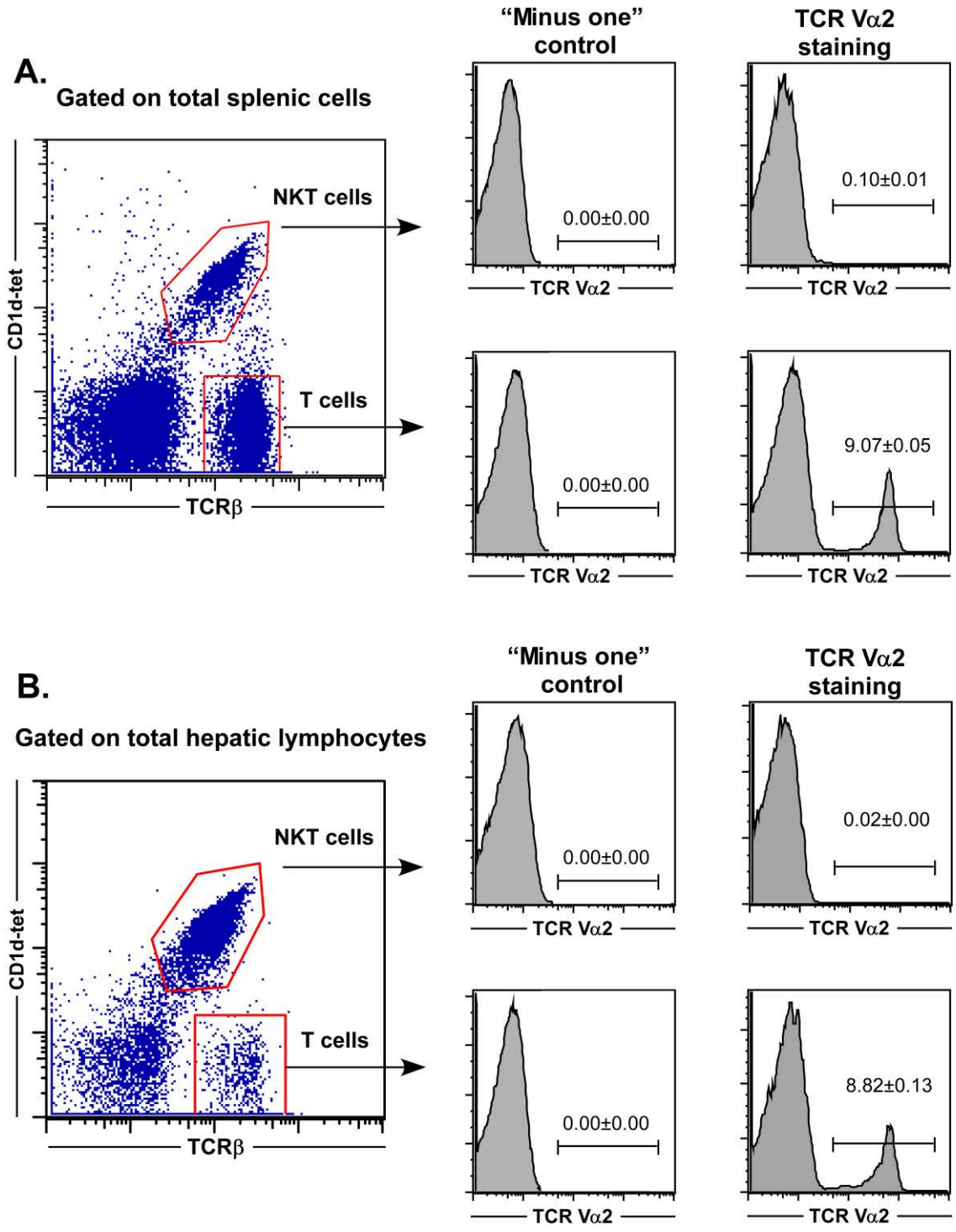


**Figure 4.14. Representative FACS plots showing gating strategy to identify the proportions of Vα2$^+$ splenic, hepatic T and NKT cells.** Splenic (A) and hepatic (B) cells were stained with antibodies for flow cytometric analysis. Mature T and NKT cells were gated from the spleens and livers of NOD.*Vα14*Tg mice. The proportions of mature T and NKT cells expressing TCR Vα2$^+$ were identified in histogram plots (right). The lower gate boundary for Vα2$^+$ cells was set based on "minus one" fluorescence controls (left histogram plots). The same strategy was applied to identify the proportions of Vα3.2$^+$ and Vα8.3$^+$ cells.

Flow cytometric analysis revealed that very few (less than 2%) immature thymic T cells expressed TCR Vα2, Vα3.2 and Vα8.3 chains on their cell surface (Table 4.3; Fig. 4.15 A). For example, only 1.23±0.04%, 0.47±0.01% and 0.30±0.01% of immature DP T cells expressed TCR Vα2, Vα3.2 and Vα8.3, respectively (Table 4.3; Fig. 4.15 A).

With regard to NKT cells, less than 0.6% of immature DP NKT cells expressed TCR Vα2 or Vα3.2 and Vα8.3 accompanied with TCR Vα14-Jα18 chain (Table 4.3; Fig. 4.15 A). The same pattern was seen with the frequencies of immature CD4 NKT cells and immature DN NKT cells (Table 4.3; Fig. 4.15 A). The proportions of immature DP NKT cells, immature CD4 NKT cells and immature DN NKT cells expressing TCR Vα2, Vα3.2 and Vα8.3 chains were significantly lower than those of immature DP T cells ($p<0.01$; Mann-Whitney U test), except for a slight increase in proportion of immature CD4 NKT cells expressing Vα8.3 compared to the other subsets (Table 4.3; Fig. 4.15 A).

**Table 4.3. Proportions of immature thymic T cells and NKT cells expressing TCR V2, V3.2 and V8.3** (Mean ± SEM)

| TCR chain | Immature DP T cells | Immature DP NKT cells | Immature CD4 NKT cells | Immature DN NKT cells |
|---|---|---|---|---|
| **Vα2** | 1.23±0.04% | 0.41±0.02% | 0.55±0.06% | 0.10±0.02% |
| **Vα3.2** | 0.47±0.01% | 0.18±0.02% | 0.18±0.05% | 0.02±0.00% |
| **Vα8.3** | 0.30±0.01% | 0.17±0.01% | 0.48±0.06% | 0.08±0.02% |

With regard to mature NKT and T cells, the proportions of mature thymic, splenic and hepatic NKT cells expressing Vα2, Vα3.2 and Vα8.3 chains were much lower than those of immature thymic T and NKT cells (Table 4.4; Fig. 4.15 B). Almost none of the mature NKT cells expressed either Vα2, Vα3.2 or Vα8.3 accompanied with Vα14-Jα18 chain. In contrast, there were large numbers of mature T cells expressing the tested Vα chains.

In the thymus, 7.12% of mature T cells expressed Vα2, 2.84% expressed Vα3.2 and 2.45% expressed Vα8.3, while in the livers the proportions were 8.82%, 3.41% and 2.71%, and in the spleens there were 9.07%, 3.44% and 2.80%, respectively (Table 4.4; Fig. 4.15 B). The significantly lower proportion of immature DP NKT cells and mature NKT cells expressing Vα2, Vα3.2 and Vα8.3 compared to immature DP T cells is consistent with the proposed effects of TCR Vα allelic exclusion on that cell population.

**Table 4.4. Proportions of mature thymic, hepatic, splenic T cells and NKT cells expressing TCR Vα2, Vα3.2 and Vα8.3** (Mean±SEM)

| TCR chain | Thymic NKT cells | Hepatic NKT cells | Splenic NKT cells | Thymic T cells | Hepatic T cells | Splenic T cells |
|---|---|---|---|---|---|---|
| **Vα2** | 0.10±0.02 | 0.02±0.00 | 0.10±0.01 | 7.12±0.11 | 8.82±0.13 | 9.07±0.05 |
| **Vα3.2** | 0.04±0.01 | 0.05±0.04 | 0.04±0.00 | 2.84±0.04 | 3.41±0.09 | 3.44±0.04 |
| **Vα8.3** | 0.08±0.02 | 0.03±0.01 | 0.09±0.01 | 2.45±0.04 | 2.71±0.06 | 2.80±0.03 |

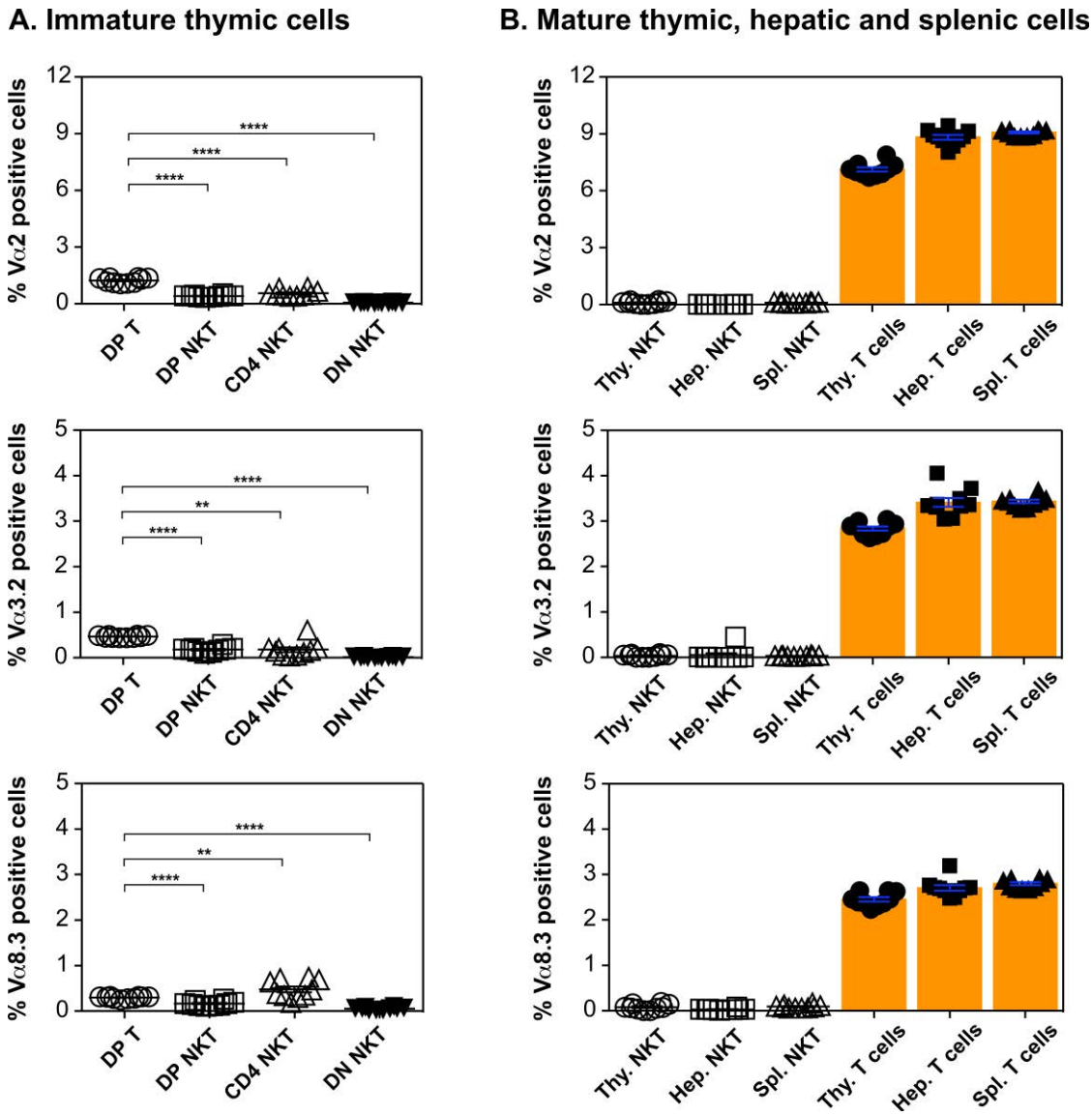


**Figure 4.15. Flow cytometric comparison of TCR Vα2, Vα3.2 and Vα8.3 co-expression by NKT cells. A.** Dot plots show the proportion of immature DP T cells, immature DP NKT cells, immature CD4 NKT cells and immature DN NKT cells expressing TCR Vα2, Vα3.2 and Vα8.3 in the thymi of NOD.*Vα14*Tg mice. **B**. Dot plots show the proportion of mature NKT and T cells expressing TCR Vα2, Vα3.2 and Vα8.3 in the thymi, livers and spleens of NOD.*Vα14*Tg mice. n= 10; Mann-Whitney U test, ** p<0.01,**** p<0.0001.

## 4.3. SUMMARY OF MAJOR FINDINGS

Transcriptional analysis by microarray of immature DP T cells and immature DP NKT cells raised the possibility that immature DP NKT cells had a higher proliferation rate than immature DP T cells. The reduction in expression of the electron transport genes *Nd1, Nd2, Nd4l, Nd5, Cox1, Cox2, Cox3* and *Atp6* suggested reduced mitochondrial oxidative

phosphorylation. This is a characteristic of the Warburg phenomenon, in which cancerous or proliferative cells predominantly rely on glycolysis and lactate fermentation instead of oxidative phosphorylation as a source of ATP (Vander Heiden *et al*., 2009; 2011; Lunt *et al*., 2011).

The possibility of increased proliferation in DP NKT cells was also supported by the up-regulated expression of genes associated with cell cycle and division. A large number of genes encoding Centromere proteins and genes involved in the E2F signalling pathway and DNA replication were significantly up-regulated by immature DP NKT cells compared to immature DP T cells. E2F1, E2F2 and E2F3 act in a functionally redundant manner to enhance the expression of many genes required for the $G_1$ to S phase transition of the cell cycle, including *Cdc6*, *Ccne1* and *Myb* (Johnson *et al*., 1993; Wu *et al*., 2001). The growth regulatory function of the RB tumour suppressor protein is mediated by its binding to E2F transcription factors; so, while overexpression of E2F1, unopposed by increased RB expression, results in increased proliferation, so too does *E2f1* gene deletion (Yamasaki *et al*., 1996; Field *et al*., 1996). Consistent with increased *E2f1* expression, E2F1-regulated genes encoding proteins critical for S phase entry, such as *Cdc6* and *Ccne1*, genes encoding proteins critical for spindle formation and chromosome segregation (such as *Cenpm*) and DNA replication (such as *Rpa2*) were also up-regulated.

The role of the E2F pathway in mediating T cell proliferation has been studied in *E2f1/E2f2* double knockout mice. E2F1 and E2F2 appear to play a role in homeostatic proliferation (HP), but not in proliferative responses to exogenous antigen, as HP in *E2f1/E2f2* double knockout mice is severely reduced (DeRyckere and DeGreori, 2005, Zhu *et al*., 2001). It is reported that homeostatic proliferation of naive T cells requires both IL7 signalling (Schluns *et al*., 2000, Surh and Sprent, 2008) and TCR stimulation by MHC-self peptide (Ernst *et al*., 1999), that of memory CD4 T cells is dependent on TCR stimulation by MHC-self peptide alone (Tan *et al*., 2002). As homeostatic proliferation of all T cells,

including memory CD4 T cells, is impaired in *E2f1/E2f2* double knockout mice, the E2F pathway play a role in mediating T cell proliferation in the context of homeostatic proliferation (Field *et al*., 1996).

Although Benlagha *et al.* (2005) previously reported that their $DP^{low}$ $CD24^{high}$ $NK1.1^{-}$ NKT cells in the thymi of newborn mice were non-dividing, our functional validation by *in vivo* BrdU incorporation confirmed our hypothesis that the immature thymic $DP^{high}$ NKT cells in NOD.*Vα14*Tg mice have increased proliferation. The proliferation bust following TCR validation seems to have some similarities to HP.

There was widespread down-regulation of non-NKT cell TCR Vα genes in immature DP NKT cells. These findings provide further evidence at the transcriptional level of allelic exclusion of competing TCR Vα chains. Alpha chain allelic exclusion is also reported to be mediated via other mechanisms. During the DP stage of thymocyte development, both alleles of the α-chain gene rearrange until a positively selectable heterodimer is formed with the previously rearranged β-chain. Positive selection causes the end of α–chain rearrangement by halting transcription of the *Rag1* and *Rag2* genes (Borgulya *et al.,* 1992; Brandle *et al*., 1992; Kouskoff *et al.,* 1995; Alam *et al*., 1995b). A post-translational mechanism ensures that only one α-chain is generally present on the cell surface of mature T cell (Alam and Gascoigne, 1998; Gascoigne and Alam, 1999, Rybakin *et al*., 2014). Different models have been proposed to explain post-translational regulation of α-chain allelic exclusion (Niederberger *et al.,* 2003). The simplest model proposed that allelic exclusion depended on the competition between two α-chain proteins for a limited supply of β-chain protein, when the TCR level on the cell surface increases during positive selection (Alam *et al*., 1995b; Alam and Gascoigne, 1998; Gascoigne and Alam, 1999). In the second model, it was proposed that the CD3z chain (a component of

the TCR-CD3 complex) may play an important role in phenotypic allelic exclusion of the α-chain by stabilizing preformed αβ pairs thereby preferentially stabilizing the αβ combination with the highest interchain affinity available to the cell. In the selective retention model, it was indicated that the positively selected αβ combination is maintained on the cell surface while the nonselected α-chain is down-regulated or retained within the cell by regulation of TCR degradation via ibiquitination (Boyd *et al*., 1998). However, those models do not explain how the widespread down-regulation of non-NKT cell TCR Vα genes occurs in immature DP NKT cells compared to immature DP T cells. One possibility is that the relatively early expression of Vα14 prevents further recombination of endogenous alleles thereby eliminating any chance of generating other viable transcripts. Therefore, the down-regulation of alpha chain families' transcripts in DP NKT cells likely reflects transcriptional evidence of allelic exclusion of competing TCR Vα chains in immature DP NKT cells at the allele level. This finding was confirmed by the very low number (almost none) of immature DP NKT cells and mature NKT cells expressing other TCR α chains, such as TCR Vα2, Vα3.2 and Vα8.3 accompanied by the TCR Vα14-Jα18 chain. The proportion of immature DP NKT cells, as well as mature NKT cells expressing the tested TCR Vα chains, was significantly lower than in immature DP T cells or mature T cells.

The Transition 1 concept strongly suggests that αβ TCR engagement of a DP T cell induces the formation of a DP NKT cell, however, the data also seem compatible with an alternative scenario, in which DP NKT cells and DP T cells might be on separate developmental pathways. At the (preceding) DN3 stage, any successfully rearranged TCRβ chain can pair with pre-Tα to form an autonomous dimerization of the pre-TCR then undergo the process called β selection. β selection signals the successful completion of TCRβ chain

gene rearrangement and results in cellular expansion, CD4 and CD8 expression, termination of further TCRβ chain gene rearrangement, initiation of TCRα chain gene rearrangement, migration into the cortex, and development of positive and negative selection "competence" factors (Michie *et al*., 2002; Neilson *et al*., 2004; Pang *et al*., 2010; Kreslavsky *et al*., 2012).

Expression of the pre-TCR upon productive rearrangement of the *Tcrβ* gene results in a burst of proliferation and in progression to the DP stage - a hallmark of αβ T cell lineage commitment. Although it is sometimes assumed that cells proliferating during β selection do not express cell surface TCR, this assumption may be incorrect as time course experiments in Lucas *et al*. work revealed low-level of TCRβ expression on the cell surface of ~20% of $BrdU^+$ thymocytes 2 hours after BrdU injection, which increases to 78.2% at 24 hours. The results showed that TCR is expressed at a low density by most thymocytes undergoing proliferation and acquisition of the $CD4^+CD8^+$ phenotype (Lucas *et al*., 1993). The DP NKT cells detected in our work appear to have variable surface TCR expression ranging from low to high, based on staining with tetramer and anti-TCRβ. Moreover, there is evidence that the TCR may transduce a signal in cells proliferating during β selection. Separation of thymocytes by centrifugal elutriation has shown that $CD4^+8^+$ large cells are enriched for cells that have not yet undergone positive selection, while many $CD4^+8^+$ small cells are presumably on the way to death by neglect (Guidos *et al*., 1990; Lundberg and Shortman, 1994). In TCRβ-transgenic mice, deletion of self-reactive thymocytes (tetramer-binding) was already detectable in large DP thymocytes (with high "forward scatter"; Baldwin *et al.,* 1999). In Baldwin *et al.* study, the large size of the cells suggests they were proliferating and the DP phenotype suggests they were undergoing β selection.

Whenever the DP population was subdivided into 2 subsets, if that subdivision delineates DP subsets of different ages, proliferation will be increased in the "younger" subset due to β selection-induced proliferation during the DN-DP transition. The data presented in

this chapter do not exclude the possibility that DP NKT cells are "younger", on average, than DP T cells. If it is admitted that DP NKT cells may be "younger" than DP T cells, then this can also explain the finding that many *Trav* genes are expressed at lower levels in the DP NKT population compared to the DP T cell population. This is because progressive recombination at the TCRα locus would be expected to incorporate a greater variety of *Trav* segments over time as the DP population ages.

In *Vα14*Tg$^{+}$ DN3 cells, assembly of a TCRβ chain that permits CD1d engagement might lead to the formation of a DP NKT cell, whereas assembly of a TCRβ chain that precludes CD1d engagement might lead to the formation of a DP T cell. Since these considerations raise a caveat about the "Transition 1" concept, unfortunately, they apply equally to its derivative, the "TCR validation" concept. This may not strongly support for the stage at which the DP NKT population and DP T cell population might diverge? However, the down-regulation of genes related to the electron transport chain, the activation of the E2F pathway, T cell signalling and the down-regulation of non-NKT associated TCR Vα genes strongly suggests that successful TCR validation occurred across Transition 1.

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# CHAPTER 5

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# TRANSITION 2 -

# THYMIC SELECTION OF NKT CELLS

## 5.1. INTRODUCTION

Thymic selection ensures that the resulting T cell populations are responsive to foreign antigens, but not self-antigens, presented by antigen presenting cells (APCs; Starr *et al*., 2003). In conventional T cells, thymic selection is primarily determined by the avidity of the interaction between the newly formed TCR on DP thymocytes and peptide-major histocompatibility complex (MHC) products on the APCs (Hogquist *et al*., 1994; Alam *et al*., 1999; Klein *et al*., 2014). When DP thymocytes have a moderate avidity interaction, they are positively selected and, subsequently, differentiate into mature CD4 or CD8 single positive conventional T cells. In contrast, if there is an insufficient or high avidity interaction, they are negatively selected and die through neglect or apoptosis, respectively. The net result of these interactions allows for the generation of T cells with low recognition for self and elimination of potentially auto-reactive cells (Starr *et al*., 2003).

The interactions between the newly formed TCRs and endogenous ligands induce a series of intracellular signalling cascades, which will direct the DP thymocytes to positive or negative selection (Starr *et al*., 2003). Upon engagement of the TCRs by antigens presented on MHC molecules, the Src family kinases Lck and Fyn are activated and phosphorylate immunoreceptor tyrosine-based activation motifs (ITAMs) on CD3 (Kane *et al*., 2000). Phosphorylated ITAMs promote the recruitment and subsequent activation of another tyrosine kinase, ZAP-70, which, in turn, phosphorylates the tyrosine residues on the adapter molecule LAT (Samelson, 2002). The phosphorylation of LAT results in recruitment of several components of the TCR signal transduction pathway, including GADs, SLP-76, PLCε1 and Grb2 (Koretzky *et al*., 2006; Wange, 2000). In the presence of a moderate-avidity TCR ligand, partial LAT phosphorylation recruits PLCε1 and GADs/SLP-76/ITK, resulting in activation of the Ras pathway and calcium signalling. The Ras pathway stimulates sustained,

low-level ERK activation, while the calcium flux activates NFAT (Brownlie *et al.,* 2013). These signals ultimately result in thymocyte survival, cessation of TCR gene recombination, antigen receptor tuning, cell migration into a new thymic environment and lineage commitment. In contrast, a high-avidity TCR interaction results in full phosphorylation of LAT. Fully phosphorylated LAT, in turn, recruits Grb2/SOS1 and strong, transient activation of ERK coupled with p38 and JNK activation and leads to programmed cell death (Starr *et al*., 2003).

Although less well understood than conventional T cells, it is believed that NKT cells also undergo positive and negative selection events. These selection processes allow for the elimination of highly auto-reactive cells while preserving a functional pool of cells. Unlike conventional T cell precursors, which are selected by peptide antigens presented in the context of MHC class I or II molecules on cortical epithelial cells, NKT cells are selected by glycolipid antigens presented by CD1d on cortical DP thymocytes (Bendelac, 1995; Cole and Raulet, 2000; Wei *et al*., 2005; Godfrey and Berzins, 2007). NKT cells are absent in both *Cd1d*$^{-/-}$ mice (Chen *et al.*, 1997; Mendiratta *et al.*, 1997; Smiley *et al.*, 1997; Gapin *et al.*, 2001) and bone marrow chimeric mice that lack CD1d expression on DP thymocytes (Chun *et al.*, 2003). In contrast, NKT cells develop normally in mice bearing a targeted mutation of the endogenous *Cd1d* gene if rescued by a human *Cd1d* transgene (Schumann *et al.*, 2005). The lack of AP-3 and prosaposin, which are involved in trafficking of CD1d to lysosomes and the loading of glycolipids into the CD1d groove, markedly impaired NKT cell development. These findings suggested that CD1d expression on cortical DP thymocytes is a critical requirement for the positive selection of NKT cells (Chun *et al*., 2003).

While the requirements for positive selection of NKT cells are gradually being clarified, the question of whether iNKT cells are subject to negative selection remains controversial (Godfrey and Berzins, 2007). The most compelling evidence has indicated that the early introduction of α-GalCer, both *in vivo* (by fetal thymic organ culture) and *in vitro*

(i.p. injection of α-GalCer from day 3 to day 14), could specifically ablate NKT cell development. However, it could not do so if the challenge was delayed after NKT cells had differentiated (Pellicci *et al.*, 2003). The study of fetal thymic organ cultures (FTOC) has shown that NKT cells could not be generated in thymic lobes cultivated in the presence of α-GalCer, while they develop normally in cultures with β-GalCer or DMSO (Pellicci *et al.*, 2003; Chun *et al.*, 2003). In these studies, the presence of α-GalCer, a potent antigen, was proposed to mediate negative selection of iNKT cells. Unfortunately, α-GalCer is not a mammalian self-antigen for the semi-invariant TCR; thus these studies do not address the question of whether iNKT cells can be negatively selected under physiological conditions.

In addition, by creating mice expressing a transgenic TCR-β chain that pairs with the canonical Vα14-Jα18 TCR-α chain, Bedel *et al*. (2014) created a model with high affinity for self-lipid/CD1d complexes. Their results indicated that effective functional maturation of invariant natural killer T cells is constrained by negative selection and T-cell antigen receptor affinity. Analysis with PBS57-loaded CD1 tetramers revealed a large reduction in, but not complete loss of, the proportion and total number of iNKT cells in the thymus, spleen, and liver of the 2A3-D Tg mice compared with the wild type mice. While the impairment occurs at the transition from stage 1 to stage 2 of NKT cell development the remained iNKT cells that avoid negative selection in these mice express natural sequence variants of the canonical TCR-α and decreased affinity for self/CD1d (Bedel *et al*., 2014). These results directly demonstrate that the TCR signaling by agonist self-ligands is the trigger of the iNKT cell differentiation pathway. It also emphasizes that only a limited range of TCR affinity for self is actually compatible with iNKT cell development versus death by neglect or negative selection.

In contrast, other studies have examined the effects of negative selection on NKT cell development through altering CD1d density on ligand expressing cells. Overexpression of

CD1d, derived from both mice (Chun *et al.*, 2003) and humans (Schumann *et al.*, 2005), on thymic stromal, dendritic cells or DP thymocytes in transgenic mice impaired NKT cell development and led to reduced NKT cell numbers. This effect is biased toward the higher affinity TCR Vβ8.2 but not the TCR Vβ2, which is known to have a lower affinity for α-GalCer in the context of CD1d (Chun *et al.*, 2003). However, the aforementioned studies all showed a failure of NKT cell development, as opposed to the deletion of formed iNKT cells. If negative selection does occur in the NKT cell lineage, it likely affects the earliest stages, possibly around the same time as positive selection, as acute treatment of the adult thymus (mostly mature NKT cells) by intrathymic injection of α-GalCer has little effect on NKT cells (Pellicci *et al.*, 2003). Additionally, Nur77, a transcription factor associated with negative selection, is selectively expressed by very immature NKT cells (Stanic *et al.*, 2004b). Therefore, further studies are required to better understand whether, or when, iNKT cells actually undergo positive and negative selection.

In Chapter 3, flow cytometric analysis of *Vα14* transgenic mice revealed that they produced large numbers of thymic $DP^{high}$ $CD24^{high}$ $NK1.1^{-}CD1d\text{-}tet^{+}TCR\beta^{int}$ (immature DP NKT) cells. Phenotypic analysis indicated that these cells exhibited a phenotype consistent with the earliest progenitors of NKT cells or pre-selection DP NKT cells. In Chapter 4, transcriptional analysis over the course of iNKT cell differentiation from immature DP T cells to immature DP NKT cells indicated that allelic exclusion and successful TCR validation occur at Transition 1, but did not show any sign of T cell selection. Based on the principal component and network analyses, the successful TCR validation by immature DP NKT cells might be a signal for thymic selection of this cell population. Therefore, in this chapter, gene expression will be compared between immature DP NKT cells and immature CD4 NKT cells to identify transcriptional evidence for thymic selection and lineage commitment of NKT cells.

The following hypothesis was tested: **"Positive selection occurs across Transition 2 between immature DP NKT cells and immature CD4 NKT cells during thymic development".**

Specifically, the aims of this chapter were:

1. To compare gene expression between immature DP NKT cells and immature CD4 NKT cells.

2. To identify significant changes in gene expression related to positive selection of NKT cells.

3. To functionally validate gene expression changes occurring during thymic selection.

## 5.2. RESULTS

### 5.2.1. Transcriptional analysis between immature DP NKT cells and CD4 NKT cells

Gene expression comparison was performed between immature DP NKT cells and immature CD4 NKT cells across Transition 2 to identify significant gene expression changes. Of the 35,556 transcripts analysed by microarray, 6,791 transcripts generated a Mann-Whitney U score of zero. Of these, 2,143 were highly differentially expressed (HDE; T test $p < 1.4x10^{-6}$). This gene list was split into transcripts, which were up-regulated (1,216 transcripts), and those which were down-regulated (930 transcripts). These up-regulated and down-regulated HDE genes were separately submitted to DAVID Bioinformatics Resources for functional annotation clustering.

#### 5.2.1.1. Transcriptional analysis of NKT cell lineage commitment

Gene Ontology analysis was performed on 1,216 up-regulated HDE transcripts in DAVID Bioinformatics Resources. The top up-regulated annotation cluster (Enrichment Score 9.6) was dominated by integral plasma membrane proteins and contained the annotation categories (SP_PIR_KEYWORDS) Membrane (representing 40 of all up-regulated HDE genes; Bonferroni corrected $p<1.6x10^{-17}$) and Glycoprotein (Bonferroni corrected $p<1.1x10^{-14}$). Several functional families of integral plasma membrane proteins dominated the gene list, including Toll-like receptors (TLR1, 3, 6 and 12), receptors for cytokines (IL-1, 2, 6, 7, 10, 18, 21, 27; IFN-α, TGF-β and TNFR super-family members 1b, 9, 18 and 26), receptors for chemokines (CCR2, 4, 7, 8, 10; CXCR2 and 6), G protein coupled receptors (114, 171, 18, 65, 68, 83, 97, C5B), integrins (alpha 2, 4, E, L, V and FG-GAP repeat containing 3; beta 2, 3 and 7) and leukocyte differentiation markers (CD2, 5, 7, 25, 37, 38, 40LG, 44, 48, 52, 53, 59A, 79B, 82, 101, 160, 226, 274; Ly6A, C1, 6G and Ly9). The differential expression of several transcripts encoding integral plasma membrane proteins at the transition 1 (between

DP NKT cells and CD4 NKT cells) is shown in Figure 5.1 (the first and third rows) and confirmed by flow cytometry (the second and the fourth rows).

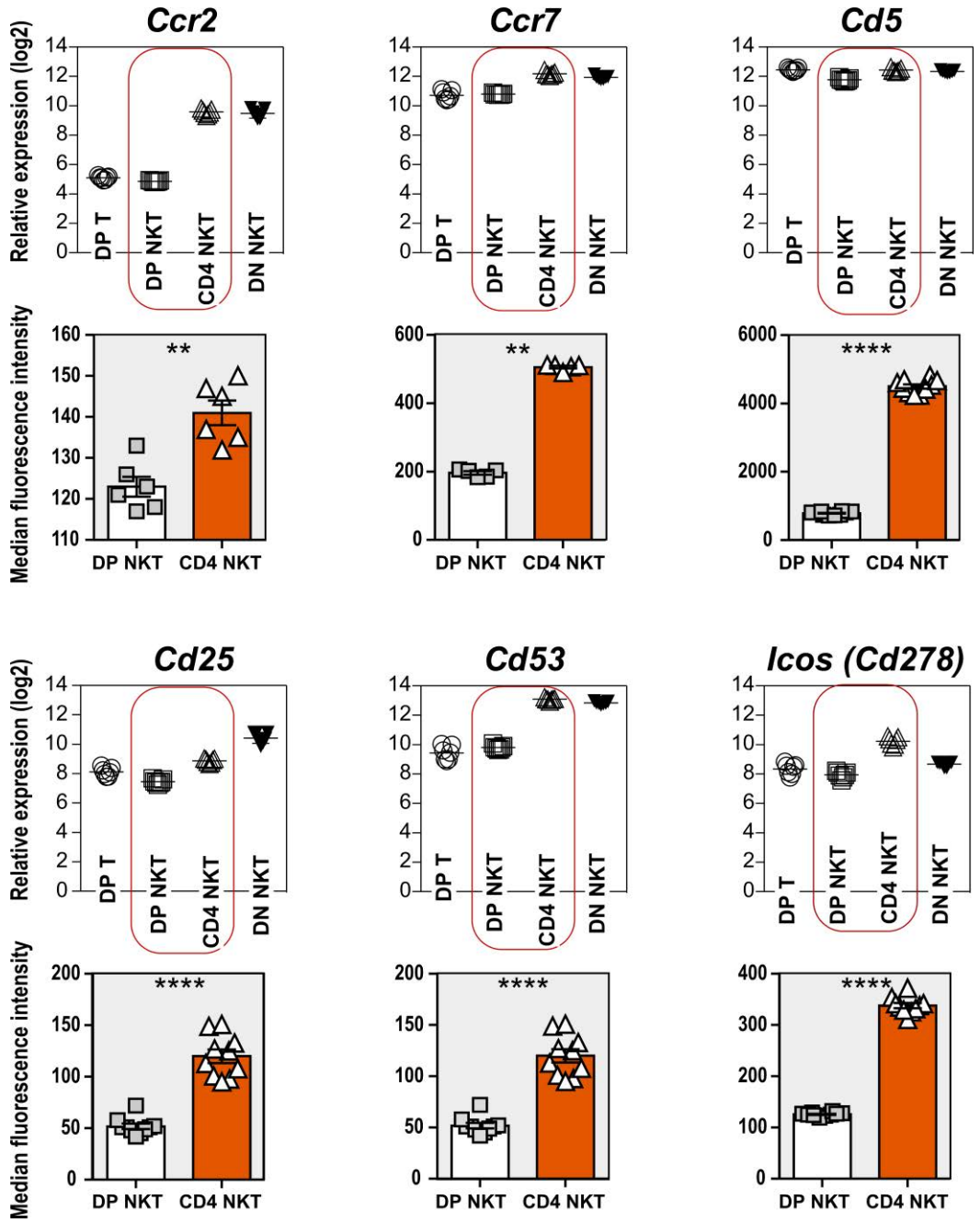


**Figure 5.1. Expression profiles of genes encoding integral plasma membrane proteins across Transition 2.** Gene expression of *Ccr2, Ccr7, Cd5* (the first row)*, Cd25, Cd53* and *Cd278 (Icos*; the third row) as determined by microarray (data were generated with assistance from Dr. Margaret Jordan and Prof. Alan Baxter). The differences in protein expression levels were confirmed by flow cytometry (the second row and the last row; n=5-6 mice per group in CCR2 and CCR7 expression experiments, n=10 mice per group in CD5, CD25, CD53 expression experiments; Mann-Whitney U test, ** $p<0.01$, **** $p<0.0001$).

Of the up-regulated HDE transcripts, amongst the most strongly differentially expressed (Mann-Whitney U statistic =0), there were genes associated with the immunomodulatory and innate-like characteristics of NKT cells, such as: *Tlr1, Nkg7, Sema4a, Art2b, S1pr1* and *Zbtb16* (Fig. 5.2).

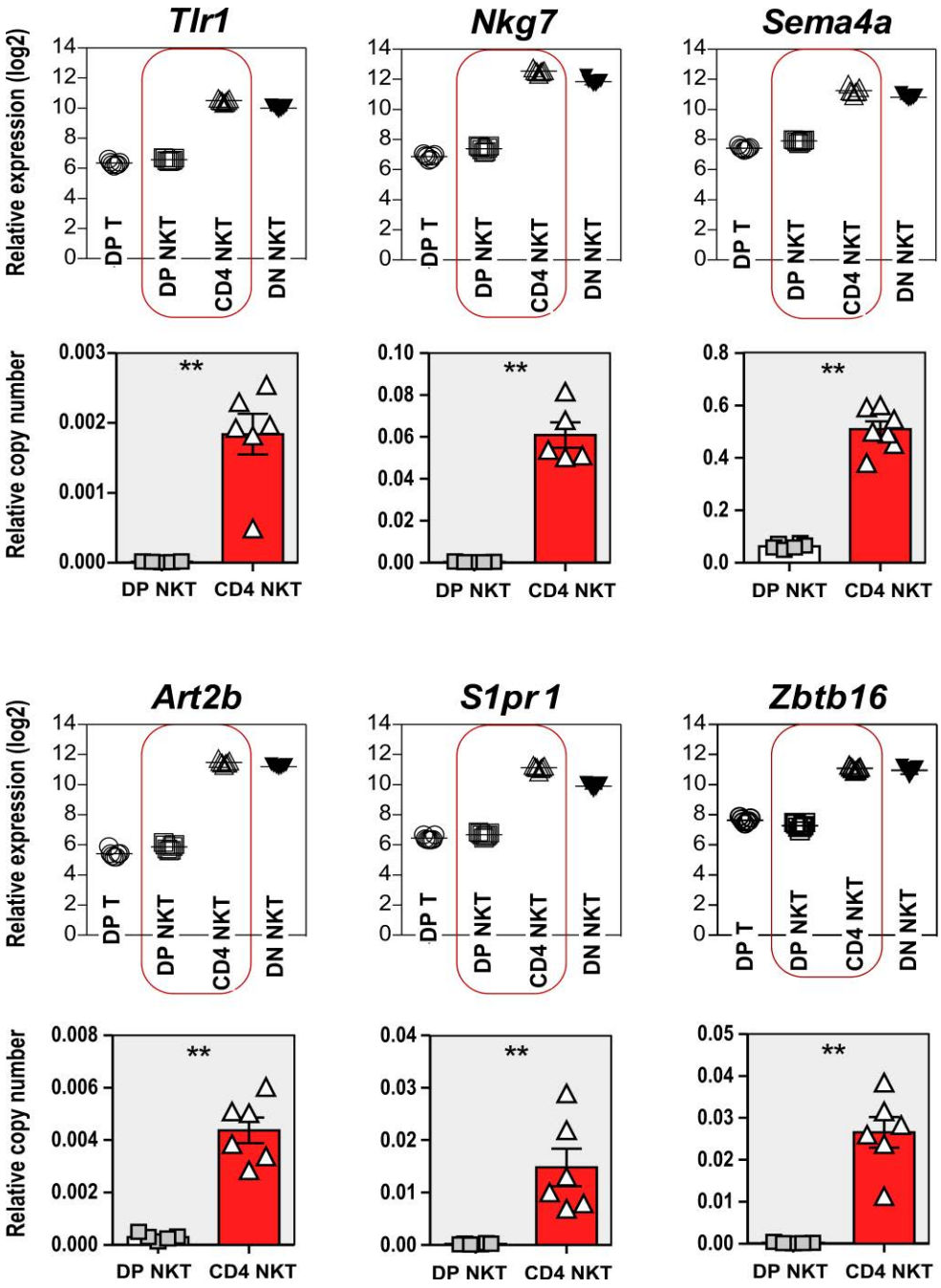


**Figure 5.2. Expression profiles of genes related to lineage commitment across Transition 2.** Gene expression of *Tlr1, Nkg7, Sema4a* (the first row), *Art2b, S1pr1, Zbtb16* (the third row) as determined by microarray (data were generated with assistance from Dr. Margaret Jordan and Prof. Alan Baxter). The differences in transcriptional levels were confirmed by qPCR (the second row and the last row; n=5-6 for DP NKT cell samples, n=5-7 for CD4 NKT cell samples; data were generated with assistance from Dr. Morgane Moreau; Mann-Whitney U test, ** $p<0.01$).

*Tlr1* (encoding Toll-Like Receptor 1-TLR1) was up-regulated 15-fold in CD4 NKT cells compared to DP NKT cells ($p<1.1x10^{-16}$, t-Test). TLR1 plays a fundamental role in pathogen recognition and activation of innate immunity (Takeuchi *et al*., 2002; Vallois *et al*., 2007). *Nkg7* (encoding Natural Killer Cell Granule Protein 7) was up-regulated 36-fold in CD4 NKT cells compared to DP NKT cells ($p<3.8x10^{-15}$, t-Test). NKG7 is associated with the cell mediated cytotoxic synapse. *Sema4a* (encoding Semaphorin 4A) was up-regulated 10-fold in CD4 NKT cells compared to DP NKT cells ($p<9.6x10^{-10}$; t-Test). SEMA4A is a type I integral membrane protein required for Th1 deviation and T-bet expression in T cells (Kumanogoh *et al*., 2005)

In addition, a number of other NKT cell-associated surface receptors were up-regulated in this transition. *Art2b* ($p<1.5x10^{-14}$; Fold change (FC) 49) encoding ADP-ribosyltransferase 2b that mediates apoptotic deletion of T-cell subsets (Seman *et al*., 2003), particularly $CD4^+$ NKT cells (Chen *et al*., 2006); *S1pr1* ($p<5x10^{-16}$; FC 22) encoding Sphingosine-1-Phosphate Receptor 1, which plays an important role in lymphocyte egress from lymphoid tissues; and *Zbtb16* ($p<6.5x10^{-14}$; FC 14) encoding Promyelocytic Leukaemia Zinc Finger (PLZF), a transcription factor that drives differentiation into NKT cells and human MR1-specific MAIT cells. These data indicated that a wide range of functional NKT cell-associated surface receptors were up-regulated in immature CD4 NKT cells compared to immature DP NKT cells and confirm the findings of Savage *et al*. (2008) that PLZF expression in NKT cells is up-regulated between Stage 0 and 1 of development (Sevage *et al*., 2008; Fig. 5.2).

The strong up-regulation of such a wide range of functional lymphocyte-associated, surface expressed, integral membrane proteins suggests that Transition 2 is associated with NKT cell lineage commitment. Consistent with this is the up-regulation of *Zbtb16*, which is responsible for driving the innate-like differentiation of NKT cells. Savage *et al*. (2008)

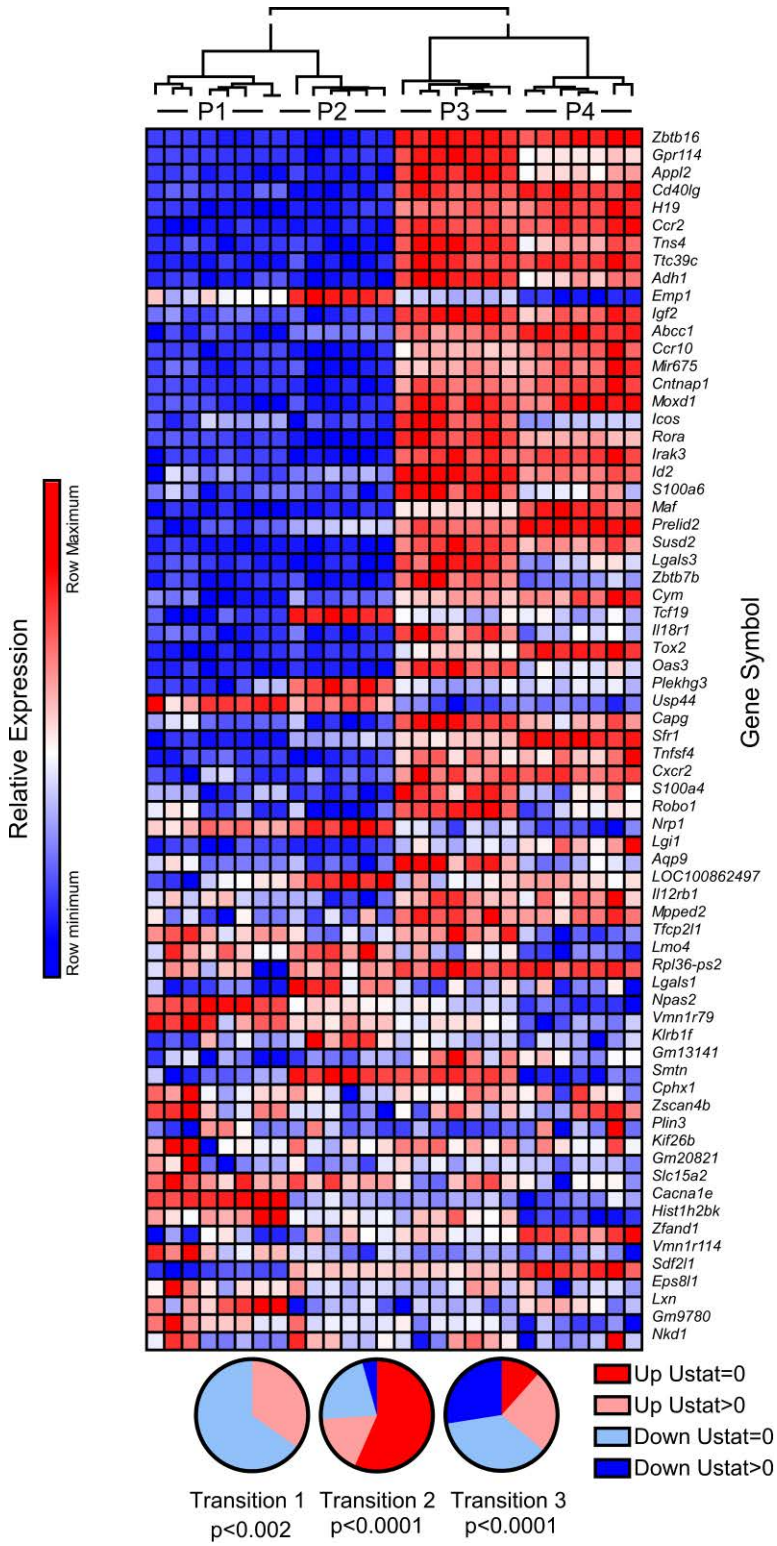


**Figure 5.3. Expression profiles of 69 transcripts, which were reported by Savage *et al*. (2008) to be modulated by PLZF.** The heat map shows the expression of these 69 transcripts by 4 different cell types (P1: DP T cells, P2: DP NKT cells, P3: CD4 NKT cells, P4: DN NKT cells), 7 biological samples per cell type, across 3 transitions. Pie charts show proportion of transcripts with 4 different statistic categories (up-regulated and U statistic = 0, up-regulated and U statistic > 0, down-regulated and U statistic = 0, and down-regulated and U statistic > 0) across each of the 3 transitions.

conducted a comparison of genome-wide transcriptions in PLZF-deficient *Luxoid* mutant with that in WT mice. The authors identified 69 genes being dependent on PLZF expression. To test whether the up-regulation of *Zbtb16* across Transition 2 was sufficient to modulate the expression of PLZF targeted genes during NKT cell development, we examined the transcript levels of the 69 genes identified by Savage *et al*. (2008; Fig 5.3). Gene expression comparison revealed that 42 of the 69 genes had a U statistic of zero across the transition 2 ($p<0.0001$; Chi-square contingency table), the vast majority of which (39 genes) were up-regulated (Fig. 5.3). These data are consistent with PLZF expression playing a significant role in NKT cell development across Transition 2.

Overall, the coordinated expression of the innate-like lymphocyte-associated transcription factor PLZF and the subsequent up-regulation of a wide range of cell-surface functional receptors associated with NKT cell immunobiology provide evidence that NKT cell lineage commitment occurred across Transition 2, between Stage 0 and 1, as previously reported by Savage *et al*. (2008).

#### 5.2.1.2. Transcriptional evidence of NKT cell selection

The association of NKT cell TCR validation with Transition 1 and Lineage commitment with Transition 2 raised the issue of the timing of NKT cell selection, which presumably could not occur later than Transition 2. Huang *et al*. (2004) published a custom microarray analysis of transcriptional changes during T cell selection by comparing transcripts between DP thymocytes that had not received a TCR signal from $MHC^{-/-}$ mice (C57BL/6.$B2m^{-/-}$.$Ab^{-/-}$.$E^{null}$) with those from positively selecting TCR transgenic mice (5CC7 and either F5 or P14). We were able to identify equivalent Affymetrix transcript cluster IDs for 32 of the transcripts reported by Huang *et al*. (2004) as being up-regulated by conventional T cell selection (Fig. 5.4). Of these, 8 transcripts had a U statistic of zero across

Transition 1 (NS, Chi-square contingency table), 24 did across Transition 2 ($p<0.0001$) and 12 did across Transition 3 ($p<0.02$). Of the transcripts that showed a U Statistic of 0 across transitions, 21 transcripts were up-regulated in immature CD4 NKT cells compared to immature DP NKT cells, across Transition 2. In contrast, only two were up-regulated across Transition 1, two were up-regulated across Transition 3, and none were increased in expression by >25%. These data are consistent with NKT cell selection occurring across Transition 2.

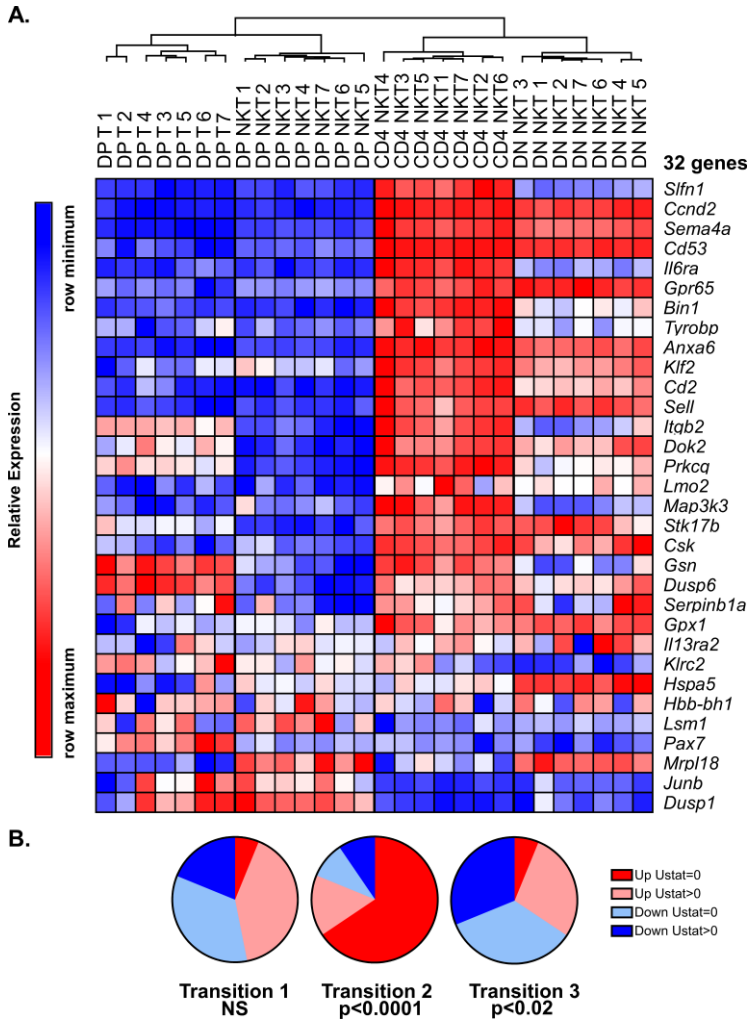


**Figure 5.4. Expression profiles of 32 transcripts, which were reported by Huang *et al*. (2004) to be involved in positive selection of conventional T cells. A**. The heat map shows the expression of these 32 transcripts by 4 different cell types, 7 biological samples per cell type. **B**. Pie charts show proportions of 32 transcripts with 4 different statistic categories (up-regulated and U statistic = 0, up-regulated and U statistic > 0, down-regulated and U statistic = 0, and down-regulated and U statistic > 0) across each of the 3 transitions.

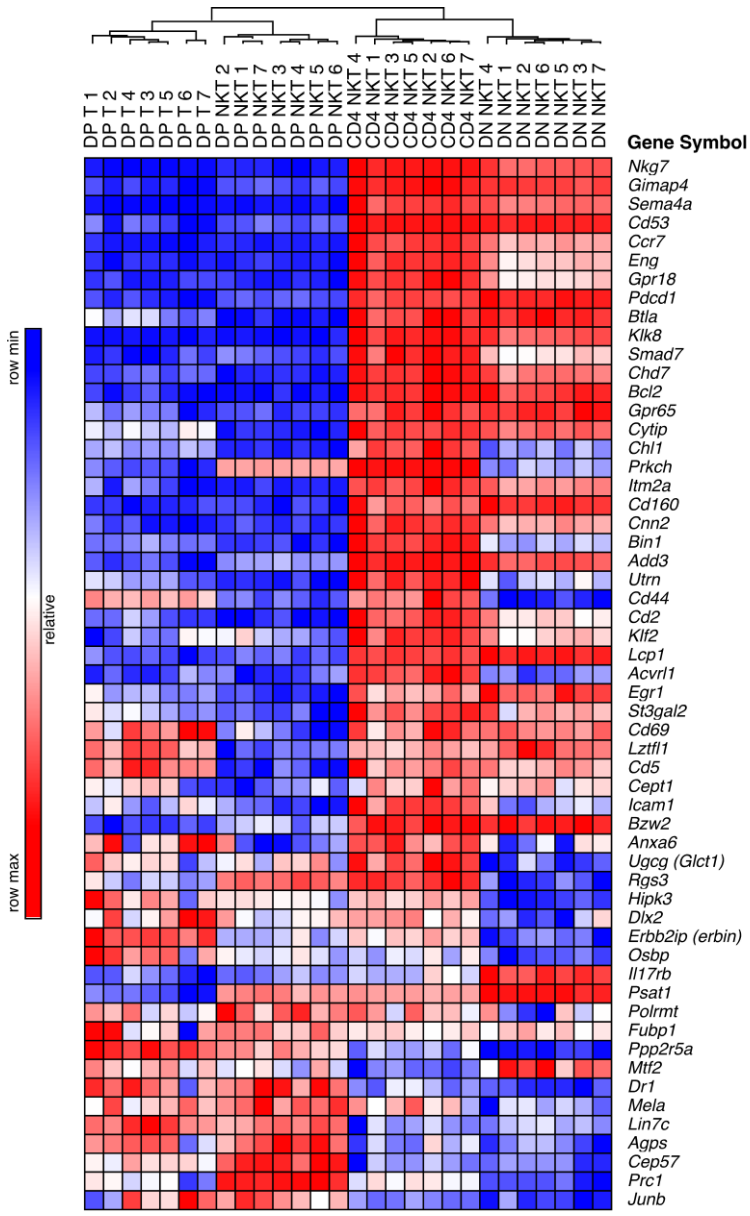


**Figure 5.5. Expression profiles of 56 transcripts, which were reported by Mick *et al*. (2004) to be associated with positive selection of conventional T cells.** The heat map shows the expression profiles of these 56 transcripts by 4 different cell types, 7 biological samples per cell type.

Mick *et al.* (2004) investigated genes regulated during thymic positive selection by comparing gene expression changes in $CD4^{+}CD8^{+}$ (double positive – DP) thymocytes from OT-I transgenic mice that have received a TCR signal with those from OT-I/$TAP^{-/-}$ transgenic mice that had not received a signal. Their data indicated that 66 genes were up-regulated during positive selection of conventional T cells. Compared to these 66 genes, we identified 56 genes in our microarray data. Expression comparison of these 56 genes across the 3 transitions indicated that 35 genes were significantly up-regulated across Transition 2 (Mann-

Whitney U statistic =0; Fig. 5.5). In contrast, only 11 genes were significantly up-regulated across Transition 1 and 8 genes across Transition 3. These data further supported the hypothesis that positive selection of NKT cells occurs across Transition 2, between DP NKT cells and CD4 NKT cells.

Taken together, the above findings suggested that positive selection of NKT cells is occurring between DP NKT cells and CD4 NKT cells and that DP NKT cells represent a pre-selection population.

### 5.2.2. Functional validation of positive and negative selection

In order to functionally validate the findings from the microarray analysis that positive selection of NKT cells occurs across Transition 2, we examined the effects of positive and negative selection on immature NKT cells.

#### 5.2.2.1. Effects of failed positive selection on NKT cells in *Vα14*Tg mice

Thymic positive selection is the preferential differentiation of thymocytes with moderate avidity for the endogenous ligands of their TCR (Zuñiga-Pflucker *et al*., 1989). It is well demonstrated that CD1d functions as an antigen-presenting molecule to present endogenous glycolipid ligands for positive selection of NKT cells (Bendelac, 1995; Cole and Raulet, 2000; Wei *et al*., 2005; Godfrey and Berzins, 2007). Thus, in order to test the hypothesis that positive selection occurs across Transition 2 and that immature DP NKT cells in the thymi of NOD.*Vα14*Tg mice represent a pre-selection population, we examined the effects of targeted deletion of CD1d on the development of immature NKT cells in *Vα14* transgenic mice.

##### 5.2.2.1.1. Effects of failed positive selection on NKT cells in NOD.*Vα14*Tg mice

Targeted deletion of CD1d was performed on NOD.*Vα14*Tg mice by crossing NOD.*Vα14*Tg mice with NOD.*Cd1d*$^{-/-}$ mice. The F1 mice were intercrossed to generate

NOD.*Cd1d*$^{-/-}$.*Vα14*Tg mice. Thymi and spleens were collected from 6-8 week old NOD WT, NOD.*Cd1d*$^{-/-}$, NOD.*Vα14*Tg and NOD.*Cd1d*$^{-/-}$.*Vα14*Tg mice and single cell suspensions were prepared. Cells were then stained with fluorescent conjugated antibodies and analysed using flow cytometry.

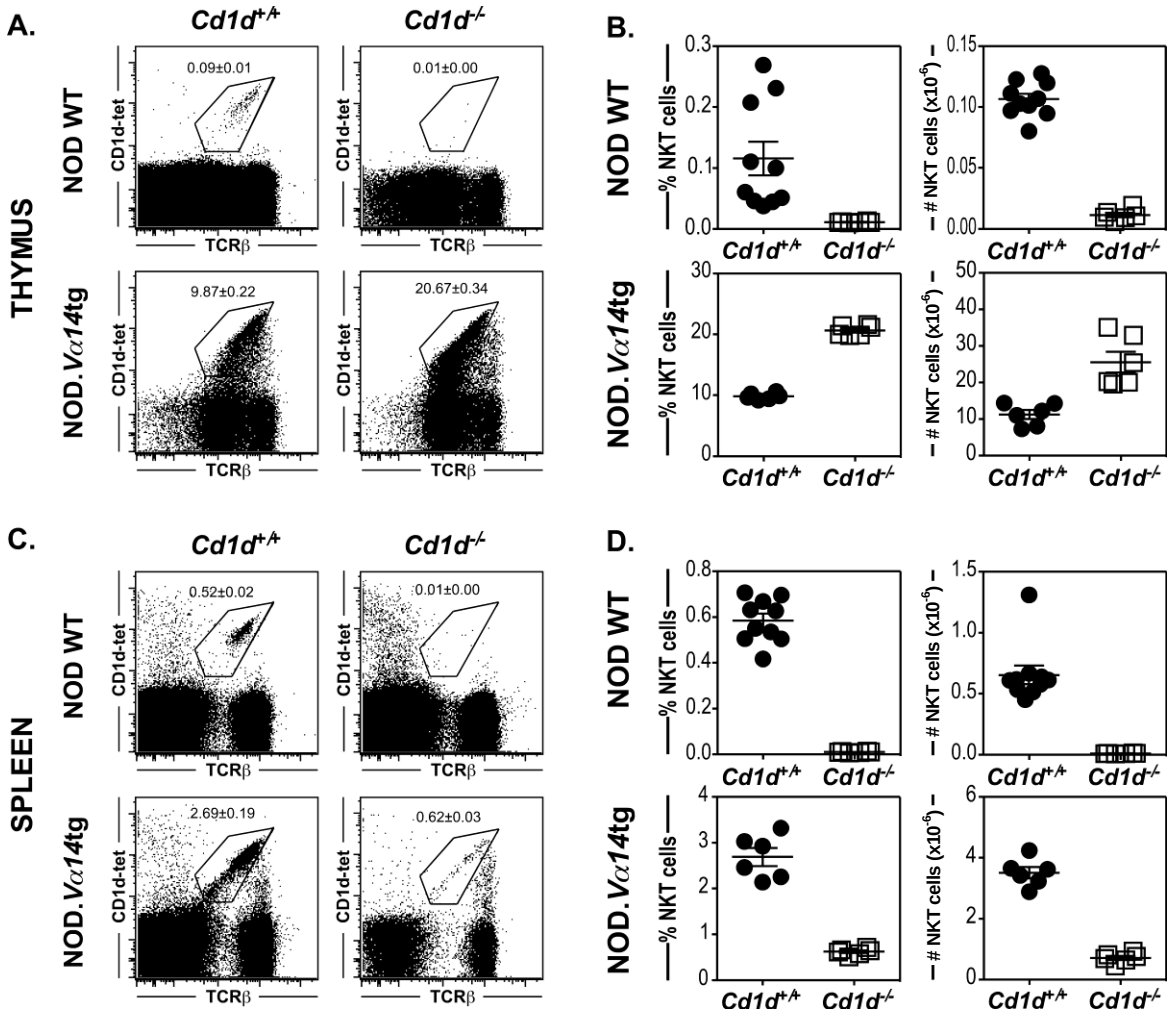


**Figure 5.6. Effects of targeted deletion of CD1d on NKT cells from NOD.*Vα14*Tg mice. A.** Representative FACS plots show the mean frequencies (±SEM) of thymic NKT cells in total thymocytes of NOD WT, NOD.*Cd1d*$^{-/-}$, NOD.*Vα14*Tg and NOD.*Cd1d*$^{-/-}$.*Vα14*Tg mice. **B**. Dot plots show mean frequencies and absolute numbers of thymic NKT cells. **C.** Representative FACS plots show the mean frequencies (±SEM) of splenic NKT cells in total splenocytes of NOD WT, NOD.*Cd1d*$^{-/-}$, NOD.*Vα14*Tg and NOD.*Cd1d*$^{-/-}$.*Vα14*Tg mice. **D.** Dot plots show mean frequencies and absolute numbers of splenic NKT cells. n=10 for NOD WT group, n=6 for NOD.*Cd1d*$^{-/-}$, NOD.*Vα14*Tg and NOD.*Cd1d*$^{-/-}$.*Vα14*Tg groups.

Flow cytometric analysis indicated that in NOD WT mice, targeted deletion of CD1d resulted in the loss of almost all NKT cells (identified as $TCR\beta^+$ CD1d-$tet^+$), in both the thymus and the periphery, in accordance with previous findings (Fig. 5.6; Bendelac, 1995; Cole and Raulet, 2000; Wei *et al*., 2005).

In sharp contrast, thymic NKT cells from NOD.*Cd1d*$^{-/-}$.*Vα14*Tg mice were significantly increased compared to NOD.*Vα14*Tg mice, although NKT cells in the periphery were severely depleted (Fig. 5.6). Both the proportions and the absolute numbers of thymic NKT cells were more than doubled. For example, the proportion of thymic NKT cells increased from 10% of total thymocytes in NOD.*Vα14*Tg mice to 20% in NOD.*Cd1d*$^{-/-}$.*Vα14*Tg mice ($p<0.01$; Mann-Whitney U test); whereas the absolute numbers of thymic NKT cells rose from 11.6 x $10^6$ cells in NOD.*Vα14*Tg mice to 22.8 x $10^6$ cells in NOD.*Cd1d*$^{-/-}$.*Vα14*Tg mice ($p<0.01$; Fig. 5.6).

The massive expansion of NKT cells in the thymus and their absence in the periphery of NOD.*Cd1d*$^{-/-}$.*Vα14*Tg mice suggested that thymic NKT cells were precluded from undergoing positive selection in the absence of the antigen presenting molecule CD1d. To identify the major population contributing to the increase in thymic NKT cells in NOD.*Cd1d*$^{-/-}$.*Vα14*Tg mice or identify the stage at which NKT cells undergo positive selection, thymic NKT cell subsets were examined using stage-specific markers (CD4/CD8, CD24/CD44 and CD44/NK1.1) and presented in Figure 5.7.

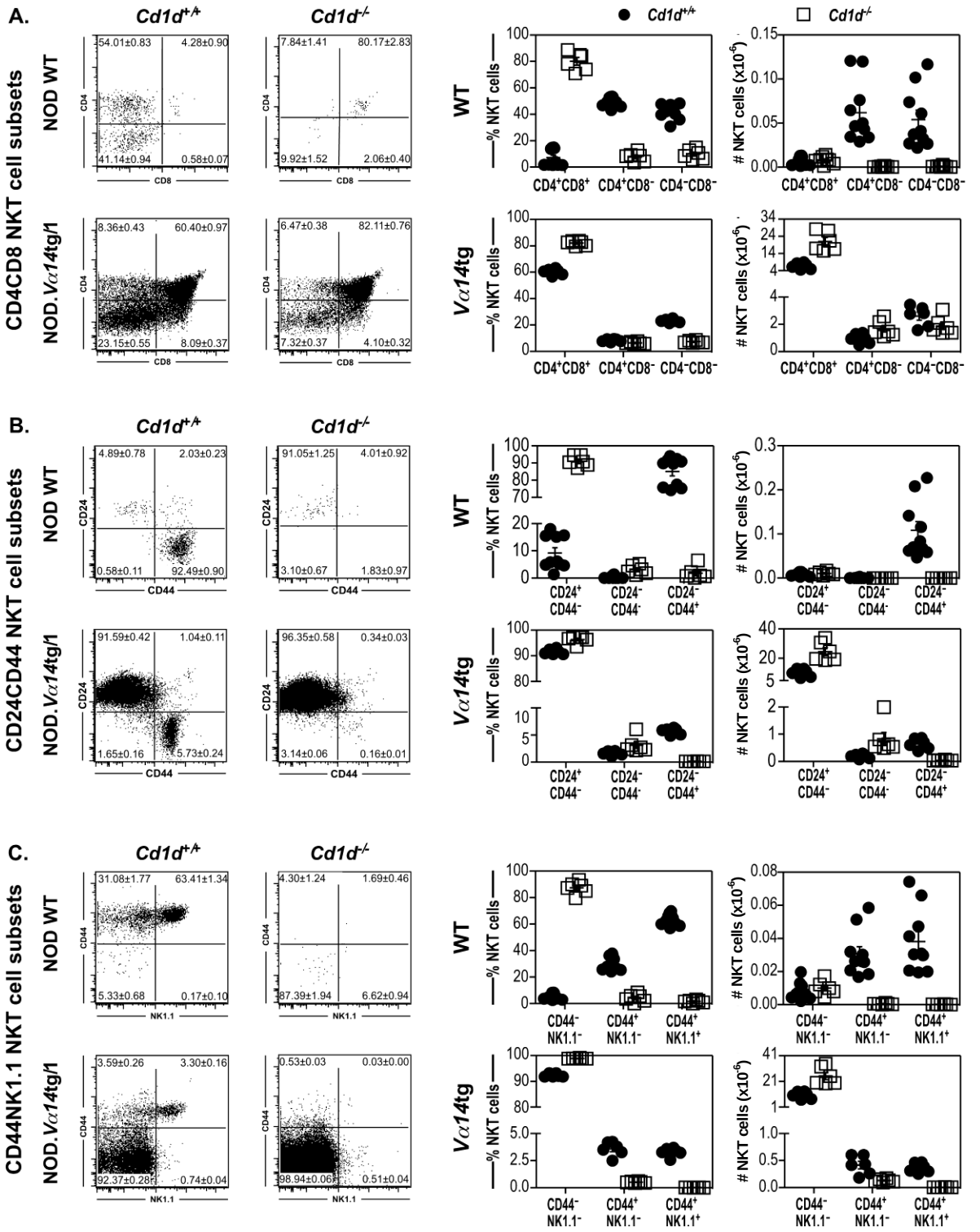


**Figure 5.7. Effects of targeted deletion of CD1d on thymic NKT cell subsets in NOD WT and NOD.*Vα14*Tg mice. A.** CD4/CD8 NKT cell subsets. **B**. CD24/CD44 NKT cell subsets. **C**. CD44/NK1.1 NKT cell subsets. For all, representative FACS plots show the mean frequencies (±SEM) of thymic NKT cell subsets in total NKT cells from the thymi of NOD WT, NOD.*Cd1d*$^{-/-}$, NOD.*Vα14*Tg and NOD.*Cd1d*$^{-/-}$.*Vα14*Tg mice. Dot plots show mean frequencies and absolute numbers of thymic NKT cell subsets in total NKT cells. The NKT cell gates applied are illustrated in Figure 5.6 A. n=10 for NOD WT group, n=6 for NOD.*Cd1d*$^{-/-}$, NOD.*Vα14*Tg and NOD.*Cd1d*$^{-/-}$.*Vα14*Tg groups.

Flow cytometric analysis of thymic NKT cell subsets revealed that, in NOD WT mice, NKT cells predominantly expressed a mature phenotype with either $CD4^+$ or DN, $CD24^{low}$ $CD44^{high}$. In sharp contrast, in NOD.*Cd1d*$^{-/-}$ mice, the few remaining NKT cells in the thymi showed an immature phenotype with 80% DP, 91% $CD24^{high}$ $CD44^{low}$ and 87% $CD44^{low}$ $NK1.1^-$ (Fig. 5.7). In NOD.*Cd1d*$^{-/-}$.*Vα14*Tg mice, the proportion of thymic DP NKT cells rose from 60% of total thymocytes in NOD.*Vα14*Tg mice to over 80% in NOD.*Cd1d*$^{-/-}$.*Vα14*Tg mice ($p<0.005$; Mann-Whitney U test; Fig. 5.7 A). The vast majority (>96%) of thymic NKT cells in NOD.*Cd1d*$^{-/-}$.*Vα14*Tg mice expressed the immature $CD24^{high}$ $CD44^{low}$ $NK1.1^-$ phenotype (Fig. 5.7 B and C).

With regard to absolute numbers, thymic DP NKT cells increased more than 3-fold, from 6.8 x $10^6$ cells in NOD.*Vα14*Tg mice to 21 x $10^6$ cells in NOD.*Cd1d*$^{-/-}$.*Vα14*Tg mice ($p<0.005$; Mann-Whitney U test; Fig. 5.7 A). NOD.*Vα14*Tg mice had 8.3 x $10^6$ $CD24^{high}$ $CD44^{low}$ NKT cells, while NOD.*Cd1d*$^{-/-}$.*Vα14*Tg mice had a more than 2-fold increase to 21 x $10^6$ cells ($p<0.005$). In contrast, mature thymic $CD24^{low}$ $CD44^{high}$ NKT cells were almost completely depleted in NOD.*Cd1d*$^{-/-}$.*Vα14*Tg mice. The numbers of mature thymic $CD24^{low}$ $CD44^{high}$ NKT cells decreased more than 20-fold, from 6.0 x $10^5$ cells in NOD.*Vα14*Tg mice to 0.29 x $10^5$ cells in NOD.*Cd1d*$^{-/-}$.*Vα14*Tg mice ($p<0.005$). These data indicate that the increase in thymic NKT cells was primarily due to an increase in numbers of immature DP NKT cells. The maturational arrest of immature DP NKT cells in the thymi of NOD.*Cd1d*$^{-/-}$.*Vα14*Tg mice suggested that these immature DP NKT cells failed to undergo positive selection; they could not develop further in the absence of the antigen presenting molecule, CD1d for positive selection. This finding supported the hypothesis that the $DP^{high}$ $CD24^{high}$ $CD44^{low}$ $NK1.1^-$ NKT cells represent a pre-selection population.

**5.2.2.1.2. Effects of failed positive selection on immature NKT cells in B6.*Vα14*Tg mice**

It has been reported that there are deficiencies in NKT cell development in NOD WT mice compared to other commonly used mouse strains, which result in the severe reduction in both NKT cell numbers and function (Gombert *et al*., 1996; Baxter *et al*., 1997; Godfrey *et al*., 1997; Poulton *et al*., 2001; Hammond *et al*., 2001). Therefore, to confirm that the maturational arrest of immature DP NKT cells in NOD.*Vα14*Tg mice is the consequence of the absence of CD1d endogenous ligand, *Cd1d* was depleted from B6.*Vα14*Tg mice by crossing B6.*Vα14*Tg mice with B6.*Cd1d*$^{-/-}$ mice. The resulting F1 mice were intercrossed to generate B6.*Cd1d*$^{-/-}$.*Vα14*Tg mice. Thymi, spleens and livers were harvested from 6-8 week old mice for flow cytometric comparison of their NKT cells to those of the background strain.

Flow cytometric analysis revealed that in B6 WT mice, the targeted deletion of CD1d also resulted in the loss of almost all NKT cells (identified as TCRβ$^{+}$ CD1d-tet$^{+}$) in the thymi, livers and spleens (Fig. 5.8). In contrast, the number of thymic NKT cells in B6.*Cd1d*$^{-/-}$.*Vα14*Tg mice was more than quadrupled, concordant with very few peripheral NKT cells. The proportion of thymic NKT cells increased from 7.8% of total thymocytes of B6.*Vα14*Tg mice to almost 21% in B6.*Cd1d*$^{-/-}$.*Vα14*Tg mice. The absolute numbers of thymic NKT cells rose from 3.4 x $10^6$ cells in B6.*Vα14*Tg mice to about 20 x $10^6$ cells in B6.*Cd1d*$^{-/-}$.*Vα14*Tg (Fig. 5.8). These data indicated that targeted deletion of CD1d in B6.*Vα14*Tg mice showed a similar effect on NKT cells as it did on those from NOD.*Vα14*Tg mice, characterised by the massive increase in thymic NKT cell numbers and the depletion of peripheral NKT cells.

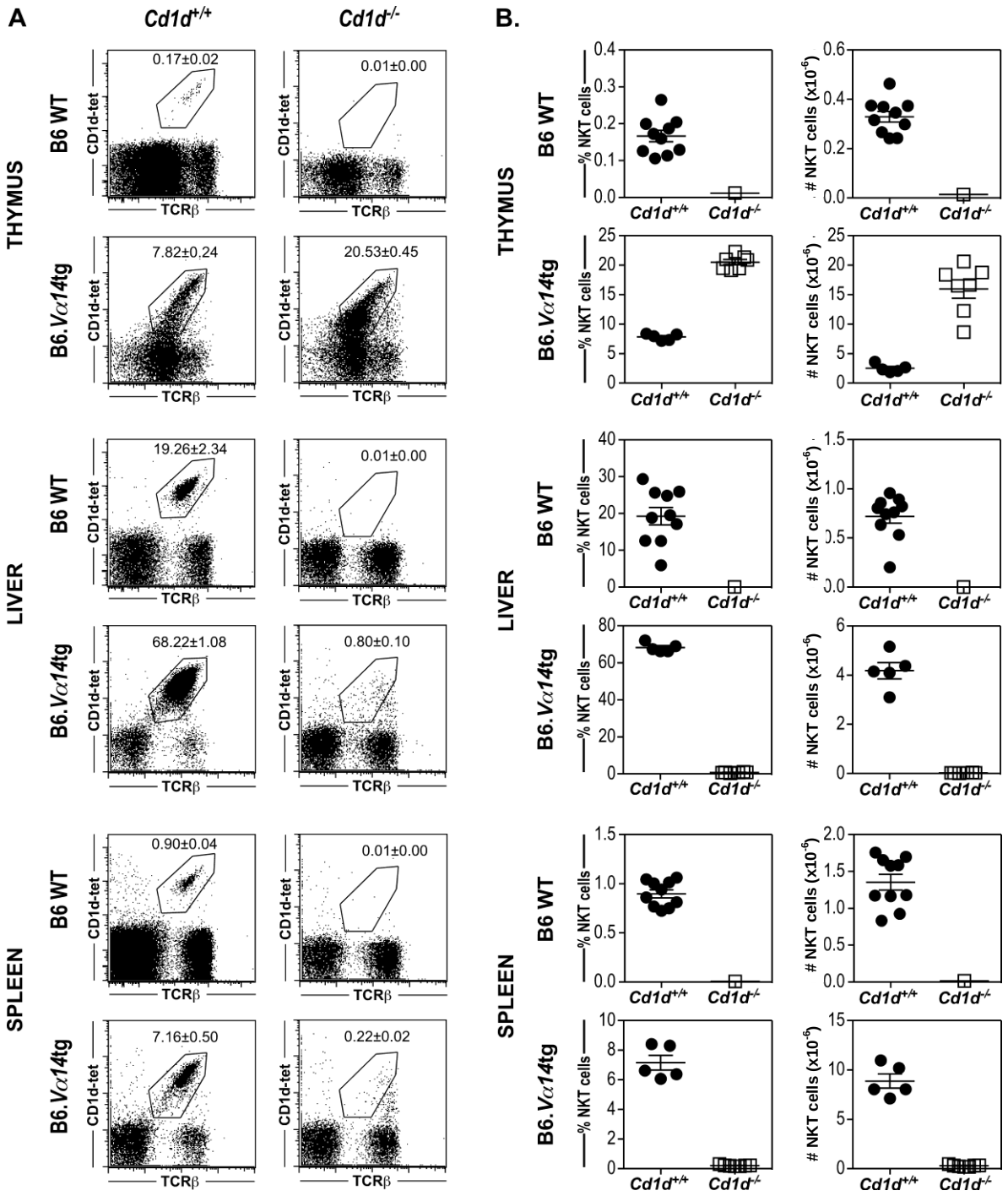


**Figure 5.8. Effects of targeted deletion of CD1d on NKT cells in B6.*Vα14*Tg mice. A.** Representative FACS plots show the mean frequencies (±SEM) of thymic, hepatic and splenic NKT cells in total cells from the thymi, livers and spleens, respectively, of B6 WT, B6.*Cd1d*$^{-/-}$, B6.*Vα14*Tg and B6.*Cd1d*$^{-/-}$.*Vα14*Tg mice. **B**. Dot plots show mean frequencies and absolute numbers of thymic, hepatic and splenic NKT cells. n=10 for B6 WT, n=1 for B6.*Cd1d*$^{-/-}$, n=5 for B6.*Vα14*Tg and n=7 for B6.*Cd1d*$^{-/-}$.*Vα14*Tg mice.

We next investigated the thymic NKT cell subsets to determine which were affected by the absence of CD1d. Thymic NKT cell subsets were compared between B6.*Vα14*Tg and B6.*Cd1d*$^{-/-}$.*Vα14*Tg mice by flow cytometric analysis using stage-specific markers CD4/CD8, CD24/CD44 and CD44/NK1.1. Our data revealed that the increase in thymic NKT cells was largely attributed to the accumulation of immature DP NKT cells. As indicated in Figure 5.9 A, the proportion of immature thymic DP NKT cells rose from 48% of total thymocytes in B6.*Vα14*Tg mice to over 85% in B6.*Cd1d*$^{-/-}$.*Vα14*Tg mice. With regard to absolute numbers, thymic DP NKT cells increased more than 10 times, from 1.2 x $10^6$ cells in B6.*Vα14*Tg mice to 13.6 x $10^6$ cells in B6.*Cd1d*$^{-/-}$.*Vα14*Tg mice ($p<0.005$; Fig. 5.9 A).

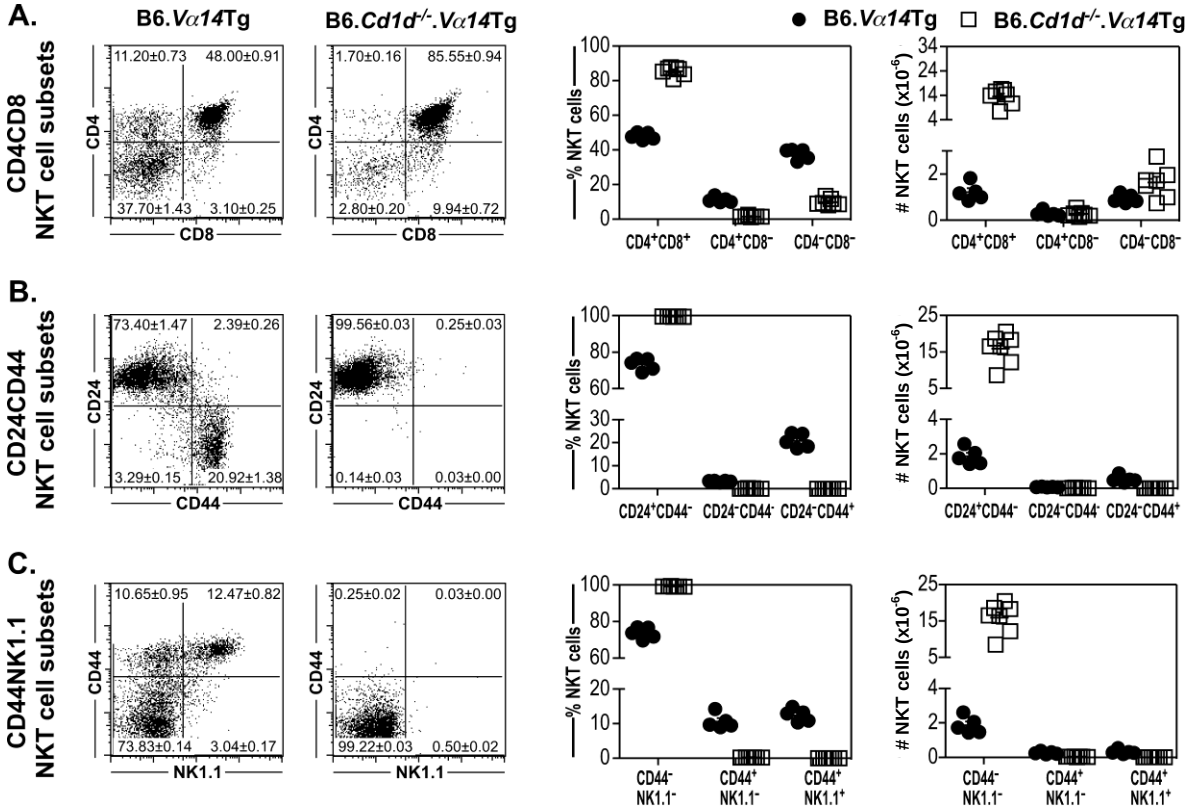


**Figure 5.9. Effects of targeted deletion of CD1d on NKT cell subsets in B6.*Vα14*Tg mice. A.** CD4/CD8 NKT cell subsets. **B**. CD24/CD44 NKT cell subsets. **C**. CD44/NK1.1 NKT cell subsets. For all, representative FACS plots (left panels) show the mean frequencies (±SEM) of thymic NKT cell subsets in total NKT cells from the thymi of B6.*Vα14*Tg and B6.*Cd1d*$^{-/-}$.*Vα14*Tg mice. Dot plots (right panels) show mean frequencies and absolute numbers of thymic NKT cell subsets in total NKT cells. The NKT cell gates applied are illustrated in Figure 5.8. n=5 for B6.*Vα14*Tg and n=7 for B6.*Cd1d*$^{-/-}$.*Vα14*Tg mice.

With regard to CD24, CD44 and NK1.1 expression, the vast majority (>99%) of thymic NKT cells expressed the immature $CD24^{high}$ $CD44^{low}$ $NK1.1^{-}$ phenotype (Fig. 5.9 B and C). While B6.*Vα14*Tg mice had 1.9 x $10^6$ $CD24^{high}$ $CD44^{low}$ NKT cells, B6.*Cd1d*$^{-/-}$.*Vα14*Tg mice had a >8-fold increase to 16 x $10^6$ cells ($p<0.005$; Mann-Whitney U test). In contrast, mature $CD24^{low}$ $CD44^{high}$ NKT cells were profoundly diminished in the thymi of B6.*Cd1d*$^{-/-}$.*Vα14*Tg. Their numbers fell more than 80-fold from 535,000 cells per thymus in B6.*Vα14*Tg mice to 6,500 cells in B6.*Cd1d*$^{-/-}$.*Vα14*Tg mice ($p<0.005$). In summary, there was a maturational arrest of immature DP NKT cells in the thymi of B6.*Cd1d*$^{-/-}$.*Vα14*Tg mice, similar to that found in NOD.*Cd1d*$^{-/-}$.*Vα14*Tg mice. The immature thymic DP NKT cells from both NOD and B6.*Vα14*Tg mice failed to undergo positive selection in the absence of CD1d. Taken together, these data also strongly support the hypothesis that the $DP^{high}$ $CD24^{high}$ $CD44^{low}NK1.1^{-}$ NKT cells represent a pre-selection population.

**5.2.2.2. Effects of enhanced negative selection by α-GalCer on NKT cells**

If immature DP NKT cells are a pre-selection population of NKT cells, they are likely subjected to negative selection. Negative selection in the thymus results in the clonal deletion (apoptosis) of thymocytes with high avidity for the endogenous ligands of their TCR. As the frequency of T precursors for any given antigen is generally low in WT animals, *in vivo* models of negative selection either involve in administration of anti-TCR/CD3 antibodies (Kishimoto and Sprent, 1997), responses to endogenous or administered superantigens that ligate whole families of TCR Vβ chains (Kappler *et al*., 1988; Jenkinson, 1989), or the use of TCR transgenic mice (Baldwin *et al.,* 2005). While α-GalCer is not technically a superantigen, it is a strong NKT cell antigen glycolipid capable of activating the vast majority of Vα14-Jα18 expressing NKT cells following presentation in the context of CD1d (Kawano *et al*., 1997), and therefore capable of causing their negative selection if administered

systemically or intrathymically (Pellicci *et al*., 2003). We made use of α-GalCer to follow negative selection in NKT cell development.

**5.2.2.2.1. Effects of intravenous (i.v.) injection of α-GalCer on NKT cells in NOD.*Vα14*Tg mice**

The effect of negative selection on $DP^{high}$ $CD24^{high}$ $CD44^{low}$ $NK1.1^{-}$ NKT cells in the thymi of NOD.*Vα14*Tg mice was examined by i.v. injection of 4 μg α-GalCer, and subsequent analysis by flow cytometry. Within 40 hours of injection, the proportion of thymic NKT cells (identified as CD1d-$tet^{+}$$TCR\beta^{+}$) reduced from 7.7% of total thymocytes in control NOD.*Vα14*Tg, injected with PBS, to 5.4% in α-GalCer injected mice (Fig. 5.10).

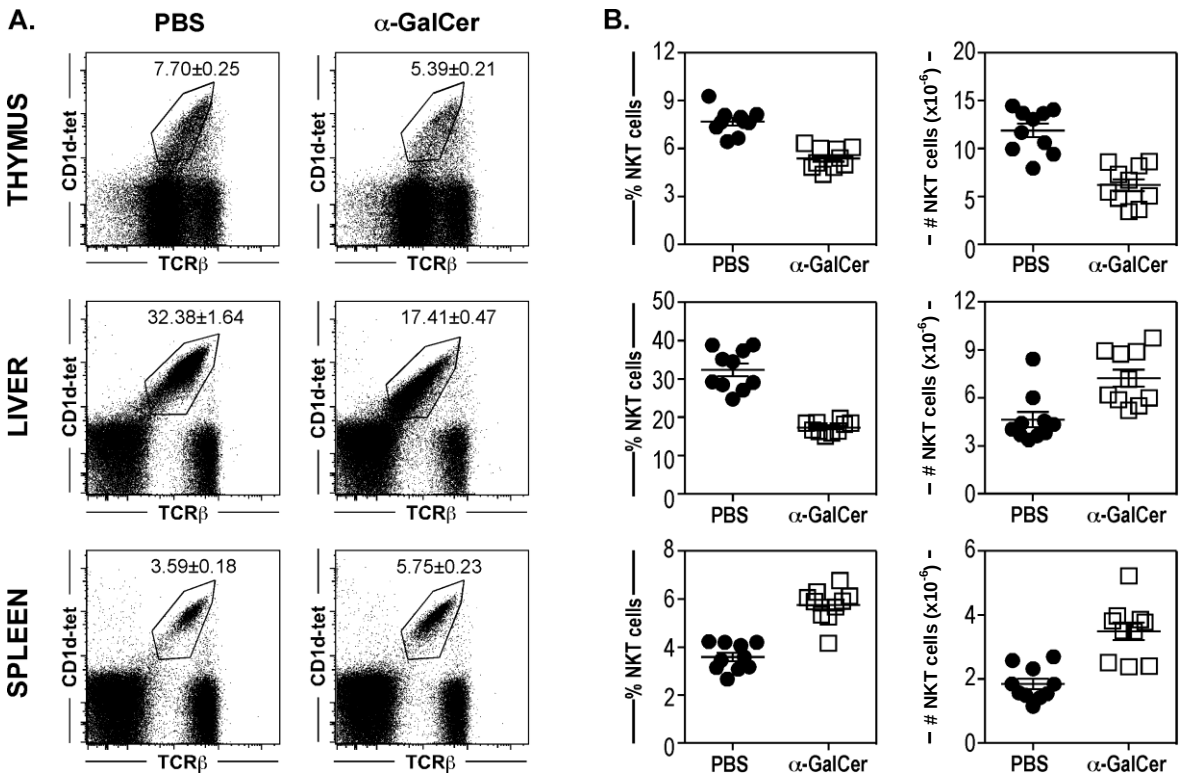


**Figure 5.10. Effects of enhanced negative selection on NKT cells in NOD.*Vα14*Tg mice by i.v. α-GalCer administration. A.** Representative FACS plots show the mean frequencies (±SEM) of NKT cells in total cells from the thymi, livers and spleens of PBS and α-GalCer injected NOD.*Vα14*Tg mice. **B**. Dot plots show mean frequencies and absolute numbers of thymic, hepatic and splenic NKT cells. n=10 for each group.

In terms of absolute numbers, thymic NKT cells had halved, from 12 x $10^6$ cells to 5.4 x $10^6$ cells after α-Galcer injection ($p<0.0001$; Mann-Whitney U test; Fig. 5.10), while total thymocytes had only slightly decreased from 1.6 x $10^8$ cells in NOD.*Vα14*Tg control mice to 1.2 x $10^8$ cells in α-GalCer injected mice ($p<0.05$). In contrast, peripheral NKT cells increased in NOD.*Vα14*Tg mice injected with α-GalCer, from 1.9 x $10^6$ cells per liver of the control mice to 3.5 x $10^6$ cells per liver of the treated mice ($p<0.005$), and from 4.7 x $10^6$ to 7.2 x $10^6$ in the spleens ($p<0.005$; Fig. 5.10). These data are consistent with peripheral antigen-induced activation and proliferation.

Flow cytometric analyses of thymic NKT cell subsets revealed that the majority of the reduction in thymic NKT cells was contributed by reduction of the DP subset, which fell from 63% of total thymic NKT cells in control mice to 48% in α-GalCer injected mice ($p<0.0001$; Mann-Whiney U test; Fig 5.11 A). In terms of absolute numbers, thymic DP NKT cells decreased from 7.5 x $10^6$ cells in control mice to 3.1 x $10^6$ cells in α-GalCer injected mice ($p<0.0001$; Fig 5.11 A). In contrast, the proportions of the more mature DN and $CD4^+$ NKT cells was significantly increased in the α-GalCer treated mice ($p<0.0001$), although they showed modest reductions in terms of absolute numbers ($CD4^+$ NS; DN $p<0.01$).

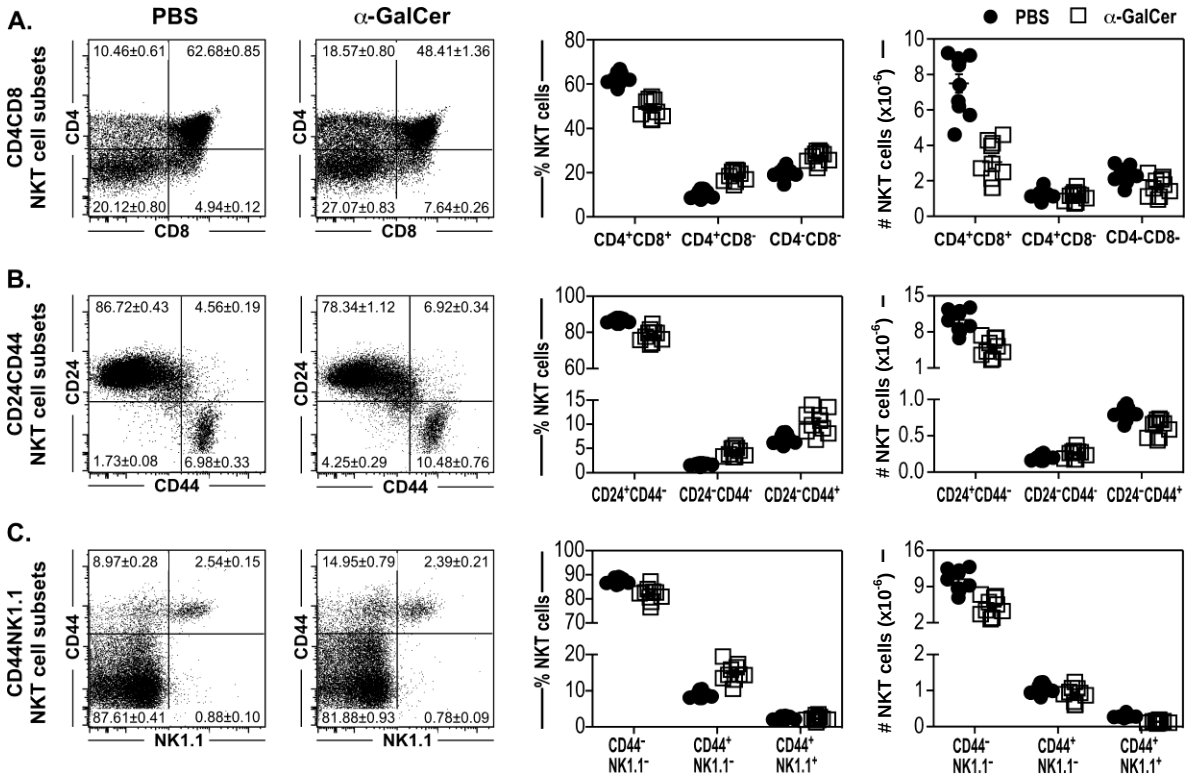


**Figure 5.11. Effects of enhanced negative selection on thymic NKT cell subsets in NOD.*Vα14*Tg mice by i.v. α-GalCer administration. A.** CD4/CD8 NKT cell subsets. **B**. CD24/CD44 NKT cell subsets. **C**. CD44/NK1.1 NKT cell subsets. For all, representative FACS plots (left panels) show the mean frequencies (±SEM) of thymic NKT cell subsets in total NKT cells from the thymi of PBS and α-GalCer injected NOD.*Vα14*Tg mice. Dot plots (right panels) show mean frequencies and absolute numbers of thymic NKT cell subsets in total NKT cells. n=10 for each group.

With regard to CD24, CD44 and NK1.1 expression, α-GalCer administration affected only the immature thymic $CD24^{high}$ $CD44^{low}$ $NK1.1^{-}$ NKT subset. There were significant reductions in both proportions and absolute numbers of $CD24^{high}$ $CD44^{low}$ and $CD44^{low}NK.1^{-}$ NKT cells in the thymi of α-GalCer treated mice. In contrast, the absolute numbers of semi-mature $CD44^{high}NK1.1^{-}$ and mature $CD44^{high}NK1.1^{+}$ NKT cells were unchanged. Taken together, the 60% reduction in DP NKT cell numbers following α-GalCer injection is consistent with this population being subjected to negative selection.

**5.2.2.2.2. Effects of intrathymic (i.t.) injection of $\alpha$-GalCer on NKT cells in NOD.*Vα14*Tg mice**

Delivery of superantigen is a preferred model of negative selection when selection ligands are exogenous. Indeed, as the negative selectable cohort of cells remains intact before encountering selecting ligands, cell death caused by negative selection can easily be visualized. It also allows the study of the fate of immature and mature cells following ligand exposure in the same system. However, thymocyte deletion can also be caused nonspecifically by the concomitant widespread activation of peripheral T cells and subsequent cytokine release (Zhan *et al.,* 2003). Thus, to minimise the effects of systemic T cell activation in our model of enhanced negative selection of immature NKT cells, NOD.*Vα14*Tg mice were subjected to intrathymic injection of 2 µg α-GalCer. NKT cell numbers and subsets from the thymus, liver and spleen were then examined 40 hours later.

Flow cytometric cell analyses indicated that the proportion of thymic NKT cells decreased from 4.1% of total thymocytes in control NOD.*Vα14*Tg mice to 2.5% in NOD.*Vα14*Tg mice injected intrathymically with α-GalCer (Fig. 5.12). In terms of absolute numbers, thymic NKT cells fell from 4.0 x $10^6$ to 2.3 x $10^6$ cells after intrathymic administration of α-GalCer. In contrast, the hepatic and splenic NKT cell numbers were unchanged between PBS controls and α-GalCer injected mice (Fig. 5.12).

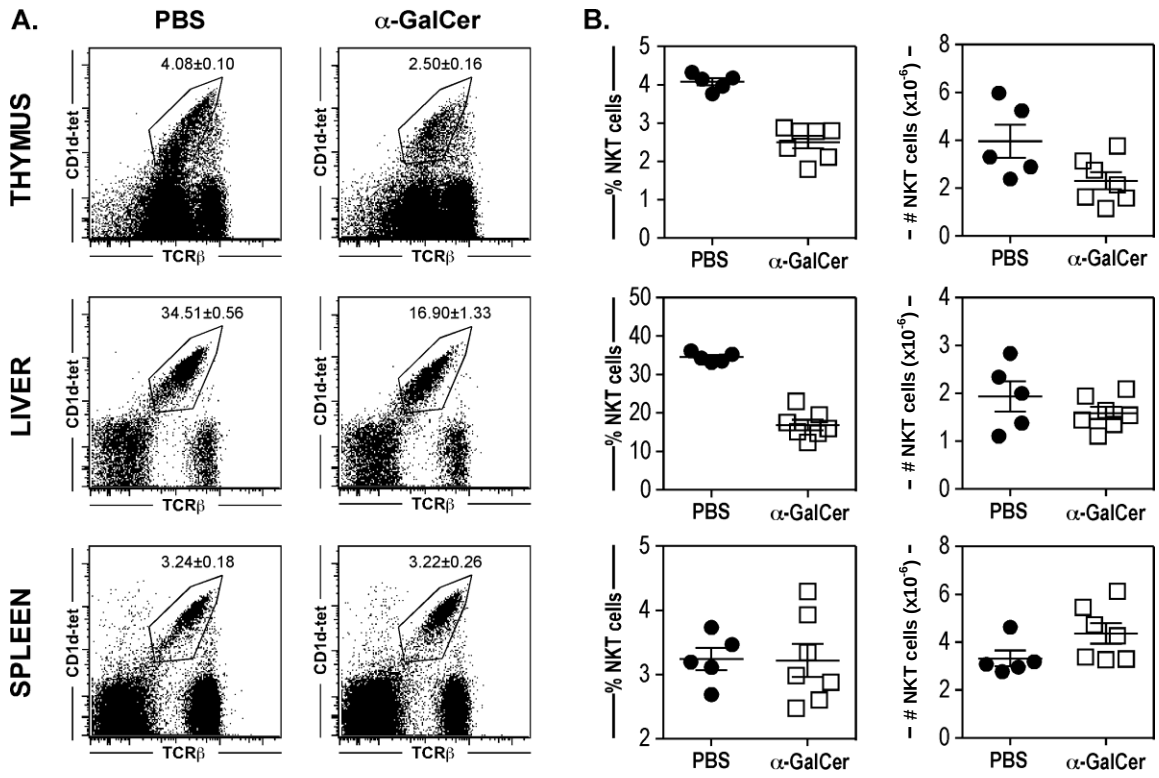


**Figure 5.12. Effects of enhanced negative selection on NKT cells in NOD.*Vα14*Tg mice following intrathymic administration of α-GalCer. A.** Representative FACS plots show the mean frequencies (±SEM) of thymic, hepatic and splenic NKT cells in total cells from the thymi, livers and spleens, respectively, of PBS and α-GalCer injected NOD.*Vα14*Tg mice. **B**. Dot plots show mean frequencies and absolute numbers of thymic, hepatic and splenic NKT cells. n=5 for PBS injected control mice and n=7 for mice injected intrathymically with α-GalCer.

With regard to NKT cell subsets, the proportion of thymic NKT cells that were DP fell from 59% of total thymocytes in PBS controls to 18% following intrathymic injection of α-GalCer ($p<0.005$; Mann-Whitney U Test; Fig. 5.13 A). Again, the proportions of the more mature CD4 SP and DN thymic NKT cells increased, from 13.7% to 29.5% ($p<0.005$) and from 23% to 47% ($p<0.005$), respectively, although their absolute numbers did not change significantly (Fig. 5.13 A). Both proportions and absolute numbers of thymic $CD24^{high}CD44^{low}$ and $CD44^{low}NK1.1^{-}$ NKT subsets significantly reduced in intrathymically injected compared to control mice (Fig. 5.13 B, C). The specificity of the deletion induced by intrathymic α-GalCer on developing NKT cells, as distinct from conventional T cells, was illustrated by the relative depletion of $DP^{high}$ $CD24^{high}$ $CD44^{low}$ NKT cells (>82%) compared

to $DP^{high}$ $CD24^{high}$ $CD44^{low}$ conventional T cells (<19%; data not shown). These data suggested that the effects of intrathymic injection of α-GalCer on NKT cells in NOD.*Vα14*Tg mice were similar to those of systemic α-GalCer administration.

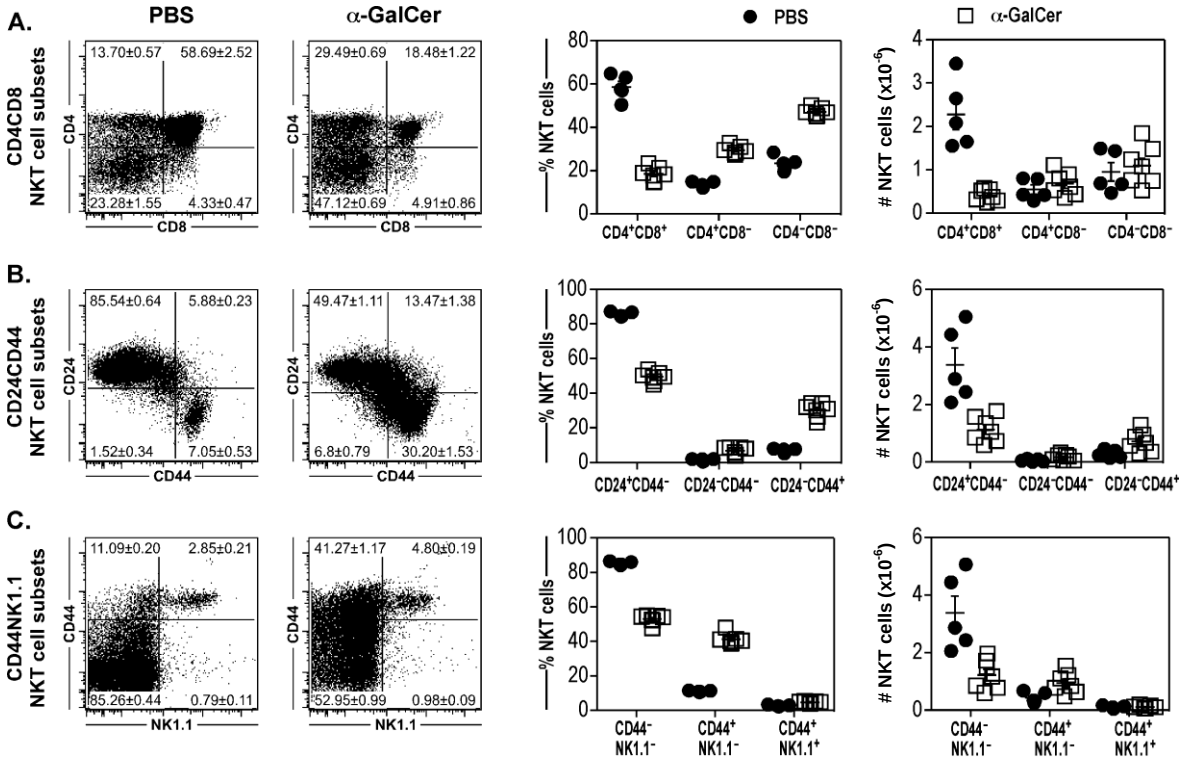


**Figure 5.13. Effects of enhanced negative selection on thymic NKT cell subsets in NOD.*Vα14*Tg mice following intrathymic administration of α-GalCer. A.** CD4/CD8 NKT cell subsets. **B**. CD24/CD44 NKT cell subsets. **C**. CD44/NK1.1 NKT cell subsets. For all, representative FACS plots (left panels) show the mean frequencies (±SEM) of thymic NKT cell subsets in total NKT cells from the thymi of PBS controls and intrathymically injected mice. Dot plots (right panels) show mean frequencies and absolute numbers of thymic NKT cell subsets in total NKT cells. n=5 for PBS injected control mice and n=7 for intrathymically injected mice.

Together, the failed positive selection and the susceptibility to negative selection of immature DP NKT cells supported the hypothesis that immature DP NKT cells represent a pre-selection population of NKT cells or represent the earliest NKT cell progenitors.

## 5.3. SUMMARY OF MAJOR FINDINGS

In this chapter, genome-wide gene expression comparison has defined transcriptional programs associated with the transformation of immature CD4 NKT cells to immature DP NKT cells. The results suggested that positive selection and lineage commitment of NKT cells occurs during this transition.

Gene Ontogeny analysis of up-regulated HDE genes across Transition 2 revealed the strong up-regulation of a wide range of functional lymphocyte-associated membrane proteins, such as Toll-like receptors, cytokine receptors, chemokine receptors, intergrins and leukocyte differentiation makers. Of these, there was a predominance of genes associated with the immunomodulatory and innate-like properties of NKT cells, such as *Tlr1, Nkg7, Sema4a, Art2b, S1pr1* and *Zbtb16*. These data suggested that Transition 2 is associated with NKT cell lineage commitment.

Commitment to the NKT cell lineage is well known to be associated with the expression of the innate-like lymphocyte-associated transcription factor Promyelocytic Leukemia Zinc Finger (PLZF, encoded by *Zbtb16*; Savage *et al*., 2008). While there was no significant difference in expression of *Zbtb16* during Transition 1 (between immature DP T cells and immature DP NKT cells), *Zbtb16* was up-regulated more than 13-fold during the subsequent transition from immature DP NKT cells to immature CD4 NKT stage (Mann-Whitney U statistic =0). This is consistent with immature CD4 NKT cells having been positively selected and having commenced lineage commitment.

The coordinated expression of PLZF and the subsequent up-regulation of a wide range of cell-surface functional receptors associated with NKT cell immunobiology combine to provide evidence that NKT cell lineage commitment occurred across Transition 2, i.e. between Stage 0 and Stage 1. However, it raised the issue of the timing of the NKT cell

selection event, which seems unlikely to take place any later than Transition 2. Many of the genes previously described as showing increased expression through positive selection of conventional T cells, were up-regulated in the immature CD4 NKT subset (Huang *et al*., 2004; Mick *et al*., 2004). These data are consistent with NKT cell selection having occurred across Transition 2.

By comparing gene expression between DP NKT cells and CD4 NKT cells, we have identified a large number of genes encoding molecules not previously known to affect the biology of type I NKT cells and many of them have been validated by qPCR and/or flow cytometry on an independent sample set.

While positive and negative selection of NKT cells has been studied previously and it is known that NKT cell development is markedly impaired by the absence of the selection molecule, CD1d (Bendelac, 1995; Chen *et al*., 1997; Mendiratta *et al*., 1997; Smiley *et al*., 1997; Cole and Raulet, 2000; Gapin *et al*., 2001; Wei *et al*., 2005) and that administration of α-GalCer at an early stage results in NKT cell development impairment (Pellicci *et al*., 2001; Chun *et al*., 2003). In these studies, due to the very low number of DP NKT cells in wild type mice, the effect of targeted deletion of CD1d and administration of α-GalCer were not directly examined on DP NKT cells but it was presumed that the effects were on the early development of NKT cells because the delay administration of α-GalCer (until day 18 of FTOC or at 8 weeks old) could not do so on differentiated NKT cells (Pellicci *et al*., 2003). The production of very large numbers of immature DP NKT cells in the thymi of our *Vα14* transgenic mice has now provided an opportunity to directly examine positive and negative selection in various subsets of NKT cells.

Targeted deletion of CD1d from both NOD and B6.*Vα14*Tg mice indicated that CD1d is required for further development of thymic immature DP NKT cells. In the absence of

CD1d, immature DP NKT cells did not progress to subsequent developmental stages and accumulated in the thymi of both NOD and B6.*Cd1d*$^{-/-}$.*Vα14*Tg mice. The almost complete absence of peripheral NKT cells in both NOD.*Cd1d*$^{-/-}$.*Vα14*Tg and B6.*Cd1d*$^{-/-}$.*Vα14*Tg mice is consistent with failed positive selection of DP NKT cells in the absence of CD1d. Additionally, immature DP NKT cells were also susceptible to negative selection mediated by the administration of α-GalCer, both intravenously and intrathymically. Together, these data are consistent with the hypothesis that DP NKT cells are a pre-selection population and that positive selection of NKT cells occurs between the immature DP and CD4$^{+}$ NKT cell development stages.

In conclusion, the data presented in this chapter provide an overall transcriptional profile of positive selection and lineage commitment of NKT cells. This is also the first time that the effects of positive and negative selection have been examined on their actual target population – the immature DP NKT cell subset.

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# CHAPTER 6

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# TRANSITION 3 -

# DIFFERENTIATION AND TRAFFICKING OF NKT CELLS

## 6.1. INTRODUCTION

NKT cells are a CD1d-dependent T cell subset that mediates potent immune-regulatory functions in settings of autoimmunity, cancer, infection and self-tolerance. NKT cells can be divided into $CD4^+$ and $CD4^-CD8^-$ double negative (DN) subsets in mice (Hammond *et al*., 1999; Eberl *et al.*, 1999), or $CD4^+$, DN, and $CD8^+$ subsets in humans (Prussin and Foster, 1997). NKT cells share their early developmental program with conventional T cells until the $CD4^+CD8^+$ (double positive - DP) stage. At this point NKT cells branch away from conventional T cells (Benlagha *et al*., 2002; 2005), and subsequently give rise to $CD4^+$ or DN NKT cells (Godfrey and Kronenberg, 2004; Godfrey *et al*., 2004). However, whether the DP NKT cells down-regulate CD4 or CD8 markers consecutively or together to become $CD4^+$ or DN NKT cells is still unknown.

NKT cells were originally reported as arising in the thymus 5 days after birth as $CD4^+$ $CD8^-$ $NK1.1^-$ NKT cells (Pellicci *et al*., 2002). They then become $CD4^+$ $CD8^-$ $NK1.1^+$ and DN $NK1.1^+$ by day 7-8, but still remained a minor subset until 3 weeks of age. By applying a CD1d-$tet^+$ enrichment strategy, Benlagha *et al*. (2005), however, identified $CD4^+$ $CD24^{high}$ $NK1.1^-$ NKT cells in the thymi of newborn mice. This cell population was demonstrated to give rise to DN NKT cells after an adoptive transfer into *Jα18*$^{-/-}$ mice. Contrary to the Pellicci study of 2002, Coquet *et al.* (2008) reported that $CD4^-NK1.1^-$ NKT cells could generate $CD4^-$ $NK1.1^+$ NKT cells in an adoptive transfer to fetal thymus organ culture (FTOC), but could not generate $CD4^+NK1.1^+$ NKT cells (Coquet *et al*., 2008). Furthermore, a low but consistent level of activation by endogenous antigen on the surface of peripheral APCs may also affect DN NKT cell differentiation in secondary lymphoid organs, because a large fraction of splenic $CD4^+$ NKT cells become DN NKT cells after repeated activation (Chen *et al*., 1997).

In addition to the developmental differences discussed above, $CD4^+$ and $CD4^-$ NKT

cells are also functionally distinct subsets. Human $CD4^+$ NKT cells were reported to make both Th1 and Th2 cytokines (such as IFN-γ, TNF, IL-4 and IL-13), whereas $CD4^-$ NKT cells primarily produce Th1 cytokines (IFN-γ and TNF; Lee *et al*., 2002; Gumperz *et al*., 2002). In contrast, mouse $CD4^+$ and DN NKT cells have no clear differences in cytokine production (Godfrey and Kronenberg, 2004). Both $CD4^+$ and DN NKT cells from mice are reported to produce large amounts of IFN-γ and IL-4 (Hammond *et al*., 1999; Matsuda *et al*., 2000; 2003; Stetson *et al*., 2003; Coquet *et al.*, 2007). However, $CD4^+$ and DN NKT cells are distinct in their ability to modulate immune responses *in vivo*. For example, hepatic DN NKT cells are able to control tumours more effectively than $CD4^+$ NKT cells from the same liver (Crowe *et al.*, 2005).

Recent work showed that, similar to Th cells, iNKT cells can be subdivided into three distinct lineages: NKT1, NKT2, and NKT17, and discriminated by the expression of cell surface markers or transcription factors (Lee *et al.,* 2016). While iNKT1 cells express T-bet ($T\text{-}bet^+$, $PLZF^{low}$, $ROR\gamma t^-$); iNKT2 cells express high levels of GATA3 and PLZF ($T\text{-}bet^-$, $PLZF^{high}$, $ROR\gamma t^-$) and iNKT17 cells have intermediate levels of PLZF, are $RoR\gamma t^+$ ($T\text{-}bet^-$, $PLZF^{int}$, $ROR\gamma t^+$) (Lee *et al.,* 2013). This method of defining iNKT cell subsets correlated precisely with cytokine production: thymic NKT1, NKT2 and NKT17 cells produced IFN-γ, IL-4, and IL-17, respectively, when stimulated with PMA and ionomycin (Lee *et al*., 2013). Each subpopulation expresses particular cytokine receptors: iNKT1 expresses IL-12R, iNKT2 expresses IL-17RB and iNKT17 expresses IL-23R (Watarai *et al*., 2012). When stimulated with the corresponding cytokine, each subset produced only the cytokine corresponding to their subset (Watarai *et al*., 2012).

Recent studies have also uncovered alternatively polarized NKT sublineages, including $T\text{-}bet^+$ NKT1, $GATA3^{high}$ NKT2 (Terashima *et al*., 2008; Watarai *et al*., 2012) and

RORγt$^{+}$ NKT17 cells (Michel., 2007; *et al* Coquest *et al*., 2008; Doisne *et al*., 2009). T-bet$^{high}$ NKT1 cells capable of producing large amounts of IFN-γ are the main terminal differentiation product in B6 mice (Benlagha *et al*., 2002; McNab *et al*., 2005). However, it should be stress that these cells are not fully polarized. They produce both IFN-γ and IL-4 on a single cell basis, although the amount of IL-4 is comparatively low compared with previous developmental stages. The GATA3$^{high}$ NKT2 and RORγt$^{+}$ NKT17 sublineages are rare in B6 mice but can be dominant in other strains or in various mutant mice. NKT2 cells, which are more abundant in BALB/c mice, predominate in the lung while NKT17 cells are rare and mostly found in peripheral lymph nodes draining the skin; NKT1 cells predominate in spleen and liver.

However, it is reported that certain cytokines can also cause cells to produce IL-4. For example, Terashima *et al.* previously showed that CD4$^{+}$ NK1.1$^{-}$ iNKT cells (similar to NKT2) express IL-17RB, a receptor for IL-25 (Terashima *et al*., 2008). IL-25 is a cytokine produced by stromal cells that can promote the Th2 immune response (Saenz *et al*., 2010). Indeed, hCD2 positive (IL-4 producing) cells were found within the IL17RB positive population both in B6 and BALB/c mice. Watarai and colleagues showed that IL-17RB$^{+}$ iNKT cells could produce IL-4 when exposed to exogenous IL-25 and that IL-25 receptor deficiency partially prevented the development of NK1.1 negative iNKT cells (Watarai *et al*., 2012). However, B6 NKT2 cells produce less IL-4 than BALB/c despite comparable level of IL17RB expression, and B6 NKT17 cells also express IL17RB indicating that IL17RB is not sufficient to drive IL-4 production (Lee *et al*., 2013).

Furthermore, it was showed that stage 1 and stage 2 iNKT cells are composed of two populations, IL-17RB$^{+}$ cells that make up iNKT2 and iNKT17 subsets, and IL-17RB$^{-}$ cells that are progenitors of the iNKT1 subset (Watarai *et al*., 2012) while iNKT1 subset is equivalent to stage 3 iNKT cells. It is worth noting that each subset has a tendency to produce

particular cytokines, yet under antigen stimulation iNKT cells might have the potential to produce all cytokines. For example, when stimulated by phorbol 12-myristate 13-acetate (PMA)/ionomycin or αGC, a large proportion of these cells will co-produce cytokines of other subsets, particularly IL-4 and IL-13 (Yoshimoto, 1994; Watarai *et al*., 2012), and each subset expressed some cytokines of other subsets (Engel *et al*., 2016; Georgiev *et al*., 2016). Kronenberg and colleagues also found that the iNKT2 subset was composed of two subpopulations, in which one expressed a large amount of genes involved in cell cycle progression, indicating that it is likely a progenitor of other iNKT cells (Engel *et al*., 2016).

There are also diversified functions among NKT cells from different organs. For example, purified $NK1.1^+$ $TCR^+$ thymic NKT cells produced a higher amount of cytokines, especially IL-4, than splenic $NK1.1^+$ $TCR^+$ NKT cells (Yang *et al*., 2003). Yang *et al*. (2003) also indicated that thymic and splenic NKT cells from both $NK1.1^+$ and $NK1.1^-$ mouse strains differ in the expression of activation markers and requirements for co-stimulatory signals. While splenic NKT cells required both TCR and CD28 mediated signals to be fully activated, engagement of CD28 had no detectable effect on the activation of thymic NKT cells (Yang *et al*., 2003). Similarly, the engagement of CD40 ligand (CD40L) on the surface of thymic NKT cells did not affect their cytokine production, although the CD40 signal was important for the cytokine profile of splenic NKT cells (Hayakawa *et al*., 2001; Yang *et al*., 2003).

The functional diversity between organs mentioned above is likely a consequence of differentiation of $CD4^+$ and DN NKT cell subsets as they exhibit different chemokine receptor patterns (Slauenwhite and Johnston, 2015). Kim *et al*. (2002) indicated that NKT cell subsets differ substantially in their expression of some chemokine receptors, even though they share expression of many others. For example, most NKT cells express CCR1, CCR2, CCR5, CXCR3, CXCR4, and CXCR6. Among these, CCR1, CCR2, CCR5, CXCR3, and CXCR6 are highly related to “tissue” T cells found in extra-lymphoid tissues. However,

CD4 NKT cells (which produce high levels of IL-2 and IL-4) express CCR4 and respond strongly to CCR4 ligand (TARC), while CD8 and DN NKT cell subsets (low IL-2 and IL-4 producers) express CCR1, CCR6 and CXCR6 and respond well to their ligands MIP-1a and LARC (Kim *et al*., 2002). The specific combinations of adhesion molecules and chemokine receptors that NKT cell subsets express determine their trafficking behaviours and correlate with the wide distribution of NKT cells in tissues (Slauenwhite and Johnston, 2015). These data support the idea that the tissue-specific microenvironments determine the differentiation of thymic NKT cells into subsets of peripheral cells that are functionally and phenotypically distinct.

While tremendous progress has been made in the identification of iNKT cell developmental intermediates and the various signaling pathways and transcription factors involved in early development, the molecular mechanisms that control the differentiation of the different iNKT cell at later stages subsets remain mysterious. What are the original signals that determine which and to what extent a given signaling pathway and/or transcription factor expression is turned on or off to determine which developmental pathway iNKT precursors choose? Further investigation to seek transcriptional evidence of NKT cell differentiation is necessary to answer the following questions: 1) What are the developmental signals that result in differential branching of the $CD4^+$ and DN NKT cell subsets? and 2) What is the stage at which they differentiate? Based on our data presented in Chapter 4, TCR validation is associated with Transition 1 and positive selection and lineage commitment are associated with Transition 2 as presented in Chapter 5; these findings raise the possibility that **functional differentiation might be associated with Transition 3, between immature $CD4^+$ NKT cells and immature DN NKT cells**.

Specifically, the aims of this chapter were:

1. To profile gene expression between immature $CD4^+$ NKT cells and immature DN NKT cells.

2. To identify significant changes in gene expression related to the differentiation of NKT cells.

3. To functionally validate gene expression changes during NKT cell differentiation.

## 6.2. RESULTS

### 6.2.1. Transcriptional analysis of $CD4^+$ NKT cells and DN NKT cells

To test the hypothesis that functional differentiation of NKT cells occurs across Transition 3, transcriptional comparison between immature $CD4^+$ NKT cells and immature DN NKT cells was performed. The expression levels of all 35,556 transcripts across Transition 3 were compared pairwise by Mann-Whitney U test, and those with a U statistic of zero (i.e. no overlap between groups) were shortlisted for ranking by Student t Test. Across this transition, 6,904 transcripts generated a Mann-Whitney U score of zero, and of these, 1,849 were highly differentially expressed (HDE; $p < 1.0 \times 10^{-6}$, by t-Test). Of the HDE transcripts, 1,270 were up-regulated in DN NKT cells compared to $CD4^+$ NKT cells, while 579 transcripts were down-regulated.

To gain insights into the biological processes involved in the differentiation between immature $CD4^+$ NKT cells and immature DN NKT cells, the up-regulated and down-regulated HDE gene names were submitted to Gene Ontogeny analysis using DAVID Bioinformatics Resources. The top annotation cluster (Enrichment score 29) of up-regulated HDE genes was dominated by lumen membrane proteins and contained the following annotation categories: membrane-enclosed lumen (representing 160 of all up-regulated HDE genes; Bonferroni corrected $p<8.7\times10^{-42}$), intracellular organelle lumen (Bonferroni corrected $p<2\times10^{-38}$) and nucleolus (Bonferroni corrected $p<1.5\times10^{-34}$). In this gene list, there was a

predominance of genes encoding mitochondrial ribosomal proteins (*Mrpl2, 12, 13, 15, 16, 17, 19, 36, 37, 38, Mrps10, 22, 23*), polymerase RNA (*Polr1a, 1b, 1e, 2e, 3d, 3h*) and general transcription factor II (*Gtf2e1, e2, f2* and *h3*).

Gene Ontogeny analysis of the down-regulated HDE genes revealed that there was an over-representation of genes associated with cell activation (GO:0001775; representing 27 of all down-regulated HDE genes; Bonferroni corrected $p<2.3x10^{-8}$), leukocyte activation (GO:0045321; Bonferroni corrected $p<1.4x10^{-7}$) and T cell activation (GO:0042110; Bonferroni corrected $p<4.6x10^{-6}$). While genes related to cell activation were more highly expressed in $CD4^+$ NKT cells than in DN NKT cells, genes encoding membrane-enclosed lumen proteins were more highly expressed in DN NKT cells. Taken together, the data suggest differential gene expression, subsequently functional differentiation between immature $CD4^+$ NKT cells and immature DN NKT cells.

### 6.2.2. Gene expression of transcriptional factors and molecules associated with NKT cell differentiation between CD4 and DN NKT cells

To gain further insights into the functional differentiation between the two subsets, we next compared gene expression of transcriptional factors or molecules associated with NKT cell differentiation reported in the literature, such as ThPOK (Dave *et al*., 1998; He *et al*., 2005), ICOS (Akbari *et al*., 2008) and LY6A (Henderson *et al*., 1998; 2002; Fig. 6.1). ThPOK, a member of the BTB-POZ family of zinc finger transcription factors, is required for the generation of $CD4^+$ NKT cells (He *et al*., 2005). Although mice with spontaneous mutations in *Zbtb7b* (encoding ThPOK) have normal frequencies and numbers of thymic and peripheral NKT cells, their mature NKT cells completely lack CD4 expression and they express CD8 on large numbers of NKT cells (Dave *et al*., 1998; He *et al*., 2005). Additionally, ThPOK deficient mice exhibit defects in IL-4 production and to a lesser extent

IFN-γ (Dave *et al*., 1998; He *et al*., 2005). In our microarray data, *Zbtb7b* expression was 3.6-fold higher in $CD4^+$ NKT cells compared to DN NKT cells (t-Test $p<8.4x10^{-8}$; Mann-Whitney U statistic =0; Fig. 6.1A). This is consistent with the role of ThPOK in maintaining the CD4 subset of the NKT cell population.

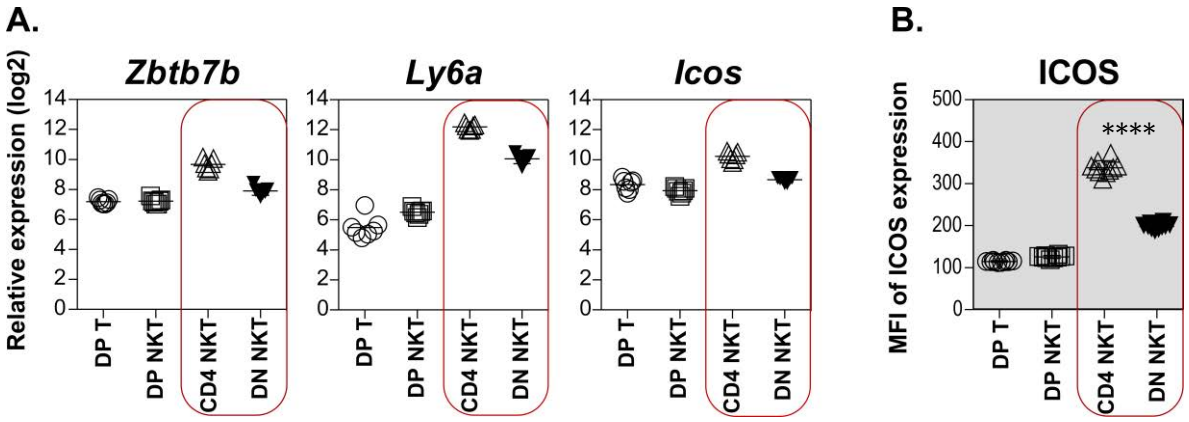


**Figure 6.1. Expression profiles of genes related to differentiation of CD4 NKT cells. A.** Gene expression of *Zbtb7b, Icos* and *Ly6a* determined by microarray (microarray data were generated with assistance from Dr. Margaret Jordan and Prof. Alan Baxter). **B.** ICOS protein expression as determined by flow cytometry. n=10, Mann-Whitney U test, **** p<0.0001

In mice, the *Ly-6* locus encodes a family of glycophosphatidylinositol-anchored, developmentally regulated cell surface proteins (Rock *et al*., 1989). Members of the *Ly-6* gene family are markers of different lineages of hematopoietic origin, including lymphocytes (Pflugh *et al*., 2000), monocytes and bone marrow cells (Patterson *et al*., 2000). Ly-6 may have a role in T cell activation (Rock *et al*., 1986), as transgenic expression of Ly-6A.2 on $CD4^+CD8^+$ thymocytes promoted thymic maturation of $CD4^+$ (but not $CD8^+$) T cells in the absence of TCR-MHC interactions (Henderson *et al*., 1998; 2002). In our microarray data, the expression of *Ly6a* was 4.6-fold higher in $CD4^+$ NKT cells compared to DN NKT cells (t-Test $p<4.8x10^{-9}$, Mann-Whitney U statistic =0; Fig. 6.1A).

Akbari *et al*. (2008) reported that Inducible T cell co-stimulator (ICOS) is highly expressed on both naive and activated iNKT cells. ICOS expression on the $CD4^+$ iNKT subset was greater than on $CD4^-$ iNKT cells. These authors also found that ICOS is required for

$CD4^+$ NKT cell survival and homeostasis in the periphery, as $CD4^+$ NKT cell numbers were significantly lower in spleens and livers of ICOS deficient mice and mice lacking ICOS ligand. Survival of wild-type iNKT cells transferred into $ICOSL^{-/-}$ mice was greatly reduced due to the induction of apoptosis. Furthermore, the remaining NKT cells in $ICOS^{-/-}$ mice were unable to produce IL-4 and IL-13 and failed to reconstitute airway hyperreactivity when adoptively transferred into $J\alpha18^{-/-}$ mice (Akbari *et al*., 2008). In our work, *Icos* expression was three times higher in $CD4^+$ NKT cells compared to DN NKT cells in our microarray data (t-Test $p<4.9x10^{-8}$; Mann-Whitney U statistic =0; Fig. 6.1A), the protein expression level was confirmed by flow cytometry ($p<0.0001$, Mann-Whitney U test; Fig. 6.1B), consistent with its role in $CD4^+$ iNKT cell function, homeostasis, and survival in the periphery.

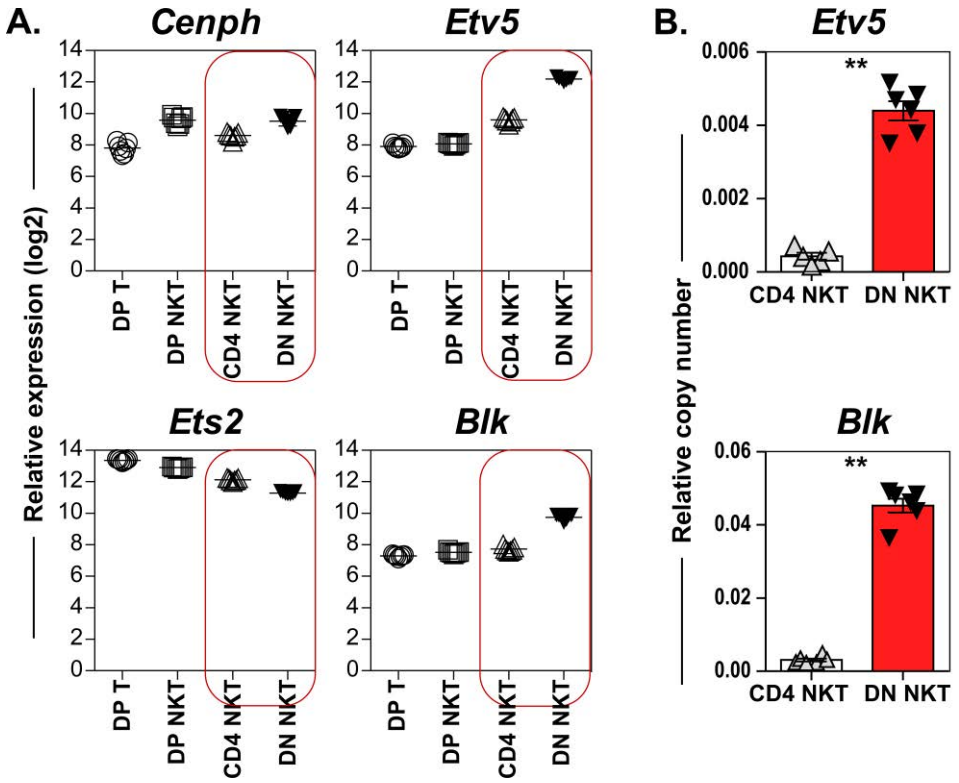


**Figure 6.2. Expression profiles of genes related to proliferation between CD4 and DN NKT cells. A.** Gene expression of *Cenph, Etv5, Cd97* and *Blk* as determined by microarray (data were generated with assistance from Dr. Margaret Jordan and Prof. Alan Baxter). **B.** *Etv5* and *Blk* expression were confirmed by qPCR. n=6 per cell population, Mann-Whitney U test, ** $p<0.01$. Data were generated with assistance from Dr. Morgane Moreau.

Transition 3 was also characterised by increased expression of genes involved in cell division and differentiation (such as *Cenph, Ets2, Etv5* and *Blk;* Fig. 6.2A). The differences in transcriptional levels of *Etv5* and *Blk* were confirmed by qPCR in an independent sample set (Fig. 6.2 B). The up-regulation of genes related to cell division by DN NKT cells is consistent with the increased proportion of proliferating ($BrdU^+$) cells in DN NKT cells, compared to that of CD4 NKT cells mentioned in Chapter 5.

### 6.2.3. Cytokine and cytokine receptor differentiation between CD4 and DN NKT cells

#### *6.2.3.1. Gene expression analysis of cytokine receptors between CD4 and DN NKT cells*

There is evidence that maturation and homeostasis of NKT cells is highly dependent on cytokines, such as IL-15 (Matsuda *et al*., 2002; Ranson *et al*., 2003), and to a lesser extent on IL-7 (Sanberg *et al*., 2004). It has been demonstrated that IL-15 regulates a large number of key molecules, such as BCL2, T-BET and $BCL\text{-}X_L$, which pertain to NKT cell survival (Gordy *et al*., 2011).

Gene expression comparison in our microarray data revealed that there were differences in expression of genes encoding cytokine receptors, such as *IL2rα, Il4rα, Il6rα, Il7r,* and *Il12rβ2* between $CD4^+$ and DN NKT cells (Fig. 6.3). For example, *Il4rα, Il7r* and *Il6rα* expression was significantly down-regulated on DN NKT cells (2.0, 2.2 and 2.8 times, respectively) compared to $CD4^+$ NKT cells (t-Test $p<3.6x10^{-7}$; Mann-Whitney U statistic =0), while *Il12rβ2* and *Il2rα* was significantly up-regulated in DN NKT cells (1.7 and 2.9-fold higher, respectively) compared to $CD4^+$ NKT cells (t-Test $p<2.8x10^{-7}$; Mann-Whitney U statistic =0; Fig. 6.3 A). In contrast, *Il2rβ/15rβ* was expressed with relatively similar levels in immature $CD4^+$ NKT cells and immature DN NKT cells after a significant up-regulation at positive selection stage (transition 2), (Fig. 6.3A). To confirm our microarray data, the differences in transcriptional levels of *Il6rα, Il12rβ2 and Il2rα* (encoding CD25) were

examined by qPCR in an independent sample set (Fig. 6.3 B) and by flow cytometry (Fig. 6.3 C), respectively. Together, these data indicated that $CD4^+$ and DN NKT cells express different cytokine receptor profiles after positive selection.

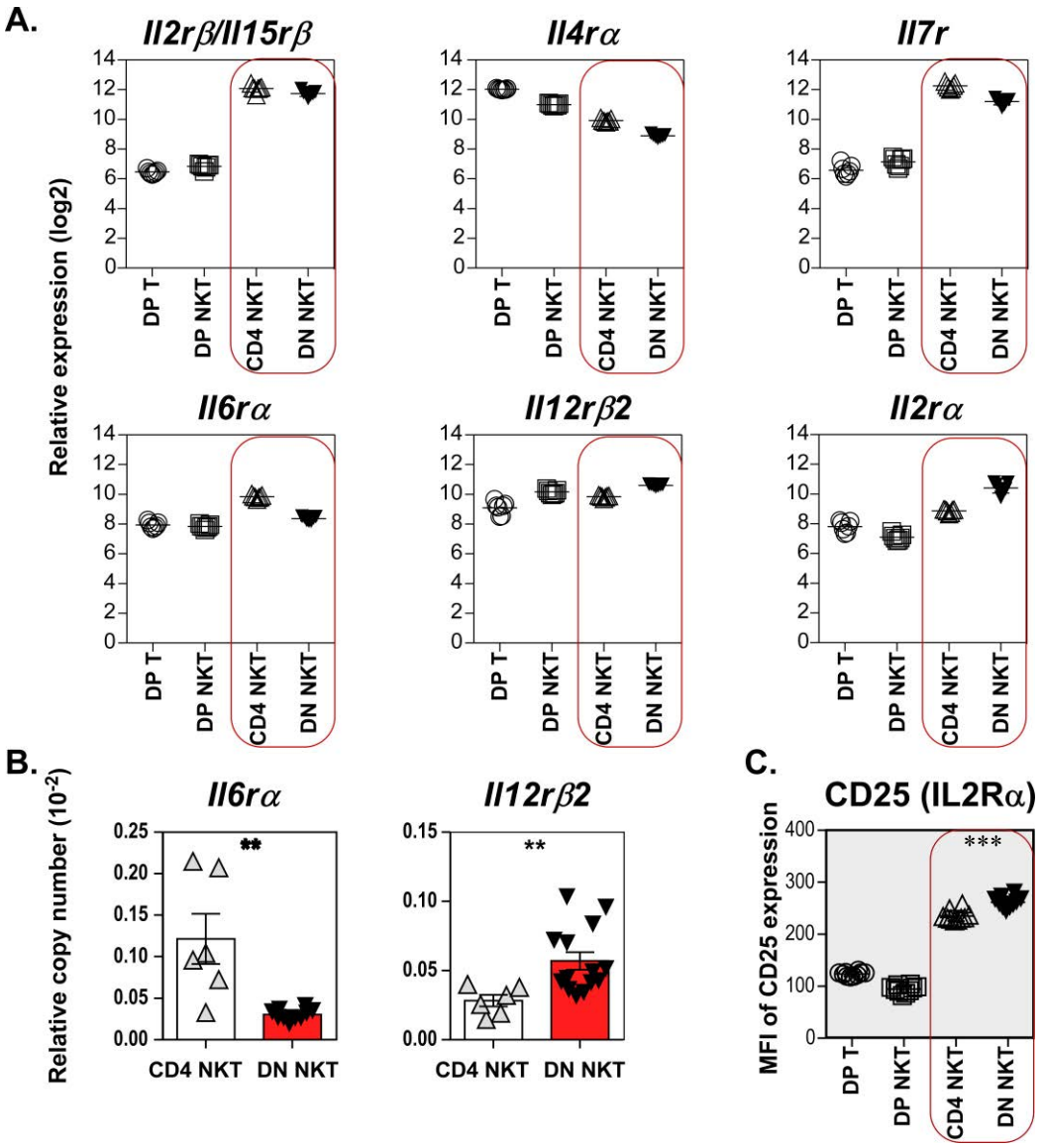


**Figure 6.3. Expression profiles of genes related to cytokine receptor between CD4 and DN NKT cells. A.** Gene expression of *IL2rα, Il4rα, Il6rα, Il7r, Il12rβ2* and *IL2rβ/15rβ* as determined by microarray (data were generated with assistance from Dr. Margaret Jordan and Prof. Alan Baxter). **B.** *Il6rα* and *Il12rβ2* expression was confirmed by qPCR (data were generated with assistance from Dr. Morgane Moreau). n=6 for CD4 NKT cell subset, n=10-14 for DN NKT cell subset. Mann-Whitney U test, ** p<0.01. **C.** CD25 (IL2Rα) protein expression as presented by flow cytometry. n = 10 per cell population, Mann-Whitney U test, *** p<0.001.

### *6.2.3.2. Validation of cytokine differentiation between immature CD4$^+$ and DN NKT cells*

An important question from the microarray analysis was whether the differences in gene expression of cytokine and cytokine receptors between immature CD4$^+$ and DN NKT cells reflected functional differentiation across transition 3. To this end, FACS sorted immature CD4$^+$ and DN NKT cells from thymi of NOD.*Vα14*Tg mice were stimulated *in vitro* by immobilised anti-CD3; supernatants were collected for cytokine analysis by bead assay. Thymic immature CD4$^+$ and DN NKT cells were sorted from 5 individual mice.

Culture supernatants were harvested for cytometric bead array (CBA) analyses of interleukin-1α (IL-1α), IL-2, IL-4, IL-5, IL-6, IL-10, IL-17, tumour necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ) and granulocyte macrophage colony-stimulating factor (GM-CSF). A striking finding was that immature CD4$^+$ NKT cells produced higher cytokine concentrations of IL-4, IL-10, IFN-γ, GM-CSF and INF-α compared to immature DN NKT cells (Fig. 6.4). The most produced cytokine was IFN-γ, which was 3 times higher in CD4$^+$ NKT cells (60 ng/ml) compared to DN NKT cells (20 ng/ml). The immature CD4 NKT cells also secreted 4 times more IL-4 and IL-10, and almost 2 times more GM-CSF and INF-α than DN NKT cells. Although IL-2 was detected in the culture supernatants, IL-2 production could not be compared, as mouse recombinant IL-2 was added to the wells at the beginning of culture. We did not detect any IL-1α, IL-5, IL-6 or IL-17 produced by both CD4 and DN NKT cells (data not shown). These findings are broadly consistent with the data reported by Chan *et al.* (2012), in which sorted CD4$^+$ NKT cells from human adult blood expressed a more extended cytokine production than CD4$^-$ NKT cells (Chan *et al.,* 2012). These data are also consistent with previous studies reporting that most thymic NKT cells from mice are very strong cytokine producers (Hammond *et al*., 1999; Berzins *et al.*, 2006) even though their studies were on different NKT cell subsets. Taken together, the difference in cytokine

production between immature CD4$^+$ NKT cells and immature DN NKT cells further supports the likelihood that these cells are functionally distinct or that functional differentiation occurs across transition 3.

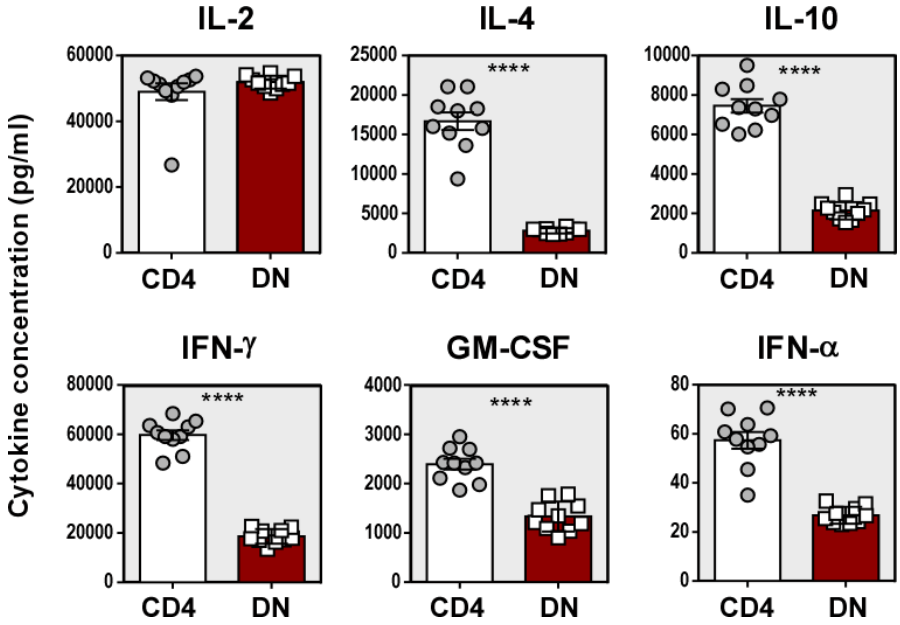


**Figure 6.4. *In vitro* stimulation of thymic NKT cells with anti-CD3e**. Thymic NKT immature CD4$^+$ and DN cells were sorted and then cultured with 100UI IL-2 in RPMI complete medium. After 72 hours, supernatants were harvested; cytokine production was determined by CBA. These dot plot graphs show means ±SEM of cytokine concentration of thymic immature CD4$^+$ NKT cells and immature DN NKT cells from NOD.*Vα14*Tg mice, 5 mice/group with duplicate cultures. Mann-Whitney U test, **** p<0.0001

### 6.2.4. Chemokine and chemokine receptor differentiation between CD4 and DN NKT cells

#### *6.2.4.1. Gene expression analysis of chemokines and chemokine receptors between CD4 and DN NKT cells*

The ability of thymocytes to migrate between particular regions of the thymus or to the periphery is critical for their maturation. It is well known that migration of conventional αβT cells is mediated by large numbers of CC chemokine receptors (CCR), such as CCR4 (Suzuki *et al*., 1999; Campell *et al*., 1999), CCR7 (Kwan and Killeen, 2004; Uneno *et al*., 2002, Mick *et al*., 2004, Huang *et al.,* 2004), and CCR9 (Uehara *et al*., 2002). However, there is little known about the effect of chemokine stimulation on the early development of NKT

cells. It has been recently reported that NKT cells in CCR5-deficient mice were resistant to activation-induced apoptosis and produced more IL-4 (Ajuebor *et al*., 2005). In addition, CCR7 plays an important role in controlling the development and migration of invariant NKT cells by enabling access to IL-15 *trans*-presentation (Cowan *et al*., 2014). In the absence of CCR7, NKT cell development was markedly impaired.

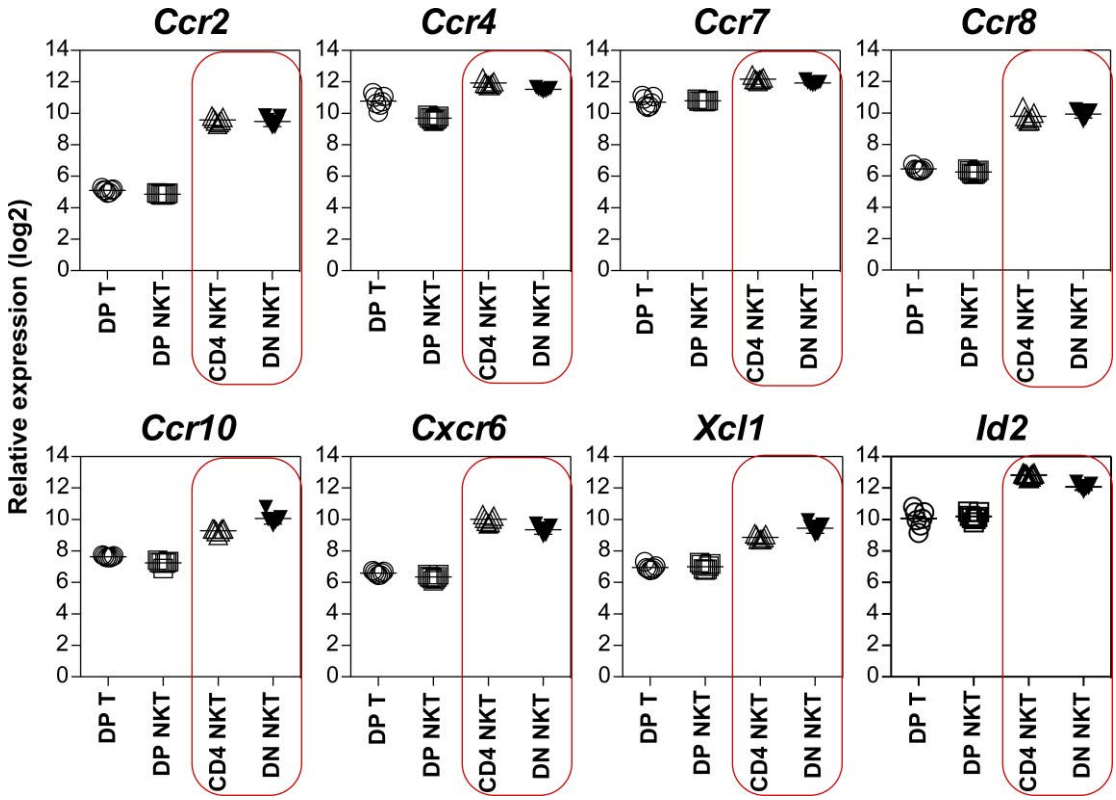


**Figure 6.5. Increased expression of genes related to chemokine receptors in CD4$^+$ and DN NKT cells.** Gene expression of *Ccr2, Ccr4, Ccr7, Ccr8* (the upper row), *Ccr10, Cxcr6, Xcl1* and *Id2* (the lower row) across 3 transitions as determined by microarray (data were generated with assistance from Dr. Margaret Jordan and Prof. Alan Baxter).

Consistent with these reports, our microarray data showed significant up-regulation of large numbers of chemokines and chemokine receptors, such as *Ccr2*, *Ccr4, Ccr7, Ccr8, Ccr10* and *Xcl1* in both immature CD4$^+$ and DN NKT cells, compared to immature DP NKT cells (Fig. 6.5 A). Upon positive selection of DP NKT cells *Ccr8* expression was increased 12-fold, *Ccr2* expression was increased almost 11-fold, and *Ccr7* exhibited a 9-fold up-regulation. However, there were little changes in expression of above chemokine receptors

across Transition 3. While the expression of *Ccr2* was similar between CD4 NKT cells and DN NKT cells, that of *Ccr4, Ccr7* was up-regulated (1.2 and 2.1 folds, respectively; t-Test $p<0.001$), that of *Ccr8, Ccr10* and *Xcl1* was slightly down-regulated (1.3, 1.6 and 1.3 folds, respectively; t-Test $p<0.05$) in CD4 NKT cells compared with DN NKT cells.

We also observed the up-regulation of genes related to homeostasis of NKT cells, such as *Cxcr6* and *Id2*. *Cxcr6* plays a critical role in homeostasis and activation of NKT cells (Germanov *et al*., 2008). Deficiency of CXCR6 was associated with the depletion of NKT cells in the liver and lungs, but accumulation in the bone marrow (Geissmann *et al*., 2005; Germanov *et al*., 2008). Similarly, NKT cell survival and homeostasis are also under the regulation of *Id2*, an inhibitor of DNA-binding protein (Monticelli *et al*., 2009). *Id2* expressed on T-BET$^{+}$, IFN-γ secreting NKT cells was required for the differentiation of this NKT cell sub-lineage (D'Cruz *et al*., 2014). Knockdown of *Id2* resulted in a drastic and selective reduction of NKT cells in the liver, while levels remained normal in thymus and spleen. In contrast, loss of *Id2* and *Id3* minimally affected iNKT cell abundance and the development of thymic NKT cells at stage 1 (D'Cruz *et al*., 2014). In our microarray data, we found that *Cxcr6* and *Id2* expression was increased 13-fold and 6-fold, respectively by both CD4 and DN NKT cells compared to DP NKT cells (Fig. 6.5). These findings suggest that the increase in expression of *Cxcr6* and *Id2* by CD4 and DN NKT cells is associated with their role in regulating their differentiation and homeostasis after positive selection.

#### *6.2.4.2. Functional validation of chemokine receptor on immature CD4 and DN NKT cells*

In order to functionally validate our microarray data on NKT cell trafficking and maturation, effects of targeted deletion of *Ccr2* and *Ccr7* on NKT cell numbers in B6 mice were examined. B6.*Ccr2*$^{-/-}$ mice were purchased from the Jackson lab (JAX), the JAX B6.*Ccr7*$^{-/-}$ mice were provided by the Cowan lab.

*6.2.4.2.1. Effects of CCR2 on NKT cell distribution*

CC chemokine ligand 2 (CCL2) is a prototypic inflammatory chemokine, which was initially described as a major chemo-attractant for monocytes (Matsushima *et al*., 1989; Rollins *et al*., 1991). However, it was recently demonstrated to be one of the most potent chemokines for both CD4 and CD8 effector-memory α/β T cells, γ/δ T cells (Carr *et al*., 1994; Roth *et al*., 1998), and NKT cells (Kim *et al*., 2002a; 2002b) that express its receptor, CC chemokine receptor 2 (CCR2). CCR2 is required for Langerhans cell migration and localization of T helper cell type 1 (Th1) - inducing Dendritic cells (DC; Sato *et al*., 2000).

CCR2 also plays an important role in NK cell migration during intranasal influenza virus infection (van Helden *et al*., 2012) and DC maturation (Jimenez *et al*., 2010). During murine cytomegalovirus (MCMV) infection, macrophage trafficking to the liver is largely dependent on CCR2 signalling (Hokeness *et al*., 2005). Crane *et al*. (2009) indicated that monocyte/macrophage trafficking from the bone marrow into circulating blood is impaired in the absence of CCR2 signalling. CCR2 activation within the bone marrow is shown to control the magnitude of monocyte/macrophage release from this compartment following MCMV infection (Crane *et al*., 2009). In support of a role for CCR2 in mediating this process, only inflammatory monocyte/macrophages expressing high levels of Ly6C, which correlates with CCR2 expression (Geissmann *et al*., 2003; Tacke and Randolph, 2006), were found to accumulate in the bone marrow during MCMV infection. Importantly, $Ly6C^{high}$ monocyte/macrophages were dramatically reduced in the blood of CCR2-deficient mice. Consistently, this cell population was also decreased in the circulation of uninfected CCR2-deficient mice, indicating a homeostatic role for CCR2 in regulating monocyte/macrophage egress from the bone marrow (Serbina and Pamer, 2006; Tsou *et al*., 2007; Engel *et al*., 2008; Qu *et al*., 2008; Yamasaki *et al.,* 2011).

In order to examine the effect of CCR2 on NKT cell development, we made used of the B6.*Ccr2*$^{-/-}$ mouse strain. This strain has a monomeric red fluorescent protein (RFP) sequence replacing the coding sequence of the chemokine (C-C motif) receptor 2 gene, which abolishes the gene function. B6.*Ccr2*$^{-/-}$ mice were backcrossed to inbred C57BL/6 mice for 5 generations prior to use in experiments. Flow cytometric analysis of B6.*Ccr2*$^{-/-}$ and B6 WT control mice revealed that both the proportions and absolute numbers of NKT cells in the thymi and spleens were unchanged (Fig. 6.6).

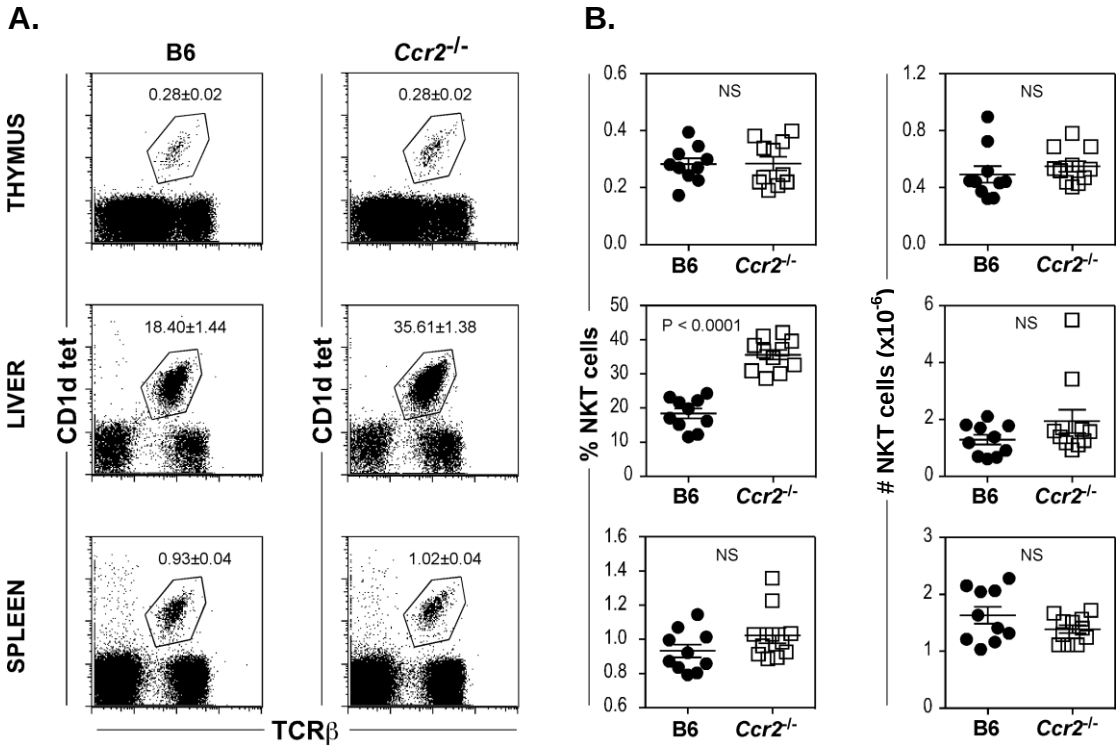


**Figure 6.6. Effects of CCR2 deficiency on NKT cells from B6 WT mice**. **A**. Representative FACS plots showing the mean proportions (±SEM) of thymic, hepatic and splenic NKT cells between B6 WT and B6.*Ccr2*$^{-/-}$ mice. **B**. Dot plot graphs show the mean frequencies and absolute numbers of thymic, hepatic and splenic NKT cells between B6 WT and B6.*Ccr2*$^{-/-}$ mice. n=10 for B6 WT group, n=11 for B6.*Ccr2*$^{-/-}$ group. Mann-Whitney U test, NS= Not significant.

In contrast, the proportion of NKT cells drastically increased in the livers of B6.*Ccr2*$^{-/-}$ mice, yet overall absolute numbers were normal. In particular, the proportion of hepatic NKT cells significantly increased from 18.40±1.44% in B6 WT controls to 35.61±1.38% in B6.*Ccr2*$^{-/-}$ mice ($p<0.0001$), whereas the absolute numbers of hepatic NKT cells were not significantly

different between B6 mice (1.29±0.17 x $10^6$ cells) and B6.*Ccr2*$^{-/-}$ mice (1.94±0.41 x $10^6$ cells). The increased frequency of hepatic NKT cells in the liver of B6.Ccr2$^{-/-}$ mice, in the absence of a change in the absolute number of these cells, likely reflects depletion of some other leucocyte subset in the liver. However, another possible explanation is that in absence of CCR2, NKT cells cannot receive signals of recruitment to inflammatory sites and therefore accumulate in the livers of B6.*Ccr2*$^{-/-}$ mice. A corollary of this hypothesis is that CCR2 expression is required for the recruitment of NKT cells.

*6.2.4.2.2. Effects of CCR7 on thymic NKT cell numbers*

To examine the effects of CCR7 expression on NKT cell development, we made use of the B6.*Ccr7* knockout mouse strain. This mouse strain harbours a targeting vector, which was designed to replace a fragment of the third exon of the *Ccr7* gene (encompassing amino acids Ser-139 to Asp-309) with a neomycin resistance gene, which abolishes the gene function. CCR7 mutant mice were backcrossed to inbred C57BL/6 mice for 5 generations prior to use in experiments.

Flow cytometric analysis of NKT cells and subsets revealed that in CCR7-deficient mice, NKT cell numbers were reduced in both the thymus and the periphery (Fig. 6.7). In the thymi, the proportion of NKT cells was significantly decreased from 0.34±0.01% in WT mice to 0.06±0.01% in B6.*Ccr7*$^{-/-}$ mice ($p<0.0001$). The absolute numbers of thymic NKT cells fell 6-fold, from 0.54±0.05 x$10^6$ in B6 WT mice to 0.09±0.01 x$10^6$ cells in B6.*Ccr7*$^{-/-}$ mice. The decrease in thymic NKT cells in B6.Ccr7$^{-/-}$ mice could be due, at least in part, to a decreased medullary volume in B6.Ccr7$^{-/-}$ mice (Ueno *et al*., 2004). In the periphery, similar patterns were seen: the proportion and numbers of NKT cells showed a 3-fold decrease in the livers and spleens of CCR7 KO mice (Fig. 6.7).

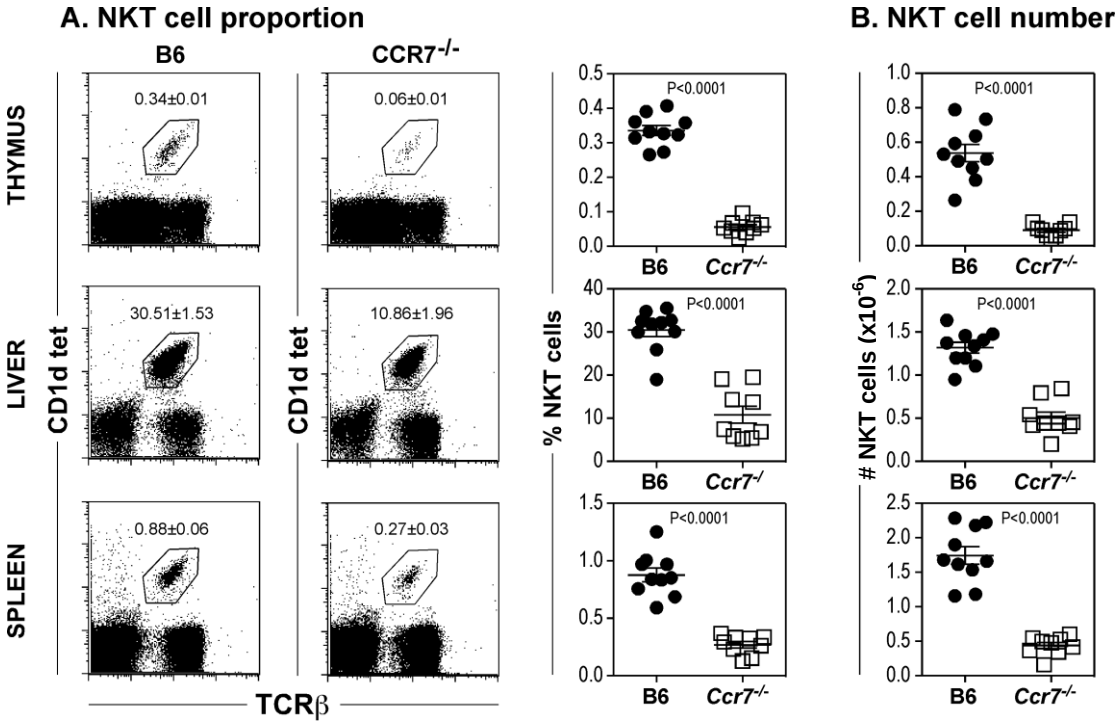


**Figure 6.7. Effects of CCR7 deficiency on NKT cells from B6 WT mice**. **A**. Representative FACS plots showing the mean proportions (±SEM) of thymic, hepatic and splenic NKT cells between B6 WT and B6.*Ccr7*$^{-/-}$ mice. **B**. Dot plot graphs show the mean frequencies and absolute numbers of thymic, hepatic and splenic NKT cells between B6 WT and B6.*Ccr7*$^{-/-}$ mice. n=10 for B6 WT group, n=9 for B6.*Ccr7*$^{-/-}$ group. Mann-Whitney U test.

There were a number of changes in thymic NKT cell subsets in CCR7-deficient mice (Fig. 6.8). While the proportions of CD4$^{+}$ NKT cells were significantly increased, those of DN NKT cells were markedly decreased in B6.*Ccr7*$^{-/-}$ mice. In terms of CD44/NK1.1 expression, there was a significant increase in the proportions of immature CD44$^{-}$NK1.1$^{-}$ and semi-mature CD44$^{+}$NK1.1$^{-}$ NKT cell subsets in B6.*Ccr7*$^{-/-}$ mice compared to WT controls. In contrast, the proportion of mature CD44$^{+}$NK1.1$^{+}$ NKT cells was reduced (Fig. 6.8). These results confirm those of Cowan *et al*. (2014), and are consistent with a role for CCR7 in thymic migration of NKT cells.

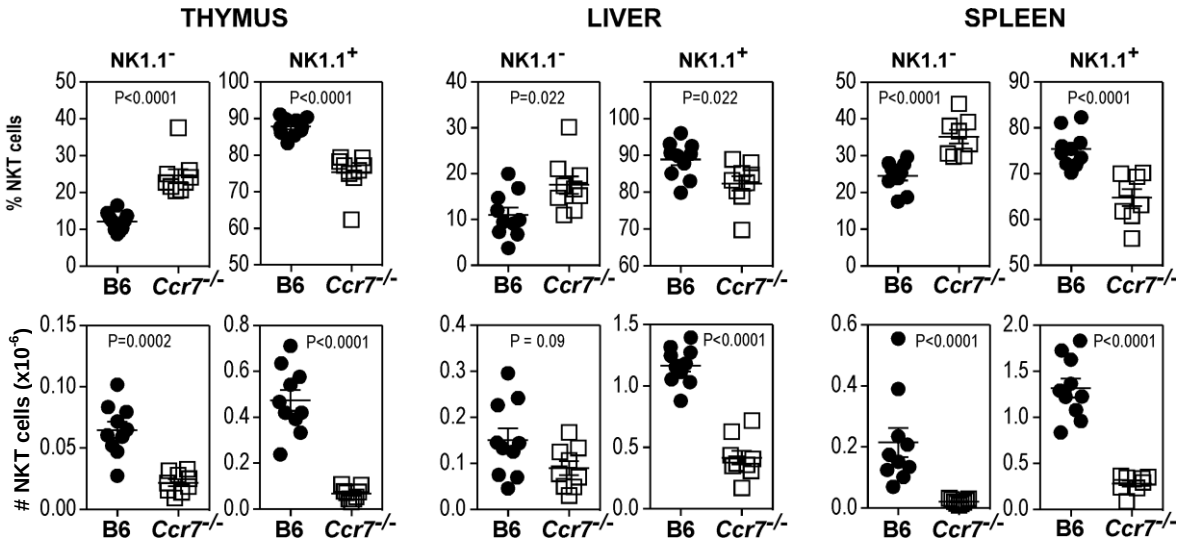


**Figure 6.8. NK1.1$^-$ and NK1.1$^+$ NKT cell subsets in CCR7KO mice.** Dot plots show mean proportions (upper row) and absolute numbers (lower row) of NK1.1$^-$ and N1.1$^+$ NKT cells in the thymi, livers and spleens of B6 WT and CCR7 KO mice. n=10 for B6 WT group, n=9 for B6.*Ccr7*$^{-/-}$ group. Mann-Whitney U test.

There was a significant increase in the proportions of thymic CD4$^+$CD8$^+$ (DP) NKT cells, which rose from 1.54±0.06% of total thymic NKT cells in B6 WT mice to 4.29±0.72% in B6.*Ccr7*$^{-/-}$ mice (Fig. 6.9). This cell population is normally excluded from analyses due to its rarity. Previously, many studies reported that CCR7 ligation by SLC/CCL21 is partly responsible for thymic export (Norment and Bevan, 2000; Ueno *et al*., 2002) and NK1.1$^-$ NKT cells, but not mature NK1.1$^+$ NKT cells, are reported to traffic in response to SLC/CCL21 (Johnston *et al*., 2003). However, in this chapter, the increased proportion of DP NKT cells, along with the expansion of immature CD44$^-$NK1.1$^-$ NKT cells mentioned previously, suggests an effect of CCR7 deficiency on further development of DP NKT cells following positive selection.

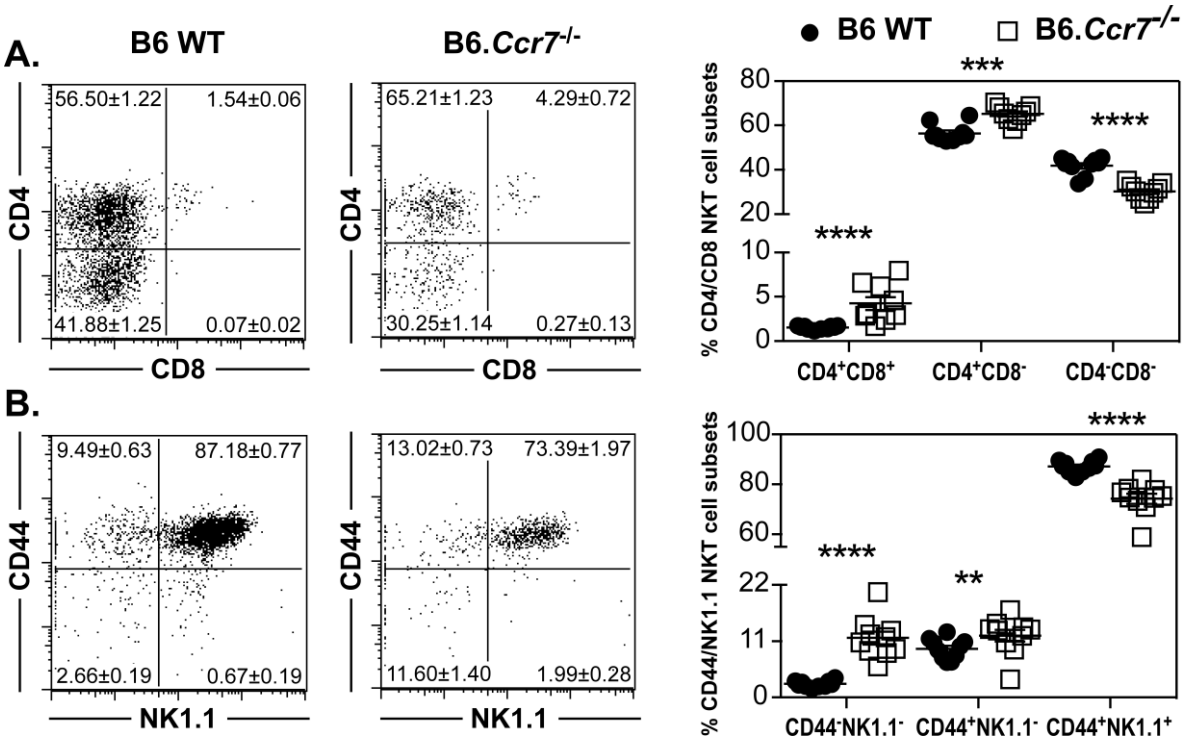


**Figure 6.9. Effects of CCR7 deficiency on thymic NKT cell subsets in B6 mice**. **A**. Representative FACS plots and dot plot graphs showing the mean frequency (±SEM) and numbers of thymic CD4/CD8 NKT cells from B6 WT and B6.*Ccr7*$^{-/-}$ mice. **B**. Representative FACS plots and dot plot graphs showing the mean frequencies (±SEM) and numbers of thymic CD44/NK1.1 NKT cells from B6 WT (n=10) and B6.*Ccr7*$^{-/-}$ mice (n=10). Mann-Whitney U test, ** p<0.01, *** p<0.001 and **** p<0.0001.

In order to examine the effect of CCR7 deficiency on pre-selection DP NKT cells, a CCR7 deficient line of B6.*Vα14*Tg mice was created. Briefly, B6.*Vα14*Tg mice were crossed to B6.*Ccr7*$^{-/-}$ mice. The resulting F1 mice were then inter-crossed. Pups were genotyped to select for mice of interest of B6.*Ccr7*$^{-/-}$.*Vα14*Tg$^{+/-}$ and B6.*Ccr7*$^{+/+}$.*Vα14*Tg$^{+/-}$ (also called B6.*Vα14*Tg$^{+/-}$) mice.

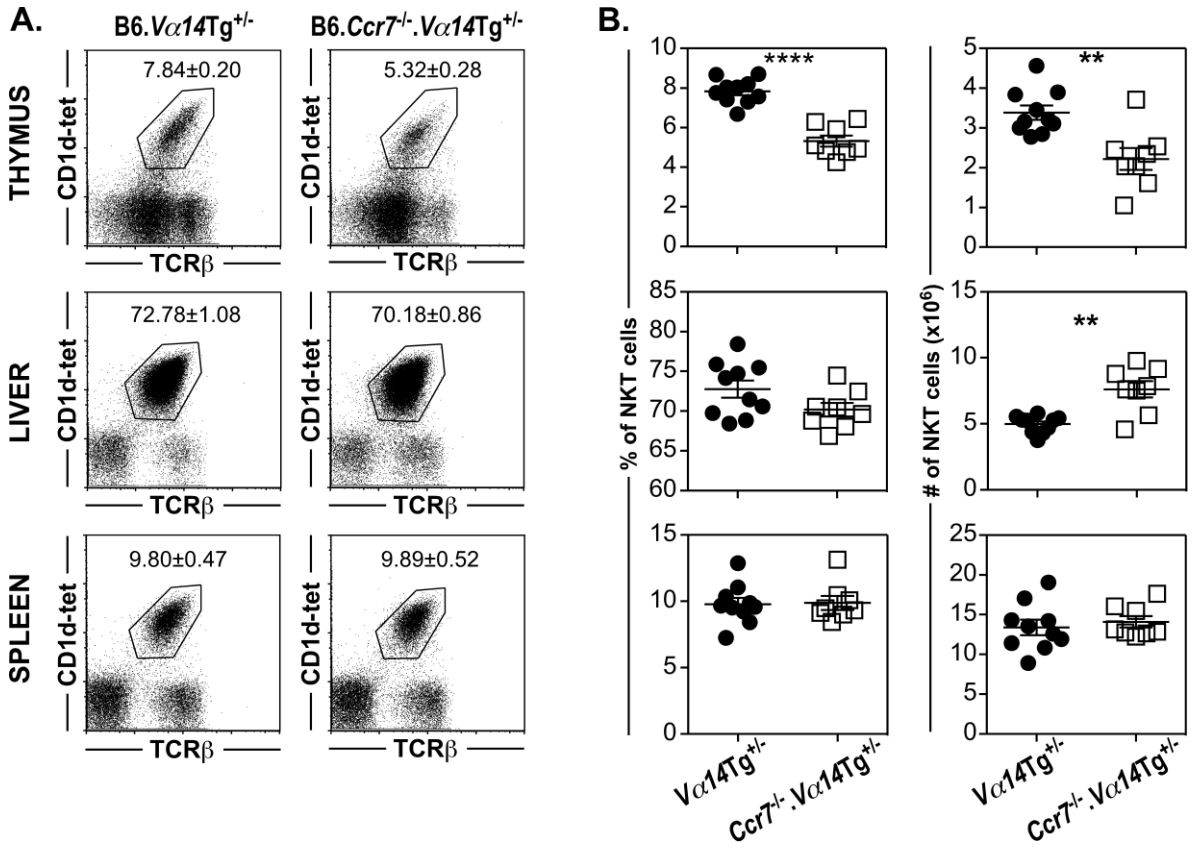


**Figure 6.10. Effects of CCR7 deficiency on NKT cells from B6.*Vα14*Tg$^{+/-}$ mice**. **A**. Representative FACS plots showing the mean frequency (±SEM) of thymic, hepatic and splenic NKT cells between B6.*Vα14*Tg$^{+/-}$ and B6.*Ccr7*$^{-/-}$.*Vα14*Tg$^{+/-}$ mice. **B**. Dot plot graphs show the mean frequency and absolute numbers of thymic, hepatic and splenic NKT cells between B6.*Vα14*Tg$^{+/-}$ (n=10) and B6.*Ccr7*$^{-/-}$.*Vα14*Tg$^{+/-}$ mice (n=9). Mann-Whitney U test, ** p<0.01, *** p<0.001 and **** p<0.0001

Flow cytometric analysis of thymic, hepatic, and splenic cells from B6.*Ccr7*$^{-/-}$.*Vα14*Tg$^{+/-}$ and B6.*Vα14*Tg$^{+/-}$ control mice revealed a significant reduction in both proportions (p<0.0001; Mann-Whitney test) and absolute numbers (p<0.001) of thymic NKT cells in B6.*Ccr7*$^{-/-}$.*Vα14*Tg$^{+/-}$ mice compared with the controls (Fig. 6.10). In contrast, the proportions and absolute numbers of splenic NKT cells were unchanged between B6.*Ccr7*$^{-/-}$.*Vα14*Tg$^{+/-}$ and B6.*Vα14*Tg$^{+/-}$ mice (Fig. 6.10). The proportions of hepatic NKT cells were similar, while the absolute numbers were significantly increased from 4.98±0.21 x $10^6$ cells in B6.*Vα14*Tg$^{+/-}$ control mice to 7.62±0.62 x $10^6$ cells in B6.*Ccr7*$^{-/-}$.*Vα14*Tg$^{+/-}$ mice (Fig. 6.10). These data imply that CCR7 deficiency affected thymic and hepatic NKT cell numbers, but had no effect on splenic NKT cells in B6.*Vα14*Tg$^{+/-}$ mice. Even though the mechanism is unclear, it is noteworthy that the effect of

CCR7 deficiency in B6.*Vα14*Tg$^{+/-}$ mice is opposite to the 3-fold decrease in hepatic NKT cell numbers in B6.Ccr7$^{-/-}$ mice compared to B6 mice (Fig. 6.7 page 187).

Flow cytometric analysis of NKT cell subsets from the thymi of B6.*Ccr7*$^{-/-}$.*Vα14*Tg$^{+/-}$ mice identified an increase in both the proportions and absolute numbers of immature DP NKT cells (Fig. 6.11).

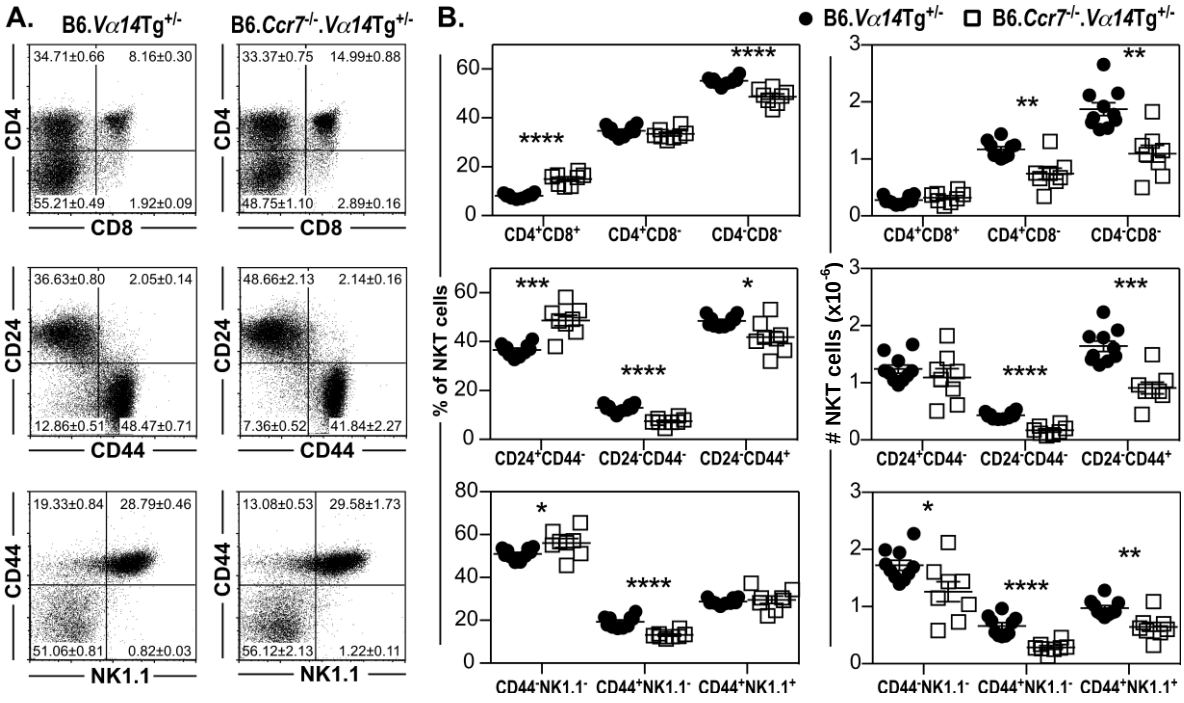


**Figure 6.11. Effects of CCR7 deficiency on thymic NKT cell subsets from B6.*Vα14*Tg mice**. **A**. Representative FACS plots showing the mean proportions (±SEM) of thymic CD4/CD8, CD24/CD44 and CD44/NK1.1 NKT cell subsets from B6.*Vα14*Tg$^{+/-}$ and B6.*Ccr7*$^{-/-}$.*Vα14*Tg$^{+/-}$ mice. **B**. Dot plot graphs show the mean proportions and absolute numbers of thymic CD4/CD8, CD24/CD44 and CD44/NK1.1 NKT cells between B6.*Vα14*Tg$^{+/-}$ (n=10) and B6.*Ccr7*$^{-/-}$.*Vα14*Tg$^{+/-}$ mice (n=8). Mann-Whitney U test, ** p<0.01, *** p<0.001 and **** p<0.0001.

The proportions of DP NKT cells rose from 8.16±0.30% of total thymic NKT cells in B6.*Vα14*Tg$^{+/-}$ control mice to 14.99±0.88% in B6.*Ccr7*$^{-/-}$.*Vα14*Tg$^{+/-}$ mice. In contrast, the proportions of thymic CD4$^{+}$ NKT cells were unchanged, and those of DN NKT cells decreased. With regard to their absolute numbers, DP NKT cells were similar between B6.*Ccr7*$^{-/-}$.*Vα14*Tg$^{+/-}$ and B6.*Vα14*Tg$^{+/-}$ control mice, whereas the numbers of CD4$^{+}$ and DN NKT cells were significantly reduced in B6.*Ccr7*$^{-/-}$.*Vα14*Tg$^{+/-}$ mice. A similar pattern

was seen with the numbers of CD24/CD44 NKT cells. While the numbers of immature $CD24^{high}CD44^{low}$ NKT cells were not different between B6.*Vα14*$Tg^{+/-}$ and B6.*Ccr7*$^{-/-}$.*Vα14*$Tg^{+/-}$ mice, numbers of mature $CD24^{low}CD44^{high}$ NKT cells declined in B6.*Ccr7*$^{-/-}$.*Vα14*$Tg^{+/-}$ mice. Similarly, the numbers of $CD44^{low}NK1.1^{-}$, $CD44^{high}$ $NK1.1^{-}$ and $CD44^{high}$ $NK1.1^{+}$ NKT cells also decreased in B6.*Ccr7*$^{-/-}$.*Vα14*$Tg^{+/-}$ mice. Although the proportions of immature ($CD4^{+}CD8^{+}$, $CD24^{high}CD44^{low}$ and $CD44^{low}NK1.1^{-}$) thymic NKT cells were increased (Fig. 6.11), the significant reduction of total thymic NKT cell numbers in KO mice, as indicated in Figure 6.10, resulted in decreased absolute numbers of all NKT cell subsets.

Together, the differences in expression levels of genes related to some transcriptional factors, cytokine receptors, chemokine receptors suggest functional differentiation between immature CD4 and DN NKT cells.

## 6.3. SUMMARY OF MAJOR FINDINGS

Transcriptional analysis of immature $CD4^{+}$ NKT cells and immature DN NKT cells indicated that there were large numbers of changes in gene expression across Transition 3. Gene Ontogeny analysis of highly differentially expressed genes revealed that there was widespread up-regulation of genes encoding proteins associated in lumenal membranes in DN NKT cells compared to $CD4^{+}$ NKT cells, including membrane-enclosed lumen and intracellular organelle lumen proteins. In addition, Gene Ontogeny analysis of genes down-regulated in DN NKT cells indicated a prevalence of genes related to cell activation in the most significant annotation categories. These differences in gene expression between $CD4^{+}$ NKT cells and DN NKT cells suggest differences in responses to signals and environmental cues.

The functional differentiation between $CD4^{+}$ and DN NKT cells was first confirmed by their distinct expression of *Zbtb7, Icos* and *Ly6a*. As microarray analysis revealed a significant up-regulation of *Zbtb7, Icos* and *Ly6a* transcripts in $CD4^{+}$ NKT cells compared to

DN NKT cells. The ICOS/ICOSL interaction is required for NKT cell homeostasis and function, as CD4 NKT cell differentiation, as well as IL-4 and IL-13 production, is impaired in $ICOS^{-/-}$ mice (Dave *et al*., 1998; He *et al*., 2005; Akbari *et al*., 2008). Furthermore, survival of WT NKT cells transferred into $ICOSL^{-/-}$ mice is reduced (Akbari *et al*., 2008).

NKT cells require a range of growth factors and survival signals, such as IL-2Rβ (Ohteki *et al*., 1997), IL-7Rα (Boesteanu *et al*., 1997), and IL-15R α and β chains (Lodolce *et al*., 1998; Chang *et al*., 2011) to support their homeostatic proliferation and long-term survival (Kennedy *et al*., 2000; Ranson *et al*., 2003; McNab *et al*., 2005). In particular, thymic NKT cell numbers and proliferation are impaired in IL-15 deficient mice but are not completely abolished (Kennedy *et al*., 2000; Matsuda *et al*., 2002). A possible explanation is that distinct NKT cell subsets require different cytokines during their development. For example, IFN-γ secreting NKT cells express CD122 (the IL-2/IL-15 receptor β chain) and require IL-15 for their development and homeostasis, while IL-17R$\beta^{+}$ NKT cells develop normally in the absence of IL-15 (Watarai *et al*., 2012). In accordance with these findings, the differences in cytokine receptor expression observed between $CD4^{+}$ NKT cells and DN NKT cells is consistent with the hypothesis that functional differentiation occurs at Transition 3.

Perhaps the most important characteristic that distinguishes NKT cells from other cells in the immune system is their ability to rapidly secrete cytokines upon stimulation. In this chapter, cytokine analysis of culture supernatant from FACS sorted immature $CD4^{+}$ and DN NKT cells revealed that immature $CD4^{+}$ NKT cells produced significantly higher level of many cytokines, including IL-4, IL-10, IFN-γ, GM-CSF and INF-α, when compared to immature DN NKT cells. Previously, *in vitro* studies have reported differences in cytokine production between splenic (but not thymic) $CD4^{+}$ and $CD4^{-}$ NKT cells from mice (Hammond *et al*., 1999) and human blood NKT cells (Gumperz *et al*., 2002; Lee *et al.,* 2002; Rogers *et al.,* 2004; Lin *et al*., 2004). However, these studies were performed on mature NKT

cells, therefore, our study is the first *in vitro* demonstration of immature $CD4^+$ and DN NKT cell subsets being functionally distinct.

Previously, many chemokine and chemokine receptors have been reported to be expressed by NKT cells and be involved in NKT cell survival and trafficking. Kim *et al*. (2002) reported that human $CD4^+$ NKT cells express low levels of CCR5 but high levels of CCR4 compared to the $CD4^-$ ($CD8^-$ or DN) NKT cell subsets. In contrast, the $CD4^-$ NKT cell subsets characteristically express CCR1, CCR6 and CXCR6. However, all NKT cells express high levels of CXCR3 and CXCR4 (D'Andrea *et al*., 2000; Motsinger *et al*., 2002). In mice, CXCR3 and its ligand, CXCL9, are essential for the homing of mature NKT cells to the periphery from the thymus. NKT cells exhibited robust migration to the inflammatory chemokines MIG/CXCL9 and IP-10/CXCL10 (Johnstons *et al*., 2003).

Additionally, CXCR6 and its trans-membrane ligand CXCL16 are important for the accumulation of NKT cells in the liver. NKT cells account for most hepatic $CXCR6^+$ cells. They were found to crawl within hepatic sinusoids and to stop upon T cell antigen receptor activation. CXCR6-deficient mice exhibited a selective and severe reduction of CD1d-reactive NKT cells in the liver and decreased susceptibility to T-cell-dependent hepatitis (Geissmann *et al*., 2005). CXCR6 and its ligands also play a critical role in homeostasis and activation of CD1d-restricted NKT cells (Matloubian *et al*., 2000; Shimaoka *et al.,* 2007; Germanov *et al*., 2008). A study on heterozygous mice with a targeted replacement of CXCR6 by eGFP showed that thymic NKT cells up-regulated the chemokine receptor CXCR6 following positive selection and migrated toward CXCL16 *in vitro* (Germanov *et al*., 2008). Liver and lung NKT cells were depleted in $CXCR6^{+/-}$ and $CXCR6^{-/-}$ mice, however, there was a redistribution of NKT cells into their bone marrow. The neutralization of CXCL16 reduced the accumulation of mature $NK1.1^+$ in the liver of wild-type mice, but not immature $NK1.1^-$ NKT cell recent thymic emigrants. Cytokine production by liver and spleen NKT cells was impaired in $CXCR6^{-/-}$ mice following *in vivo* stimulation with α-galactosylceramide. These findings suggest an additional

role for CXCR6/CXCL16 in maturation or survival of immature liver NKT cells and a novel role for CXCR6 in NKT cell activation.

In this chapter, microarray analyses of immature thymic CD4 and DN NKT cells from NOD.*Vα14*Tg mice revealed that both $CD4^+$ and DN NKT cells highly expressed large numbers of chemokine receptors after the positive selection events, including CCR2, CCR4, CCR7, CCR8, CCR10 and CXCR6. Of these chemokine receptors, the role of CCR7 was validated on B6 WT and B6 *Vα14*$Tg^{+/-}$ mice. The deficiency of CCR7 resulted in 6- and 3-fold decrease in thymic and peripheral NKT cell numbers, respectively, in B6 WT mice. However, it presents a different effect on B6.*Vα14*$Tg^{+/-}$ mice. While the CCR7 deletion on B6.*Vα14*$Tg^{+/-}$ mice leaded to a significant reduction in both proportions and absolute numbers of thymic NKT cells in B6.*Ccr7*$^{-/-}$, those of splenic NKT cells were unchanged but the absolute numbers of hepatic NKT cells were significantly increased. The mechanisms for this discrepancy of CCR7 role on B6 WT and B6.*Vα14*$Tg^{+/-}$ mice are unclear. The effect of CCR7 on NKT cells from B6 WT mice seen in this chapter were confirmed in Cowan *el al.* work.

*Id2* is a transcriptional regulator that plays a critical role in NKT cell homeostasis and hepatic localization. *Id2*-deficient NKT cells fail to accumulate in the liver and bone marrow. Monticelli *et al*. (2009) reported that the absence of *Id2* does not affect NKT cell migration but results in reduction in *Bcl-2* and *Bcl-*$X_L$ mRNA levels and increased apoptosis in these mice. Both hepatic and splenic NKT cells from *Id2*$^{KO}$ mice show dramatic reduction in CXCR6 mRNA and protein expression compared to *Id2*$^{WT}$ NKT cells (Moticelli *et al*., 2009; Geissmann *et al*., 2005; Germanov *et al*., 2008).

In conclusion, transcriptional comparison between immature $CD4^+$ and DN NKT cells indicated that the two populations exhibit different gene expression profiles, particularly differences in their cytokine receptor and chemokine receptor profiles. These differences in cytokine production support the hypothesis that functional differentiation occurs at transition 3, between $CD4^+$ NKT cells and DN NKT cells.

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# CHAPTER 7

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# GENERAL DISCUSSION

### *7.1. Production of a novel model Vα14 TCR transgenic strain*

*Vα14-Jα18* transgenic mice have been previously developed to aid functional and developmental studies of invariant NKT cells (Bendelac *et al*., 1996; Griewank *et al*., 2007). In this work, a validated transgenic construct containing an NKT-associated Vα14-Jα18 TCR α chain cDNA was expressed on the NOD mouse genetic background, which confers a partial defect in NKT cell selection and development (Baxter *et al*., 1997; Poulton *et al*., 2001; Griewank *et al*., 2007).

Flow cytometric analysis of CD1d-tet$^{+}$TCRβ$^{+}$ lymphocytes from NOD.*Vα14*Tg mice indicated that thymic, hepatic and splenic NKT cell numbers were significantly increased. Transgenic lines 1 and 3 had ~60-90-fold increases in thymic NKT cell numbers while lines 2 and 4 had ~3-fold increases; numbers of hepatic NKT cells were raised ~4-fold in lines 1 and 3, and by 25-50% in lines 2 and 4; lines 1 and 3 resulted in ~7-fold increases in splenic NKT cell numbers, while lines 2 and 4 had similar numbers to the non-transgenic parental strain. Both the CD4 SP and DN populations of mature NKT cells could be identified in the thymi, livers and spleens of WT mice and mice from the NOD.*Vα14*Tg lines. Virtually, all (>99%) NKT cells in the livers and spleens of NOD.*Vα14*Tg mice were either CD4$^{+}$ or DN. In both peripheral organs of NOD.*Vα14*Tg mice, the proportions of the two major NKT cell subsets that express the relatively mature CD24$^{low}$ CD44$^{high}$ phenotype were similar to WT, as were the proportions of CD44$^{high}$ NKT cells of either subset that expressed NK1.1. These peripheral NKT cells were functional and responded to *in vivo* stimulation with α-GalCer by robust cytokine production.

The expression of the *Vα14* transgene on both NOD and B6 genetic backgrounds has resulted in increased numbers of mature peripheral NKT cells. From a developmental and evolutionary perspective, the results imply that expression of the Vα14-Jα18 TCR $\alpha$ chain

alone might be sufficient information to bias the differentiation of a thymocyte towards the NKT cell pathway. However, it remains to be tested whether expression of the Vα14-Jα18 TCR α chain itself is genetically programmed in iNKT cells, or whether it occurs randomly.

### *7.2. Increased production of very immature NKT cells*

As presented in this work, one of the most striking characteristics of the thymic NKT cell population in both NOD and B6.*Vα14*Tg mice was the presence of increased numbers of the DP subset. In particular, this population constituted between 35 and 65% of all thymic CD1d-tet$^{+}$TCRβ$^{+}$ NKT cells in the various lines of NOD.*Vα14*Tg mice, while it accounted for less than 5% of thymic NKT cells in NOD WT mice. The DP NKT cells were predominantly CD24$^{high}$ CD44$^{low}$ NK1.1$^{-}$. Compared with previously published data (Benlagha *et al*., 2002, Godfrey and Berzins, 2007), it suggests that the thymic DP$^{high}$ CD24$^{high}$ CD44$^{low}$ NK1.1$^{-}$ CD1d-tet$^{+}$TCRβ$^{+}$ population represents NKT cells that have not yet undergone thymic selection because: 1) The population lacks expression of the developmental markers typical of mature NKT cells, viz: NK1.1 and CD44; 2) They express the CD24 marker, which is characteristic of conventional T cells prior to selection; 3) Cells of this phenotype were not found outside the thymus, despite very large numbers of them being found within it; and 4) Quantitative PCR analysis of Vα14-Jα18 encoded transcripts of sorted DP$^{high}$ thymocytes in a previous study by Gapin and colleagues found equivalent proportions in WT and *Cd1d*$^{-/-}$ mice (Gapin *et al*., 2001). Similarly, only a single *Vα14-Jα18* rearrangement could be detected in the DP$^{dull}$ population of WT mice, in contrast with the multiple rearrangements detected in DP$^{high}$ thymocytes (Gapin *et al.,* 2001). Therefore, the transgenic expression of an NKT-associated Vα14-Jα18 TCR α chain cDNA in this work resulted in greatly expanded numbers of immature DP NKT cells with the phenotype of the earliest identifiable NKT cells.

In NKT cell development, much attention has been paid to mature NKT cells and the acquisition of the NK1.1 marker, primarily because prior to the generation of CD1d-tet (Benlagha *et al*., 2000; Matsuda *et al*., 2000), the expression of NK1.1 on $CD3^+$ cells was used as a surrogate marker of NKT cells and almost all of NKT cells express $CD44^{high}$. Therefore, CD44 and NK1.1 were widely used to classify NKT cell developmental subsets. Two thymic populations of NKT cells that have been widely studied are: $CD44^{high}NK1.1^+$ NKT cells, which appear to be non-dividing and long-lived, and do not contribute significantly to thymic export (Gapin *et al.*, 2001; Pellicci *et al*., 2002; Benlagha *et al*., 2002), and recent thymic emigrant NKT cells, which are primarily $CD44^{high}NK1.1^-$ and acquire NK1.1 expression between 24 hours and a week after thymic egress (Benlagha *et al.,* 2002; McNab *et al*., 2005). The up-regulation of NK1.1 on $CD44^{high}$ NKT cells is dependent on the cell-intrinsic expression of the transcription factor T-bet (Townsend *et al*., 2004).

The identification of the $HSA^{high}$ $DP^{low}$ and $HSA^{high}$ $CD4^+$ stages by *in vitro* enrichment of NKT precursors from pools of up to 60 new-born thymi with CD1d-αGalCer tetramers and MACS sorting, essentially completed the map of the enigmatic developmental pathway of NKT cells (Gapin *et al*., 2001; Benlagha *et al*., 2002; Pellicci *et al*., 2002; Gadue *et al*., 2002). However, the very low frequency of very immature NKT cells (estimated at less than $1/10^6$) has precluded physical identification. The presence of greatly expanded numbers of very immature NKT cells in NOD.*Vα14*Tg mice provided us with an opportunity to study the earliest identifiable stages of NKT cell commitment and differentiation, and to help dissect factors controlling the numbers and function of this important immunoregulatory population. Therefore, our NOD.*Vα14*Tg mice provided a novel model of NKT cell selection in which sufficient pre-selection cells were available that the stages and processes involved could be clearly distinguished.

### *7.3. Supporting early stages of previously established developmental pathway of NKT cells*

In contrast to extensive work made on mature and relatively mature NKT cells, there were very few studies that focus on early development of NKT cells. With an attempt to identify the earliest CD1d-tet$^{+}$ thymic NKT cells, Gapin and colleagues stained C57BL/6 mouse thymi with CD1d-tet-phycoerythrin (PE), and isolated positive cells with anti-PE magnetic beads (Gapin *et al*., 2001). Of the NKT cells obtained from thymi of nine-day old mice, 25% were "DP$^{dull}$", a population whose frequency declined with age, was not seen in the periphery at any time, and was absent in CD1d$^{-/-}$ mice. The authors postulated that this population probably represented early post-selection NKT cells (Gapin *et al*., 2001) because during conventional T cell development, TCR ligation of DP$^{high}$ thymocytes triggers CD4 or CD8 down-regulation (Swat *et al*., 1992; Page *et al*., 1993). In contrast, quantitative polymerase chain reaction analysis of Vα14-Jα18 encoded transcripts of sorted DP$^{high}$ thymocytes found equivalent proportions in wild type and *Cd1d*$^{-/-}$ mice, consistent with the DP$^{high}$ phenotype marking pre-selection NKT cells (Gapin *et al*., 2001). Further evidence of the DP$^{high}$ to DP$^{dull}$ transition representing positive selection was obtained by CDR3 spectratyping in WT mice: Only a single peak could be detected in the DP$^{dull}$ population, which corresponded to the canonical *Vα14-Jα18* rearrangement. This contrasted with the multiple rearrangements detected in DP$^{high}$ thymocytes (Gapin *et al*., 2001).

In 2005, Benlagha and colleagues applied a similar tetramer-based enrichment strategy to identify DP CD24$^{high}$ NK1.1$^{-}$ NKT cell precursors in the thymi of new-born mice. These cells were non-dividing and already exhibited the same Vβ8 TCR bias characteristic of mature NKT cells (Benlagha *et al*., 2005). They therefore are likely to be post-selection, making them the earliest population of post selection NKT cells identified to date. In contrast, the subsequent population of CD24$^{low}$NK1.1$^{-}$ CD1d-tet$^{+}$ NKT cells contained two subsets

($CD44^{low}$ and $CD44^{high}$), both of which were rapidly proliferating (Benlagha *et al*., 2002). On the basis of the changing proportions with aging from two to six weeks, Benlagha and colleagues proposed that the $CD44^{low}$ population precedes the $CD44^{high}$ population during their maturation (Benlagha *et al*., 2002). The developmental pathway of NKT cells in mice postulated by Godfrey and Berzins is therefore: $DP^{high}$ $CD24^{high}$ $CD44^{low}$ $NK1.1^{-}$ → selection → $DP^{low}$ $CD24^{high}$ $CD44^{low}$ $NK1.1^{-}$ (Stage 0) → DN or $CD4^{+}$ $CD24^{low}$ $CD44^{low}$ $NK1.1^{-}$ (Stage 1) → DN or $CD4^{+}$ $CD24^{low}$ $CD44^{high}$ $NK1.1^{-}$ (Stage 2) → DN or $CD4^{+}$ $CD24^{low}$ $CD44^{high}$ $NK1.1^{+}$ (Stage 3; Godfrey and Berzins, 2007).

Although the later part of the developmental pathway postulated by Godfrey and Berzins is currently challenged by the more recent data in the field about the development of the novel NKT1, NKT2 and NKT17 lineages, the presence of large numbers of $DP^{high}$ $CD24^{high}$ $CD44^{low}$ $NK1.1^{-}$ CD1d-$tet^{+}$ $TCR\beta^{+}$ cells in the thymus of NOD.*Vα14*Tg mice and their absence in the periphery, suggested that these cells represent a very immature, possibly pre-selection population of NKT cells. This gave us the opportunity to compare the transcriptional profiles of $DP^{high}$ $CD24^{high}$ $CD44^{low}$ $NK1.1^{-}$ CD1d-$tet^{+}TCR\beta^{+}$ cells (i.e. very immature NKT cells) with those of more mature NKT cell subsets in order to identify the transcriptional transitions associated with early development of NKT cells, such as TCR validation, positive selection and lineage commitment.

Microarray analyses were performed on four different thymic populations: $DP^{high}$ $CD24^{high}$ $NK1.1^{-}$ CD1d-$tet^{-}TCR\beta^{+}$ cells (immature DP conventional T cells), DP $CD24^{high}$ $CD44^{low}$ $NK1.1^{-}$ CD1d-$tet^{+}TCR\beta^{+}$ cells (DP NKT cells), $CD4^{+}CD8^{-}CD24^{high}$ $NK1.1^{-}$CD1d-$tet^{+}TCR\beta^{+}$ cells (CD4 NKT cells) and $CD4^{-}CD8^{-}$ $CD24^{high}$ $NK1.1^{-}$CD1d-$tet^{+}TCR\beta^{+}$ cells (DN NKT cells), which were isolated by FACS sorting from thymi of NOD.*Vα14*Tg mice. Principal Component (PC) analysis of transcript expression indicated that the three largest

PCs explained >58% of variation across all samples. Seven samples from each of the four cell types clustered together into groups, and the four groups were distributed evenly across PC1 (X axis, which explained >29% of variation) in the order: DP $CD24^{hi}$ $NK1.1^-$ T cells → DP $CD24^{hi}$ $NK1.1^-$ NKT cells → CD4 $CD24^{hi}$ $NK1.1^-$ NKT cells → DN $CD24^{hi}$ $NK1.1^-$ NKT cells. The transcriptional progression of the four NKT cell subsets therefore mirrors the early developmental progression described by Godfrey's group, based on their and other's work (Godfrey and Berzins, 2007; Coquet *et al*., 2008). Of particular interest is the possibility that our analysis of DP NKT cells might identify the transcriptional events associated with Control Point 1 of NKT cell development, which Godfrey and Berzins attributed to selection (Godfrey and Berzins, 2007).

In addition to the confirmation of NKT cell development, the results from this work indicated some other points of T lineage development. It is well demonstrated that sequential rearrangement of the T cell receptor for antigen β and α chains is a hallmark of thymocyte development. In TCR transgenic model systems, TCRα and -β chains are expressed early at the $CD4^-CD8^-$ DN stage instead of the DP stage (Terrence *et al*., 2000; Lacorazza *et al*., 2001; Wolfer *et al*., 2002). The early expression of TCRα has been suggested to affect β-selection even in the presence of the pre-TCR because TCRβ has a higher affinity for TCRα than it does for pre-Tα (Trop *et al*., 2000). In this case, the αβTCR heterodimer can mediate β-selection if expressed at the DN stage, but it is highly inefficient (Borowski *el al*., 2004). Therefore, early expression may affect αβ/γδ lineage commitment, which results in a large population of mature DN $TCR^+$ cells both in the thymus and the periphery (Bruno *et al*., 1996; Fritsch *et al*., 1998; Terrence *et al*., 2000; Lacorazza *et al*., 2001). They represent a terminally differentiated thymic population without the ability to seed the DP compartment (Lacorazza *et al*., 2001) and present properties consistent with γδ-lineage cells in the

periphery (Terrence *et al*., 2000). By creating a Cre/lox-based conditional model in which TCR expression would be delayed until the DP stage (as is the case in normal animals), Baldwin *et al.* (2005) rescued the reduced DP and increased DN cellularity typically seen in TCR transgenics because the chain was expressed at the appropriate stage.

In our work, the premature expression of transgenic *Vα14*TCR might promote DN T cell development as seen in previous studies (Baldwin *et al.,* 2005; McDonald *et al,.* 2014). The DN:CD4 inversion seen in both NOD and B6 *Vα14* transgenic mice might be due to TCR engagement in developing NKT cells occurring earlier, on average, in *Vα14* transgenic mice compared to controls. The results could be postulated that, in the normal thymus, NKT cells might bifurcate into DN and CD4 subsets due to a difference in the "age" of the DP cell when it is positively selected into the NKT lineage.

Secondly, as seen in this work, the mature NKT cells generated by NOD.*Vα14*Tg/1 mice exhibited the relatively similar characteristics of TCR Vβ usage as NKT cells from WT mice, however, 70-80% of $CD44^-$ NKT cells and ~38% of $CD44^+$ NKT cells in the thymus of NOD.*Vα14*Tg mice use TCR Vβ chains other than Vβ8, Vβ7 or Vβ2, exceeding by far the frequency detected in the thymus of WT NOD mice (~0%). These findings suggest that the choice of TCRβ chain affects the probability that a αGalCer/mCD1d tetramer-binding thymocyte will transition from a $CD44^-$ to a $CD44^+$ phenotype.

### *7.4. TCR validation was associated with the transition from DP T to DP NKT cells*

NOD.*Vα14*Tg mice, a novel *Vα14* TCR transgenic strain bearing greatly expanded numbers of $CD24^{high}CD44^-$ NKT cells provided a model of NKT cell selection in which sufficient pre-selection cells were available that the stages and processes involved could be clearly distinguished. Evidence supporting the use of the model comes from the relatively large numbers of transcripts differentially expressed between thymic $TCR\beta^+DP^{high}$ $CD24^{high}$

populations that either did or did not bind CD1d-tet. This comparison provided evidence of successful TCR signalling in thymic $DP^{high}$ $CD24^{high}$ $NK1.1^{-}$ NKT cells, including TCRα allelic exclusion and *Rag1* down-regulation.

Transcriptional analysis comparing immature DP T cells and immature DP NKT cells revealed a reduction in expression of the electron transport genes, such as NADH Dehydrogenase (*Nd1, Nd2, Nd4, Nd5*), Cytochrome C Oxidase (*Cox1, Cox2, Cox3*) and ATP Synthase 6 (*Atp6*) suggesting reduced mitochondrial oxidative phosphorylation and raising the possibility that immature DP NKT cells had a higher proliferation rate. This is a characteristic of the Warburg phenomenon, in which cancerous or proliferative cells increase aerobic glycolysis at the expense of oxidative phosphorylation (Vander Heiden *et al*., 2009; 2011).

Transcriptional evidence was sought to support the hypothesis that the increase in proliferation across Transition 1 was due to TCR signalling. Genes encoding almost all the proximal components of the TCR signalling cascade were significantly down regulated (U stat = 0): *Lck* ($p<6.6 \times 10^{-8}$; t-Test), *Fyn* ($1.4 \times 10^{-7}$), *Zap70* ($5.4 \times 10^{-5}$), *Vav1* ($1.3 \times 10^{-6}$) and *Plcg1* ($1.2 \times 10^{-10}$). Although not previously reported, the effects of this transition would be akin to other mechanisms of TCR tuning, such as down regulation of the TCR and CD4/CD8 co-receptors, regulation of accessory molecules such as CD5, CD2 and CD28, and phosphorylation of SHP-1 (Grossman and Paul, 2015), suggesting that the TCR had been ligated and successfully signalled – constituting validation of a functionally rearranged (in this case transgenic) receptor.

The evidence of allelic exclusion of endogenous alpha chain loci supports the hypothesis that the proliferation observed across Transition 1 was due to successful TCR signalling and validation. TCR alpha chains dominated the most significantly differentially expressed transcripts, constituting ten of the top 20 most significant. Highly significantly

down-regulated TCR variable-alpha gene segments included: *Tcra-V22.1, Trav8d-1, Trav7d-4, Trav12-2, Trav9-4, Trav6-5, Trav9n-1, Trav6-3, Trav7d-3, Trav9d-3, Trav13d-1, Trav7d-2, Trav6-7-dv9, Trav7-6, Trav6d-5, Trav9-4, Trav13-2, Trav12-2, Trav12n-2, Trav14-1, Trav7-6, Trav5-4, Trav6d-3, Trav1, Trav14n-2, Trav12-2, Trav12-1, Trav7d-4, Trav4-4-dv10, Trav9d-3, Trav7n-5, Trav14d-3-dv8, Trav8-1* and *Trav12-1* (all U=0; $p<1.4x10^{-6}$, t-Test). The loss of expression of the great majority of non-NKT cell associated Vα chains is consistent with successful validation of the transgene-encoded TCR initiating the mechanism for allelic exclusion of competing TCR.

The possibility of increased proliferation in DP NKT cells was also supported by the up-regulated expression of genes associated with the cell cycle and cell division. A large number of genes involved in the E2F signalling pathway, DNA replication and Centromere proteins were significantly up-regulated by immature DP NKT cells compared to immature DP T cells. The most strongly differentially expressed gene identified across Transition 1 encoded the transcription factor *E2f1*, which was highly significantly up-regulated, lending support for increased proliferation in DP NKT cells. E2F1, E2F2 and E2F3 act in a functionally redundant manner to enhance the expression of many genes required for $G_1$ to S phase cell cycle progression, proliferation and development, including *Cdc6*, *Ccne1* and *Myb* (Johnson *et al*., 1993; Wu *et al.,* 2001). The growth regulatory function of the Rb tumour suppressor protein is mediated by its binding to E2F transcription factors, so that while overexpression of E2F1 unopposed by increased RB expression results in increased proliferation, so does *E2f1* gene deletion (Yamasaki *et al*., 1996; Field *et al*., 1996). Consistent with increased *E2f1* expression, E2F-regulated genes, such as *Cdc6* and *Ccne1* were also significantly up-regulated, as were genes encoding proteins critical for spindle formation and chromosome segregation (such as *Cenpm)* and DNA replication (such as *Rpa2*) consistent with E2F1 up-regulation driving cell proliferation.

The role of the E2F pathway in mediating T cell proliferation has been studied in *E2f1/E2f2* double knockout mice. It appears to play a role in homeostatic proliferation (HP) but not in proliferative responses to exogenous antigen, as HP in *E2f1/E2f2* double knockout mice is severely reduced (DeRyckere and DeGregori, 2005), but proliferation in response to exogenous antigen is not (Zhu *et al.,* 2001; DeRyckere and DeGregori, 2005). While HP of naive T cells requires both IL7 signalling (Schluns *et al*., 2000) and TCR stimulation by MHC-self peptide (Ernst *et al*., 1999), HP of memory CD4 T cells is dependent on TCR stimulation by MHC-self peptide alone (Tan *et al*., 2002). As HP of all T cells, including memory CD4 T cells is impaired in *E2f1/E2f2* double knockout mice, the E2F pathway must play a role in mediating T cell proliferation in the context of HP (Field *et al*., 1996). The early processes of thymocyte TCR validation and T cell selection resemble those of HP, in that they are mediated by MHC-self peptide recognition and stimulate proliferation. Activation of the E2F pathway in Transition 1 therefore suggests that T cell signalling, and therefore TCR validation, has occurred.

While Benlagha and colleagues previously reported that their $DP^{low}$ $CD24^{high}$ $NK1.1^{-}$ NKT cells in the thymi of newborn mice were non-dividing (Benlagha *et al.*, 2005), our functional validation by *in vivo* BrdU incorporation confirmed our hypothesis that the immature thymic $DP^{high}$ NKT cells in NOD.*Vα14*Tg mice have increased proliferation. Transcriptional evidence of allelic exclusion of competing TCR Vα chains in immature DP NKT cells was provided by the widespread down-regulation of non-NKT cell TCR Vα genes in Transition 1. This finding was confirmed by the almost complete absence of immature DP NKT cells and mature NKT cells expressing other TCR Vα chains, such as TCR Vα2, Vα3.2 and Vα8.3 accompanied with TCR Vα14-Jα18 chain. Together, down-regulation of genes related to the electron transport chain, the activation of the E2F pathway, T cell signaling and

the down-regulation of non-NKT associated TCR Vα genes suggests that successful TCR validation occurs across Transition 1.

### *7.5. Positive selection and lineage commitment were associated with the transition 2 from immature DP NKT cells to immature CD4 NKT cells*

Transcriptional analysis across Transition 2 suggests that positive selection and lineage commitment of NKT cells occurs during the transition from immature DP NKT cells to immature CD4 NKT cells. Gene Ontogeny analysis of up-regulated HDE genes revealed the strong up-regulation of a wide range of functional lymphocyte-associated membrane proteins, such as Toll-like receptors, Cytokine receptors, Chemokine receptors, Intergrins and Leukocyte differentiation markers. Of these, there was a predominance of genes associated with the immunomodulatory and innate-like properties of NKT cells, such as *Tlr1, Nkg7, Sema4a, Art2b, S1pr1* and *Zbtb16*.

Commitment to the NKT cell lineage is well known to be associated with the expression of the transcription factor PLZF, a transcription factor that drives differentiation into NKT cells and human MR1-specific MAIT cells, encoded by *Zbtb16* (Savage *et al*., 2008). Transactivation of PLZF appears to be the molecular switch that differentiates a T cell into an innate-like effector T cell. Physiologically, only Vα14-Jα18 TCR-expressing DP thymocytes and a few others (such as Vγ1.1Vδ6.3 γδ NKT cells, innate lymphoid cells in mice and mucosal-associated invariant T (MAIT) cells, NK cells, γδ T cells in humans) are able to up-regulate PLZF and acquire this phenotype. In the absence of PLZF, these Vα14-Jα18 TCR-expressing cells join the heterogeneous pool of naïve conventional helper T cells, migrating through secondary lymphoid tissue and being reliant on primary activation before being able to express effector functions. However, the complex phenotype of these residual cells hints at the necessity of other PLZF functions or interactions with other pathways

engaged during NKT cell-like positive selection. Ectopic expression of PLZF on cells that normally do not induce expression confers a robust and coherent effector program similar to that of NKT cells, indicating that PLZF is a lineage commitment factor for the innate-like phenotype. The close apposition of evolutionarily conserved TCRs, non-polymorphic ligands, and the unique effector program regulated by PLZF is a tantalizing clue as to the necessity of these rare, atypical T cell subsets for protective immunity.

Previous studies indicated that PLZF does not seem to have a dominant impact on the DP pool or thymic selection and does not significantly alter negative selection or the TCR repertoire (Savage *et al*., 2011). Analyses of PLZF transgenic cells in the presence or absence of the Marilyn TCR transgene of male mice (those express the H-Y antigen and as a consequence negatively select T cells bearing the anti-H-Y specific Marilyn TCR) indicated that in the presence of PLZF, I-$A^b$-HY-specific Marilyn TCR transgenic thymocytes underwent negative selection at the DP stage of development as expected (Savage *et al*., 2011). PLZF did not seem to alter the TCR signaling threshold of DP thymocytes as judged by CD69 induction upon graded TCR stimulation by a range of antigen concentrations. In addition, assessment of retroviral super antigens-mediated deletion of cells bearing Vβ families associated with recognition of BALB/c super antigens indicated that the presence of the PLZF transgene provided a partial rescue from deletion in BALB/c x PLZF transgenic (B6) F1 mice (Savage *et al*., 2011). Given such limited information on PLZF activity in T cells, it is difficult to speculate on how ectopic expression could be preserving cells normally deleted. However, the defect on numbers of NKT cells found in PLZF-mutant mice is localized to the intrathymic Stage 1 ($CD24^{low}CD44^{low}NK1.1^-$) to Stage 2 ($CD24^{low}CD44^{high}NK1.1^-$) transition. Mixed bone marrow chimeras clearly revealed normal frequencies of Stage 0 ($CD24^{high}CD44^{low}NK1.1^-$) and Stage 1 populations with a sharp decline of the subsequent stage 2 cells (Savage *et al*., 2011).

In our work, there was no significant difference in expression of *Zbtb16* between immature DP T cells and immature DP NKT cells, consistent with the immature DP NKT cells not yet having undergone positive selection, while *Zbtb16* was up-regulated more than 13-fold during the subsequent transition from immature DP NKT cells to immature CD4 NKT stage. These findings are consistent with immature CD4 NKT cells having been positively selected and commenced lineage commitment. The strong up-regulation of such a wide range of functional lymphocyte-associated, surface expressed, integral membrane proteins at Transition 2 is also consistent with this transition being associated with NKT cell lineage commitment and confirms the finding by Savage and colleagues that PLZF expression in NKT cells is up-regulated between Stage 0 and Stage 1 in development and responsible for driving the innate-like differentiation of NKT cells (Savage *et al*., 2008).

By comparison of genome-wide transcription in PLZF-deficient *Luxoid* mutant mice with that in WT mice, Savage and colleagues identified 69 genes being dependent on PLZF expression (Savage *et al*., 2008). The examination of transcript levels of these 69 genes in our microarray data confirmed that the up-regulation of *Zbtb16* across transition 2 was sufficient to modulate expression of PLZF target genes. Forty-two of the 69 genes identified by Savage and colleagues had a U statistic of zero across the transition ($p<0.0001$; Chi-square contingency table), and the vast majority (39/42) were up-regulated, consistent with PLZF expression playing a significant role in Transition 2 of NKT cell development.

The coordinated expression of the innate-like lymphocyte-associated transcription factor PLZF and the subsequent up-regulation of a wide range of cell-surface functional receptors associated with NKT cell immunobiology combine to provide evidence that NKT cell lineage commitment occurred across Transition 2, between Stage 0 and 1. However, it raised the issue of the timing of the NKT cell selection event, which could not take place later than lineage commitment in Transition 2. Of the genes showing up-regulation in immature

CD4 NKT cells, many have been previously described as increased in expression during positive selection of conventional T cells (Huang *et al*., 2004; Mick *et al*., 2004). These data are consistent with NKT cell selection occurring across Transition 2.

Positive and negative selection of NKT cells has been previously studied. NKT development is markedly impaired by the absence of the antigen presenting molecule, CD1d for positive selection (Bendelac, 1995; Chen *et al*., 1997; Smiley *et al*., 1997; Mendiratta *et al*., 1997; Coles and Raulet, 2000; Gapin *et al*., 2001; Wei *et al*., 2005) or by the early administration of α-GalCer (Pellicci *et al*., 2003; Chun *et al*., 2003). However, in these studies, due to the very low number of DP NKT cells in wild type mice, the effect of targeted deletion of CD1d and administration of α-GalCer were not directly examined on DP NKT cells. It was assumed that negative selection affected the early development stage cells as delayed treatment with α-GalCer *in vivo* did not deplete NKT cells (Pellicci *et al*., 2003). In contrast, the production of very large numbers of immature DP NKT cells in the thymi of *Vα14* transgenic mice provided us with an opportunity to directly examine the positive and negative selection of NKT cells.

Targeted deletion of CD1d from NOD.*Vα14*Tg mice indicated that CD1d is required for further development of thymic immature DP NKT cells. In the absence of CD1d, immature DP NKT cells did not progress to subsequent developmental stages and accumulated in the thymi of these mice. The almost complete absence of peripheral NKT cells in NOD.*Cd1d*$^{-/-}$.*Vα14*Tg mice is consistent with failed positive selection of DP NKT cells in the absence of CD1d. Additionally, the immature DP NKT cells were also susceptible to negative selection mediated by the administration of α-GalCer both intravenously and intrathymically. The behaviour of DP NKT cells under these experimental conditions was consistent with them not yet having undergone selection, as: 1) DP NKT cells were not found outside the thymus, despite very large numbers of them being found within it; 2) Their

numbers were more than doubled by targeted deletion of CD1d, which is the antigen presentation glycoprotein required for NKT cell positive selection; 3) They were depleted to about half their numbers by systemic or intrathymic injection of the strong NKT cell glycolipid antigen α-GalCer in a CD1d-dependent manner; and 4) DP NKT cells showed only three of the 44 transcriptional hallmarks of positive selection. Together, these data are consistent with the hypothesis that DP NKT cells are a pre-selection population and that positive selection of NKT cells occurs between immature DP NKT cells and CD4 NKT cells.

NKT cells are positively selected by ligating CD1d expressed on DP cortical thymocytes, which account for >80% of all thymocytes. If this was sufficient for positive selection, as distinct from merely necessary, the accumulation of pre-selection DP NKT in NOD.*Vα14*Tg mice can probably not be explained. On the contrary, these results suggest that positive selection of NKT cells is not just dependent on ligation of conventional DP thymocytes, as these are abundant. Instead this model shows evidence of a backlog of positive selection when numbers of pre-selection NKT cells are increased by transgenesis, consistent with a rate-limiting step.

CD1d is required for NKT cell positive selection (Bendelac *et al*., 2007; Godfrey and Berzins, 2007). The absence of CD1d on thymocytes abrogates the DP-DP interaction, which is required for selection of the lineage (Bendelac, 1995). However, an additional signal such as the SLAM-SLAM homotypic interaction between immature NKT cells and selecting DP thymocytes might be a reasonable candidate for a necessary second signal. SAP mediates the SLAM receptor signaling that has been shown to occur upon DP-DP interactions and is required for NKT cell development (Chan *et al*., 2003; Griewank *et al*., 2007; Jordan *et al.* 2011). In $SAP^{-/-}$ mice, CD1d-tet-reactive cells never mature past the transitional $CD24^{high}$ stage (Griewank *et al*., 2007). These studies highlight the unique selection event that drives commitment of the NKT cell lineage: positive selection by ligand-bearing DP thymocytes

coincident with SLAM receptor signaling. However, in addition to the SLAM receptor signaling other co-stimulators or cytokines, such as IL7 or IL15, may be required.

Although the approach used in this thesis to study positive and negative selection is not new, our findings elucidate some long-standing issues regarding the positive and negative of NKT cells by directly testing on a real DP NKT cell population. Additionally, it is showed in our work and others that CD4 NKT and DN NKT cells are very rare in the thymus of $CD1d^{-/-}$ mice (Gapin *et al*., 2001 and in this thesis). By contrast, it is surprising and interesting that CD1d-$tet^{+}$ cells attain non-DP phenotypes in the absence of CD1d. These cells were either CD4 or DN and still expressed the $CD24^{+}CD44^{-}$ phenotype. They are present in the thymus of *Cd1d*$^{-/-}$*.Vα14*Tg mice, with population sizes similar to *Cd1d*$^{+/+}$*.Vα14*Tg mice. Although the mechanism for the appearance of these non-DP phenotypes is unclear, the possible explanation is that they might develop in a CD1d-independent manner. Their ontogeny is related to the premature expression of the Tg TCRα chain that binds to the TCRβ chains expressed at the DN3 stage. It is believed that, by modifying the signals that normally emanate from the pTα-TCRβ complexes, the TCRα transgene bypasses the subsequent need for TCR-mediated positive selection (von Boehmer *et al*., 1991; Bruno *et al*., 1996; Eberl *et al.*, 1999b; Terrence *et al.,* 2000; Borowski *et al.,* 2004; Baldwin *et al.,* 2005). Therefore, similar to a subset of mature NKT cells, these Tg cells do not express CD8 or both CD4 and CD8. In TCRβ rearranging cells, TCRα proteins are expressed so early that they mimic the pre-TCRα chain with regard to induction of cell maturation as well as allelic exclusion (Buer *et al*., 1997). It will be useful to identify these cells at an early stage of development and to determine whether they represent a CD1d-independent NKT cells.

Secondly, the checkpoints imposed on developing T cells mandate that they generate TCR α and β chains that form an αβ TCR capable of positive selection (Goldrath and Bevan, 1999). TCR α and β chain gene assembly is precisely modulated within the context of normal

thymocyte development. TCRβ chain genes are rearranged in DN thymocytes (Godfrey and Zlotnik, 1993; Starr *et al.,* 2003). Expression of a TCRβ chain, as a pre-TCR, results in signals that promote cellular expansion and developmental progression to the DP stage of thymocyte development (Godfrey and Zlotnik, 1993; Michie and Zuniga-Pflucker, 2002; Starr *et al.,* 2003). Pre-TCR signals also promote TCRα chain gene rearrangement and prohibit further TCRβ chain gene assembly through the process of allelic exclusion (Aifantis *et al*., 1997; von Boehmer and Fehling, 1997; Khor and Sleckman, 2002). Because allelic exclusion prohibits DP thymocytes from generating new TCRβ chains, these cells must generate TCRα chains that form an αβ TCR capable of positive selection if they are to become mature T cells. Data presented in this thesis confirms that the requirements to complete NKT development *in vivo* extend beyond the mere capacity to bind αGalCer/mCD1d tetramer. Two potential requirements are (i) a "selectable" TCRβ chain sequence and (ii) TCR engagement with a particular glycolipid inside CD1d.

TCRβ chain repertoire of NKT cells is generated through the step-wise assembly and subsequent selection of TCRβ variable region exons during thymocyte development. Earlier studies using TCRβ chain transgenic mice (on a TCRβ$^{-/-}$ background) showed that the Vβ segment utilized is critical for NKT cell development (Ohteki and MacDonald, 1996). Vβ usage analysis in our work revealed that NKT cells produced by *Vα14* transgenic mice present the bias toward Vβ2, Vβ7 and Vβ8. However, it also showed that 70-80% of CD44– NKT cells and ~38% of CD44+ NKT cells in the thymus of NOD.*Vα14*Tg mice use TCR Vβ chains other than Vβ8, Vβ7 or Vβ2, exceeding by far the frequency detected in the thymus of WT NOD mice (~0%). These findings suggest that the choice of TCRβ chain affects the probability that a αGalCer/mCD1d tetramer-binding thymocyte will transition from a CD44$^{-}$ to a CD44$^{+}$ phenotype.

Some recent studies suggested that the bias toward the NKT lineage is generally associated with CDR3β (complementarity-determining region 3β) sequences. Analyses of the length and sequence heterogeneity of CDR3β domains in $V\alpha14^+$ NKT cells by Ronet *et al*. (2001) and Matsuda *et al*. (2001) concluded that CDR3 lengths of permissive TCRβ chains (Vβ2, Vβ7 and Vβ8) were equally heterogeneous for NKT cells and conventional T cells. The distinctive CDR3β sequence motifs are not apparent for NKT cells, it remains possible that certain TCRβ chain sequences may bias positive selection in favor of NKT cells. By expressing a TCRβ chain (Vβ8.2–Jβ1.4) derived from a transgenic $V\alpha14^+$ NKT hybridoma, Viret *et al*. (2000) indicated that canonical *Vα14–Jα281* rearrangements were increased in the thymus of these transgenic mice in a CD1d-dependent fashion and NKT cells (defined as $NK1.1^+ TCR\alpha\beta^+$) were twice as frequent as in control mice. These findings suggested that certain CDR3β motifs are critical for CD1d recognition during NKT cell development.

In terms of antigens, NKT cells can recognize CD1d molecules bound to a number of different glycolipids. Because some of these glycolipids are microbial in origin, it is thought that NKT cells contribute to responses to invading bacteria. However, NKT cells also react with CD1d-expressing antigen presenting cells (APCs) in the absence of infectious organisms, suggesting that NKT TCRs can recognize CD1d plus endogenous lipids and thus play a role in autoimmunity or indirectly play a role in innate response to infections. Several studies have investigated the nature of the endogenous lipid antigen(s) presented by CD1d molecules in various immunological contexts. More than 170 lipid species, comprising glycerophospholipids and sphingolipids, have been identified from human CD1d monomers produced by the human B cell line 721.221 (Cox *et al*., 2009). Another study revealed that phosphatidylcholine, lysophospholipids, and sphingomyelin represent the main lipids associated with human CD1d molecules expressed in this cell line (Yuan *et al*., 2009). Several glycosphingolipids, including GM1a, GD1a, and GM2, are also found bound by mouse CD1d

expressed by myeloid APCs (Muindi *et al.,* 2010). Finally, various molecular species of phosphatidylcholine with different fatty acid compositions are found loaded into mouse CD1d molecules produced by insect cells. These studies reveal the great diversity of self-lipids that can be found associated with CD1d molecules when produced in different systems

The structures of several mouse and human NKT TCRs in complex with CD1d bound to the marine sponge-derived antigen α-galactosylceramide (α-GalCer) (Borg *et al.,* 2007; Pellicci *et al.,* 2009), α-GalCer analogs (Wun *et al*., 2011), or related microbial glycolipids (Li *et al*., 2010) have recently been solved. These structures show that NKT TCRs, regardless of their precise TCRβ sequence, dock similarly on CD1d. The location and details of the docking are quite different from those seen in the engagement of conventional TCRs to their MHC plus peptide ligands (Godfrey *et al.,* 2008). A ‘‘hot spot’’ on the NKT TCR, composed only of germline encoded residues, is essential for recognition of CD1d plus several structurally diverse antigens, including isoglobotrihexosylceramide (iGb3), which has been proposed to be one of the self-antigens responsible for NKT cell autoreactivity (Zhou *et al*., 2004). These studies also suggest that the diverse CDR3β loop can modulate and fine tune the interaction of NKT TCRs with CD1d-glycolipid antigen complexes (Mallevaey *et al*., 2009). In this thesis, TCR engagement with a well known a-GalCer glycolipid inside CD1d, in vivo demonstrated negative selection of NKT cells and also confirm the requirement for the involvement of CD1d.

Early T cell development leads to at least five major outcomes, including thymocyte survival, allelic exclusion, migration into a new thymic microenvironment, TCR tuning of antigenic responsiveness and lineage commitment. The first outcome of positive selection is regulation of survival and cell death. Survival signals received by developing thymocytes during positive selection prevent programed cell death and allow the thymocytes to continue along their maturation pathway. The anti-apoptotic factor (Bcl-2) has been reported to

maintain DP thymocyte survival. *Bcl-2* gene expression was found to be up-regulated during DP stage of conventional T cells (Linette *et al*., 1994) and these levels are maintained in post-selection thymocytes (Mick *et al*., 2004). In our microarray data, *Bcl-2* expression was relatively similar between DP T cells and DP NKT cells, but was differentially up-regulated by immature CD4 and DN NKT cells, suggesting these post-selection NKT cells need *Bcl-2* expression to survive.

In wild type mice, the *Vα14* and *Jα18* gene segments are distally located in the TCRα gene and it has been shown that the *Vα14*-to-*Jα18* re-arrangement requires an extended period at the DP stage to take place, and is dependent on the expression of a number of factors including Bcl-$x_L$ (a Bcl-2 homologue, encoded by *Bcl2l1;* Bezbradica *et al*., 2005; Egawa *et al.,* 2005). In contrast, our microarray data showed that the anti-apoptotic factor *Bcl2l1* was down-regulated across Transition 1 from immature DP T cells to immature DP NKT cells. The exact mechanism remains to be determined, however, one possible explanation is that the expression of the rearranged *Vα14-Jα18* gene segments by transgenesis may have rendered the up regulation of *Bcl2l1* unnecessary.

The protein Programmed Cell Death 1 (PD-1) also plays an important role in thymocyte survival by controlling the threshold of positive selection in conventional T cells. The effects of the PD-1 deficiency on the thymocyte differentiation were examined at the clonal level on transgenic mice crossed between TCR-Vβ8 and TCR-α/β (H-Y and 2C) transgenic mice and PD-$1^{-/-}$ mice on neutral genetic backgrounds (Nishimura *et al*., 2000). It was reported that these mice exhibited a selective increase in the $CD4^+CD8^+$ population with little impact on other thymocyte subsets. The absence of PD-1 significantly facilitated the transition of thymocytes from DN to DP stage in the TCR transgenic lines as well as in RAG-$2^{-/-}$ mice injected with anti-CD3 mAb, suggesting that PD-1 controls the threshold of

selection, probably by affecting the threshold of pre-TCR/CD3 complex–mediated signalling (Nishimura *et al*., 2000). In contrast, H-Y or 2C transgenic PD-$1^{-/-}$ mice with the positively selecting background revealed significantly reduced efficiency for the generation of $CD8^{+}$ single positive cells bearing the transgenic TCR-α/β in spite of the increased DP population. The results collectively indicate that PD-1 negatively regulates the β selection and modulates the positive selection (Nishimura *et al*., 2000).

In our microarray, *Pd-1* gene expression levels were lowest on immature DP T cells, showing a 1.5 fold increase on DP NKT cells, but were 6 times higher in immature CD4 and DN NKT cells compared to immature DP NKT cells. This is in good agreement with previous studies, which found that PD-1 was not expressed on pre-selection DP thymocytes, but highly expressed on post-selection T cells (Nishimura *et al*., 1996; Mick *et al*., 2004). Consistent with studies mentioned above, our findings indicated an association between PD-1 expression and thymic selection of NKT cells.

Allelic exclusion is the second outcome of early T cell development by which a product of single allele is selected for expression. Allelic exclusion of T cell receptor genes is regulated differently between the α and β chains (Gascoigne and Alam, 1999). For the β chain, the presence of a productively rearranged β chain gene generally prevents further rearrangement of the other β chain genes, therefore, only one of the β chain loci is able to produce full-length, correctly rearranged β chain protein (Uematsu *et al.*, 1988; Krimpenfort *et al.,* 1989). In contrast, both alleles of the α chain rearrange until a positively selectable heterodimer of TCRαβ is formed and undergoes positive selection (Malissen *et al*., 1992), leading to Rag1/2-turnoff, which stops further rearrangement (Borgulya *et al*., 1992; Brandle *et al*., 1992; Petrie *et al.,* 1993; Kouskoff *et al*., 1995). Thus, immature ($TCR^{low}$) DP thymocytes have the potential to produce two α chain messages and express dual TCR α

chain proteins on the cell surface. Therefore, some T cells express two functionally rearranged and expressed α chains, but most express a single α chain on their cell surface (Alam *et al.,* 1995; Boyd *et al.,* 1998). It is reported that the number of peripheral T cells expressing two different α chains as part of two functional and independent TCRs vary widely from 5% to 15% in mice (Heath *et al*., 1995; Elliott and Altmann, 1995; Alam and Gascoigne, 1998; Corthay *et al*., 2001; Rybakin *et al*., 2014) and about 30% in human (Padovan *et al*., 1993). Cell surface expression of two α chains has also been detected in TCR transgenic mice (Heath and Miller, 1993; Hardardottir *et al*., 1995).

In contrast, although mouse T cell clones frequently carry two in-frame *Vα* rearrangements and express two mRNA species, they have not been found to express two cell surface α chains (Malissen *et al*., 1988; Kuida *et al.,* 1991; Couez *et al.,* 1991). In addition, Rybakin and colleagues indicated that transgenic mice bearing Vα3.2 and Vα2 miniloci showed low frequency of cells in these transgenic mice that actually rearranged the α chain miniloci (Rybakin *et al*., 2014). However, once expressed, both the Vα3.2 and Vα2 containing α chains had a strong ability to be positively selected with either a rearranged Vβ5 containing transgene or the natural repertoire of TCR β chains, the authors reported the failure to find a distinct population of cells that expressed dual TCRs, even intracellularly.

In our work, microarray analyses show the widespread down-regulation of non-NKT cell TCR Vα genes in immature DP NKT cells. This finding likely provides further evidence of allelic exclusion of competing TCR Vα chains at the transcriptional level. The primary mechanism of α-chain allelic exclusion described in literature is mediated at the protein level. During the DP stage of thymocyte development, both alleles of the α chain gene rearrange until a positively selectable heterodimer is formed with the previously rearranged β chain. Positive selection ceases α-chain rearrangement by

shutting off the *Rag1* and *Rag2* genes (Borgulya *et al.,* 1992; Brandle *et al.,* 1992; Kouskoff *et al.,* 1995; Alam *et al.,* 1995). A post-translational mechanism ensures that only one α chain is generally present on the cell surface of mature T cells (Alam and Gascoigne, 1998; Gascoigne and Alam, 1999, Rybakin *et al.,* 2014). Different models have been proposed to explain the post-translational regulation of the α chain allelic exclusion (Niederberger *et al.,* 2003). The simplest model proposed that allelic exclusion depended on the competition between two α chain proteins for a limiting supply of β chain protein, when the TCR level on the cell surface increases during positive selection (Alam *et al.,* 1995; Alam and Gascoigne, 1998; Gascoigne and Alam, 1999). In the second model, the CD3z chain (a component of the TCR-CD3 complex) might play an important role in phenotypic allelic exclusion of α chain by stabilizing preformed αβ pairs and thus preferentially stabilizing the αβ combination with the highest interchain affinity available to the cell. In the selective retention model, it was indicated that the positively selected αβ combination is maintained on the cell surface while the nonselected α-chain is down-regulated or retained within the cell by regulation of TCR degradation via ubiquitylation (Boyd *et al.,* 1998). However, those models do not explain how the widespread down-regulation of non-NKT cell TCR Vα genes occurs in immature DP NKT cells compared to immature DP T cells. The possibility is that the relatively early expression of Vα14 stops further recombination of endogenous alleles so that they have a greater chance of not having generated a viable transcript. Therefore, the down-regulation of alpha chain families' transcripts in DP NKT cells is likely the transcriptional evidence of allelic exclusion of competing TCR Vα chains in immature DP NKT cells at allele level. This finding was confirmed by the very low number (almost none) of immature DP NKT cells and mature NKT cells expressing other TCR α chains, such as TCR Vα2, Vα3.2 and

Vα8.3 accompanied with TCR Vα14-Jα18 chain. The proportion of immature DP NKT cells, as well as mature NKT cells expressing the tested TCR Vα chains, was significantly lower than immature DP T cells and mature T cells.

Regulation of migration and adhesion is also a critical outcome of thymic selection. Following positive selection, there are two significant changes in thymocyte motility and localisation: thymocytes begin to migrate rapidly and they relocate to the thymic medulla (Akiyama *et al.,* 2008; Bonito *et al.,* 2013). Chemokine receptor signaling is the most important mechanism that enables thymocytes to enter the medulla and accumulate therein. Numerous studies have implicated CCR7 as a key chemokine receptor responsible for the accumulation of SP thymocytes within the medulla (Suzuki *et al.,* 1999; Bleul and Boehm, 2000; Ehrlich *et al.*, 2009; Cowan *et al.,* 2014). It is reported that SP thymocytes undergo *in vitro* chemotaxis toward CCR7 ligands, whereas CCR7 mediates chemotaxis of SP thymocytes from the cortex toward the medulla (Suzuki *et al.,* 1999; Bleul and Boehm, 2000; Ehrlich *et al*., 2009). Overexpression of CCR7 leads to inappropriate translocation of DP thymocytes to the medulla (Kwan and Killeen, 2004), whereas lack of CCR7 or its ligands results in decreased accumulation of SP thymocytes within the medulla, impaired TCR signalling, negative selection and general autoimmunity (Davalos-Misslitz *et al.,* 2007; Cowan *et al.,* 2014).

In our microarray data, we found a large number of changes in gene expression of chemokine receptors, which have been previously reported as being related to positive selection of conventional T cells (Campell *et al*., 1999; Mick *et al*., 2004; Huang *et al*., 2004; Kwan *et al*., 2004). For example, we observed increased expression of *Ccr4* and *Ccr7* upon selection. *Ccr4* expression was slightly down-regulated by immature DP NKT cells compared to immature DP T cells but showed four times higher expression in immature CD4 and DN NKT cells compared to immature DP NKT. *Ccr7* expression was equal between DP T cells

and DP NKT cells, but was increased almost nine fold in immature CD4 and DN NKT cells. This is concurrent with the role of *Ccr7* in medulla migration and immigration from the thymus (Kwan and Killeen, 2004; Ueno *et al*., 2002).

CCR4 also affects medullary entry, however, it is up-regulated first on postpositive selection $CD69^+$ DP and $CD69^+$ CD4 thymocytes, before the up-regulation of CCR7 at the SP stage (Suzuki *et al.,* 1999; Cowan *et al*., 2014; Ki *et al.,* 2014). In the absence of CCR4, thymocyte negative selection is impaired, which causes the accumulation of autoreactive naïve T cells in secondary lymphoid organs and autoimmunity ensues (Hu *et al*., 2015). In addition to promoting thymocyte chemokinesis and chemotaxis, chemokine receptors likely mediate efficient interactions of SP thymocytes with medullary APCs. Both CCR4 and CCR7 have been shown to facilitate interactions of T cells with APCs in secondary lymphoid organs as well as interactions of SP thymocytes with medullary APCs (Tang and Cyster, 1999; Friedman *et al.,* 2006, Hu *et al*., 2015).

The fourth outcome of early T cell development is the tuning of T cell antigen receptor responsiveness, which allows immature thymocytes to be positively selected by complexes of self-peptide and MHC molecules (peptide-MHC) of lower affinity but prevents mature T cells from responding to similar complexes in the periphery. Negative selection prevents generalized T cell aggression towards healthy tissue, while preserving a naïve T cell repertoire available to produce T cell responses against foreign peptide-MHC when they are summoned to fight infection or malignant cell transformation (Palmer, 2003, Fu *et al.,* 2014). This task is fulfilled only when the survival during thymic development of immature T cells to recognize self peptide-MHC with low-to-moderate strength in a process termed positive selection is ensured. Positive selection generally precedes negative selection in the thymus, signaling survival of the positively selected thymocytes enables these cells to distinguish foreign antigen in the context of their own self-MHC (i.e., MHC restriction).

CD5, a monomeric cell surface glycoprotein expressed on thymocytes, T cells, and a subset of B cells, has been shown to negatively modulate signaling through both the B and T cell antigen receptors (Tarakhovsky *et al*., 1995, Bikah *et al.,* 1996). Although the precise mechanism by which CD5 inhibits TCR signaling has not been elucidated, it is reported that after TCR engagement, CD5 is tyrosine-phosphorylated and associates with several effector molecules that could potentially influence the TCR signaling response, including Cbl, rasGAP, SHP-1, casein kinase 2, and TCR-ζ/ZAP-70 (Davies *et al*., 1992; Pani *et al.*, 1996; Gary-Gouy *et al.*, 1997; Raman *et al.*, 1998; Dennehy *et al.*, 1998). CD5 deficiency in wild-type mice has no obvious effects on T cell development (Tarakhovsky *et al.*, 1994). However, the analysis of thymocytes expressing a transgenic αβTCR revealed that thymocyte selection was dramatically affected in mice lacking CD5 (Tarakhovsky *et al.*, 1995, Pena-Rossi *et al.*, 1999). The decrease in DP thymocyte numbers in TCR transgenic/$CD5^{-/-}$ mice compared to TCR transgenic and $CD5^{+/+}$ mice is suggestive of enhanced TCR signaling. However, the impact of CD5 deletion on thymocyte selection varies depending on the transgenic TCR analyzed, ranging from a mild to a significant shift from positive toward negative selection. CD5 surface expression is regulated by TCR signal intensity during development. CD5 surface expression levels on mature thymocytes and T cells parallel the avidity of the positively selecting TCR/MHC/ligand interaction. In another study, Azzam and colleagues manipulated CD5 expression in mutant mice during thymocyte development to examine the effect of CD5 overexpression or CD5 deletion on selection of thymocytes that express TCR transgenes (Azzam *et al.,* 2001). Their findings revealed that the impact of altering CD5 expression on thymic selection depends on the avidity of the selecting interaction and, subsequently, the level of endogenous CD5 surface expression. Substitution of endogenous CD5 with a transgene encoding a truncated form of the protein failed to rescue the $CD5^{-/-}$ phenotype, which demonstrated that the cytoplasmic domain of CD5 is required for its

inhibitory function. Together, these results indicate that inducible regulation of CD5 surface levels during thymocyte selection functions to fine tune the TCR signaling response.

Previous findings in conventional T cells indicated that TCR tuning at the proximal stage of conventional T cells requires up-regulation of *Cbl-b* and CD5-associated *Shp-1* to block *Vav* activation (Sprent and Surh, 2011). In this case, CD5 acts as a negative modulator of TCR signalling due to the preferential shift from positive to negative selection in CD5-deficient mice (Azzam *et al*., 2001). In our microarray data, it was reported that *Cd5, Btla* and *Cbl-b* expression was increased by positive selection of NKT cells. *Cd5* expression was up-regulated by positively selected (immature CD4 and DN) NKT cells. A 6.2 fold higher in expression of *Btla* (encoding B and T lymphocyte-associated gene; Han *et al.,* 2004) and a 2.5 fold greater expression of *Cbl-b* was also found on these post-selection NKT cell populations compared to the two pre-selection populations (immature DP T and immature DP NKT cells). Taken together, these data suggest that the changes in *Cd5*, *Btla* and *Cbl-b* levels might relate to TCR tuning processes during NKT cell development

The commitment to the NKT lineage, as well as other lineages of innate-like lymphocytes is associated with the expression of Promyelocytic Leukemia Zinc Finger (PLZF, encoded by *Zbtb16*; Savage *et al*., 2008), and T-bet (encoded by *Tbx21*; Townsend *et al*., 2004). Our microarray data indicated that there was no significant difference in expression of *Zbtb16* between immature DP NKT cells and immature DP conventional thymocytes, but there was a more than 13-fold up-regulation across Transition 2, consistent with lineage commitment occurring at this point. *Tbx21* was significantly, but only slightly higher expressed in immature DP NKT cells compared to immature DP conventional thymocytes. In contrast, the immature CD4 NKT cells showed reduced expression of *Tbx21*. This slight increase in the expression of *Tbx21* in immature DP NKT cells suggests a possibility for an earlier commencement of lineage commitment.

In 2012, Cohen *et al*. reported gene expression profiles of $CD44^{-}NK1.1^{-}$ iNKT cells at stage 1 (shortly after the DP branch point). Of the top 100 genes, they found differentially up-regulated by the stage 1 NKT cells compared to DP thymocytes, 41 genes were in our top 100 gene list which were highly significantly up-regulated by immature CD4 NKT cells compared to immature DP NKT cells ($p<0.05$) and another forty-three genes also had significantly increased expression ($p<0.05$; Supplementary Table 5.5). These datasets are comparable in terms of addressing changes during positive selection of NKT cells and the results provide evidence that our iNKT cell transgenic mouse strain is a valid model for early NKT cell development study.

In conclusion, the data presented in this work provided, for the first time, an overall transcriptional profile during TCR validation, positive selection and lineage commitment of NKT cells. These findings have further confirmed phenotypic changes during NKT cell development observed by previous studies and suggest that immature DP NKT cells are pre-selection progenitors of NKT cells. Our data also offer a new view of gene-expression programs at early stage of NKT cells through a large number of novel gene changes observed during positive selection of NKT cells. The transcriptional analyses in this chapter indicated that TCR validation occurs prior to positive selection in NKT cells. Our transcriptional regulatory network approach mapped TCR validation to the transition from DP T to DP NKT cells, while positive selection and lineage commitment were associated with the transition from DP NKT to CD4 NKT. This is the first time that the effects of positive and negative selection have been examined on their actual population – the immature DP NKT cells. Here we confirm by *in vivo* experimentation that both positive and negative selection occur at the latter transition, separating for the first time in any T cell population the events associated with TCR validation from those associated with positive selection. NOD.*Vα14*Tg mice provide an opportunity to study the earliest identifiable stages of NKT cell commitment and differentiation, and will help dissect factors controlling the numbers and function of this important immunoregulatory population.

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# APPENDIX A

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# SUPPLEMENTARY DATA

**Table S-1: List of genes that are overlapped between our research and Cohen *et al.* data**

| CD44-NK1.1-NKT cells/ DP Thymocytes (Cohen *et al.*, 2012) | | | Gene # | CD4 NKT cells/DP NKT cells (from this research) | | |
|---|---|---|---|---|---|---|
| **Gene Symbol** | **FC** | **p-value** | | **Gene Symbol** | **FC** | **p-value** |
| 9930111J21Rik | 9.20 | 0.00036 | 1 | 9930111J21Rik2 | 7.60 | 2.57E-13 |
| *Adh1* | 8.54 | 0.00819 | 2 | *Adh1* | 7.02 | 1.72E-13 |
| *Art2b* | 29.40 | 0.01128 | 3 | *Art2b* | 50.93 | 1.15E-15 |
| *Atp8b4* | 17.12 | 0.00000 | 4 | *Atp8b4* | 8.09 | 1.79E-14 |
| *Ccr2* | 8.13 | 0.00780 | 5 | *Ccr2* | 10.95 | 4.06E-13 |
| *Ccr8* | 9.97 | 0.00011 | 6 | *Ccr8* | 11.67 | 3.14E-12 |
| *Cd226* | 11.99 | 0.00001 | 7 | *Cd226* | 9.19 | 6.47E-16 |
| *Cd40lg* | 8.86 | 0.00202 | 8 | *Cd40lg* | 10.83 | 4.74E-12 |
| *Cd53* | 15.33 | 0.00064 | 9 | *Cd53* | 9.70 | 3.09E-15 |
| *Cd7* | 10.33 | 0.00578 | 10 | *Cd7* | 16.95 | 1.70E-13 |
| *Chn2* | 31.51 | 0.00039 | 11 | *Chn2* | 18.82 | 9.39E-16 |
| *Ctsw* | 10.20 | 0.00343 | 12 | *Ctsw* | 11.31 | 1.31E-15 |
| *Cxcr6* | 12.37 | 0.00829 | 13 | *Cxcr6* | 12.76 | 8.51E-14 |
| *Ephx1* | 14.52 | 0.00001 | 14 | *Ephx1* | 9.08 | 4.89E-13 |
| *Fam129a* | 23.81 | 0.00025 | 15 | *Fam129a* | 11.79 | 8.17E-13 |
| *Folr4* | 11.21 | 0.00046 | 16 | *Folr4* | 14.52 | 8.94E-15 |
| *Gimap4* | 25.98 | 0.00011 | 17 | *Gimap4* | 12.62 | 1.03E-14 |
| *Gm4759* | 16.22 | 0.00086 | 18 | *Gm4759* | 7.37 | 1.05E-11 |
| *Gm8995* | 21.81 | 0.00004 | 19 | *Gm8995* | 8.55 | 6.93E-14 |
| *Id2* | 33.65 | 0.00060 | 20 | *Id2* | 6.21 | 4.34E-12 |
| *Igfbp4* | 10.29 | 0.01018 | 21 | *Igfbp4* | 27.77 | 1.72E-16 |
| *Il2rb* | 21.41 | 0.00498 | 22 | *Il2rb* | 37.79 | 3.20E-15 |
| *Il7r* | 45.14 | 0.00003 | 23 | *Il7r* | 34.76 | 5.43E-14 |
| *Ipcef1* | 11.31 | 0.00000 | 24 | *Ipcef1* | 6.23 | 1.13E-11 |
| *Itgae* | 17.37 | 0.00028 | 25 | *Itgae* | 6.74 | 2.16E-12 |
| *Itgb3* | 7.22 | 0.00003 | 26 | *Itgb3* | 12.80 | 1.06E-13 |
| *Lfng* | 7.06 | 0.00003 | 27 | *Lfng* | 11.11 | 6.36E-15 |
| *Ly6a* | 43.88 | 0.00027 | 28 | *Ly6a* | 55.80 | 4.00E-15 |
| *Nrgn* | 8.99 | 0.00950 | 29 | *Nrgn* | 11.56 | 3.41E-12 |
| *Nt5e* | 18.56 | 0.00122 | 30 | *Nt5e* | 7.24 | 5.87E-12 |
| *Oasl2* | 6.71 | 0.00108 | 31 | *Oasl2* | 7.53 | 6.66E-13 |
| *Parp8* | 9.15 | 0.00211 | 32 | *Parp8* | 11.40 | 7.93E-15 |
| *Pdcd1* | 11.94 | 0.00046 | 33 | Pdcd1 | 6.41 | 3.37E-14 |
| *Plac8* | 14.48 | 0.02293 | 34 | Plac8 | 11.54 | 2.03E-13 |
| *S1pr1* | 10.02 | 0.00683 | 35 | S1pr1 | 23.08 | 2.07E-15 |
| *Sema4a* | 9.14 | 0.00003 | 36 | Sema4a | 11.31 | 2.10E-13 |
| *Slfn1* | 13.15 | 0.00218 | 37 | Slfn1 | 8.97 | 1.29E-11 |
| *Smpdl3a* | 21.13 | 0.00886 | 38 | Smpdl3a | 7.31 | 2.75E-14 |
| *St8sia6* | 8.38 | 0.00121 | 39 | St8sia6 | 7.03 | 3.48E-11 |
| *Tagap* | 11.45 | 0.04544 | 40 | Tagap | 8.38 | 1.20E-12 |
| *Zbtb16* | 37.64 | 0.00016 | 41 | Zbtb16 | 14.07 | 2.62E-14 |

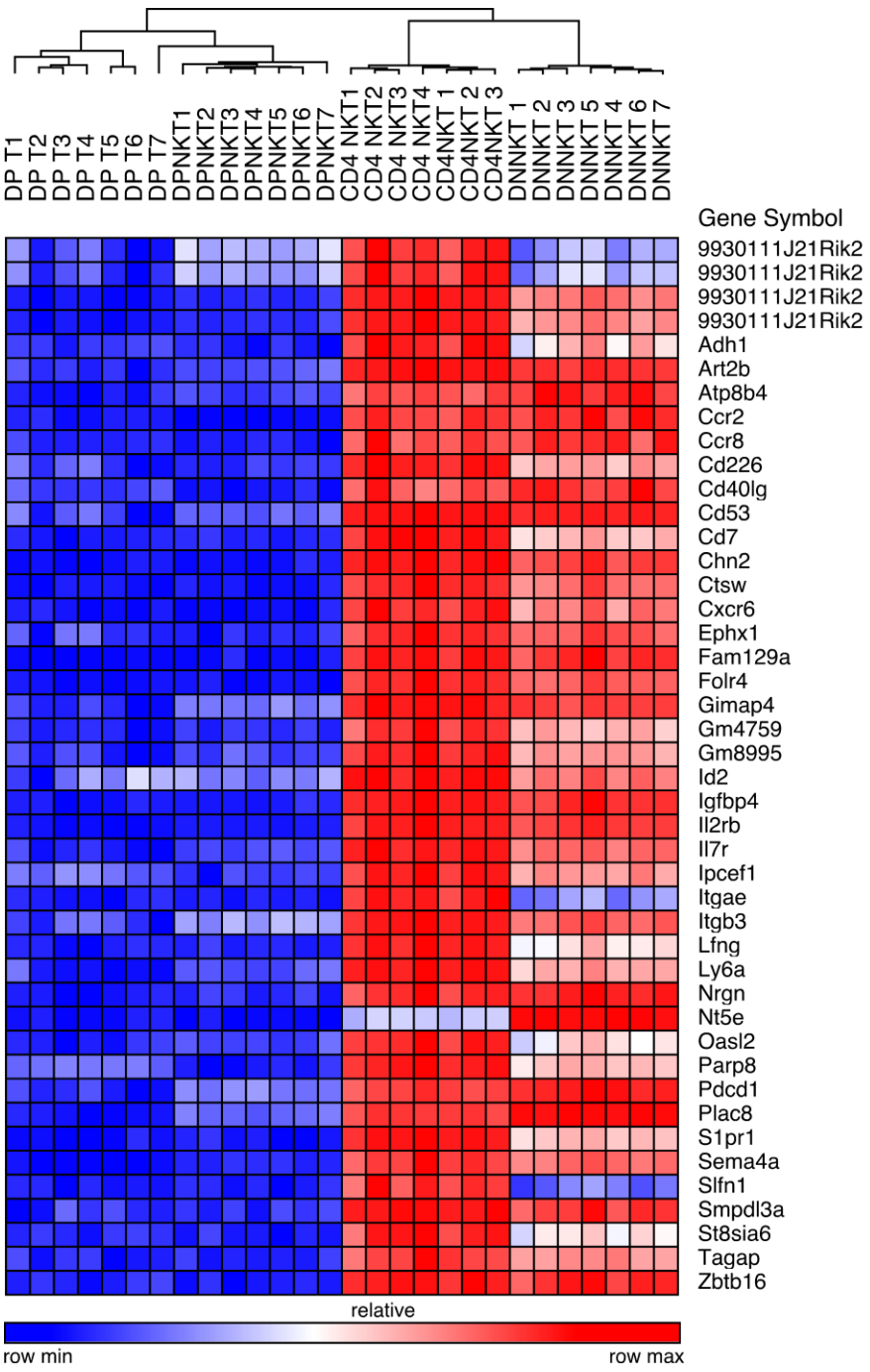


**Figure S-1. Expression profiles of 41 genes that overlap with Cohen's data**

**Table S-2: Genes were found in Cohen's top 100 up-regulated gene list and also significantly increased expression in our microarray analysis of immature CD4 NKT cells compared to DP NKT cells.**

| CD44⁻NK1.1⁻NKT cells/ DP Thymocytes (Cohen *et al.*, 2012) | | | Gene # | CD4NKT cells/DPNKT cells (from this research) | | |
|---|---|---|---|---|---|---|
| **Gene Symbol** | **FC** | **p-value** | | **Gene Symbol** | **FC** | **p-value** |
| *Icos* | 32.42 | 0.00000 | 1 | *Icos* | 4.96 | 5.47E-10 |
| *Il18r1* | 25.02 | 0.00097 | 2 | *Il18r1* | 2.57 | 8.91E-10 |
| *Ifi30* | 18.08 | 0.00060 | 3 | *Ifi30* | 1.8 | 4.17E-08 |
| *Capg* | 14.72 | 0.00835 | 4 | *Capg* | 2.57 | 7.74E-09 |
| *Inpp4b* | 13.14 | 0.00000 | 5 | *Inpp4b* | 5.6 | 2.37E-15 |
| *Myo1e* | 11.48 | 0.00775 | 6 | *Myo1e* | 5.08 | 8.04E-12 |
| *Rbpms* | 11.46 | 0.00034 | 7 | *Rbpms* | 4.82 | 4.00E-11 |
| *Fam134b* | 11.15 | 0.00006 | 8 | *Fam134b* | 4.81 | 4.01E-13 |
| *Pde3b* | 10.96 | 0.00006 | 9 | *Pde3b* | 4.17 | 7.76E-11 |
| *Akr1c18* | 10.63 | 0.06761 | 10 | *Akr1c18* | 1.47 | 8.63E-08 |
| *Gm1966* | 10.37 | 0.00073 | 11 | *Gm1966* | 4.95 | 1.16E-11 |
| *Rexo2* | 10.18 | 0.00096 | 12 | *Rexo2* | 2.33 | 2.78E-11 |
| *Endod1* | 9.72 | 0.00754 | 13 | *Endod1* | 5.61 | 3.30E-10 |
| *Cd9* | 9.5 | 0.00453 | 14 | *Cd9* | 2.44 | 3.36E-08 |
| *Gm5068* | 9.17 | 0.00004 | 15 | *Gm5068* | 1.91 | 1.68E-05 |
| *Rtp4* | 8.58 | 0.00107 | 16 | *Rtp4* | 4.6 | 4.15E-10 |
| *Adam19* | 8.53 | 0.00002 | 17 | *Adam19* | 2.43 | 5.91E-09 |
| *Gm4955* | 8.45 | 0.00207 | 18 | *Gm4955* | 2.52 | 9.94E-08 |
| *S100a6* | 8.22 | 0.00926 | 19 | *S100a6* | 2.16 | 1.78E-08 |
| *Gpr65* | 8.1 | 0.00035 | 20 | *Gpr65* | 4.02 | 5.23E-11 |
| *Ndfip1* | 8.1 | 0.00005 | 21 | *Ndfip1* | 2.31 | 2.80E-13 |
| *Il4* | 7.95 | 0.03278 | 22 | *Il4* | 1.24 | 4.49E-05 |
| *Gm5921* | 7.88 | 0.00413 | 23 | *Gm5921* | 1.14 | 0.036846 |
| *Plekha1* | 7.84 | 0.00468 | 24 | *Plekha1* | 2.78 | 1.29E-08 |
| *Srgn* | 7.79 | 0.00001 | 25 | *Srgn* | 1.38 | 3.62E-07 |
| *B2m* | 7.73 | 0.00210 | 26 | *B2m* | 1.35 | 1.73E-07 |
| *Trpm6* | 7.69 | 0.00074 | 27 | *Trpm6* | 1.32 | 1.59E-06 |
| *Il17rb* | 7.61 | 0.00042 | 28 | *Il17rb* | 1.17 | 0.004988 |
| *Irgm1* | 7.6 | 0.00047 | 29 | *Irgm1* | 2.71 | 8.94E-11 |
| *Ass1* | 7.56 | 0.00404 | 30 | *Ass1* | 1.32 | 9.26E-07 |
| *Gramd1b* | 7.54 | 0.00004 | 31 | *Gramd1b* | 4.6 | 1.27E-11 |
| *Lgmn* | 7.52 | 0.00945 | 32 | *Lgmn* | 3.23 | 3.20E-10 |
| *Ubash3b* | 7.37 | 0.00029 | 33 | *Ubash3b* | 3.98 | 9.81E-14 |
| *Ddx60* | 7.31 | 0.00031 | 34 | *Ddx60* | 5.04 | 6.37E-11 |
| *Cd82* | 7.26 | 0.00137 | 35 | *Cd82* | 2.5 | 1.39E-14 |

| | | | | | | |
|---|---|---|---|---|---|---|
| *Ugcg* | 6.98 | 0.00002 | 36 | *Ugcg* | 1.22 | 0.000359 |
| *Tmem64* | 6.88 | 0.01191 | 37 | *Tmem64* | 2.01 | 1.70E-08 |
| *Cnn2* | 6.85 | 0.00005 | 38 | *Cnn2* | 2.87 | 2.13E-12 |
| *Mapre2* | 6.8 | 0.00010 | 39 | *Mapre2* | 2.17 | 5.53E-09 |
| *Cap2* | 6.74 | 0.00736 | 40 | *Cap2* | 3.55 | 1.45E-11 |
| *Tmem173* | 7.02 | 0.00397 | 41 | *Tmem173* | 4.11 | 1.12E-11 |
| *Stk39* | 7.03 | 0.01051 | 42 | *Stk39* | 5.5 | 4.67E-12 |
| *Ttc39b* | 7.14 | 0.00400 | 43 | *Ttc39b* | 2.11 | 7.36E-11 |

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# APPENDIX B

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# BUFERS AND SOLUTIONS

**BUFFER FOR CELL SUSPENSION PREPARATION AND FLOW CYTOMETRY**

**MACS Buffer**

500 ml of 1X DPBS

5% (v/v) Bovine Serum

2mM EDTA

Mix well. Stored at 4°C

**DNA EXTRACTION BUFFERS:**

**Digest Buffer**

| ***Stock*** | ***Final*** | ***Add*** |
|---|---|---|
| 1M Tris pH8.0 | 100 mM | 10 ml |
| 0.5M EDTA | 5 mM | 1 ml |
| 10% SDS | 0.2% | 2 ml |
| 5M NaCl | 200mM | 4 ml |
| dH2O | Up to 100 ml | 84 ml |

**Proteinase-K**

Final Add

100mM 10ml 5mM 1ml 0.2% 2ml 200mM 4ml upto 100ml 84ml

10mg dissolved in 1ml d H2O. Store at -20°C in aliquots.

1. Add 500µl digest buffer to approx. 1cm of tail in an eppendorf tube.

2. Add 10µl [10mg/ml] proteinase-K.

3. Incubate tails 56°C at least 3hrs (or O/N).

## BUFFERS FOR GEL ELECTROPHORESIS

### 50X TAE Buffer (Tris-Acetate-EDTA)

242 g Tris base

57.1 ml Glacial Acetic acid

100ml 0.5M EDTA

DI $H_2O$ to 1 liter

1. Weigh out 242 g Tris base (FW=121.14) and dissolve in 750 mL deionized water.

2. Carefully add 57.1 ml glacial acetic acid and 100 mL of 0.5 M EDTA (pH 8.0) and adjust the solution to a final volume of 1 L.

3. This stock solution can be stored at room temperature. The pH of this buffer is not adjusted and should be about 8.5.

### 1X TAE Buffer (working solution)

The working solution of 1X TAE buffer is made by simply diluting the stock solution by 50x in deionized water. Final solute concentrations are 40mM Tris acetate and 1mM EDTA. The buffer is now ready for use in agarose gel electrophoresis.

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# APPENDIX C

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# COMMUNICATIONS ARISING FROM THIS WORK

## Invited talks and posters at conferences

Annual Meeting of the Australian Society for Immunology, 2012, Melbourne (Poster)

Townsville festival of life sciences, 2013, 2014 and 2015 (Poster).

Annual Meeting of the Australian Society for Immunology, 2013, Wellington, (Poster)

Annual Meeting of the Australian Society for Immunology, 2014, Wollongong, (Poster)

Brisbane Immunology Group 15th Annual Retreat, 2014, Gold Coast (Poster)

6$^{th}$ Congress of FIMSA, 2015, Singapore (Talk)

Annual Meeting of the Australian Society for Immunology, 2015, Canberra, (Poster)

## Primary publications

TCR validation distinct from selection and lineage commitment in NKT cells. Xuyen T Dinh, Margaret A Jordan, Dragana Stanley, Morgane Moreau, Stuart Berzins, Adrian Gemiarto, and Alan G Baxter. (manuscript under preparation: Journal of Nature Immunology)

## Other publications

MicroRNA-146a regulates ICOS–ICOSL signalling to limit accumulation of T follicular helper cells and germinal centres Alvin Pratama, Monika Srivastava, Naomi J. Williams, Ilenia Papa, Sau K. Lee, Xuyen T. Dinh, Andreas Hutloff, Margaret A. Jordan, Jimmy L. Zhao, Rafael Casellas, Vicki Athanasopoulos & Carola G. Vinuesa. Nature communication 6:6436. DOI: 10.1038/ncomms7436.

Biological processing of dinuclear ruthenium complexes in eukaryotic cells. Xin Li, Kirsten Heimann, Xuyen Thi Dinh, F. Richard Keene and J. Grant Collins. Mol. BioSyst. 2016, 12, 3032-3045. DOI: 10.1039/C6MB00431H.

### Awards

Australian Development Scholarship: Doctor of Philosophy at JCU

Robert Logan memorial grant: FIMSA conference, Singapore, 2015.

FIMSA travel Bursary: FIMSA/IIS Advanced Immunology course, Singapore, 2015.

ASI conference travel award: Annual Meeting of ASI, Canberra, 2015.