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Dok proteins and CD28 regulate the development of γδ NKT cells

By

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"We are just an advanced breed of monkeys on a minor planet of a very average star. But we can understand the Universe. That makes us something very special."

— Stephen Hawking

ABSTRACT

In T cells, two members of the Dok family of adaptor proteins, Dok-1 and Dok-2, are predominantly expressed. Recent evidence suggests that they play a negative role in T cell signaling. In order to define whether Dok proteins regulate T cell development, we have generated transgenic mice overexpressing Dok-1 in thymocytes and peripheral T cells. For some experiments we also used mice deficient for the expression of both Dok-1 and Dok-2. In this research project we show that Dok overexpression negatively regulates the activity of the key components downstream of TCR signaling such as LAT, ZAP-70, PLC-γ and Erk. We also show that the overexpression of Dok-1 causes a dramatic reduction in the numbers of total, DP and SP thymocytes with an increase in the numbers of thymocytes in the DN stage, compared to WT controls. Further investigations indicated that this reduction is not due to an increased apoptosis in DP thymocytes in Dok-1 transgenic mice but rather a delay in the transition of thymocytes from the $CD4^-CD8^-$ (DN) to $CD4^+CD8^+$ (DP) stage. More precisely, Dok-1 overexpression results in a block inside the DN stages by arresting thymocytes transition from DN3a to DN3b which are considered to be respectively the pre- and post-β selection stages of T cell development. Moreover, Dok-1 overexpression promotes the development of γδ T cells in the thymus, spleen, and liver of the transgenic mice. This developmental promotion correlates with the level of Dok-1 overexpression and it is mainly due to the specific expansion of the $V\gamma1.1^+$ $V6.3^+$ subset of γδ T cells. Similar to their small population in WT mice, this expanded population of $V\gamma1.1^+$ $V6.3^+$ T cells in Dok-1 Tg mice have also innate properties including rapid IL-4 production following stimulation. They express the transcription factor PLZF and they require SLAM-associated adaptor protein (SAP) for their development. Therefore, we concluded that Dok-1 promotes the development of γδ NKT cells. Moreover, Dok-1 overexpression

promotes the generation of an innate-like $CD8^+$ T cell population. These cells express the transcription factor Eomesodermin, upregulate memory markers like CD44 and CD122 and produce IFN-γ upon first stimulation. Thymic overproduction of IL-4 by γδ NKT cells is likely responsible for the innate conversion of $CD8^+$ thymocytes in Dok-1 Tg mice.

We pursue our study to further characterize NKT cells by studying the signaling pathways that might be important for their development and maturation. We investigated the contribution of CD28 signaling in the functional development of αβ and γδ NKT cells in mice. We show that CD28 signaling promotes the thymic maturation of $PLZF^+$ IL-4 producing NKT cells without any effect on their positive selection. Our results also show that CD28 signaling positively regulates the LFA-1 expression on both αβ and γδ NKT cells. Using mixed bone marrow chimeric mice, we demonstrated that the developmental defect of γδ NKT cells in CD28-deficient mice is cell autonomous. Moreover, in both wild type C57BL/6 and Dok-1 transgenic mice (with increased number of γδ NKT cells), we show that the CD28-mediated regulation of thymic IL-4 producing NKT cells promotes the differentiation of $Eomes^+$ $CD44^{high}$ innate-like $CD8^+$ T cells. Taken together, these findings reveal previously unappreciated mechanisms by which Dok-1 and CD28 can be considered as important regulators of the innate immune response via controlling the NKT cell homeostasis and consequently the size of the innate-like $CD8^+$ T cell pool.

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It is to my family that I dedicate this thesis!

TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

CHAPTER 1: INTRODUCTION

CHAPTER 2: PUBLICATIONS

Publication No.1

LIST OF ABBREVIATIONS

7-AAD: 7-amino-actinomycin D

α-GalCer: α-Galactosylceramide

Ab: Antibody

Abl: Abelson murine leukemia viral oncogene

AP-1: Activator protein-1

APC: Antigen presenting cell

BCR: B-cell receptor

BM: Bone marrow

BrdU: Bromodeoxyuridine

CBP: CREB binding protein

CD: Cluster of differentiation

CDR3: Complementarity determining region 3

CXCL12: CXCL12 chemokine (C-X-C motif) ligand 12

CXCR4: CXCR4 chemokine (C-X-C motif) receptor 4

DAG: Diacylglycerol

DN: Double negative

Dok: Downstream of tyrosine kinases

DP: Double positive

EBV: Epstein-Barr virus

Egr: Early growth response

Eomes: Eomesodermin

Erk: Extracellular signal regulated kinases

FACS: Fluorescence-activated cell sorting

FasL: Fas ligand

FTOC: Fetal thymic organ cultures

Fyn: Proto-oncogene tyrosine-protein kinase

GEF: Guanine nucleotide exchange factors

GEMs: Glycolipid-enriched membrane micro-domains

Grb2: Growth factor receptor-bound protein 2

GSL: Glycosphingolipid

HBV: Hepatitis B virus

Hexb: Hexosaminidase b

i.p: Intraperitoneal

ICAM: Intracellular adhesion molecule

Id3: Inhibitor of DNA binding 3

IFN-γ: Interferon-γ

iGb3: Isoglobotrihexosylceramide

IL: Interleukine

iNKT: invariant natural killer T

IP3: Inositol-1,4,5-triphosphate

IRS-4: Insulin receptor substrate 4

ISP: Immature single positive

ITAM : Immunoreceptor tyrosine activation motifs

Itk: IL-2-inducible T-cell kinase

ITSM: Immunoreceptor tyrosine-based switch motifs

JNK: JUN amino-terminal kinase

KLF-2: Kruppel-like factor 2

LAT: Linker for activation of T cells

LCR: Locus control region

LFA-1: Lymphocyte function associated antigen-1

Lfa-1: Lymphocyte function associated antigen-1

MAIT: Mucosa-associated invariant T

MAPK: Mitogen-activated protein kinas

M-CSF: Macrophage colony-stimulating factor

MFI: Median of fluorescent intensity

MHC: Major histocompatibility complex

Nck: Non-catalytic region of tyrosine kinase adaptor protein 1

NFAT: nuclear factor of activated T cells

NKT: Natural killer T

NOD mice: Nonobese diabetic mice

ns: not significant

PCR: Polymerase chaine reaction

PH: Pleckstrin homology

PI3K: Phosphatidylinositol 3-kinase

PIP2: Phosphatidylinositol 4,5-bisphosphate

PIP3: Phosphatidylinositol (3,4,5)-triphosphate

PKC: Protein kinase C

PLC-γ: Phospholipase C-γ

PLZF: Promyelocytic leukemia zinc finger

PTB: Phospho-tyrosine binding

PTEN: Phosphatase and tensin homolog deleted on chromosome 10

PTK: Protein tyrosine kinase

Q-RT-PCR: Quantitative Reverse Transcription PCR

RAG: Recombination activating gene

RasGAP: Ras GTPase activating protein

RasGRP: Ras-guanyl-releasing protein

SAP: SLAM-associated protein

SD: Standard deviation

SDF-1: Stromal cell-derived factor-1

SEM: Standard error of mean

SH2: Src homology 2

SH3: Src homology 2

SHIP: SH2 domain-containing inositol 5'-phosphatase

SHP-1/2: SH2 domain-containing phosphatase-1/2

SLAM: Signaling lymphocytic activation molecule

SLP-76: SH2 domain containing leukocyte protein of 76kDa

SP: Single positive

TCR: T cell receptor

TD: Thymus Dependent

TEC: thymic epithelial cell

Tg: Transgenic

Th: T helper

TNF: Tumor necrosis factor

Treg: Regulatory T cells

WASp: Wiskott–Aldrich syndrome protein

WT: Wild type

XLP: X-linked lymphoproliferative

ZAP-70: Zeta-chain-associated protein kinase-70

CHAPTER 1

INTRODUCTION

1. Innate versus adaptive immunity

In our body, a wide range of molecules, cells and tissues work together to protect us from foreign infection and help us to maintain our health. The resistance to a disease (we call it immunity) is the result of the combined roles of the two arms of the immune system that work cooperatively in response to an antigen challenge. These two arms are generally described as innate immunity (immunity that we are born with) and adaptive immunity (immunity that we acquire). The innate immune system is known to use a small number of germ line–encoded receptors that detect a limited set of conserved antigens and provides rapid and non-specific responses to invading pathogens without granting any memory. On the other hand, adaptive immunity using variable immune receptors provides delayed but specific responses to pathogens which will eventually create memory and long lasting protective immunity to that particular pathogen (Table 1). The innate immunity comprises a large group of cells with various functions and tissue distributions such as phagocytes (including neutrophils, monocytes, macrophages, dendritic cells, and mast cells), basophils, eosinophils and NK cells. However the adaptive immunity is composed of "conventional" αβ or γδ T cells and B cells (Figure 1) (Borghesi and Milcarek 2007).

Table 1) Distinguishing characteristics of innate versus adaptive immunity

	Innate immunity	**Adaptive immunity**
Receptors	Invariant	Variable
Distribution	Non-clonal	Clonal
Memory	NO	YES
Specificity	Degenerate	Specific
Response kinetics	Fast	Delayed

Adapted from (Borghesi and Milcarek 2007)

However, recent studies have proven that the distinction between the innate and the adaptive immunity is less clear cut than it seemed to be, especially with the discovery of new features in the distinct subsets of T cells showing the characteristics of both arms of the immune system. These specific T cells are called unconventional or innate-like T cells (in contrast to conventional T cells). They consist of TCR$\alpha\beta^+$ or TCR$\gamma\delta^+$ natural killer (NK) T cells called $\alpha\beta$ NKT (type I and type II) and $\gamma\delta$ NKT cells which are the prototypes of innate-like T cell population. There are also a few other subtypes of innate-like T cells which share similar innate-like characteristics with NKT cells, these cells consist of Mucosa-associated invariant T (MAIT) cells, H2-M3-restricted $CD8^+$ T cells, CD8$\alpha\alpha$ intraepithelial T cells, innate-like $CD4^+$ and $CD8^+$ T cells (Figure 1) (Prince, Yin et al. 2009, Das, Sant'Angelo et al. 2010). These unconventional T cells show distinct features that make them different from their conventional counterparts. For example they have a more restricted TCR repertoire than conventional T cells. Unlike conventional T cells, they have no naïve but activated or memory-like phenotype and can exert their effector function rapidly upon infection/stimulation (Berg 2007, Veillette, Dong et al. 2007, Prince, Yin et al. 2009, Das, Sant'Angelo et al. 2010). The differences in the phenotype and function of conventional and unconventional or innate-like T cells might be the result of different signaling requirements during their selection and development.

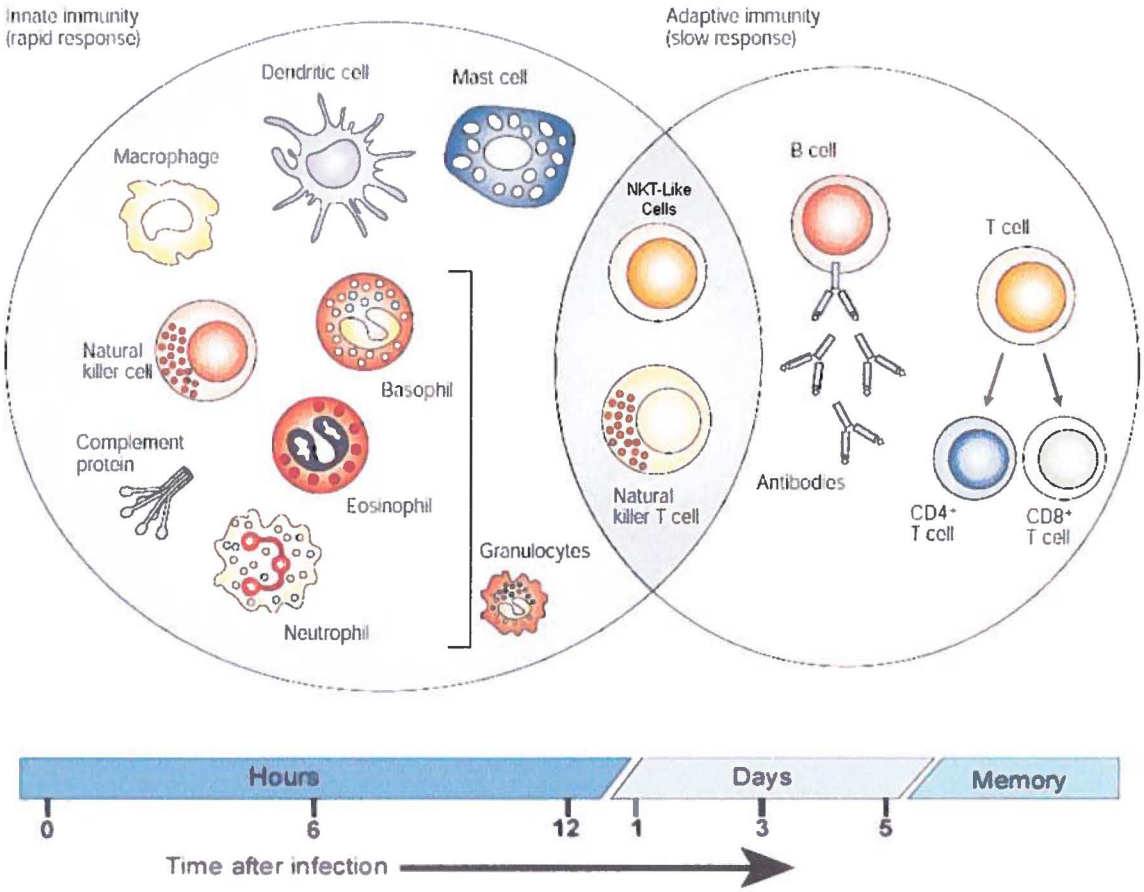

Figure 1) Cells of the adaptive and innate immune system. Innate cells respond within the first few hours upon infection but adaptive cells which can recognize specific pathogens during infection have a delayed response because only a few cells can recognize a specific pathogen and need to expand, but upon clearance of the infection adaptive cells can form memory. However, "Innate-like" lymphocytes such as NKT and NKT-like cells have characteristics of both innate and adaptive immune cells since they can respond to infection rapidly and have a memory-like phenotype. They make an interface between the two arms of the immune system. Adapted from (Dranoff 2004).

2. T cell development

In spite of their characteristic differences, both conventional and innate-like T cells have common precursors called early T cell progenitors (ETPs) entering the thymus after migration from the bone marrow (Prince, Yin et al. 2009). The thymus is seeded with progenitor cells as early as embryonic day 11.5 (E11.5) in mice (Owen and Ritter 1969). These progenitors have the potential to start the developmental program, characterized by discrete stages of extensive proliferation, increasing lineage commitment, and selection (Figure 2). This highly ordered process is governed by a multitude of molecular signals and environmental cues. Distinguished by the type of T cell receptor (TCR) molecule expressed on their surface, all the T cell sublineages fall into two main classes of αβ and γδ T cells. It is believed that after commitment to the T cell lineage, becoming either αβ or γδ T cell is the first lineage decision made by the progenitors. Sequential developmental stages achieved by these T cell precursors are distinguished by the expression of the differentiation markers such as CD4, CD8, CD25, and CD44. According to the expression level of these markers, thymocytes can be divided into different subsets in the order of their maturation (Lee, Stadanlick et al. 2010). The process starts with precursor cells in the Double Negative (DN) stage which lack the expression of both markers CD4 and CD8. Because of the heterogeneity of the DN thymocyte compartment it has been subdivided into different subsets according to the expression of differentiation markers on their surface (DN1a-DN1e, DN2, DN3a-DN3b and DN4) (Ciofani and Zuniga-Pflucker 2010). $CD44^{+}CD25^{-}$ (DN1) cells possess multi-lineage potential and can differentiate into B cells, T cells, myeloid cells, NK cells and dendritic cells. If multi-potential DN1 cells start to upregulate CD25 expression and become $CD44^{+}CD25^{+}$ (DN2), their commitment to the T lineage is guaranteed since in the transition from DN1-DN2 they start to rearrange 3 TCR genes: *Tcrb* or

Tcrg and *Tcrd*, a process which is called TCR "gene rearrangment" and is completed in DN3 stage ($CD44^{-}CD25^{+}$). Those DN cells that successfully completed rearranging TCRβ genes have undergone β-selection and express TCRβ molecules in complex with germline-encoded pre TCRα (pre-Tα) chain which is called pre-TCR. These cells will undergo further maturational stages which are accompanied by the upregulation of CD5 and CD27 and an increase in cell size, and the transition from the pre-selection DN3a to the post-selection DN3b stage (Hoffman, Passoni et al. 1996, Taghon, Yui et al. 2006) Thereafter, αβ T lineage cells downregulate CD25 and upregulate CD4 and CD8 to generate double-positive (DP) cells that constitute the majority of thymocytes (~80%). DP cells undergo TCRα gene rearrangement and the resulting TCRαβ heterodimer then undergoes MHC-mediated selection to yield mature CD4 or CD8 single-positive T cells. (Starr, Jameson et al. 2003, Kreslavsky, Gleimer et al. 2010). In parallel, those cells that do not start rearranging TCRβ genes but successfully rearrange their TCRγ and δ genes are entitled to become γδ T cells (γδ-selection). These cells usually remain as DN and undergo a smaller proliferative burst compared to αβ lineage (i.e., pre-TCR^{+}) DN thymocytes. They downregulate expression of CD24 following maturation and leave the thymus for peripheral organs and epithelial surfaces. It is not precisely delineated when the two αβ and γδ lineage of T cells diverge from each other but there is some evidence arguing that this divergence is completed at DN3 stage when cells at DN2 start to downregulate CD44 expression and become $CD44^{-}CD25^{+}$ (Figure 2) (Ciofani and Zuniga-Pflucker 2010, Lee, Stadanlick et al. 2010). Besides conventional T cells, innate-like αβ and γδ NKT cells (among other innate-like T cells) are also the outcomes of T cell development process, all originating from common early T cell progenitors (Figure 2). These unconventional T cells have evolutionarily conserved, semi-invariant or invariant αβ or γδTCRs. The development of αβ and γδ NKT cells is distinct from

that of conventional αβ and γδ T cells even though they originate from common DP and DN precursors respectively (Coles and Raulet 1994, Bendelac, Lantz et al. 1995). It has been shown at least for αβ NKT cells that they become selected on other hematopoietic cells (cortical DP thymocytes) rather than thymic epithelial cells and through interaction with non-peptide Ag in complex with non-classical MHC molecules (Ohteki 2002, Berg 2007, Horai, Mueller et al. 2007, Veillette, Dong et al. 2007). They have a signature transcription factor, the promyelocytic leukaemia zinc finger (PLZF) which belongs to the family of Krüppel-like zinc finger proteins. This transcription factor is essential for their innate properties. (Benlagha, Kyin et al. 2002, Pellicci, Hammond et al. 2002, Dao, Guo et al. 2004, Kreslavsky, Savage et al. 2009, Das, Sant'Angelo et al. 2010). Unlike conventional T cells, signalling lymphocyte activation molecule (SLAM) receptors and their associated adaptor protein SAP play a crucial role in the development of both αβ and γδ NKT cells (Chung, Aoukaty et al. 2005, Nichols, Hom et al. 2005, Alonzo, Gottschalk et al. 2010). Being the subjects of this PhD research project, the selection, development, maturation and function of these two subtypes of innate-like T cells will be discussed further in the next parts.

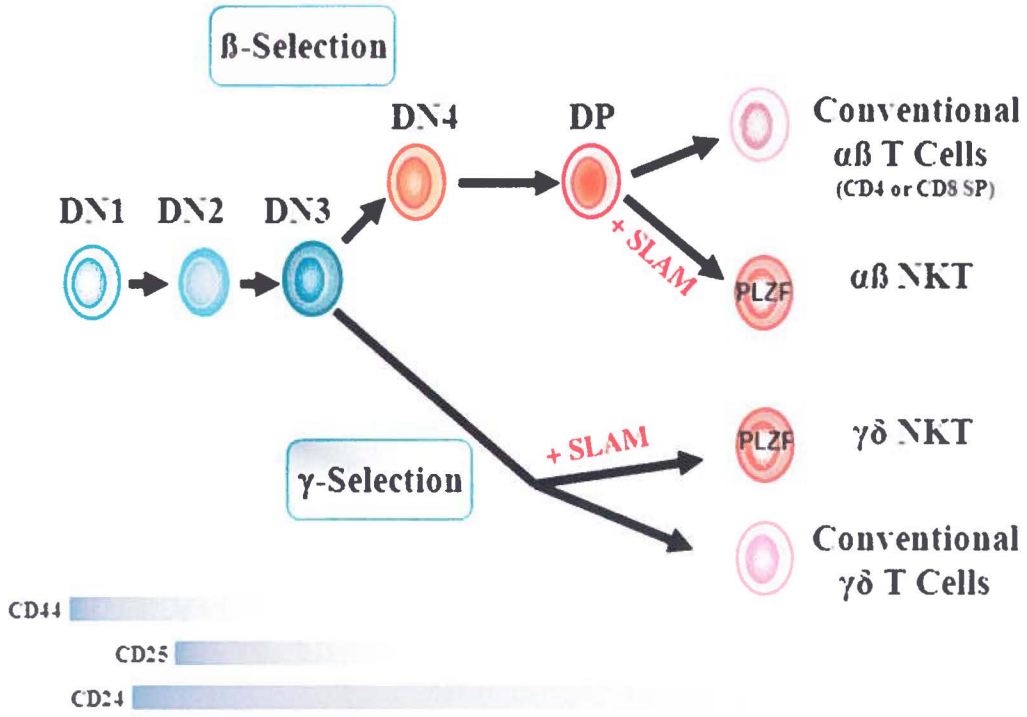

Figure 2) Schematic of the discrete stages of T cell development within the thymus. $CD4^-CD8^-$ double negative (DN) cells are the most primitive subset in the thymus. These cells can undergo β-selection, give rise to the subsequent $CD4^+CD8^+$ double positive (DP) cells and become eventually conventional αβ T cells or αβ NKT cells. Alternatively, DN cells can undergo γ-selection, stay in the $CD4^-CD8^-$ double positive (DN) stage and become eventually conventional γδ T cells or γδ NKT cells.

2.1. Pre-T cell receptor versus T cell receptor

Pre-TCR and TCR molecules are the two essential receptor complexes expressed on developing and mature T cells. Signaling molecules that modulate the pre-TCR and TCR signaling strength are the key determinants in the regulation of T cell development and homeostasis. Pre-TCR is similar to the TCRαβ since it is composed of two components: a β-chain and an α-component. In the TCRαβ, the TCRα chain is the protein product of a successful and productive TCRα locus rearrangement, but in the pre-TCR, the α-component is a germline-encoded surrogate of the TCRα chain, termed the pre-Tα. The pre-Tα is unique and it is not interchangeable with TCRα during T lymphocyte development, as it contains unique features that are responsible for optimal proliferation, survival, differentiation, and commitment in developing DN3 cells (Borowski, Li et al. 2004). These differences allow the pre-TCR to signal cell-autonomously (Yamasaki, Ishikawa et al. 2006) at a lower threshold unlike its more mature counterpart, the TCRαβ, which requires engagement with peptide-major histocompatibility complex (p-MHC) to initiate signaling. These abilities of the pre-TCR are due to a unique extracellular domain of pre-Tα that contains specific charged residues that promote spontaneous dimerization (Yamasaki, Ishikawa et al. 2006), leading to its autonomous signaling capacity. Many knockout and overexpression studies have been used to determine the key signaling molecules downstream of the pre-TCR. It has been shown that similar to what has been found for the TCR, the pre-TCR also requires CD3 components, as well as TCR-associated tyrosine kinases, Lck and Fyn, and scaffolding proteins, SLP-76, and LAT, and other downstream molecules in order to signal differentiation at the β-selection checkpoint (Mombaerts, Anderson et al. 1994, van Oers, Lowin-Kropf et al. 1996, Pivniouk, Tsitsikov et al. 1998, Zhang, Sommers et al. 1999). Despite the activation of the same transcription factors as TCR signaling, the

consequences are different. This probably reflects the differential accessibility of specific genes in thymocytes at different stages of maturation (von Boehmer 2005).

2.2. TCR signaling cascade

One of the first signaling events that occurs upon the interaction of the TCR with the peptide-MHC complex is the stimulation of two members of the Src family of intracellular tyrosine kinases, Lck and Fyn (Nel 2002, Mustelin and Tasken 2003) leading to the phosphorylation of immunoreceptor tyrosine activation motifs (ITAMs) on the intracellular domains of the TCR ζ (also called CD3 ζ) and CD3-γ, -δ and -ε subunits (Pitcher and van Oers 2003) resulting in the recruitment of Syk-family kinase ZAP-70. This protein binds to the phosphorylated ITAMs and become phosphorylated by Lck, which goes on to phosphorylate the adaptors LAT and SLP-76 (Pitcher and van Oers 2003, Houtman, Houghtling et al. 2005, Au-Yeung, Deindl et al. 2009, Smith-Garvin, Koretzky et al. 2009, Wang, Kadlecek et al. 2010). Phosphorylated adaptor proteins LAT and SLP-76 serve as a platform for recruitment of Tec family kinase Itk through its SH3 and SH2 domains and this recruitment allows for Lck phosphorylation of Itk (Heyeck, Wilcox et al. 1997). Upon activation and recruitment of Itk to the membrane, Itk phosphorylates and activates phospholipase Cγ1 (PLCγ1) which hydrolyzes phosphatidylinositol 4,5- bisphosphate (PI(4,5)P2) into inositol-1,4,5-triphosphate (IP3) and diacylglycerol (DAG) (Rhee 2001). Following cleavage of PIP2, DAG activates members of the protein kinase C (PKC) family and RasGRP. PKC leads to the activation of JUN amino-terminal kinase (JNK) thereby regulating the transcription factor activator protein-1 (AP-1) which leads to specific gene expression and particular effector function (Schaeffer, Broussard et al. 2000, Miller and Berg 2002, Newton 2004). RasGRP via activating Ras leads to the activation of Erk1/2

(Schaeffer, Debnath et al. 1999, Schaeffer, Broussard et al. 2000). Among all the different pathways and molecules that can be activated downstream of Erk signaling is the expression of the transcription factor, inhibitor of DNA binding 3 (Id3) which is through the induction of the Egr expression (Carleton, Haks et al. 2002, Engel and Murre 2004). Id3 is the antagonist of E2A and together they play important roles in different checkpoints of T cell development downstream of TCR signaling (Bain, Cravatt et al. 2001, Miyazaki, Rivera et al. 2011, Naito, Tanaka et al. 2011). On the other hand IP3 enables intracellular $Ca2^{+}$ release and sustained calcium influx results in activation of downstream effectors including dephosphorylation and translocation of the transcription factor, nuclear factor of activated T cells (NFAT) to the nucleous which leads to particular effector functions in a T lymphocte (Figure 3) (Lewis and Cahalan 1995, Crabtree and Olson 2002).

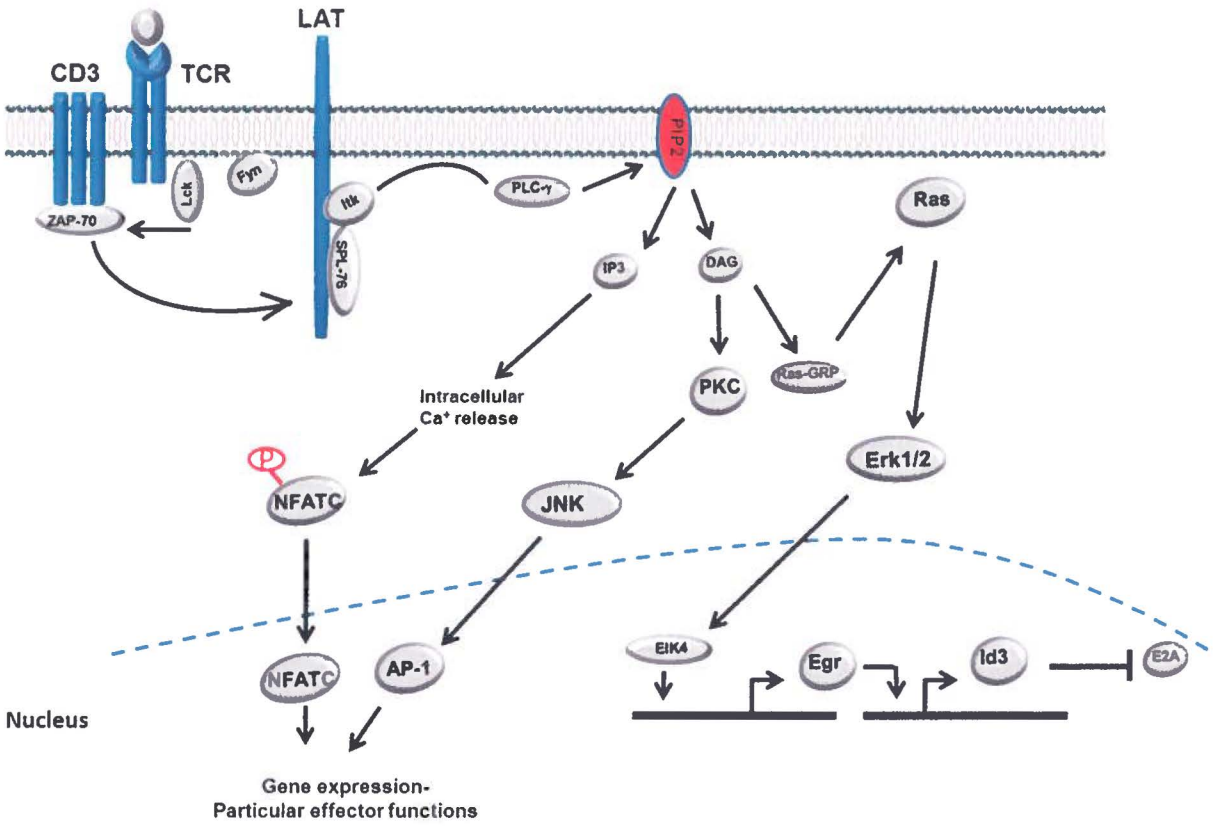

Figure 3) T cell receptor signaling. Stimulation through the T cell receptor results in the recruitment and activation of both Lck and ZAP-70. Lck and ZAP-70 phosphorylate and activate SLP-76 and LAT resulting in the formation of a signaling complex for recruiting Itk which goes on to phosphorylate PLCγ. Activated PLCγ catalyzes PIP2 to IP3 and DAG. IP3 induces $Ca2^{+}$ mobilization activation resulting in the activation and translocation of NFAT to the nucleus and DAG induces Erk and JNK pathways.

2.3. Role of TCR signaling strength in T cell development

Several studies have reported that the quality of TCR signaling (strength/duration) is an important determinant for αβ versus γδ fate decision, positive versus negative selection and CD4/CD8 lineage differentiation and also conventional versus innate-like T cell lineage choice (Liu and Bosselut 2004, Hayes, Li et al. 2005).

2.3.1 αβ vs. γδ fate decision

Showing that the bifurcation of αβ and γδ T cell lineages occurs around the DN2-DN3 stage of T cell development was a big achievement in the world of T cell development study (Petrie, Scollay et al. 1992, Ciofani, Knowles et al. 2006). But how this divergence happens and what are the main determinants still remained to be answered.

There have been several proposed models to explain the mechanism by which an immature DN thymocyte decides to become either αβ or γδ T cell (Kang and Raulet 1997, MacDonald and Wilson 1998). Two models are currently popular: the "stochastic model" and the "signal strength model" (Figure 4). "Stochastic model" proposes that the fate of the cells is randomly determined before the expression of either αβ or γδ TCR and cells will survive and mature if the isoform of the TCR they are going to express is matched with their predetermined fate (Figure 4A). A piece of evidence supporting this model is that the progenitor cells which give rise to αβ or γδ T cells show heterogeneous expression of intracellular and surface proteins including transcription factors and cytokine receptors. This heterogeneity may reflect the activation of αβ or γδ lineage-specific molecular programs prior to the expression of a functional TCR isoform (Kang, Volkmann et al. 2001, Melichar, Narayan et al. 2007). On the other hand the "signal strength model" proposes that the strength of the signal delivered by the antigen receptor actually

instructs lineage choice, with immature DN thymocytes receiving a strong signal choosing the γδ T cell fate and those receiving a weak signal choosing the αβ T cell fate (Figure 4B) (Hayes, Shores et al. 2003). According to this model, the signal that instructs the lineage choice can potentially be delivered by any TCR isoform under normal conditions, and γδ TCR transduces the strong signal while the pre-TCR delivers the weak signal (Haks, Lefebvre et al. 2005, Hayes, Li et al. 2005). Evidence supporting this model is coming from the results of an experimental system using mice expressing a γδ TCR transgene of defined antigen specificity, KN6. The KN6 TCR recognizes the non-classical MHC class Ib molecule T22d, whose surface expression is β2-microglobulin (β2M)-dependent (Bonneville, Ito et al. 1989, Crowley, Fahrer et al. 2000). Using this transgenic model, it was clearly indicated that the absence of the γδ TCR ligand and the kinase Lck leads to a dramatic increase in DP cell numbers at the expense of DN γδ TCR^+ cells showing stronger TCR signal favors γδ and weaker signal favors αβ lineage development (Haks, Lefebvre et al. 2005, Hayes, Li et al. 2005). But it was still probable that TCR signal strength does not determine lineage fate but instead confirms the fate decision of pre-committed immature DN thymocytes. A more recent study has cleared up all the doubts by studying the lineage potential of early TCR-expressing thymocytes in the OP9-DL1 co-culture system. They demonstrated that TCR $γδ^+$ DN3 cells which can give rise to both αβ and γδ lineages, developed only into the γδ lineage when they received a strong signal from the TCR indicating that pre-commitment does not occur prior to TCR signaling (Kreslavsky, Garbe et al. 2008). These results suggest that, regardless of which molecules are expressed in immature thymocytes, or their potential to influence the decision process, the strength of the TCR has the last word.

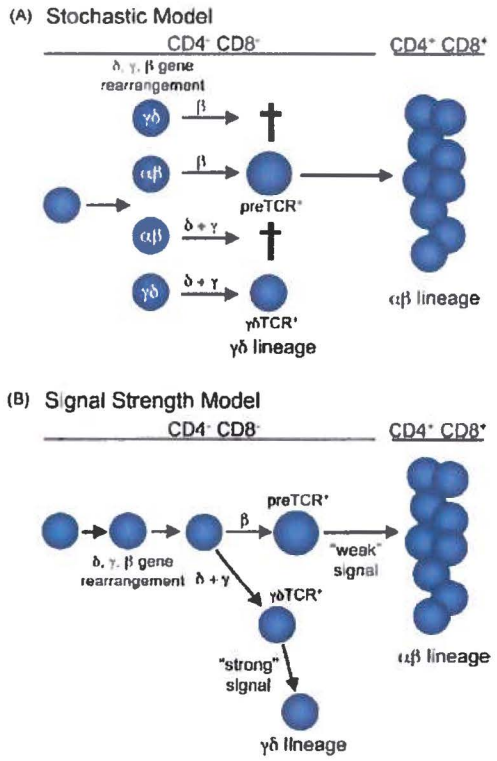

Figure 4) Current models of αβ/γδ lineage commitment (A) Stochastic model: in this model, the lineage fate decision occurs randomly and prior to the expression of either the pre-TCR or the γδ TCR. (B) Signal strength model: in this model, the strength of the TCR signal dictates the fate of the cell, with cells receiving a strong signal choosing the γδ T cell fate and cells receiving a weak signal choosing the αβ T cell fate (Hayes, Laird et al. 2010).

2.3.2. Positive vs. negative selection

After αβ vs. γδ lineage decision, the next important check point during T cell development is once TCRαβ$^+$ cells upregulate CD4 and CD8 and reach the DP stage of development. After becoming DP, thymocytes move deep into the thymic cortex where they are exposed to and interact with the self-antigens which are presented by MHC-I or MHC-II molecules. The strength of the TCR signal is critical for the decision of a cell to continue maturation which is called positive selection. At this stage the TCR signal should be strong enough for the cells to become positively selected otherwise they die from neglect (Starr, Jameson et al. 2003). Later a process called negative selection removes thymocytes that are capable of strong binding with self-antigen/MHC complexes. These T cells having a very strong

TCR signaling undergo cell death otherwise they will eventually enter the peripheral organs as autoreactive T cells and are capable of inducing autoimmune diseases in the host (Starr, Jameson et al. 2003, Prince, Yin et al. 2009).

2.3.3. CD4/CD8 lineage commitment

A further checkpoint in the fate decision during the course of T cell development is when cells decide to differentiate into either $CD4^+$ or $CD8^+$ T cells depending on the TCR specificity for either MHC class II or class I molecules. Since two decades ago different hypothesises have been proposed explaining the coordination of $CD4^+$ or $CD8^+$ phenotype with MHC recognition. "Instructional" model which was later modified to a "Quantitative instructional" model (Itano, Salmon et al. 1996, Matechak, Killeen et al. 1996) proposes that a relatively strong signal, usually accompanied by CD4–MHC class II interactions, instructs DP thymocytes to differentiate into $CD4^+$ T cells and that a weaker signal, usually accompanied by CD8–MHC class I interactions, results in $CD8^+$ T cell lineage commitment.

Further investigation indicated that in DP-confined TCR signaling model, in which the expression of the essential tyrosine kinase ZAP-70 downstream of TCR signaling is restricted to only DP thymocytes (Liu, Adams et al. 2003), there is a normal CD8 but not CD4 lineage differentiation. Interestingly, it was also demonstrated that this model redirects even MHC class II–restricted thymocytes into the CD8 lineage (Liu and Bosselut 2004). These findings demonstrate that not only the strength of TCR signal but also the duration of signaling *in vivo* is a critical determinant of CD4-CD8 lineage differentiation with longer lasting TCR signaling (here defined by the presence of ZAP-70) favoring CD4 lineage differentiation and TCR signaling with less duration (here defined by the absence of ZAP-70) favoring CD8 lineage choice (Liu and Bosselut 2004).

2.3.4. Conventional vs. innate-like tymphocyte lineage choice

As it was mentioned earlier, innate-like thymocytes consist of different subtypes of T cells with common properties. These subsets of T cells show heterogeneity regarding the role of TCR signaling strength in their selection and development. For example the results from several studies indicate that in αβNKT cells, the invariant TCR binds to complexes of ligand-MHC (αGalCer-CD1d) with a relatively high affinity and a particularly long half-life in the order of minutes rather than seconds which is characteristic of conventional peptide-MHC I/II reactive TCRs (Sidobre, Naidenko et al. 2002, Cantu, Benlagha et al. 2003, Sim, Holmberg et al. 2003, Sidobre, Hammond et al. 2004). On the other hand the story is the opposite for H2-M3-restricted innate-like T cells where peptide studies have determined that only the weakest agonists can induce the positive selection of H2-M3-restricted T cells while strong agonists induce cell death (Chiu, Wang et al. 1999, Berg, Irion et al. 2000).

Interestingly, γδ NKT cells which also have a conserved TCR molecule like αβ NKT cells, and likewise are SLAM receptor signaling dependent are accumulated in Itk-deficient mice, while αβ NKT cells are diminished (Au-Yeung and Fowell 2007, Felices, Yin et al. 2009, Qi, Xia et al. 2009). Since Itk regulates TCR signaling, these two subsets of NKT cells may differ in their TCR requirements in the selection process. Thus, the strength of the TCR signal plays a major role in different checkpoints in the course of T cell development and lineage differentiation. This role of TCR signaling appears to be important for the development of both conventional and unconventional innate-like T cells (Prince, Yin et al. 2009).

2.4. The importance of the Erk-Egr-Id3 axis in T cell development

The p42 and p44 mitogen-activated protein kinases (MAPKs), also known as extracellular signal regulated kinases (Erk)2 and (Erk)1, respectively, have been implicated in proliferation as well as in differentiation programs. The first checkpoint in the T cell development process occurs between the DN and DP stages and is associated with formation of the pre-TCR. In 1999 for the first time it was reported that Erk1/2 are phosphorylated and activated upon engagement of the pre-TCR, indicating that the Erk signaling cascade is initiated by the pre-TCR. It was also shown that the activation of this pre-TCR signaling cascade is mediated through Lck (Michie, Trop et al. 1999). Erk signaling is considered as an important determinant in TCR signaling and T cell development because it regulates the activity of essential transcription factors in T cell development called E proteins. E proteins E2A (E12, E47) and HEB are indispensible transcription factors early in T cell development. E proteins are basic helix-loop-helix (bHLH) family members (Murre, McCaw et al. 1989) that bind the consensus E-box DNA sequence (CANNTG) with their basic region, and they can interact with other proteins via their helix-loop-helix domain (Murre, McCaw et al. 1989, Voronova and Baltimore 1990). In thymocytes, E2A and HEB work cooperatively as heterodimers to promote the expression of genes critical for thymocyte development and differentiation (Takeuchi, Yamasaki et al. 2001, Schwartz, Engel et al. 2006). In E2A- or HEB-deficient mice, thymocyte development is partially arrested, with αβ-lineage cells more severely affected than cells of the γδ-lineage (Bain, Engel et al. 1997, Barndt, Dai et al. 2000). Mice with double deficiency in E2A and HEB undergo a severe developmental block before pre-TCR expression and a dramatic reduction of pre-Tα expression (Wojciechowski, Lai et al. 2007). The arrested thymocytes also have increased proliferation and expansion, suggesting a role for these E proteins that is beyond T cell

differentiation, and encompass cell cycle arrest before pre-TCR expression as well. E proteins play an important role as regulators of thymocyte selection, functioning as gate-guards at critical checkpoints during thymocyte development (Figure 5) (Engel, Johns et al. 2001, Engel and Murre 2004, Wojciechowski, Lai et al. 2007). How Erk pathway regulates E protein activation? Signaling through the pre-TCR activates the Erk pathway, leading to the expression of early growth response family members Egr1-4 (Carleton, Haks et al. 2002). Egr transcription factors are required for the development of DN3 cells across the β-selection checkpoint to the DP stage. Inhibiting Egr function induces impairment in this progression, while its ectopic expression allows for DN3 cells to bypass β-selection without having a functional pre-TCR (Carleton, Haks et al. 2002).

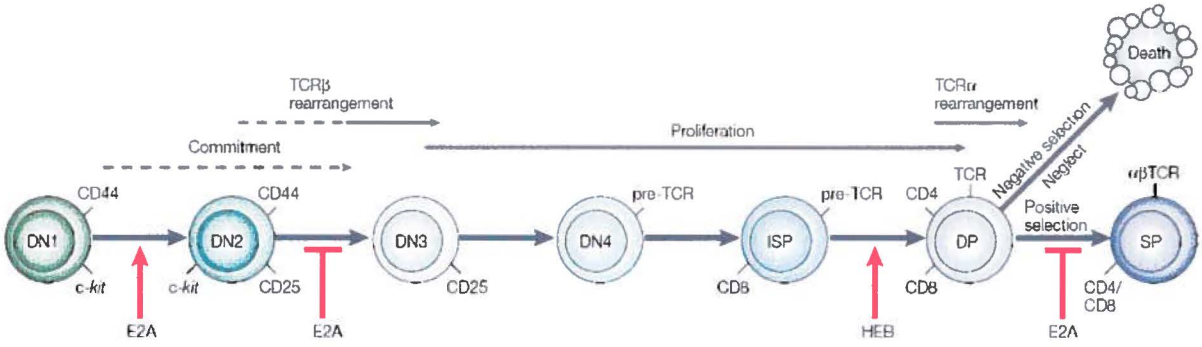

Figure 5) Schematic model of the role of E proteins in T cell development. Developmental stages are presented as labelled circles, with the names of significant surface marker molecules attached. The roles of E2A or HEB proteins are indicated in red. DN, $CD4^-$ and $CD8^-$ double-negative thymocyte; DP, $CD4^+$ and $CD8^+$ double-positi e thymocyte; ISP, immature single-positive $CD8^+$ thymocyte; SP, $CD8^+$ or $CD4^+$ single-positive thymocyte (Engel and Murre 2001).

Among the earliest transcriptional changes induced by pre-TCR signaling is the expression of Egr transcription factor (Xi and Kersh 2004, Xi and Kersh 2004). Studies on this pathway have suggested that Egr expression is important at this stage because it serves to control thymocyte proliferation in response to pre-TCR signals (Xi, Schwartz et al. 2006). It also induces the expression of the transcription factor Id3 (Engel and Murre 2004). Id proteins (4 members in mammals) are HLH-only proteins (Benezra, Davis et al. 1990) that make heterodimers with E proteins and prevent them from binding DNA. Id protein expression is induced upon pre-TCR signaling-mediated activation of the Erk pathway (Engel, Johns et al. 2001) leading to elevated Id3 gene expression levels at β-selection (Taghon, Yui et al. 2006). This critical step allows Egr proteins to mediate DN3 differentiation across the β-selection checkpoint. By inducing Id3 expression, the developmental arrest imposed by E2A function is removed as a result of the Id3/E2A heterodimerization (Rivera, Johns et al. 2000).

2.4.1. αβ vs. γδ fate decision

Different studies on the role of TCR signaling strength in αβ versus γδ lineage choice suggested that the Erk-Egr-Id3 axis might be capable of transforming an analog TCR signal strength into a binary lineage choice meaning that TCR signal is digitized by this axis, with strong signal instructing the γδ and weak signal instructing the αβ fate choice (Haks, Lefebvre et al. 2005, Hayes, Li et al. 2005, Kreslavsky, Gleimer et al. 2010). The most obvious evidence is the higher phosphorylation level of Erk in TCRγδ– compared to pre-TCR-expressing thymocytes (Hayes, Li et al. 2005) and also in KN6 TCRγδ transgenic thymocytes in the presence of the ligand (Haks, Lefebvre et al. 2005).Moreover, the Egr transcription factors and their target, inhibitor of E protein function Id3 were induced in KN6 cells more strongly when the ligand was

present (Haks, Lefebvre et al. 2005). Since the Egr family consists of several members with overlapping functions, more studies have been focused on the role of Id3 downstream of Erk signaling pathway in αβ versus γδ lineage choice. First of all, Lauritsen et al showed that the ability of ectopically expressed Egr1 to simultaneously promote γδ and oppose αβ lineage development was dependent on the Id3 since these effects were markedly diminished in Id3-deficient cells (Lauritsen, Wong et al. 2009). They also show that in KN6 TCR transgenic mice in the absence of Id3, large numbers of DP cells were generated even when the TCR γδ ligand was expressed suggesting that Id3 upregulation is required for γδ versus αβ lineage commitment (Lauritsen, Wong et al. 2009). Not only in TCR transgenic mice but also in TCR non-transgenic mice it was shown that, Id3 deficiency resulted in a dramatic decrease in Vγ5 T cells in the skin and in Vγ4 T cells in the spleen (Blom, Heemskerk et al. 1999). To explain how TCR signal strength can be directed into a binary lineage choice, it has been proposed that the duration of the signal is more important than the signal intensity per se (Lee, Stadanlick et al. 2010). According to this hypothesis Erk activation is sustained in presence of the ligand in the KN6 system and this in turn leads to stabilization of the immediate early gene Egr1 and consequently Id3 expression leading to reduce E protein activity (Lee, Stadanlick et al. 2010).

2.5. CD28

The coreceptor CD28 is constitutively expressed on T cells. CD28 is also a member of the immunoglobulin superfamily and is expressed as a disulphide-linked homodimer. This coreceptor is involved in T cell proliferation, IL-2 production, prevention of T cell anergy, induction of the anti-apoptotic factor Bcl-xL and differentiation of naïve T cells into Th1 and Th2 type of helper T cells (Lenschow, Walunas et al. 1996, Chambers and Allison 1997, Harris

and Ronchese 1999, Alegre, Frauwirth et al. 2001, Bour-Jordan and Blueston 2002). Upon stimulation, CD28 activates phophatidylinositol 3-kinase (PI3K) and induces intracellular signaling leading to cytokine production, proliferation and survival (Alegre, Frauwirth et al. 2001). A primary costimulatory signal is delivered through the CD28 receptor after engagement of its ligands, B7-1 (CD80) or B7-2 (CD86) which are respectively expressed after induction or constitutively on antigen presenting cells (APCs). Engagement of cytotoxic lymphocyte associated molecule-4 (CTLA-4 or CD152) to the same B7-1 or B7-2 ligands results in attenuation of T cell responses so the CD28/CTLA-4/B7-1/B7-2 family provides a paradigm to define related immune pathways in T cells (Greenfield, Nguyen et al. 1998, Chambers, Kuhns et al. 2001, Salomon and Bluestone 2001). Therefore, CD28 costimulation is necessary for the initiation of most of T cell responses, and its blockage results in ineffective T cell activation.

2.5.1 Integration of CD28 and TCR signals

When TCR signal is strong enough (for example in vitro conditions when T cells are stimulated with TCR specific antibodies) it can mediate all of the main gene expression programs downstream of its signaling cascade (Diehn, Alizadeh et al. 2002). In contrast, CD28 stimulation alone results in a transient expression of only a few genes with no obvious biological consequences, indicating an accessory role for its signaling (Diehn, Alizadeh et al. 2002, Riley, Mao et al. 2002). Since under normal circumstances, only a few TCR molecules are ligated at once, it is likely to generate short lasting or incomplete activation events which cannot lead to any cell proliferation and differentiation, but rather to T cell anergy or death (Irvine, Purbhoo et al. 2002). But if engaged together the coreceptor CD28 strongly amplifies a weak TCR signal (Michel, Attal-Bonnefoy et al. 2001). So far, no unique signaling pathways or effectors have been identified exclusively for CD28. Instead, all of the proteins identified as components of the

CD28 signaling pathway constitute a small subset of those that are implicated in TCR signaling pathway (Figure 6). The main components downstream of CD28 signaling are PI3K (Rudd 1996), two members of Tec family of PTKs, Tec (Yang, Ghiotto et al. 1999) and IL-2-inducible T-cell kinase (Itk) (August, Gibson et al. 1994), guanine nucleotide exchange factor Vav1 (Klasen, Pages et al. 1998, Michel, Grimaud et al. 1998) and the serine/ threonine kinase Akt (also known as protein kinase B, PKB) (Parry, Reif et al. 1997, Michel, Attal-Bonnefoy et al. 2001).

- Activation of PI3K:

Upon CD28 ligation, it becomes tyrosine phosphorylated by Lck and/or Fyn at the Tyr-Met-Asn-Met motif in its cytoplasmic tail and it binds the p85 regulatory subunit of PI3K (Michel, Attal-Bonnefoy et al. 2001). PI3K recruitment by CD28 contributes to or complements TCR-dependent PI3K signaling. This kinase phosphorylates lipid substrates and generates phosphatidylinositol-3,4,5-triphosphate (PI(3,4,5)P3) at the plasma membrane. Then, the locally generated PIP3 serves as a docking site for the PH domains of PDK1 and its target, the kinase PKB/AKT (Appleman, van Puijenbroek et al. 2002, Diehn, Alizadeh et al. 2002). Activation of AKT links CD28 to pathways that are crucial for controlling the expression of many genes during T cell activation. One of them involves the NF-κB pathway. AKT cooperates with PKCθ to induce translocation of NFκB to the nucleus and transcription of NFκB target genes including IL-2 (Jones et al., 2000; Narayan et al.,2006; Park et al., 2009; Sanchez-Lockhart et al., 2004) and Bcl-xL (Burr et al., 2001; Chen et al., 2000; Khoshnan et al., 2000). AKT also phosphorylates and inactivates glycogen synthase kinase 3α (GSK3α) and GSK3β, which inhibits the nuclear export of NFAT (Beals, Sheridan et al. 1997) and promotes T cell survival

(Kane, Andres et al. 2001). Thus, AKT-mediated GSK-3 inactivation might be responsible for prolonged NFAT nuclear localization and thus IL-2 transcription following CD28 costimulation.

- Activation of Tec kinases:

The two members of the Tec family of thyrosine kinases, Tec and Itk become activated in two steps. First with their PH domain interaction with the products of PI3K or other binding partners like heterotrimeric G-protein subunits, PKCs or F-actin, they are translocated to the plasma membrane or specific intracellular microenvironments required for activation. Once at the membrane, Tec kinases are phosphorylated on a tyrosine residue in their catalytic domain by Src Family of tyrosine Kinases Lck and Fyn and become activated (Takesono, Finkelstein et al. 2002). Tec and Itk are crucial for the production of secondary messengers through positive regulation of PLC-γ1 and therefore, contribute to an increase in intracellular calcium concentration, diacylglycerol (DAG)-dependent activation of PKC and the activation of Ras/Raf/Erk through RasGRP. Upon CD28 ligation both Itk and Tec become activated via binding to the proline-rich regions in the CD28 amino- and carboxyl terminus (August, Gibson et al. 1994, Yang, Ghiotto et al. 1999, Michel, Attal-Bonnefoy et al. 2001). In contrast to the TCR, ligation of CD28 alone does not induce the phosphorylation of PLC-γ1 and increased intracellular calcium concentration (Michel, Attal-Bonnefoy et al. 2001), or activation of Erk and PKC-θ (Coudronniere, Villalba et al. 2000), indicating that functional effects of CD28-mediated activation of Tec and Itk are depending on the TCR signaling, which provides the signaling platform of LAT–SLP76 complex (Figure 6).

- Activation of Vav1:

Vav1 is a guanine nucleotide exchange factor for Rho-family GTPases and key signal transducer downstream of the TCR by regulating the activation of PI3K (Tybulewicz, Ardouin et al. 2003). Membrane recruitment and activation of Vav1 upon TCR ligation requires the phosphorylation of SLP-76 (Tuosto, Michel et al. 1996). Since SLP-76 is not tyrosine phosphorylated upon CD28 ligation (Acuto, Mise-Omata et al. 2003) the two receptors have different strategies for activating Vav1. T-cell development is markedly reduced in Vav1-deficient mice, and the few thymocytes or T cells that do develop are defective in TCR induced phosphorylation of AKT, and Tec kinases and consequently PLC-γ1 and show poor recruitment of SLP-76 to LAT (Reynolds, Smyth et al. 2002). Also, nuclear import of NFAT, increased concentrations of intracellular calcium, and activation of NF-κB and Ras/Raf/Erk pathway are strongly affected Vav1-deficient mice (Turner and Billadeau 2002). Interestingly, CD28 deficiency impairs Vav1 activation without altering TCR induced phosphorylation of ZAP-70 and LAT, but affects SLP-76, PLC-γ1 and Itk phosphorylation and ATK activation (Michel, Attal-Bonnefoy et al. 2001). This phenotype which is remarkably similar to that of Vav1-deficient T cells (Reynolds, Smyth et al. 2002) indicates a crucial role for Vav1 as an important signaling effector in CD28 costimulation.

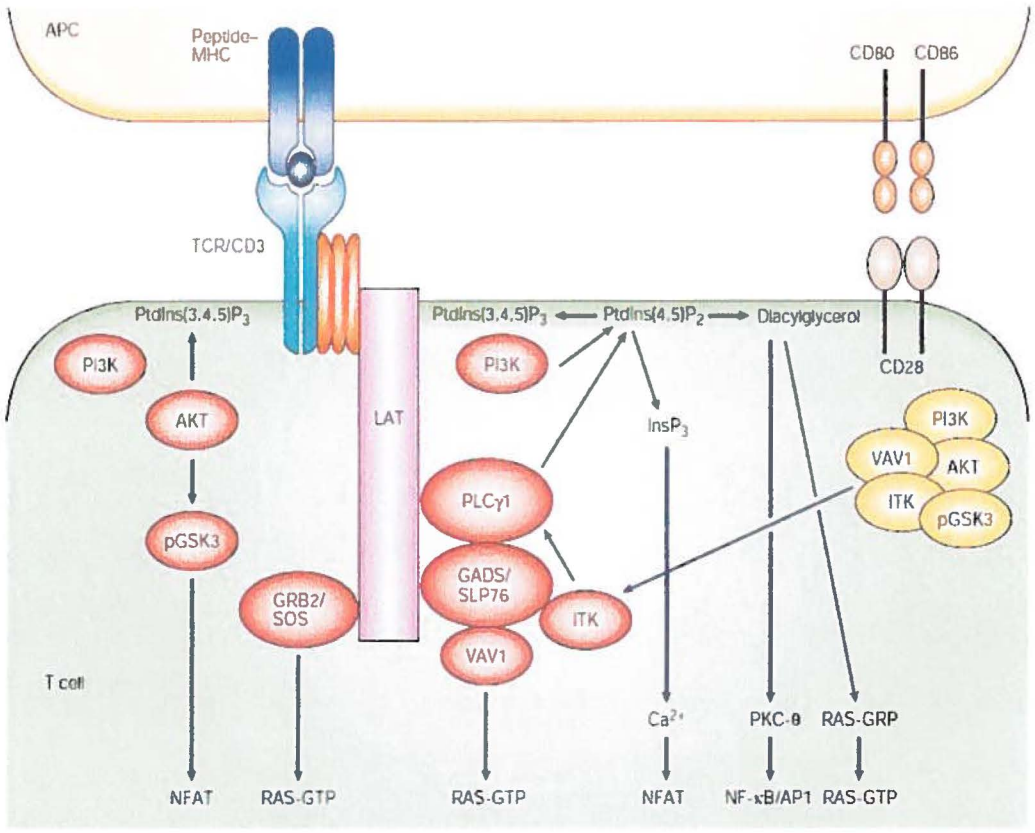

Figure 6) TCR–LAT and CD28 signaling pathways. Ligation of TCR by peptide–MHC on an antigen presenting cell (APC) triggers the recruitment of signaling elements (red) that are organized in complexes around the membrane scaffold protein linker for activation of T cells (LAT) that is resident in glycolipid-enriched membrane micro-domains (GEMs). These signaling components ensure connection to the main pathways that control nuclear transcriptional and gene activation. This scaffold provides a structure on which CD28 signaling elements (yellow) can bind to and enhance TCR signals (Acuto and Michel 2003).

2.5.2. CD28 and T cell development

- Conventional αβ T cells (negative and positive selection):

Role of CD28 signaling on thymocyte positive and negative selection is quite controversial. In 1998 impairment in negative selection was reported in CD28-deficient mice. According to this *in vitro* study the elimination of DP thymocytes was found to be significantly reduced in response to either antigen or antibody crosslinking of the TCR complex in CD28-deficient animals. It was not due to a defect in thymocyte survival as thymocytes from CD28-deficient mice displayed similar sensitivity to apoptosis initiated by either γ-irradiation or dexamethasone compared to wild type controls (Noel, Alegre et al. 1998). Moreover, other studies indicated that B7/CD28 interaction enhances clonal deletion of autoreactive T cells (Kishimoto and Sprent 1999, Li and Page 2001, Buhlmann, Elkin et al. 2003). However, later a more precise study was performed using HY-specific TCR transgenic RAG-2-deficient (HY-rag) mice which are either deficient in CD28 (HY-rag CD28KO) or both B7 antigens (HY-rag B7DKO). This study demonstrated that there is no alteration in thymic negative selection in thymi of HY-rag males in the presence or absence of either CD28 or its antigens (Vacchio, Williams et al. 2005).

Interestingly in the absence of either CD28 or B7, HY-rag female mice had an increased number of CD8 SP thymocytes in their thymi which were mature and functionally competent. Moreover DP thymocytes from CD28- or B7.1/B7.2- deficient females had higher levels of both CD5 and TCR compared to WT female controls, suggesting a stronger selecting signal. Fetal thymic organ cultures proved that this elevated number of thymic CD8 SP cells is due to an increased thymic differentiation rather than recirculation of peripheral T cells in CD28-deficient mice. Finally, in non-TCR-Tg mice there was an increased selection of mature CD4 and CD8 SP T cells in the

absence of CD28 or B7 antigens compared to WT mice (Vacchio, Williams et al. 2005). Consistent with this report, previous *in vitro* studies had also demonstrated that CD28 costimulation promotes differentiation of DP thymocytes into SP T cells (Cibotti, Punt et al. 1997, Groves, Parsons et al. 1997). So far, the role of CD28 in the development of conventional T cells is considered as minor, leading to relatively normal numbers of conventional $CD4^+$ and $CD8^+$ T cells in thymus and periphery compared to wild type controls.

- ***Regulatory T cells (Tregs):***

Compared to conventional αβ T cell development, CD28 signaling has a huge impact on the development of unconventional $CD25^+CD4^+$ regulatory T cells (Tregs). CD28 deficiency leads to a dramatically reduced numbers of Tregs in both thymus and periphery (Salomon, Lenschow et al. 2000, Bour-Jordan and Blueston 2002, Lohr, Knoechel et al. 2003). For the first time in 2000 Salomon et al showed that CD28 deficiency which leads to a profound decrease of the immunoregulatory $CD4^+CD25^+$ T cells induces exacerbated diabetes in nonobese diabetic (NOD) mice and the transfer of this regulatory T cell subset from control NOD animals into CD28 deficient animals can delay or prevent diabetes (Salomon, Lenschow et al. 2000).

- ***iNKT cells (Type I αβ NKT cells):***

Another group of unconventional T cells which is developmentally dependent on CD28 signaling is type I αβ NKT (invariant or iNKT) cells. In 2008 three independent studies showed the critical role of CD28-B7 interaction during intrathymic development, expansion and function of iNKT cells (Chung, Nurieva et al. 2008, Williams, Lumsden et al. 2008, Zheng, Zhang et al. 2008). Chung et al showed that in the absence of the costimulation ligands B7-1 and B7-2 there is a defective intrathymic development of iNKT cells which leads to a lower expansion of these cells in the thymus and periphery as well as a postselectional impairment in both phenotypical

and functional maturation of these cells (Chung, Nurieva et al. 2008). By transferring WT and B7-deficient bone marrow (BM) cells to WT and B7-deficient recipients, this study also revealed that costimulatory molecules on both hematopoietic and nonhematopoietic cells are required for iNKT cell development (Chung, Nurieva et al. 2008). In the other study Zheng et al, using mice with targeted mutations of B7-1/2 and CD28, showed that the NKT subset is significantly reduced in the thymus, spleen and liver which is mainly due to decreased cell proliferation and increased cell death in the thymi of CD28-deficient mice (Zheng, Zhang et al. 2008). This study also showed that CD28-deficient mice were less susceptible to Con A induced murine hepatitis (Zheng, Zhang et al. 2008). In this model autocrine IL-4 produced by liver NKT cells plays a critical role in cell mediated cytotoxicity (Kaneko, Harada et al. 2000, Takeda, Hayakawa et al. 2000). Williams and colleagues also indicated that B7-CD28 signaling does not affect early iNKT cell lineage commitment, but exerts its influence on the subsequent intrathymic expansion and differentiation of iNKT cells. Using CD28 wild-type/CD28-deficient mixed bone marrow chimeras they show that CD28 by using both cell-autonomous and non-cell-autonomous mechanisms play critical roles during iNKT cell development. Surprisingly transgenic mice in which thymic expression of B7 is elevated also showed essential defects in thymic iNKT cell development which indicates that both disruption and/or augmentation of this costimulatory interaction has substantial effects on iNKT cell development in the thymus (Williams, Lumsden et al. 2008).

2.6. SLAM signaling

2.6.1. SLAM family members

The family of signaling lymphocytic activation molecule (SLAM) receptors belongs to the immunoglobulin receptor super-family. This family consists of six members, named SLAM (CD150), 2B4 (CD244), NTB-A (Ly108 in mice), CRACC (CS1, CD319), CD84, and Ly-9 (CD229), which are expressed and mediate diverse outcomes on different hematopoietic cells (Table 2) (Veillette and Latour 2003, Veillette 2006, Veillette, Dong et al. 2007, Claus, Meinke et al. 2008). All the members of the family are type I transmembrane receptors with an extracellular part consisting of an N-terminal V-type Ig-domain plus a membrane-proximal C2-type Ig-domain (Figure 7) (Sandrin, Gumley et al. 1992, Engel, Eck et al. 2003). The cytosolic part of the SLAM receptor family members contains two to four tyrosine-based signaling motifs that become phosphorylated upon receptor engagement and are the basis for SLAM signaling (Ma, Nichols et al. 2007, Claus, Meinke et al. 2008). The tyrosine of these motifs is embedded in a consensus sequence TxYxxV/I, where x represents any amino acid. These motifs have been termed immunoreceptor tyrosine-based switch motifs (ITSM), because they can recruit different signaling molecules that promote activating or inhibitory signals (Figure 7) (Shlapatska, Mikhalap et al. 2001). ITSMs can bind to a family of adapter proteins which mediate SLAM signaling such as, SLAM-associated protein (SAP), Ewings sarcoma-Fli1-activated transcript 2 (EAT-2) and EAT-2 related transducer (ERT) (Engel, Eck et al. 2003, Latour and Veillette 2004, Roncagalli, Taylor et al. 2005, Veillette 2006, Ma, Nichols et al. 2007). These adapter proteins are small, comprising a Src homology 2 (SH2)-domain and a short C-terminal extension. The importance of SLAM and SAP function in immunity was highlighted by the finding that the severe immune disorder X-linked lymphoproliferative disease (XLP) is caused by the absence or

dysfunctionality of SAP (Coffey, Brooksbank et al. 1998, Nichols, Harkin et al. 1998, Sayos, Wu et al. 1998). XLP disease is a rare inherited human immunodeficiency, primarily characterized by an impaired immune response to Epstein-Barr virus (EBV) infection (Morra, Howie et al. 2001, Latour and Veillette 2003, Ma, Nichols et al. 2007). The defects in lymphocyte function that lead to the pathogenesis of XLP are not fully understood, but in recent years several findings helped to understand the underlying mechanisms. The development of the majority of lymphocytes seems to be not affected by SAP deficiency, as the numbers of NK, T and B cells are normal in XLP patients. Only the subset of NKT cells does not develop properly in these patients (Nichols, Hom et al. 2005, Pasquier, Yin et al. 2005). SH2 domain–containing protein tyrosine phosphatase (SHP)-1 and -2 and also inositol-phosphatase SHIP can bind to the phosphorylated ITSM and mediate inhibitory signals through SLAM receptor signaling (Figure 8) (Tangye, Lazetic et al. 1999, Shlapatska, Mikhalap et al. 2001, Eissmann, Beauchamp et al. 2005).

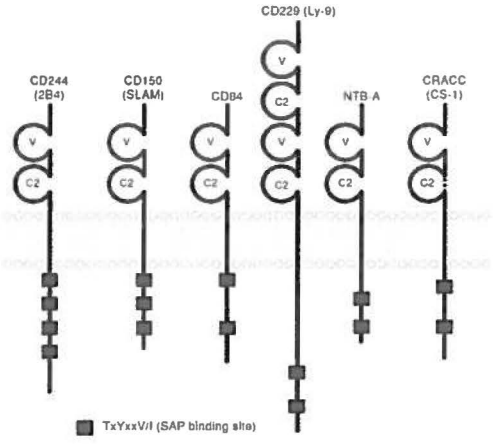

Figure 7) SLAM family of receptors. Typical SLAM receptor consists of an Ig like V- and C2-like domain. The cytoplasmic domain contains 2-4 ITSMs that are binding sites for the adaptor SAP. A number of splice variants exist for these receptors that can vary the number of ITSMs (Ma, Nichols et al. 2007).

Table 2) Expression and function of SLAM family members in hemopoietic cells*

SLAM family molecule	Cellular distribution	Function
SLAM (CD150 IPO-3)	Thymocytes, naive B cells, memory T cells, in vitro activated T and B cells, mature DCs, platelets, HSCs	• IL-4 secretion by $CD4^+$ T cells • IL-12, TNF-α production by macrophages • MV receptor • Self-ligand
2B4 (CD244)	NK cells, γδ T cells, memory $CD8^+$ T cells, monocytes, basophils, eosinophils	• NK cell cytokine secretion, cytotoxicity • Immune synapse formation in $CD8^+$ T cells • May also signal through its ligand CD48
NTB-A (Ly108 SF2000)	NK cells, T cells, NKT cells, T_{FH} cells, B cells, eosinophils	• Human NK cell cytokine secretion, cytotoxicity • IL-4 secretion by $CD4^+$ T cells • Neutrophil function • Self-ligand
Ly9 (CD229)	Some thymocytes, T cells, T_{FH} cells, B cells, NKT cells, NK cells (dim)	• ? Negative regulator of TcR signaling • Self-ligand • Minimal phenotype of *ly9*$^{-/-}$ mice
CD84	Most thymocytes, HSCs, B cells, T cells, T_{FH} cells, NKT cells, mast cells, monocytes, macrophages, DCs, neutrophils, basophils, eosinophils, platelets	• ? T cell proliferation, cytokine secretion • Platelet spreading • Self-ligand
CRACC (CS1 mouse novel Ly9)	NK cells, $CD8^-$ T cells, some $CD4^+$ T cells, B cells, mature DCs	• NK cytotoxicity (? SAP-independent) • Self-ligand

*The expression data apply largely to human cells since the analysis of the expression of SLAM family receptors in mouse has not been as comprehensive as in human, and thus there may be some species-specific differences. Adapted from (Ma, Nichols et al. 2007).

2.6.2. SLAM signaling cascade

SAP can bind to ITSMs in the cytoplasmic domains of all SLAM family receptors with the exception of CRACC (Sayos, Wu et al. 1998, Shlapatska, Mikhalap et al. 2001). This protein can associate with ITSM of SLAM in its unphosphorylated state, although the status of binding is improved upon phosphorylation (Li, Gish et al. 1999, Poy, Yaffe et al. 1999, Finerty, Muhandiram et al. 2002, Hwang, Li et al. 2002). In contrast, SAP association with ITSMs of other SLAM family members like 2B4 (Tangye, Lazetic et al. 1999, Parolini, Bottino et al. 2000), Ly108 (Zhong and Veillette 2008), Ly9 (Sayos, Martin et al. 2001) and CD84 (Sayos, Martin et al. 2001, Tangye, Nichols et al. 2003) requires pre-phosphorylation. Several studies indicated that SAP also competed with SHP-1 and /or SHP-2 for recruitment to ITSMs of SLAM, CD84, 2B4, NTB-A, and Ly9 (Sayos, Wu et al. 1998, Castro, Hauser et al. 1999, Tangye, Lazetic et al. 1999, Parolini, Bottino et al. 2000, Bottino, Falco et al. 2001, Sayos, Martin et al. 2001, Shlapatska, Mikhalap et al. 2001, Zhong and Veillette 2008). Apart from this phosphatase competition, SAP can also mediate positive signal transduction from SLAM family receptors. Data from different studies demonstrated that SAP recruits Fyn to the membrane proximal ITSM of SLAM, allowing Fyn activation and tyrosine phosphorylation of remaining ITSMs on SLAM (Latour, Gish et al. 2001, Chan, Lanyi et al. 2003, Chen, Relouzat et al. 2004).

- SAP-Fyn dependent SLAM signaling:

Studies on SLAM signaling suggest that an inducible SAP-Fyn interaction is initiated by a conformational change of SAP when it binds to SLAM (Chen, Latour et al. 2006). In this model, the SH3 domain of Fyn interacts with an arginine-based motif (R78) in SAP that lies outside of the phosphotyrosine binding pocket. This permits SAP to simultaneously interact with SLAM as well as Fyn resulting in a SLAM-SAP-Fyn complex (Figure 8) (Chan, Lanyi et al.

2003, Latour, Roncagalli et al. 2003, Li, Iosef et al. 2003). According to this model, mutation of the critical arginine (R78) of SAP dramatically reduces the ability of SAP to recruit Fyn and induce SLAM phosphorylation (Chan, Lanyi et al. 2003, Latour, Roncagalli et al. 2003, Li, Iosef et al. 2003). Although Fyn is considered as the primary kinase to phosphorylate SLAM family receptors, potential interactions of SAP with Lck have also been shown (Simarro, Lanyi et al. 2004). Activation and recruitment of Fyn result in phosphorylation of the remaining ITSMs and the recruitment of the lipid phosphatase SHIP. SHIP binds to the adaptor proteins, downstream of tyrosine kinases (Dok)-1 and Dok-2, allowing recruitment of RasGTPase activating protein (RasGAP) which is one of the most important interacting partners of the Dok adaptor proteins which will be discussed later in this chapter (Latour, Gish et al. 2001, Latour, Roncagalli et al. 2003).

- SAP-Fyn independent SLAM signaling:

Apart from Fyn, SAP can also interact with other proteins at its R78 motif. Studies on the effect of SLAM signaling on cytokine secretion show that SLAM engagement on $CD4^+$ T cells can increase recruitment of PKCθ to the immune synapse and it is accompanied with an increase in IL-4 expression (Cannons, Yu et al. 2004). Further molecular examination of this signaling pathway revealed that the formation of SAP-PKCθ complex occurs in the absence of Fyn (Cannons, Wu et al. 2010).

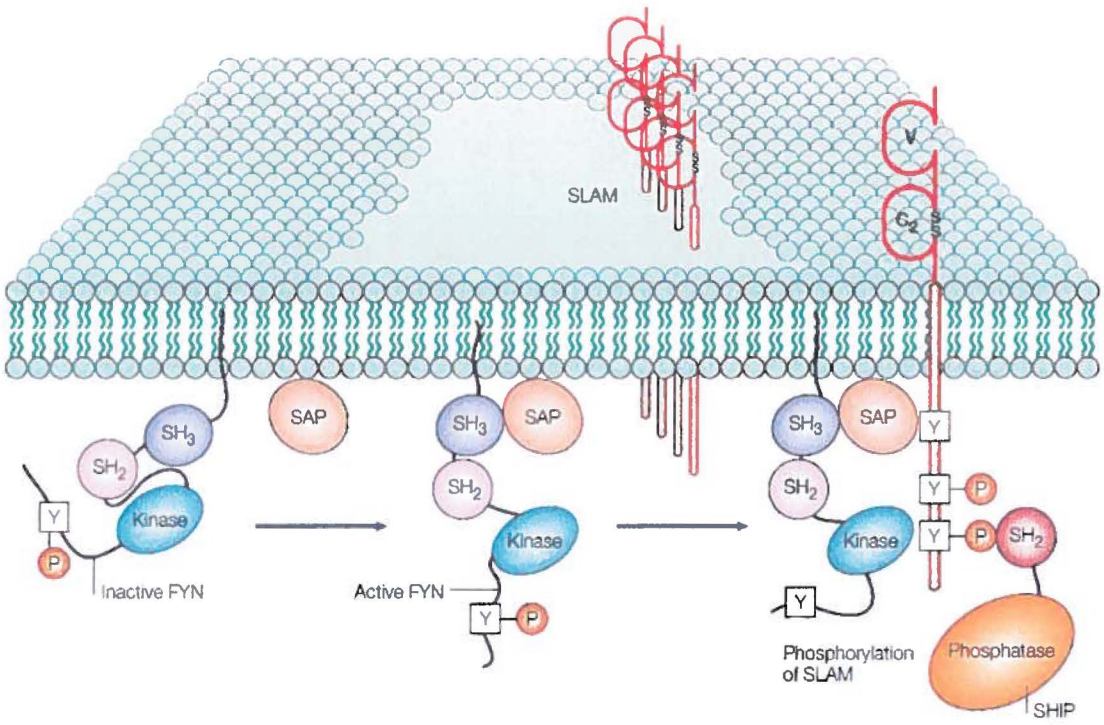

Figure 8) Model of FYN activation and recruitment by SAP. Binding of SAP to SLAM promotes its association with Fyn and induces its activation. Subsequently, receptor-associated Fyn can phosphorylate tyrosine residues in the cytoplasmic tail of the receptor. This creates docking sites for phosphatases SHP2 or SHIP (Engel, Eck et al. 2003).

2.6.3. SLAM receptors and thymocyte development

As previously mentioned, SAP-mediated signaling is crucial for the development of a class of innate-like cells called NKT cells. SAP-deficient mice (and XLP patients) exhibit a complete absence of αβ iNKT cells, showing the importance of SAP-mediated signaling in the development of these cells. Upstream of SAP, SLAM family receptors are implicated as they are expressed mostly on DP thymocytes (iNKT selecting cells), but not on the thymic stroma (Griewank, Borowski et al. 2007). Elegant mixed bone marrow chimera experiments have demonstrated Ly108 along with SLAM as the two members of SLAM family receptors required for αβ iNKT cell development (Griewank, Borowski et al. 2007). However, how Ly108/SLAM signaling promotes or allows for the development of these cells is still unknown. iNKT cells progress through four stages of development that can be tracked as stage 0, 1, 2 and 3, identified by the expression of maturation markers, and SAP-deficient mice display a block at very early stage in iNKT cell development (just following their invariant TCR rearrangement at stage 0) (Nunez-Cruz, Yeo et al. 2008, Alonzo, Gottschalk et al. 2010). NKT cells also require and express the key transcription factor, PLZF that is crucial for their development and activated phenotype. PLZF was shown to be sufficient to provide memory/effector phenotype to naïve T cells, but failed to rescue NKT cell development in SAP or Fyn-deficient mice (Kovalovsky, Alonzo et al. 2010), arguing that reconstitution with PLZF alone does not compensate the complex outcome of SLAM/SAP signaling, which is required for their selection. Factors regulating PLZF expression are not completely understood. While it has been previously shown that in immature γδ T cells and NKT cells, PLZF can be induced by strong TCR stimulation (Kreslavsky, Savage et al. 2009, Seiler, Mathew et al. 2012) very recently it has been shown that costimulation of DP thymocytes through Ly108 significantly enhances PLZF expression (Dutta,

Kraus et al. 2013). Costimulation with Ly108 increased expression Egr-2 and its binding to the promoter of *Zbtb16*, which encodes PLZF, and resulted in PLZF levels similar to those seen in NKT cells indicating a potential role for Ly108 in the induction of PLZF (Dutta, Kraus et al. 2013). The SAP R78-Fyn signaling axis has also been shown to affect iNKT cell ontogeny. While components in this pathway are yet to be elucidated, it has been shown that Fyn-deficient and SAPR78A knock-in mice exhibit a significant reduction in NKT cell numbers (Eberl, Lowin-Kropf et al. 1999, Gadue, Morton et al. 1999, Nunez-Cruz, Yeo et al. 2008). Other than classical αβ iNKT cells there is another group of innate-like T cells, called γδ NKT cells. These cells also express and invariant type of TCR like αβ iNKT cells showing innate properties and the lack of the adapter protein SAP has been shown to negatively affect their development (Alonzo, Gottschalk et al. 2010) suggesting that SAP might regulate the development of both TCRαβ and TCRγδ expressing NKT cells.

2.7. Dok family of adaptor proteins

In mammals, downstream of tyrosine kinase (Dok) protein family has seven members (Dok-1–Dok-7) (Mashima, Hishida et al. 2009). Among all the members, Dok-1 was the first to be identified. In 1990, this protein was originally identified as a tyrosine- phosphorylated protein in cells transformed by oncoproteins with protein tyrosine kinase (PTK) activity or in cells treated with epidermal growth factor, whose receptor had integrated PTK activity (Ellis, Moran et al. 1990). This protein was initially referred to as p62 due to its apparent molecular mass, 62 kDa. After the original discovery, more biochemical studies revealed that p62 is readily tyrosine-phosphorylated downstream of a wide range of PTKs and it is associated with p120 RasGAP upon tyrosine phosphorylation (Ellis, Moran et al. 1990, DeClue, Vass et al. 1993)

Several cell activating events had been reported in which p62 was rapidly tyrosine phosphorylated, such as the stimulation of the B-cell receptor (BCR) or macrophage colony stimulating factor (M-CSF) receptor (Heidaran, Molloy et al. 1992, Gold, Crowley et al. 1993) The diversity of the PTK-associated cellular events in which p62 was found to be tyrosine phosphorylated suggested that p62 plays an important role as a common substrate of PTKs. Further studies, mainly performed by two groups finally announced an adapter role for p62 (Carpino, Wisniewski et al. 1997, Yamanashi and Baltimore 1997) which was then named Dok (downstream of tyrosine kinases) and later Dok-1 as the Dok family expanded to seven different members in mammals (Figure 9).

2.7.1 Expression pattern

Among all the seven members Dok-1, Dok-2 and Dok-3 are preferably expressed in the cells of the immune system (Table 3) and phylogenetically comprise one of the three subgroups in the Dok protein family concerning their primary structure (Figure 9) (Mashima, Hishida et al. 2009).

Table 3) Expression of Dok-1/2/3 in hematopoietic cells

Dok	B cells	T cells	Myeloid cells
Dok-1	+	+	+
Dok-2	-	+	+
Dok-3	+	-	+

+, High expression; -, Little or no expression
Adapted from (Mashima, Hishida et al. 2009)

Dok-1 mRNA is expressed in B and T cells as well as in myeloid cells like macrophages and neutrophils. Similarly, Dok-2 mRNA is preferably expressed in T cells and myeloid cells, with little or no expression in B cells (Yasuda, Shirakata et al. 2004, Yasuda, Bundo et al. 2007). By contrast, Dok-3 mRNA is preferentially expressed in myeloid cells and B cells, with little or no expression in T cells (Lemay, Davidson et al. 2000). Other than hematopoietic cells the expression of Dok-1 has been reported in numerous types of non-hematopoietic cells, indicating their non-immunological function, giving Dok a central role in cell signaling mediated by a wide range of protein tyrosine kinases. For example Dok-1 has been reported to be a potential target downstream of insulin-like growth factor-1 (IGF-1) receptor in neurons (Smith, Wang et al. 2004) and in platelets, Dok-1 is reported to be expressed and independently attenuates

Ras/mitogen-activated protein kinase pathways to inhibit platelet-derived growth factor-induced mitogenesis (Zhao, Janas et al. 2006).

2.7.2 Structure

All the seven members of the Dok protein family share structural similarities characterized by the NH2-terminal pleckstrin homology (PH) and phospho-tyrosine binding (PTB) domains followed by SH2 target motifs in the COOH-terminal moiety, indicating their adapter function. Phylogenetic analysis using full-length sequences of the seven members of the family shows that mouse Dok proteins cluster in three subgroups, namely Dok-1/2/3, Dok-4/5/6, and Dok-7. The phylogenic tree is showed in Figure 9 (Mashima, Hishida et al. 2009).

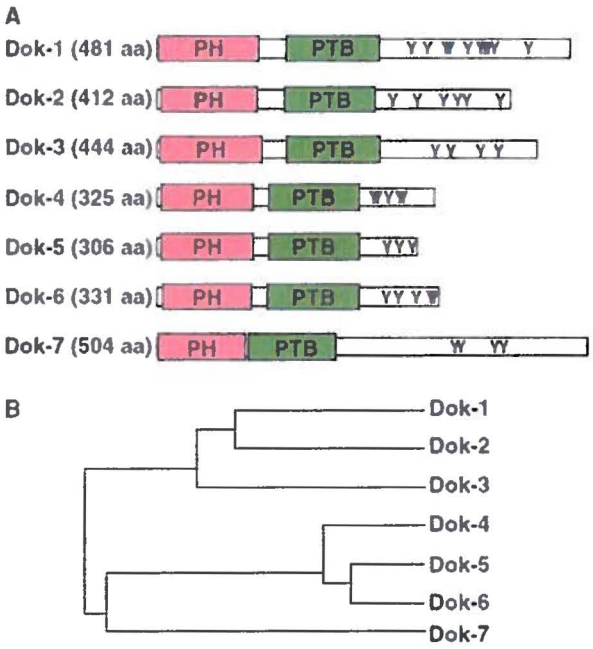

Figure 9) The Dok family proteins. (A) Schematic representation of mouse Dok proteins. Y denotes a tyrosine residue in the COOH-terminal region. (B) A phylogenetic tree of mouse Dok proteins (Mashima, Hishida et al. 2009).

- Pleckstrin homology (PH) domain:

PH domains are relatively small modules comprising about 120 amino acids. These domains play an essential role in the localization or translocation of many proteins to cellular membranes through their interaction with phospholipids (Rebecchi and Scarlata 1998, Lemmon and Ferguson 2000). This domain enables Dok to have a better interaction with membrane associated PTKs which phosphorylate this adaptor protein. Like many other PH domains, the Dok-1 PH domain is capable of binding with multiple phosphorylated phosphoinositides, such as phosphatidylinositol bisphosphate (PIP2) and phosphatidylinositol triphosphate (PIP3) (Noguchi, Matozaki et al. 1999). As expected the lack of a PH domain in Dok-1 showed the dissociation of this protein from the subcellular membrane compartment in addition to impaired tyrosine phosphorylation upon the activation of PTKs (Noguchi, Matozaki et al. 1999). So, other than PTKs the activities of Dok proteins can be regulated by phosphoinositide kinases like Phosphatidylinositol 3-kinase (PI3K) which have essential roles in the metabolic generation of PIP2 and PIP3 (Zhao, Schmitz et al. 2001). On the other hand phosphatase enzymes like SH2-containing inositol phosphatases (SHIPs) and phosphatase and tensin homolog deleted on chromosome 10 (PTEN) which are able to generate PIP2 by dephosphorylation of PIP3 can regulate the activity of Dok proteins (Lemmon 2008). All these findings strongly suggest that the activities of the Dok are regulated not only by PTKs but also by phosphoinositide kinases / phosphatases through its PH domain.

- Phosphotyrosine-binding (PTB) domain:

PTB domains are small modules found in many adapter proteins and they are responsible for protein–protein interactions. These small modules in many cases interact with short peptide sequences including an NPXY motif upon its tyrosine phosphorylation (NPXYp) and thereby centralize the formation of multi-protein complexes (Forman-Kay and Pawson 1999,

Uhlik, Temple et al. 2005). A relatively limited number of proteins have been reported to associate with the PTB domain of Dok-1, Dok-2, and/or Dok-3 (Mashima, Hishida et al. 2009). Different studies showed that the PTB-binding protein does not always have a strong 'consensus' sequence. For example other sequences like NXLpY have been reported to interact with Dok-1 PTB domain *in vitro* condition (Songyang, Yamanashi et al. 2001). Therefore further unbiased studies are necessary to make a complete list of the proteins targeted by the PTB domains of the Dok family.

- SH2-target motif:

There are several SH2-containing proteins that can interact with SH2-target motif of Dok proteins. Some of those are Abl (Cardell, Tangri et al. 1995, Kadri, Blomqvist et al. 2008), PI3K and PLC-γ (Kadri, Blomqvist et al. 2008), Tec (Gerard, Favre et al. 2004, Zhou, Mattner et al. 2004, Kadri, Blomqvist et al. 2008), Nck (Noguchi, Matozaki et al. 1999, Gugasyan, Quilici et al. 2002, Zhou, Mattner et al. 2004, Hao, Dong et al. 2011) and p120 RasGAP (Carpino, Wisniewski et al. 1997, Yamanashi and Baltimore 1997, Noguchi, Matozaki et al. 1999, Schaeffer, Debnath et al. 1999, Songyang, Yamanashi et al. 2001, Gugasyan, Quilici et al. 2002, Miller and Berg 2002, Hao, Dong et al. 2011, Miyazaki, Rivera et al. 2011).

Molecular interaction of Dok-1 and Dok-2 with RasGAP (Ras GTPase-activating protein and a potent inhibitor of Ras) is the most extensively studied interaction of these two members of the Dok family. In the resting state, cells mostly have the inactive form of Ras in their cytoplasm (a GDP-bound form) but upon stimulation (like engagement of a ligand to its PTK receptor), guanine nucleotide exchange factors (GEFs) such as SOS and Ras-guanyl-releasing proteins (RasGRPs) mediate the GDP to GTP conversion and activate Ras. Meanwhile, there are some proteins like p120 RasGAP which negatively regulate this conversion and results in Ras

inactivation. It is hypothesized that negative regulatory role of Dok-1/2 in the Ras–Erk pathway is associated with their ability to interact and recruit p120 RasGAP to inhibit Ras downstream of PTKs (Campbell, Khosravi-Far et al. 1998). More precisely, the interaction between p120 RasGAP and Dok-1 or Dok-2 occurs between the SH2 domains of p120 RasGAP and their target motifs (YXXP) in the COOH-terminal moiety of Dok-1/2. In mouse Dok-1, the phosphorylation of tyrosines 295 and 361 provides actual docking sites for the SH2 domains of p120 RasGAP (Songyang, Yamanashi et al. 2001).

2.7.3. Dok-1 and Dok-2 in TCR signaling pathway

Dok-1 and Dok-2, but not Dok-3, are preferentially expressed in T cells. For the first time the two adapter proteins have been shown to become tyrosine phosphorylated upon TCR signaling (by CD3 stimulation) in primary human T cells (Dong, Corre et al. 2006). Their negative regulatory role in TCR signaling was found in mice lacking both Dok-1 and Dok-2 which showed enhanced IgG responses to Thymus Dependent (TD) antigens, indicating that Dok-1 and Dok-2 play a negative role in T cell dependent immune responses (Yasuda, Bundo et al. 2007). It was also reported that $CD4^+$ T cells from these mice show enhanced proliferation and IL-2 production upon *in vitro* TCR stimulation (Yasuda, Bundo et al. 2007). In another independent study a negative regulatory role of Dok proteins was demonstrated where overexpression of Dok-2 in bone marrow cells followed by transplantation into lethally irradiated mice revealed a remarkable impairment of thymocyte transition from $CD4^-CD8^-$ (DN) to $CD4^+CD8^+$ (DP) in the thymus (Gugasyan, Quilici et al. 2002). By contrast, in the absence of both Dok-1 and Dok-2 the number of DP but not DN thymocytes increased (Yasuda, Bundo et al. 2007).

Interestingly, the expression level of both Dok-1 and Dok-2 could affect the activation (phosphorylation) level of different key components downstream of TCR signaling. In mice lacking both Dok-1 and Dok-2, upon TCR stimulation $CD4^+$ T cells show enhanced phosphorylation of ZAP-70, LAT, and Erk (Yasuda, Bundo et al. 2007). Although the mechanisms are not clear, Dok-1 and Dok-2 appear to inhibit ZAP-70 activation during TCR-mediated signaling. In a different study siRNA-mediated downregulation of Dok-1 and Dok-2 expression also enhanced the activation of ZAP-70 and its downstream signaling (Dong, Corre et al. 2006). Even though the exact molecular mechanisms are unclear, there are possible modes of action of Dok proteins explaining the Dok-mediated inhibition of TCR signaling. The PTB domain of both Dok-1 and Dok-2 can interact with the short peptide motifs NQLY and NPDY within the ITAMs of TCRζ and CD3ε. Since these ITAMs are essential for binding ZAP-70 and consequently their activation, interference between ZAP-70 and the Dok proteins might happen through their binding to the ITAMs and this accordingly reduces their phosphorylation (Figure 10). An evidence for this theory is a report that says PH and PTB domains of Dok-1 and Dok-2 are essential for the negative regulation of TCR signaling and SH2 target motifs seemed to be dispensable (Yasuda, Bundo et al. 2007). Consistently another group of scientists reported that the loss of the PH domain blocks the inhibitory effects of Dok-1 and Dok-2 in T cells (Guittard, Gerard et al. 2009) suggesting the importance of the localization of the Dok proteins in the cellular membrane. There is also evidence indicating that the adapter proteins Dok-1 and Dok-2 can inhibit Erk pathway downstream of TCR signaling. Studies on Jurkat cells reported that upon CD2 stimulation phosphorylated forms of Dok-1 and Dok-2 are able to make molecular complexes with RasGAP (Nemorin, Laporte et al. 2001). Therefore, this recruitment of RasGAP to the proximity of Ras which is mediated by Dok might be responsible for the attenuation of

Erk1/2 signaling (Figure 10). On the other hand, both Dok-1 and Dok-2 have been reported to be the major tyrosine-phosphorylated proteins associated to Tec, a member of Tec family of tyrosine kinases downstream of both TCR and CD28 signaling and both of these proteins could block the IL-2 promoter activity induced by Tec (Gerard, Favre et al. 2004).

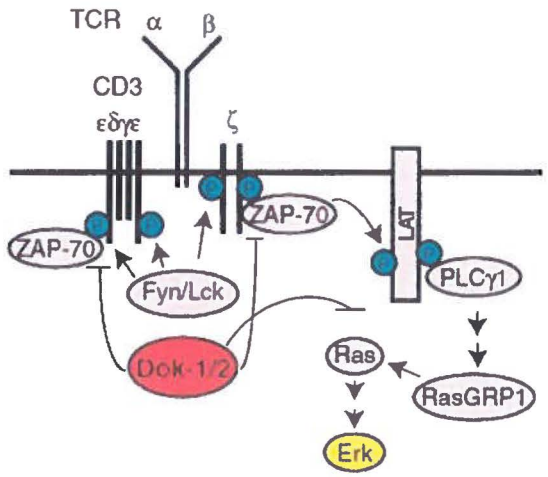

Figure 10) Simplified model of the role of Dok proteins in TCR-mediated signaling. Upon TCR engagement and activation of the Src-family PTKs Fyn and Lck, ITAMs in the TCRζ and CD3ε become phosphorylated and recruits ZAP-70, which in turn phosphorylates LAT. This event leads to the activation of the Ras–Erk pathway via RasGRP (Mashima, Hishida et al. 2009). Dok can negatively regulate downstream events of TCR signaling by inhibiting Ras or interfering with ZAP-70.

2.7.4. Dok-1 and Dok-2 in Costimulatory signaling Pathways

In T cells, Other than in TCR signaling, Dok-1 and Dok-2 have been reported to become tyrosine phosphorylated downstream of costimulatory receptor signaling pathways like CD28 in cell lines and primary T cells (Yang, Ghiotto et al. 1999, Michel, Attal-Bonnefoy et al. 2001, Dong, Corre et al. 2006). Its phosphorylation was also reported upon CD2 stimulation which was dependent on the Src-family PTK, Lck (Nemorin and Duplay 2000). It's been shown that the overexpression of Dok-1 led to the specific inhibition of CD2-mediated Erk1/2 activation (Nemorin, Laporte et al. 2001). Also the forced expression of Dok-1 or Dok-2 inhibits the activation of both Erk and NFAT upon CD2 signaling, indicating a negative regulatory function, in which the oligomerization of Dok-1 and Dok-2 appears to be critical (Boulay, Nemorin et al. 2005). However, the physiological roles of Dok proteins in CD2 or CD28 signaling are yet to be discovered. Tyrosine phosphorylation of both Dok-1 and Dok-2 is also induced in T cells upon activation of SLAM receptors (Latour, Roncagalli et al. 2003). As it was mentioned earlier, when SLAM is crosslinked on T cells, it becomes tyrosine phosphorylated through a SAP- and Fyn-dependent mechanism (Latour, Gish et al. 2001, Chan, Lanyi et al. 2003, Latour, Roncagalli et al. 2003, Li, Iosef et al. 2003). The phosphorylated ITSM on SLAM provides docking sites for the phosphatase SHIP, which is subsequently phosphorylated and binds the adaptor proteins Dok-1, Dok-2 (Latour, Gish et al. 2001). Upon recruitment of Dok to the proximity of SLAM receptor Fyn can also phosphorylate Dok (Latour, Roncagalli et al. 2003). Tyrosine-phosphorylated Dok proteins bind the SH2 domain of RasGAP (Latour, Gish et al. 2001, Veillette 2006). Although the physiological roles of Dok proteins in SLAM-mediated signaling are still unclear but this protein can recruit RasGAP and potentially inhibit Ras and Erk pathway downstream of SLAM receptor signaling (Latour, Gish et al. 2001).

Therefore, it seems that the complex of Dok-RasGAP is common downstream of three different signaling pathways in T cells which are TCR, CD28 and SLAM receptor signaling.

2.7.5. Dok-1 and Dok-2 in other signaling pathways in T cells

In addition to their role in TCR and costimulatory receptor signaling pathways, Dok proteins have been reported to be important downstream other signaling pathways like chemokine receptor CXCR4 (Okabe, Fukuda et al. 2005). It's been shown that the association of CXCR4 and its ligand stromal cell-derived factor-1 (SDF-1alpha/CXCL12) mediates Dok-1 tyrosine phosphorylation by Lck and association with RasGAP and other adaptor proteins like Nck, and Crk-L in Jurkat T cells. Chemotaxis to SDF-1/CXCL12 was reduced in the case of Dok-1 overexpression and significantly enhanced in Dok-1-deficient $CD4^+$ and $CD8^+$ splenic T cells (Okabe, Fukuda et al. 2005). IL-4 signaling is another signaling pathway that Dok proteins are involved in. It's been reported that the loss of Dok-1 impaired the proliferative response of $CD4^+$ T cells and B cells to IL-4. Conversely, the overexpression of Dok-1 in a myeloid cell line increased the IL-4 induced proliferation, indicating a positive role for Dok-1 downstream of IL-4 signaling. Tyrosine phosphorylation, and thereby the activation of Stat6 and IRS-2 both are critical for IL-4 signaling; however, in Dok-1-deficient cells stimulated with IL-4 only the activation of Stat6 was perturbed (Inoue, Yasuda et al. 2007).

3. Natural killer T (NKT) cells

3.1. αβ NKT cells

In 1987, published studies from three separate groups demonstrated the existence of a distinct subset of Vα14–Jα18 TCRα$^+$ T cells in C57BL/6 mice that expressed intermediate rather than high levels of TCR, with a two to three times higher frequency of Vβ8 expression than conventional T cells, and lack the expression of both CD4 and CD8 (Budd, Miescher et al. 1987, Ceredig, Lynch et al. 1987, Fowlkes, Kruisbeek et al. 1987). Interest in DN TCRαβ$^+$ lymphocytes increased when it was discovered that they were a potent source of immunoregulatory cytokines, including IL-4, IFN-γ and TNF (Zlotnik, Godfrey et al. 1992). Other groups reported on the existence of a subset of TCRαβ$^+$ T cells that expressed NK1.1, which was previously considered to be only expressed by NK cells (Sykes 1990, Levitsky, Golumbek et al. 1991). These NK1.1$^+$ T cells also expressed intermediate levels of TCR with a bias towards Vβ8.2 expression, and included two subsets: CD4$^+$ T cells and DN T cells and were also found to be a source of immunoregulatory cytokines (Arase, Arase et al. 1993, Yoshimoto and Paul 1994). Further investigations revealed that these Vα14–Jα18 invariant TCRα chain-expressing cells were CD1d restricted, an MHC class I like molecule which presents lipid antigens to T cells (Beckman, Porcelli et al. 1994, Bendelac, Lantz et al. 1995). Meanwhile, another population of CD1d restricted TCRαβ$^+$ cells was found which expressed a diverse set of TCR α- and β -chains instead of the invariant Vα14–Jα18 TCRα and limited numbers of β chains (Cardell, Tangri et al. 1995, Kadri, Blomqvist et al. 2008). Further investigations categorized these two subsets of CD1d restricted T cells as type I and type II subsets of αβ NKT cells respectively.

3.1.1. Type I αβ NKT cells (iNKT cells)

In mice, Type I αβ NKT cells are also known as "classical" or "invariant" NKT (iNKT) cell (Das, Sant'Angelo et al. 2010). As it was mentioned earlier iNKT cells in mice express an invariant TCRα chain (Vα14-Jα18) that possesses a conserved CDR3 region associated with Vβ8.2, Vβ2 or Vβ7 chains (Dellabona, Padovan et al. 1994, Godfrey and Berzins 2007, Godfrey, Stankovic et al. 2010). Humans have a homologous population of these T cells expressing an invariant Vα24 rearrangement (Vα24-Jα18), associated with Vβ11 (Dellabona, Padovan et al. 1994). In mice, iNKT cells consist of at least two populations based on the expression of CD4, including $CD4^+$ or $CD4^-CD8^-$(DN) subsets and based on the expression of NK1.1 there are $NK1.1^+$ and $NK1.1^-$ subsets (Godfrey, MacDonald et al. 2004, Godfrey and Berzins 2007, Godfrey, Stankovic et al. 2010). However, in human in addition to $CD4^+$ and DN subsets, a small fraction of NKT cells express CD8α marker and also mature human NKT cells from the blood express CD161 instead of NK1.1 (Baev, Peng et al. 2004, Berzins, Cochrane et al. 2005). $NK1.1^-$ population mostly exist in the thymus and less in periphery (Benlagha, Kyin et al. 2002, Pellicci, Hammond et al. 2002). It was discovered that the Vα14–Jα18 expressing NKT cells recognized the Glycosphingolipid (GSL) α-galactosylceramide (α-GalCer) derived from a marine sponge (Kawano, Cui et al. 1997). Subsequently, α-GalCer loaded on CD1d tetramers became a useful tool to study Vα14–Jα18-expressing NKT cells which significantly helped to advance the NKT cell research field (Gumperz, Miyake et al. 2002, Godfrey, Stankovic et al. 2010). Following the discovery of α-GalCer, investigations on type I NKT cells increased extensively, while the lack of specific reagents had limited the studies of the non-Vα14–Jα18 expressing type II NKT cells.

3.1.2. Tissue Distribution of iNKT Cells

In a mouse thymus, iNKT cells represent about 0.5% of all the thymocytes populations (Kim, Butcher et al. 2002) and in the liver where they are most abundant, they represent as much as 30% of the T cell population. In spleen, they represent 2.5% of all T cells and in peripheral lymph nodes and in blood they make a population of 0.5% of all thymocytes (Benlagha, Weiss et al. 2000, Matsuda, Naidenko et al. 2000, Hammond, Pellicci et al. 2001). The high expression of CD1d on liver resident Kupffer cells seems to be important for retaining the NKT cells in the liver (Geissmann, Cameron et al. 2005). Furthermore, the expression of lymphocyte function associated antigen-1 (LFA-1) and the interaction with intracellular adhesion molecule-1 (ICAM-1) expressed on NKT cells as well as production of IL-15 by hepatic stellate cells appear to be crucial for the maintenance of NKT cells in liver (Winau, Hegasy et al. 2007). Recently, a subset of iNKT cells that lack NK1.1 and produces high amounts of IL-17 and low amounts of IFN-γ and IL-4 has been identified (Michel, Keller et al. 2007). It was demonstrated that these IL-17 producing NKT cells were highly abundant in lungs. Notably, in humans, the frequencies of iNKT cells appear to be lower, approximately ten times less than the population observed in mice. In humans, most of the studies on iNKT cells have been limited to those in peripheral blood. However, there is a much lower frequency (1 %) of Vα24 iNKT cells present in the human liver compared with mice (Doherty, Norris et al. 1999). Moreover in humans three subsets ($CD4^+$, $CD8^+$ and DN) of NKT cells are not evenly distributed in different tissues. In thymus, spleen, blood and liver, $CD4^+$ NKT cells are the major NKT cell subset, whereas in other tissues including lymph node and bone marrow, the majority of NKT cells are DN and/or $CD8^+$ (Ishihara, Nieda et al. 1999).

3.1.3. Type II αβ NKT cell

Type II NKT cells (also called non-Vα14 and non-Vα24 NKT cells in mice and humans respectively) express a more diverse TCR α-chain repertoire with only some recurrent Vα3.2-Jα9 / Vβ8 or Vα8 / Vβ8 chain combination. These cells are a CD1d-restricted population but they are not activated by α-GalCer presented by CD1d (Cardell, Tangri et al. 1995, Godfrey, MacDonald et al. 2004, Godfrey, Stankovic et al. 2010). The antigens recognized by type II NKT cells are not fully characterized, but sulfatide (a self-glycolipid derived from myelin) and non-lipidic small molecules such as lyso-sulfatide (an analog of sulfatide) have been described as ligands of type II NKT cells (Jahng, Maricic et al. 2004, Van Rhijn, Young et al. 2004). Using sulfatide-CD1d tetramers showed that type II NKT cell population is significantly smaller than type I in the mouse spleen (Jahng, Maricic et al. 2004).

3.1.4. Maturation of iNKT cell

The development of αβ NKT cells is distinct from that of conventional αβ T cells even though they originate from common DP precursors. The study of αβ NKT cell development has been possible by the use of α-GalCer-CD1d tetramers specific for the iNKT cells. Due to the lack of unique reagents for type II NKT cells, most information available describes the development of iNKT cells. At the DP stage, cells to become NKT cells are positively selected by other DP thymocytes expressing CD1d-lipid complexes (Figure 11). This is in contrast to the selection of conventional αβ T cells, which become selected by cortical thymic epithelial cells (TECs), bearing MHC molecules presenting peptide antigens. The different stages of thymic NKT cell development, and important factors for each stage, are illustrated in Figure 11. The selection of NKT cells by CD1d expressing self-lipids induces an activated memory phenotype

($CD69^{high}$) already at "stage 0" of development, which is distinct from the naïve phenotype of mature conventional single positive (SP) thymocytes. Other than α-GalCer, an endogenous GSL Isoglobotrihexosylceramide (iGb3) has been identified to stimulate NKT cells (Kawano, Cui et al. 1997, Zhou, Mattner et al. 2004). Mice deficient in the lysosomal glycosphingolipid degrading enzyme -hexosaminidase b subunit ($Hexb^{-/-}$) which are consequently deficient for iGb3 were shown to exhibit a significant decrease in type I NKT cells. Cells staining positive with α-GalCer tetramers were reduced by 95% in these mice suggesting that iGb3 is involved in the thymic positive selection of NKT cells (Zhou, Mattner et al. 2004). NKT cells are only selected when CD1d is expressed on DP thymocytes (Coles and Raulet 1994, Bendelac, Lantz et al. 1995, Forestier, Park et al. 2003, Xu, Chun et al. 2003). $CD1d^{-/-}$ mice, or mice having a defect in CD1d processing and presentation, lack mature NKT cells (Godfrey and Berzins 2007). The process of positive selection by CD1d-lipid complex with TCR requires ligation of both TCR and a costimulatory molecule, SLAM that signals via SLAM-associated protein (SAP) and the downstream Src kinase (Fyn) (Nichols, Hom et al. 2005, Pasquier, Yin et al. 2005). NKT cells are deficient in mice lacking Fyn, demonstrating the importance of SLAM-SAP-Fyn signaling pathway in the developmental program of NKT cells (Eberl, Lowin-Kropf et al. 1999, Gadue, Morton et al. 1999). Also, at this point, induction of different transcription factor such as PLZF, Runx1, c-Myc and Egr2 appears to be very important (Das, Sant'Angelo et al. 2010). PLZF is expressed during the whole process of development and maturation of NKT cells, starting after the positive selection of DP thymocytes to the terminally differentiated stage of NKT cell development in peripheral tissues (Savage, Constantinides et al. 2008). Following successful CD1d-TCR ligation, NKT cells enter "stage 0" indicated by expression of CD24 and CD69, these cells do not express the memory marker CD44 nor NK1.1 ($CD24^{high}$, $CD69^{high}$,

$CD44^-$ and $NK1.1^-$). At this stage all NKT cells express CD4, they are extremely rare and they don't proliferate. Further maturation occurs through downregulation of CD24 to reach the mature $CD4^+$ NKT cell stage with low expression of CD44 in "stage 1". These "stage 1" NKT cells remain in the thymus where they continue their developmental program into "stage 2" by upregulating CD44 and IL-2 receptor (CD122) that mediate the induction of low basal transcription of Th2 followed by Th1 cytokines. Upon leaving the thymus, NKT cells start to express NK lineage receptors such as NK1.1 and enter "stage 3". The transcription factor T-bet is essential for the transition from "stage 2" to "stage 3". Some NKT cells in "stage 3" reside in the thymus (figure 11) however the function of these cells in the thymus has not been identified yet (Benlagha, Kyin et al. 2002, Godfrey, Stankovic et al. 2010). Other than surface markers defining the maturational stages of NKT cells, there are several other important changes like the level of the PLZF expression and changes in NKT cell function which accompany these transition stages. PLZF expression increases after positive selection, is highest in "stage 1" when the cells are $CD44^{low}NK1.1^-$ and decreases as differentiation proceeds to "stage 3" (Kovalovsky, Uche et al. 2008, Savage, Constantinides et al. 2008). Regarding the functional changes in the course of NKT call maturation, several studies have reported that "stage 1" and "stage 2" αβ NKT cells acquire the ability to secrete IL-4 and make little IFNγ, whereas "stage 3" αβ NKT cells produce abundant amounts of IFN-γ but little IL-4 (Pellicci, Hammond et al. 2002, Stetson, Mohrs et al. 2003). It has also been reported that NKT cells in stages 0, 1 and 2 are thought to be thymus dependent but most αβ NKT cells emigrate from the thymus before stage 3 and maturation continues out in the periphery (Franki, Van Beneden et al. 2006, Allende, Zhou et al. 2008). Upon leaving the thymus, NKT cells preferentially migrate to the liver, however they are also present in spleen, bone marrow, lung and gut with exception of an IL-17 producing subset of

iNKT cells which is abundant in the lung and lymph nodes, but hardly detectable in the liver and bone marrow (BM) of both C57BL/6 and BALB/c mice. This distinct NKT cell population expresses chemokine receptor CCR7, in contrast to other αβ NKT cells. This suggests that it might be a functionally distinct population of NKT cells that locate specifically in lung and lymph nodes (Michel, Keller et al. 2007, Watarai, Sekine-Kondo et al. 2012). Several studies reported that NKT cells also undergo negative selection in the thymus. The addition of α-GalCer to fetal thymic organ cultures (FTOC) results in the disappearance of Vα14 NKT cell and increased expression of CD1d in transgenic mice causes a decrease in the number of Vα14 NKT cells (Chun, Page et al. 2003).

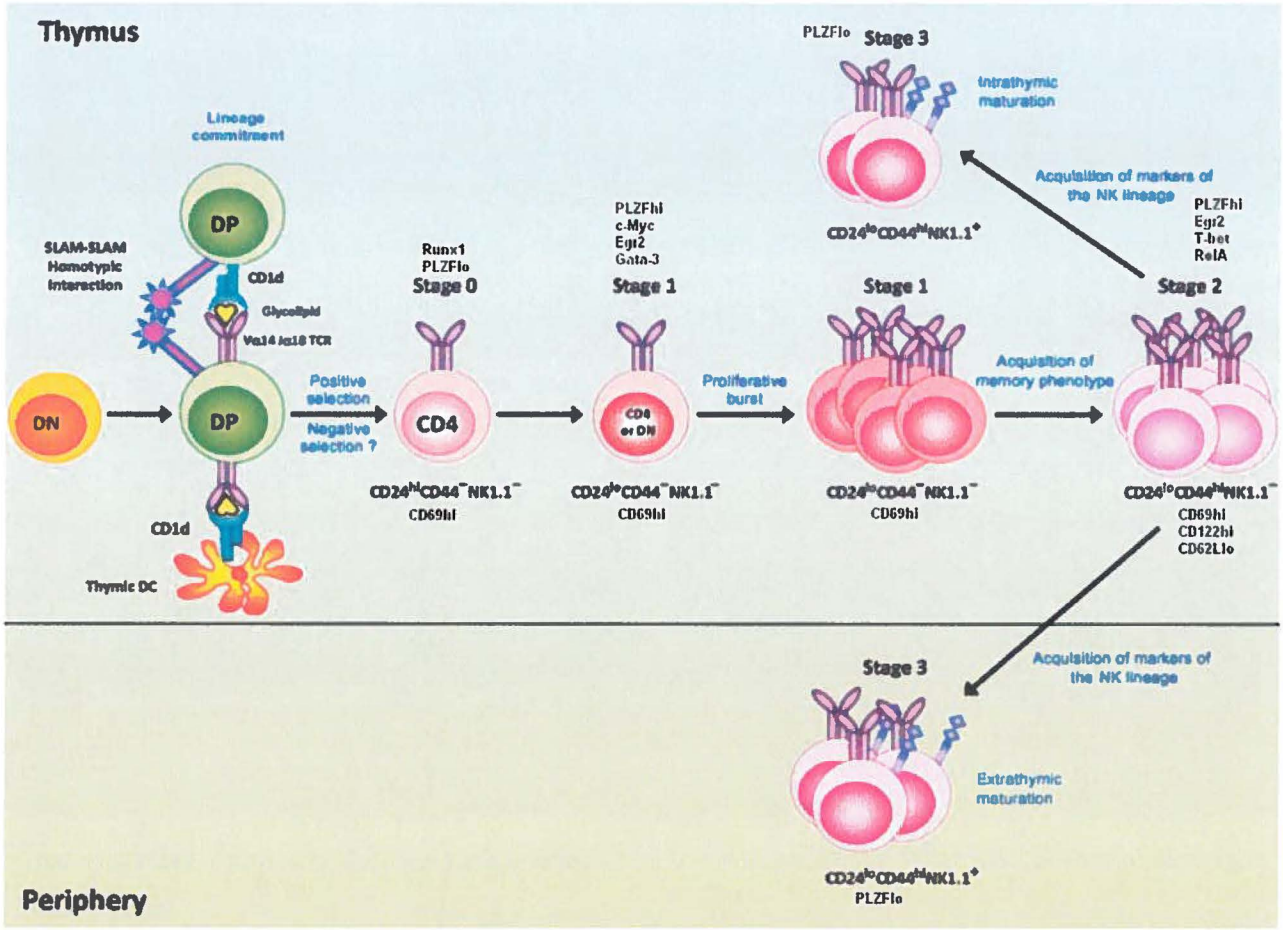

Figure 11) αβ iNKT cell development. NKT cells diverge from conventional T cell development when the TCR interact with CD1d expressing self-lipids. The positive selection event by CD1d-lipid complex with TCR requires ligation of both TCR and a costimulatory molecule, SLAM that signals via SAP and Fyn. Further development of NKT cells occurs in a stepwise manner, from "stage 0" to "stage 3", where the expression of different transcription factors as well as cell surface proteins changes. At "stage 2", NKT cells can leave the thymus and complete their development peripherally. Some NKT cells stay in thymus as "stage 3". Adapted from (Das, Sant'Angelo et al. 2010).

3.1.5. Polarized iNKT cell sublineages

Recent studies have uncovered alternatively polarized NKT sublineages including previously defined T-bet^{high} NKT1 cells, capable of producing large amounts of IFN-γ as the main terminal differentiation product in C57BL/6 mice predominate in spleen and liver (Benlagha, Kyin et al. 2002, McNab, Berzins et al. 2005) and newly defined GATA3^{high} NKT2 cells which produce IL-4 (Terashima, Watarai et al. 2008, Watarai, Sekine-Kondo et al. 2012) and RORγt^{+} IL-17-producing NKT17 cells (Michel, Keller et al. 2007, Doisne, Becourt et al. 2009, Watarai, Sekine-Kondo et al. 2012). These two sublineages are rare in C57BL/6 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. NKT17 cells are rare and mostly found in peripheral lymph nodes draining the skin. These polarized sublineages have the typical profiles of transcription factors, cytokines and chemokine receptors found in their Th1/Th2/Th17 counterparts, although their bias seems to be less absolute because, for example, NKT1 cells also produce IL-4 (Constantinides and Bendelac 2013) (Table 4). New studies proposed a modification to the early model of NKT cell development (stage 1-3) by introducing surface molecule IL-17RB (a key component of the receptor for IL-25) as a marker to distinguish true stage 2 precursors from terminally differentiated NKT2 and NKT17 cells (Watarai, Sekine-Kondo et al. 2012). *Il-17rb*$^{-/-}$ mice were compared with IL-15 KI mice (*Il15*^{L117P}) which have been previously reported to be deficient in iNKT development (due to the critical role of IL-15 in the development and homeostatic maintenance of these cells) (Matsuda, Gapin et al. 2002, Ranson, Vosshenrich et al. 2003). It was demonstrated that in contrast to IL-15 KI mice who show a severe decrease in the percentage and number of total iNKT cells in the thymus (mostly cells in stage 3), *Il-17rb*$^{-/-}$ mice show only a slight decrease which is due to a specific reduction

in the frequency and number of iNKT cells in Stage 1 and Stage 2, while the stage 3 population was unchanged. Furthermore, in C57BL/6 mice more than 80% of Stage 1/2 iNKT cells were IL-17RB^{+}, while only 2% of the Stage 3 iNKT cells were IL-17RB^{+}. In order to determine if iNKT cell subtypes arise as a distinct population in the thymus, each subtype in Stage 1 or Stage 2 was sorted and co-cultured with a fetal thymus (FT) lobe from Jα18-deficient mice. IL-17RB^{-} subtype in Stage 1 gave rise to cells in Stage 2 and Stage 3, whereas IL-17RB^{+} subtype in Stage 1 gave rise to cells in Stage 2 but not to Stage 3. In order to confirm the stability and plasticity of iNKT cell subtypes, sorted thymic iNKT cell subtypes based on the expression of CD4 and IL-17RB from WT B6 mice were transferred into iNKT cell-deficient *Ja18*$^{-/-}$ mice (Watarai, Sekine-Kondo et al. 2012). Similarly, in a very recent published study, Hogquist group demonstrated that there is a fraction of NKT2 cells which produce IL-4 in the steady state and these NKT2 cells which are the equivalents of Stage 1 and 2 of iNKT cell maturation do not give rise to NKT1 cells (equivalents of stage 3)(Lee, Holzapfel et al. 2013). It was demonstrated that among total iNKT cells in both BALB/c and C57BL/6 mice the expression of IL-17RB was limited to NKT2 cells secreting IL-4 in the steady state. Intrathymic injection of these cells with GFP reporter for T-bet gene expression (Zhu, Jankovic et al. 2012) into congenic hosts, indicated that these NKT2 cells won't express T-bet–GFP in thymus and spleen after injection. This suggests that the NKT2 cells were not the developmental intermediates that would give rise to T-bet^{+} NKT1 cells (Lee, Holzapfel et al. 2013).

Table 4) iNKT cells sublineages: NKT1, NKT2 and NKT17 and their characteristics

	PLZF	Signature transcription factor	Main cytokine	Conventional classification	Predominant location
NKT2	High	GATA-3	IL-4	Stages 1 and 2	Lung
NKT17	Intermediate	RORγt	IL-17	Stage 2	Peripheral lymph nodes
NKT1	Low	T-bet	IFN-γ	Stage 3	Liver and Spleen

Adapted from (Constantinides and Bendelac 2013, Lee, Holzapfel et al. 2013)

3.1.6. Functional roles of αβ NKT cells in the immune system

As mentioned earlier, NKT cells are functionally different from conventional MHC-restricted T cells in terms of their activation state. NKT cells have an activated or memory phenotype with high expression levels of CD44 and CD122 and fast production of high amounts of cytokines, including IFN-γ, IL-4, IL-10, IL-13, IL-17, IL-21 and tumor necrosis factor (TNF) (Gumperz, Miyake et al. 2002, Bendelac, Savage et al. 2007, Coquet, Kyparissoudis et al. 2007, Michel, Keller et al. 2007, Sakuishi, Oki et al. 2007). When NKT cells become activated they can promote or suppress the immune system (Smyth and Godfrey 2000, Matsuda, Mallevaey et al. 2008). Several factors are involved in the quality of the NKT cell response against an infection, such as the lipid antigen presented by CD1d, the activation status of antigen presenting cell (APC) and the presence or absence of inflammatory cytokines (Brigl, Bry et al. 2003, Nagarajan and Kronenberg 2007). There are several types of immune cells which have been indicated to become regulated downstream of NKT cell activation. Among those are NK cells which are reported to become activated and mediate antitumor cytotoxicity of NKT cells (Metelitsa, Naidenko et al. 2001). B cells are also interacting with activated NKT cells. For example NKT cells are indicated to be as sufficient as $CD4^+$ Th0 cells for promoting B cell proliferation and antibody production (Galli, Nuti et al. 2003). On the other hand, NKT cells can

limit the activation of autoreactive $CD1d^+$ B cells in autoimmune diseases like systemic lupus erythematosus (SLE) (Wermeling, Lind et al. 2010). NKT cells are also indicated to play roles in dendritic cell (DC) maturation (Kronenberg 2005). All these events influence downstream innate and adaptive immune responses in various pathological conditions including cancer, autoimmune and infectious diseases (Godfrey and Kronenberg 2004). By comparing wild type mice with mice deficient in iNKT cells ($J\alpha18^{-/-}$), or mice deficient in all NKT cells ($CD1d^{-/-}$), it has been possible to study the natural role of type I and type II NKT cells in several immune responses.

– αβ NKT cells and tumor immunity:

α-GalCer was originally discovered as an antitumor agent, and the role of NKT cells in tumor immunity was revealed when α-GalCer was found to activate iNKT cells which is a requirement for the antitumor activity (Kawano, Cui et al. 1997). Furthermore, IL-12 was known to possess antitumor properties (Kobayashi, Fitz et al. 1989) but the downstream effects of IL-12 was not described. Through the use of $J\alpha18^{-/-}$ mice, Cui et al were able to demonstrate the significant effect of iNKT cells in antitumor immunity driven by IL-12 (Cui, Shin et al. 1997) and it was dependent on a direct contact between iNKT cells and tumor cells. Additionally, activation of iNKT cells by α-GalCer resulted in iNKT cell mediated killing of tumor cells through a CD1d-independent, NK-like mechanism (Kawano, Cui et al. 1998).

– αβ NKT cells and autoimmunity:

The results of many studies indicate that there is a link between NKT cells to several autoimmune diseases however their role in autoimmune diseases has not always been a protective role. In some autoimmune settings, they might have a pathogenic role. A pathogenic role for NKT cells was revealed using a mouse model of rheumatoid arthritis, in which the disease is induced by immunization with heterologous type-II collagen. Mice lacking iNKT cells

have less severe rheumatoid arthritis compared to WT control mice (Chiba, Kaieda et al. 2005). As a protective role for NKT cells in autoimmune diseases, in Multiple Sclerosis (MS), decreased numbers of iNKT cells in peripheral blood of patients has been demonstrated. Interestingly, this reduction seems to correlate with relapse of the disease (Illes, Kondo et al. 2000, van der Vliet, von Blomberg et al. 2001) while numbers of iNKT cells were increased during the remission phase (Araki, Kondo et al. 2003).

– αβ NKT cells and infections:

Due to the rapid production of high amounts of inflammatory cytokines, NKT cells have a powerful capacity to increase immunity against several infections. In mouse models, NKT cells have been described to induce immunity to several pathogens such as viruses, gram-positive and gram-negative bacteria, fungi, parasites and helminths (Tupin, Kinjo et al. 2007). In 2000, it was demonstrated that *Borrelia burgdorferi* (Bb) infected $CD1d^{-/-}$ mice showed increased Bb specific IgG antibodies and developed infection-induced arthritis, suggesting that NKT cells play a role in the protection from Bb infection in mice (Kumar, Belperron et al. 2000). NKT cells can also polarize conventional $CD4^{+}$ T cells into either Th1 or Th2 and thereby modulate the immune system to several microbial infections, including bacteria and helminths. NKT cells might also have negative effects such as causing increased liver injury during Salmonella infection (Shimizu, Matsuguchi et al. 2002). In *Pseudomonas aeruginosa* and *Streptococcus pneumoniae* lung infections, iNKT cells have been shown to induce protection in mice. Protection was associated with recruitment of iNKT cells to the site of infection that subsequently promoted the recruitment of neutrophils. Moreover, activation of iNKT cells by administration of α-GalCer inhibited hepatitis B virus (HBV) replication in the liver of HBV

transgenic mice. The inhibition induced by α-GalCer was associated with induction of IFN-γ and IFN-α/β in the liver (Kakimi, Guidotti et al. 2000).

3.2. γδ NKT cells

3.2.1. An overview on γδ T cell subsets

In human, γδ T cells comprise less than 5% of total lymphocytes in the blood and peripheral organs however they have high abundance in the organs like reproductive tract, skin and intestine. More than half of the lymphocyte population in these epithelial organs are γδ T cells (Dranoff 2004). It is notable that within the epithelial tissues, γδ T cells are the first line of defense and can help in wound repair (Prince, Yin et al. 2009). γδ T cells begin to develop during embryonic day 14, which is three days before αβ T cells begin to develop. Certain γδ subsets encoded by specific Vγ gene segments are exported from the fetal and adult thymus at defined periods (Havran and Allison 1988). At days 14-17, $V\gamma3^+$ cells are the first lymphocytes found to be developed in the thymus. After day 17, development of $V\gamma3^+$ cells is completed and they are not found in the fetal or adult thymus anymore since they have migrated to the skin (Havran and Allison 1988). $V\gamma4^+$ T cells develop around day 14 through day 20 and home to the uterus, lung and tongue. $V\gamma2^+$ and $V\gamma5^+$ cells arise around day 16 and migrate to the lungs and gut, respectively (Havran, Jameson et al. 2005). Finally $V\gamma1.1^+$ cells begin to develop near the end of gestation period at day 18 and continue to develop in the adult thymus along with the $V\gamma2^+$ and $V\gamma5^+$ cells. $V\gamma1.1^+$ cells preferentially home to the liver, but can also be found in the spleen and lymph nodes (Figure 12) (Allison and Havran 1991, Carding and Egan 2002, Xiong and Raulet 2007). It has been shown that γδ T cells play diverse roles in the immune system from wound healing and tumor surveillance to having contribution to the clearance of viral,

bacterial and fungal infections (Welsh, Lin et al. 1997, Girardi, Oppenheim et al. 2001, Carding and Egan 2002, Jameson, Ugarte et al. 2002, Bonneville, O'Brien et al. 2010).

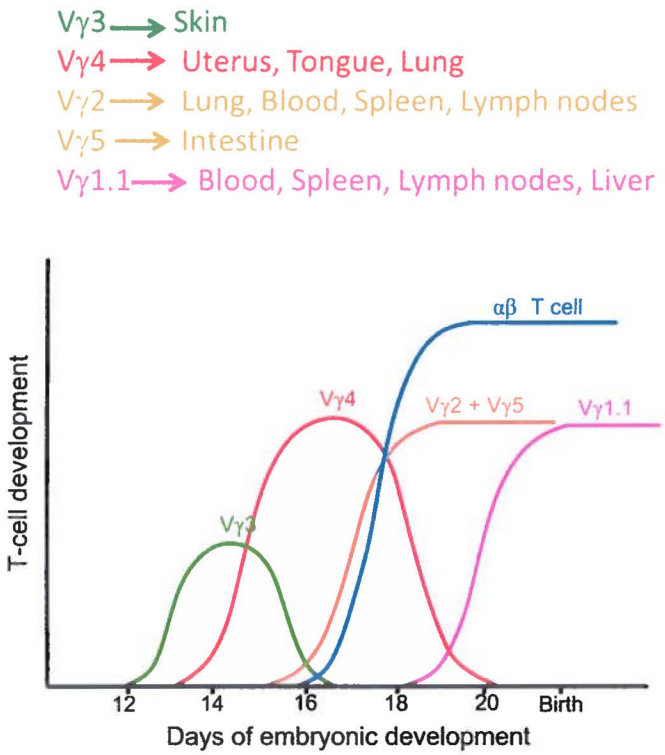

Figure 12) Generation of mouse γδ T cells. Mouse γδ T-cell generation is developmentally programmed. γδ T cell receptors are encoded by specific Vγ- gene segments and leave the thymus in waves. As illustrated here, γδ T cells are the first lymphocytes to develop in the thymus around embryonic day 14 while αβ T cells do not begin to develop until around embryonic day 17. Each γδ T cell subset preferentially migrates to specific organs upon leaving the thymus as indicated besides the graph. Adapted from (Havran and Allison 1988, Havran and Allison 1990, Havran, Jameson et al. 2005).

3.2.2. Heterogeneity in the selection of γδ T cell subsets

Unlike αβ T cells, which are known to be ligand dependent for their selection through MHC complex molecules (Klein, Hinterberger et al. 2009) only few ligands have been discovered specifically for γδ T cells, although it is not clear whether those ligand are absolutely required for their selection and development. Until now, there are a few selecting ligands known for specific γδ T cells including two independently isolated clones; KN6 and G8. For these two clones of γδ T cells, two closely related non-classical MHC class I molecules T10 and T22 have been identified as ligands. (Matis, Cron et al. 1987, Crowley, Fahrer et al. 2000, Shin, El-Diwany et al. 2005). These T10/T22 specific γδ T cells make a very small population among total γδ T cells (0.1-1%), expressing variety of Vγ chains (Shin, El-Diwany et al. 2005) showing this model of selection is not the case for the entire γδ T cell population. So far many studies used KN6 TCRγδ transgenic mice to examine γδ T cell lineage fate (Haks, Lefebvre et al. 2005, Jensen, Su et al. 2008). One study using T10/T22 model showed that the functionality of the γδ T cell is dependent on how the cells are selected. This study demonstrated that γδ T cells that were selected by the non-classical MHC molecules T10/T22 were ligand-experienced and secreted IFN-γ and γδ T cells that were not selected by the non-classical MHC molecules T10/T22 were naïve and secreted IL-17 (Jensen, Su et al. 2008). Another population of γδ T cells that are known be ligand dependent for their selection are the dendritic epidermal T cells (DETC), which express the invariant Vγ3Vδ1 receptor (Havran and Allison 1990, Havran, Chien et al. 1991). Recent studies have shown that these cells require the immunoglobulin-like protein Skint1 for their selection, which is expressed on thymic epithelial cells and in the skin (Boyden, Lewis et al. 2008, Barbee, Woodward et al. 2011). Overall, it seems that different subsets of γδ T cells may have different selection requirements which may or may not require ligands for their selection. In

addition, being ligand dependent or independent may also affect their future function as well (Janssen and Zhang 2003).

3.2.3. Vγ1.1$^+$Vδ6.3$^+$ γδ T cells as *bona fide* γδ NKT cells

Originally in mice, innate-like subset of γδ T cells were defined as Thy-1^{dull} cells that represents a very rare subpopulation in C57BL/6 (~5% of total γδ T cells) and a bigger subtype in DBA/2 mice (~30% of total γδ T cells). They were able to simultaneously secrete IFN-γ, IL-4, IL-10, and IL-3 while Thy-1$^+$ γδ thymocytes were able to secrete only IFN-γ. They were found to be MHC class I independent for their selection since they were present in β2m-deficient mice suggesting that subtype of γδ T cells may regulate immune responses to a different variety of antigens (Vicari, Mocci et al. 1996). Like αβ NKT cells, they reported to reside in the adult thymus, spleen and liver (Vicari, Mocci et al. 1996, Azuara, Levraud et al. 1997, Azuara, Lembezat et al. 1998) and express the memory marker CD44 while half of their population is CD4$^+$ and NK1.1$^+$ (Azuara, Levraud et al. 1997). These cells were found to be rare in newborn mice and they start to expand after birth during the first two weeks of postnatal life reaching homeostasis at week 3 after birth (Azuara, Levraud et al. 1997). Finally it was concluded that this Thy-1^{dull} thymocyte population is the TCR γδ$^+$ equivalent of those αβ NKT cells since both cell populations produce the same distinct pattern of cytokines upon activation, share a number of phenotypic markers originally defined for activated or memory T cells, display similar postnatal kinetics of appearance in the thymus and express a very restricted TCR repertoire (Azuara, Levraud et al. 1997). The majority of Thy-1^{dull} γδ thymocytes were found to express TCR encoded by the Vγ1 gene and a novel Vδ6 gene originally named Vδ6.4 in DBA/2 and Vδ6.3 in C57BL/6 mice (Azuara, Levraud et al. 1997, Azuara, Lembezat et al. 1998, Gerber,

Azuara et al. 1999, Azuara, Grigoriadou et al. 2001). Sequence analysis of these functionally rearranged γδ genes revealed highly restricted δ junctions, and more diverse γ junctions. The characteristics of the TCR molecules in adult Thy-1^{dull} γδ T cells were found to be very similar to those TCR molecules normally found in the fetal thymus as there was minimum or no N region diversity within Vδ6Cδ chains and there was only an invariable Dδ2-Jδ1 junction (Chien, Iwashima et al. 1987, Azuara, Levraud et al. 1997, Azuara, Lembezat et al. 1998, Azuara, Grigoriadou et al. 2001). Subsequent studies demonstrated that when adult bone marrow was transplanted into lethally irradiated mice, the Thy-1^{dull} γδ T cell population did not fully develop. In contrast, transplantation of fetal thymi into syngeneic hosts resulted in Thy-1^{dull} γδ T cell development (Grigoriadou, Boucontet et al. 2003). Therefore, it was suggested that a majority of Thy-1^{dull} γδ T cells arise from fetal precursors. Finally in 2009, for the first time it was reported that the BTB-zinc finger transcription factor PLZF is also expressed in Vγ1.1^{+}Vδ6.3^{+} γδ T cells and like αβ NKT cells it is responsible for the innate properties of this innate-like subset of γδ T cells (Kreslavsky, Savage et al. 2009). Using OP9-DL1 coculture system, Kreslavsky and colleagues showed that TCR cross-linking induced PLZF expression in all polyclonal immature γδ thymocytes, suggesting that agonist selection might be required for PLZF induction and this selection actually governs the acquisition of their ''innate'' properties. Also transgenic expression of Vγ1.1^{+}Vδ6.3^{+} TCR was sufficient to support the development of large numbers of PLZF expressing T cells, further supporting the importance of the TCR nature for PLZF induction (Kreslavsky, Savage et al. 2009). Further studies using PLZF-deficient mice revealed that unlike αβ NKT cells the frequency, number and localization of Vγ1.1^{+}Vδ6.3^{+} γδ T cells were not dramatically affected by PLZF expression but these cells were functionally impaired since they were incapable of dual IL-4 and IFN-γ secretion following stimulation, indicating that

in the absence of PLZF this repertoire of Vγ1.1$^+$Vδ6.3$^+$ γδ T cells are not real innate-like γδ NKT cells (Kreslavsky, Savage et al. 2009, Alonzo, Gottschalk et al. 2010). These data indicate that like for αβ NKT cell, PLZF is an essential transcription factor and required for the innate-like phenotype of Vγ1.1$^+$Vδ6.3$^+$ γδ T cells. Another member of the BTB-POZ family, ThPOK, was also found to be expressed in the Vγ1.1$^+$Vδ6.3$^+$ subset of γδ T cells and it's been indicated that the level of ThPOK expression increases during the maturation of Vγ1.1$^+$Vδ6.3$^+$ γδ T cells (Alonzo, Gottschalk et al. 2010, Park, He et al. 2010). Lack of ThPOK was shown to result in a decrease in the frequency of these cells whereas overexpression of ThPOK resulted in an increase in the number of Vγ1.1$^+$Vδ6.3$^+$ γδ T cells (Park, He et al. 2010). In addition, these studies showed that Vγ1.1$^+$Vδ6.3$^+$ γδ T cells highly expressing ThPOK were also PLZF$^+$ and also in the absence of ThPOK, PLZF expression was also reduced along with decreased IL-4 secretion (Alonzo, Gottschalk et al. 2010). It is still unclear what role ThPOK is exactly playing in the development and function of Vγ1.1$^+$Vδ6.3$^+$ γδ T cells but it is possible that PLZF may have a role in regulating or modulating of ThPOK expression.

3.2.4. γδ NKT cells are also dependent on SAP

Like αβ NKT cells, selection and development of γδ NKT cells is also dependent on SLAM-SAP signaling since two different studies revealed that Vγ1.1$^+$Vδ6.3$^+$ γδ T cell numbers were reduced in SAP-deficient mice, albeit not as dramatically as the numbers of αβ NKT cells (Kreslavsky, Savage et al. 2009, Alonzo, Gottschalk et al. 2010). Therefore (not directly demonstrated) it is conceivable that similar to αβ NKT cells, PLZF$^+$ γδ T cells are also selected on other thymocytes rather than thymic epithelial cells. It's been also suggested that some costimulatory interaction in the course of this selection is required for PLZF induction

(Kreslavsky, Gleimer et al. 2010). This interaction for the case of γδ NKT cells is unlikely to be mediated by SLAM receptors, as PLZF is induced in SAP-deficient Vγ1.1^{+}Vδ6.3^{+} γδ T cells although not to the levels observed in WT cells (Alonzo, Gottschalk et al. 2010).

3.2.5. Role of TCR signaling strength in γδ NKT cell development

In both αβ and γδ NKT cell subsets, PLZF expression is associated with a very restricted combination of TCR chains and both cell types are believed to undergo an alternative way of selection in which these T cells become positively selected by agonist ligands, which would normally be expected to induce T cell deletion (Baldwin, Hogquist et al. 2004). Moreover, as previously mentioned it has been demonstrated the PLZF can be induced *in vitro* by strong TCR signal in polyclonal immature γδ thymocytes, suggesting that PLZF may also be induced *in vivo* by agonist selection (Kreslavsky, Savage et al. 2009) meaning that PLZF^{+} γδ T cells also require high affinity TCR signaling for their lineage commitment. Ironically, the number of Vγ1.1^{+}Vδ6.3^{+} γδ T cells is increased in TCR signaling mutant mice such as Itk knockouts (Felices, Yin et al. 2009, Qi, Xia et al. 2009, Yin, Cho et al. 2013) as well as in mice with a mutated form of the adaptor protein SLP-76 (SLP-76:Y145F) (Alonzo, Gottschalk et al. 2010). Similarly, it was previously shown that there is an accumulation of NK1.1^{+} TCRγδ $^{+}$ thymocytes in CD3ζ-deficient mice (Arase, Ono et al. 1995) and there is an increased number of an IL-4 producing γδ T cell subtype in LAT mutant mice (Nunez-Cruz, Aguado et al. 2003). This unusual γδ NKT cell accumulation was also reported in mice deficient for Id3 expression (transcription factor induced by TCR signaling) (Lauritsen, Wong et al. 2009, Ueda-Hayakawa, Mahlios et al. 2009, Verykokakis, Boos et al. 2010). Accumulated γδ NKT cell population in TCR signaling mutant mice is actually against the established theory of their high affinity TCR

signaling requirement (Alonzo, Gottschalk et al. 2010, Kreslavsky, Gleimer et al. 2010). On the other hand, we cannot exclude the correlation between the TCR signal strength and PLZF upregulation since it might explain how its expression correlates with two totally different types of TCRs, the Vα14 TCR of αβ NKT cells and the Vγ1.1$^+$Vδ6.3$^+$ γδ TCR, and why PLZF expression is induced by high affinity TCR interaction *in vitro* (Kreslavsky, Gleimer et al. 2010). Different hypothesis have been proposed to explain this situation. According to one of them, it is possible that some branches of TCR signaling like those that require Itk and specific tyrosine residues in SLP-76 inhibit PLZF induction on γδ T cells (or let's say expansion of PLZF$^+$ γδ T cells) whereas the others promote it (Kreslavsky, Gleimer et al. 2010). An alternative explanation was also suggested by Lauritsen et al. which is applicable for both TCR signaling mutants and Id3 knockouts (Lauritsen, Wong et al. 2009). This theory states that some of the Vγ1.1$^+$Vδ6.3$^+$ γδ T cells, which might be deleted by receiving a very strong TCR signal during negative selection, get rescued when TCR signaling is somewhat attenuated.

3.2.6. Role of E- and Id proteins in γδ NKT cell development

While Id3 expression was previously demonstrated to be required for γδ T cell lineage development (Acuto and Michel 2003, Haks, Lefebvre et al. 2005, Hayes, Li et al. 2005, Lauritsen, Wong et al. 2009), the overall numbers of the γδ T cells in TCR non-transgenic Id3-deficient mice were increased due to an accumulation of γδ NKT cell population (Lauritsen, Wong et al. 2009, Ueda-Hayakawa, Mahlios et al. 2009, Verykokakis, Boos et al. 2010). It was initially proposed that similar to TCR signaling mutants, among total γδ T cell sublineages, Id3 deficiency prevented the deletion of high affinity TCRγδ bearing NKT cells, since using the KN6 TCRγδ transgenic mice, the deletion of transgenic TCRγδ$^+$ thymocytes in the presence of a 10-

fold higher-affinity ligand (Adams et al., 2008, Pereira et al., 1992) was prevented in the absence of Id3 (Lauritsen, Wong et al. 2009). This is suggesting an apparent dichotomy of Id3 function, with Id3 restricting the development of those subsets bearing high-affinity γδ TCRs while at the same time it is required for the development of the broader repertoire of non-autoreactive γδ T cells (Kreslavsky, Gleimer et al. 2010). A very recent study has developed our current knowledge on the role of the transcription factor Id3 in the development of γδ NKT cells. Studying mice with two different genetic backgrounds, demonstrated that in absence of Id3, γδ NKT cell accumulation is much stronger in C57BL/6 compared to 129/sv background. Quantitative trait linkage analysis demonstrated that another member of the Id family, Id2 is the major modifier of Id3 in limiting $V\gamma1.1^+V\delta6.3^+$ T cell expansion (Zhang, Lin et al. 2013). qPCR analysis showed that Id2 mRNA expression in $V\delta6.3^+$ γδ T cells from Id3-deficient mice on C57BL/6 background was lower than those on B6/129 mixed background, suggesting that the intrinsic weakness of Id2 transcription from Id2 C57BL/6 allele is the reason why it cannot compensate for Id3-deficiency and restrict $V\gamma1.1^+V\delta6.3^+$ T cell population. In developing T cells, if both Id2 and Id3 are completely deleted, the $V\gamma1.1^+$ $V\delta6.3^+$ γδ T cells actually fail to accumulate, possibly because of attenuated proliferation and increased cell death induced by unrestricted E protein activity (knowing that Id proteins antagonize E proteins). It was also demonstrated that deletion of all alleles of HEB, E2A, Id2, and Id3 by CD4Cre can induce accumulation of $V\delta6.3^+$ γδ T cells, while deletion by LckCre fails to induce a similar phenotype. Thus it was finally suggested that Id proteins control γδ T cell development through inhibition of E proteins in a developmental stage–specific manner. Therefore a dual safety model was proposed to indicate the role of Id3 and Id2 in the control of $V\gamma1.1^+V\delta6.3^+$ T cell development in a stage –specific manner (Zhang, Lin et al. 2013) (Figure 13).

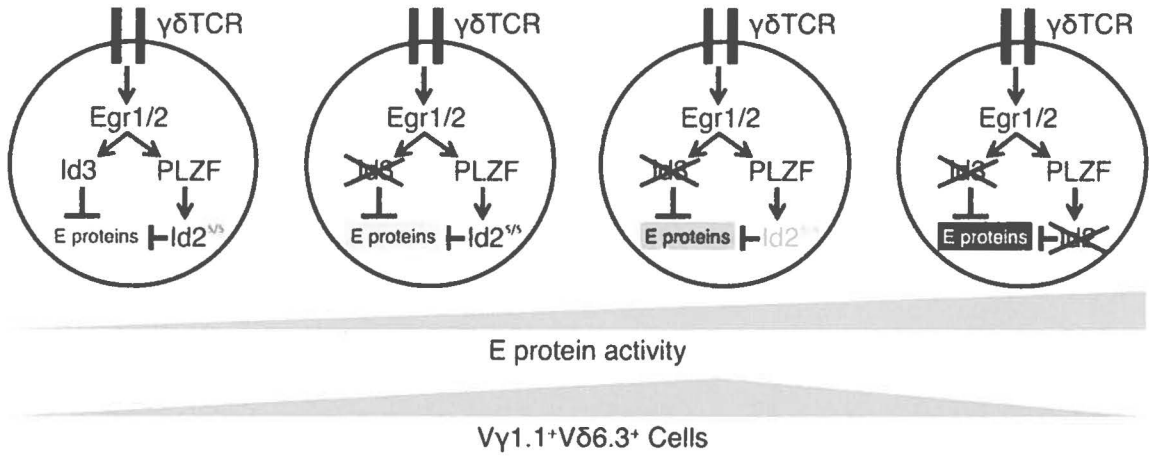

Figure 13) Schematic diagram of Vγ1.1^{+}Vδ6.3^{+} T cell developmental control by Id2 and Id3. Developing Vγ1.1^{+}Vδ6.3^{+} thymocytes receive strong TCR signaling, upregulating Id2 and Id3 through Egr1/2 and PLZF. The Id proteins inhibit activity of E proteins, affecting the survival and proliferation of Vγ1.1^{+}Vδ6.3^{+} T cells. - Id2 is expressed from a more active allele (Id2^{s}, "strong") - Id2 is expressed from a less active allele (Id2^{B}, "B6")

According to this model, Egr is the major transcription factor mediating the TCR signaling induction of Id3 expression in T cell development (Lauritsen, Wong et al. 2009) and Id2 has been shown to be activated by PLZF, which is a direct target of Egr2 in iNKT cell development (Gleimer, von Boehmer et al. 2012). When both Id2 and Id3 are present, they keep E protein activity low, and consequently prevent the expansion of γδ NKT cells. When Id3 is deleted, Id2 will play a safety role to control E protein activity but this safety role of Id2 is compromised by its hypomorphic allele in the B6 background, allowing an increase in E protein activities to an optimal level for driving $V\gamma1.1^{+}V\delta6.3^{+}$ γδ T cell expansion. When both Id2 and Id3 are completely deleted, E protein activity becomes too high and again limits the $V\gamma1.1^{+}V\delta6.3^{+}$ γδ T cell population. Therefore it's the level of E protein activity (regulated by Id2 and Id3) which is crucial for γδ NKT cell development, especially during their "maturation". This is consistent with the previously proposed model (Lauritsen, Wong et al. 2009), indicating that the developmental block imposed by high levels of Id proteins and low E protein activity can be a mechanism to limit the number of auto-reactive γδ T cells that can recognize self-Ag in the thymus, reiterating the idea that Id2 and Id3 collaboratively act as "dual safety" in limiting the expansion of γδ NKT cells (Lauritsen, Wong et al. 2009, Zhang, Lin et al. 2013).

3.2.7. Functional roles of γδ NKT cells in the immune system

Unlike αβ NKT cells, which are well studied regarding their functional role in the immune system, the role of γδ NKT cells needs to be further elucidated. However some protecting and pathogenic roles have been identified for them in different immune settings like autoimmunity, tumor immunity and infections reflecting their specific innate-like properties and ability to produce simultaneously pro- and anti-inflammatory cytokines. One study in 1996,

demonstrated that among the bulk of the γδ T cell populations, the Vδ6.3$^+$ and Vδ6.4$^+$ T cell are dominant in the response to the intracellular bacterium *Listeria monocytogenes* in BALB/c mice and they predominantly expand in the sites of infection during immune responses. It was shown that at low doses of infection, the γδ T cell response occurs late in the disease course, while at high doses, the response is earlier and of greater magnitude, particularly in the liver. At all infectious doses the Vδ6.3$^+$ cell population predominates in the γδ T cell response over the other subsets of γδ T cells (Belles, Kuhl et al. 1996). In another study a regulatory role of Vγ1$^+$ γδ T cells was shown in tumor immunity (induced by B16-F0 melanoma cells) via suppressing Vγ4$^+$ T cells (Hao, Dong et al. 2011). It was formerly shown that Vγ4$^+$ γδ T cells played a protective role in tumor immunity through Eomesodermin controlled, IFN-γ– and perforin-dependent mechanisms (He, Hao et al. 2010) but no interaction between these two subtypes of γδ T cells had been formerly reported regarding the tumor regulatory function. Using neutralizing anti–IL-4 Ab and IL-4-deficient mice, it was demonstrated that the suppressive factor derived from Vγ1$^+$ γδ T cells blocking the anti-tumor activity of Vγ4$^+$ γδ T cells was IL-4 (Hao, Dong et al. 2011). One other study has also demonstrated that Vγ1.1$^+$Vδ6.4$^+$ TCR transgenic mice on a RAG-deficient background developed spontaneous dermatitis in the tail. Closer examination of the tail in these mice showed massive infiltration by lymphocytes and granulocytes (Kreslavsky, Savage et al. 2009). It is known that epidermis is the home of Vγ5 dendritic epidermal T cells (DETC) and these cells are known to have regulatory properties. Spontaneous dermatitis that is developed in TCRγ–deficient mice on certain backgrounds can be rescued by the transfer of Vγ5 fetal thymocytes (Girardi, Lewis et al. 2002). It was previously demonstrated that in the absence of canonical Vγ5$^+$ DETCs, other γδ T cells can take over their niche (Lewis, Girardi et al. 2006). So, it is tempting to speculate that in a RAG-deficient mouse when both αβ and γδ T cells with

their regulatory properties are absent, transgenic $V\gamma1.1^+V\delta6.4^+$ T cells take over their niche in the skin and initiate uncontrolled inflammation. Whether or not the disease is related to PLZF dependent effector program of these cells needs to be elucidated. Another physiological consequence of having a larger pool of these auto-reactive γδ T cells was shown in Id3-deficient mice. These mice spontaneously develop an autoimmune disease similar to human Sjögren's syndrome (Li, Dai et al. 2004). The large population of $V\gamma1.1^+V\delta6.3^+$ γδ T cells in these mice is potentially involved in the pathogenesis. However, a previous report also showed that these cells can play a role in suppressing tissue inflammation which might be through the production of anti-inflammatory cytokines, such as IL-10 (Carding and Egan 2002). In recent years, the discovery that SAP and PLZF, which are also required for the maturation and function of $V\gamma1.1^+V\delta6.3^+$ T cells, have sparked interest in studying of this T cell subset. Interestingly, since the innate properties and localization of these cells resembles αβ NKT cells there must be similar functional properties between the two types of innate-like T cells in the immune system although not much has been revealed for γδ NKT cells. Apart from their direct functional role in the immune system by secreting different kinds of cytokines both TCR αβ and γδ-expressing $PLZF^+$ NKT cells can mediate the innate-conversion of $CD8^+$ T cells in the thymus due to their IL-4 production in the steady state. These innate-like $CD8^+$ T cells act like memory T cells and rapidly produce IFN-γ, yet antigen recognition is not required for their differentiation (Berg 2007).

4. Innate-like $CD8^+$ T cells, an alternative memory in the CD8 T cell lineage

For a long time, the term 'innate T cell' was used to describe distinct lineages of cells in the thymus showing a memory phenotype and function with rapid production of large amounts of cytokines upon TCR stimulation. Those included the MHC class-Ib molecules restricted T cells, like CD1d, H2-M3 and MR-1 (Urdahl, Sun et al. 2002, Treiner and Lantz 2006) where NKT cells were the prototype of cells belonging to this family. The initial discovery of innate-like $CD8^+$ T cells was back in 2006 when in the Tec kinase Itk-deficient mice "$CD8^+$ innate T cells" were described as a diverse population of polyclonal $CD8^+$ T cells in the thymus having the phenotype and function of memory CD8 T cells without previous exposure to the antigen (Atherly, Lucas et al. 2006, Broussard, Fleischacker et al. 2006). In these mice, in the absence of Itk, CD8 SP thymocytes revealed special phenotype regarding both surface markers and function, such as very low expression of CD24 and conversely very high expression of memory markers, CD44, CD122 and CXCR3 ($CD24^{lo}CD44^{hi}CD122^{hi}$ $CXCR3^{hi}$). These cells also were able to produce high amounts of IFN-γ upon TCR stimulation acting like "primed T cells" (Atherly, Lucas et al. 2006, Broussard, Fleischacker et al. 2006). Further investigation revealed that unlike conventional memory T cells that express T-Box transcription factor T-bet, innate-like CD8 T cells express another T-Box transcription factor named Eomesodermin which gives them their innate properties (Atherly, Lucas et al. 2006, Broussard, Fleischacker et al. 2006, Berg 2007, Prince, Yin et al. 2009).

4.1. Multiple gene deficiency models lead to increased thymic $CD8^+$ Innate-like T cells with an IL-4-dependent mechanism

In addition to those pioneering studies on Itk-deficient mice, several other mouse models deficient in either TCR signalling components or some transcription factors were found to have high abundance of thymic $CD8^+$ innate-like T cells (Lee, Jameson et al. 2011).

Those include the transcription factors Kruppel-like factor 2 (KLF2) (Weinreich, Takada et al. 2009, Weinreich, Odumade et al. 2010), CREB binding protein (CBP) (Fukuyama, Kasper et al. 2009), and inhibitor of DNA binding 3, Id3 (Verykokakis, Boos et al. 2010). Likewise mice having a mutated form of SLP-76 (SLP76:Y145F) which is not able to interact with Itk and maintain its activity (Jordan, Smith et al. 2008) were also found to phenocopy the $Itk^{-/-}$ mice in terms of accumulated thymic innate-like $CD8^+$ T cells (Table 5).

Table 5) Multiple gene deficiency models give rise to innate-like $CD8^+$ T cells.

Gene deficiency or Transgenic mice	Eomes Upregulation on $CD8^+$ Thymocytes	Cell extrinsic effect	IL-4 dependency	Effector NKT lineage
$Itk^{-/-}$	Elevated	YES	YES	γδ NKT
$Id3^{-/-}$	Elevated	YES	YES	γδ NKT
$Klf2^{fl/fl}\ Cd4^{Cre}$	Elevated	YES	YES	αβ and γδ NKT
SLP76:Y145F	Elevated	Not determined	Not determined	Not determined
$Cbp^{fl/fl}\ Lck^{Cre}$	Elevated	YES	Not determined	Not determined

Adapted from (Lee, Jameson et al. 2011)

It was unclear how all these mutations could lead to the development of this prominent population of T cells in the thymus. For the first time it was in KLF2 deficient mice, a model with accumulated αβ and γδ NKT cells (Odumade, Weinreich et al. 2010, Lee, Jameson et al. 2011) where the clues to the initial discovery of the complex mechanism leading to the development of innate-like $CD8^+$ T cells were revealed to be extrinsic effects (Weinreich, Takada et al. 2009, Weinreich, Odumade et al. 2010). When KLF2-deficient bone marrow cells were transferred into irradiated hosts, together with a minority of WT cells, the WT 'bystander' $CD8^+$ thymocytes adopted an innate $CD8^+$ T cell phenotype, similar to the KLF2-deficient thymocytes. By contrast, when WT cells were in the majority, neither population showed an innate-like CD8 phenotype. These data demonstrate that innate $CD8^+$ T cell development in the KLF2-deficient mice is caused by extrinsic factors. Using the same strategy, the generation of Eomes-expressing innate $CD8^+$ T cells in Itk-, CBP- and Id3-deficient mice was also found to be due to cell extrinsic effects, and the cytokine IL-4 was the extrinsic factor since the bystander cells that lack IL-4R did not upregulate Eomes, or show any other aspects of the innate-like phenotype, and also they fail to produce IFN-γ. Likewise when KLF2-, Itk-, or Id3-deficient mice were crossed with IL-4R-deficient mice, innate $CD8^+$ T cells did not develop in these mice (Verykokakis, Boos et al. 2010, Weinreich, Odumade et al. 2010, Lee, Jameson et al. 2011). Interestingly, double gene deficiency of PLZF together with KLF2, Itk and Id3 led to the failure of innate-like $CD8^+$ T cell development (Verykokakis, Boos et al. 2010, Weinreich, Odumade et al. 2010) suggesting a direct evidence of the role of $PLZF^+$ IL-4 producing cells in the innate conversion of CD8 T cells in these mice (Figure 14), although this direct evidence has not been determined yet for mice bearing CBP-deficiency or SLP76:Y145F mutation (Table 5).

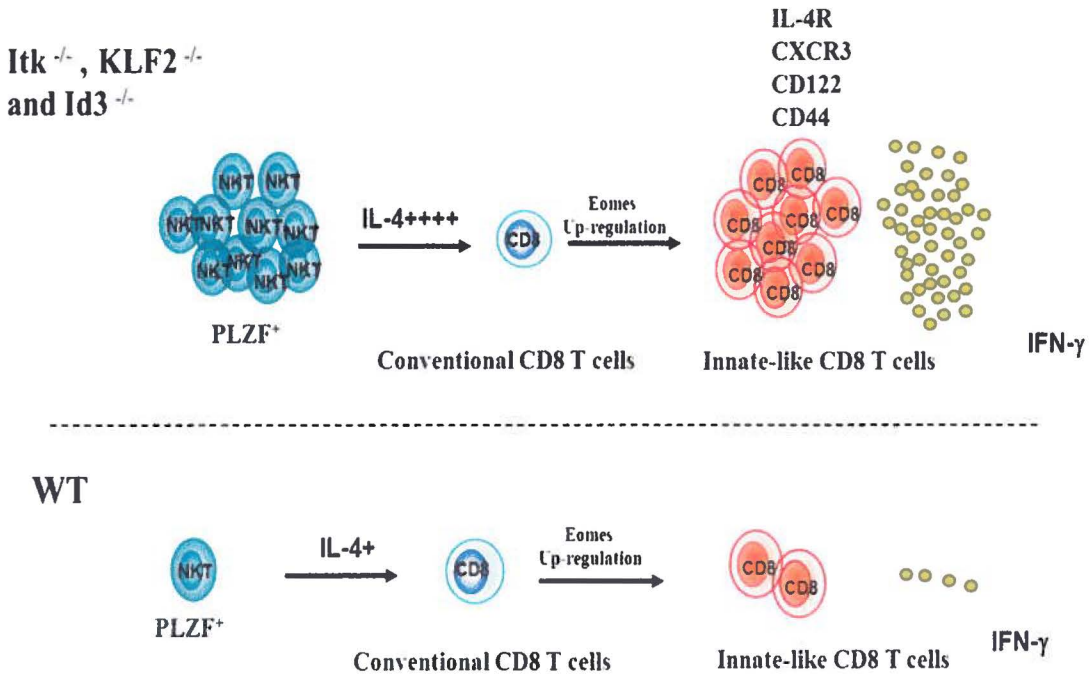

Figure 14) Innate conversion of CD8 T cells by IL-4 produced by NKT cells. TCR signaling mutants, KLF2-, Itk- or Id3-deficient mice have an expanded $PLZF^+$ NKT cells population that are the source of IL-4 which influences the innate inversion of CD8 T cells in trans. Due to the paucity of NKT cells in WT mice, this innate conversion most likely occurs but it is less apparent.

4.2. Natural occurrence of Innate-like $CD8^+$ T cells

It has been shown that wild-type BALB/c mice have a distinct population of innate-like $CD8^+$ T cells that are dependent on iNKT cells and IL-4 (Weinreich, Odumade et al. 2010, Lai, Zhu et al. 2011). Interestingly, inbred strains of mice were shown to vary in their frequency of iNKT cells, with BALB/c mice on the high end of the spectrum and C57BL/6 mice on the low end (Hammond, Pellicci et al. 2001, Rymarchyk, Lowenstein et al. 2008). BALB/c mice have three to five times greater numbers of $PLZF^+$ cells in the thymus compared to C57BL/6 mice, with the majority of them being iNKT cells (Rafei, Hardy et al. 2011). As in the various gene-deficient models, this innate phenotype is dependent on IL-4 produced by NKT cells, since they become eliminated in BALB/c IL-4R-deficient and/or BALB/c Cd1d-deficient mice (Weinreich, Odumade et al. 2010). Therefore, the developmental regulation of innate-like $CD8^+$ T cells by $PLZF^+$ NKT cell population is not only a phenotype of some gene-deficient mice but also a physiological process in inbred mouse strains (Lee, Jameson et al. 2011). In a very recent study, Hogquist group using KN2 IL-4-reporter mice, in which the first two exons of the endogenous *Il4* gene were replaced with sequence encoding human CD2 (where the expression of human CD2 on the cell surface reports IL-4 secretion *in vivo*) (Mohrs, Wakil et al. 2005) show that, up to 40% of thymic iNKT cells from KN2 mice of the BALB/c strain (BALB/c-KN2 mice) expressed human CD2 on the cell surface, whereas iNKT cells from KN2 mice of the C57BL/6 strain (B6-KN2 mice) were largely negative for human CD2 proving that more IL-4 is being produced in the thymus of a BALB/c mouse compared to a C57BL/6 strain in the steady state (Lee, Holzapfel et al. 2013). They also compared six different commonly used inbred strains of mice (C57BL/6, NOD, DBA2, CBA, 129SvJ, and BALB/c) and determining the relative proportion of NKT1, NKT2 and NKT17 cells in each they showed that C57BL/6 mice were at

one end of the spectrum, with more NKT1 cells and fewer NKT2 cells, whereas BALB/c were at the other end of the spectrum with more NKT2 and less NKT1 cells. The six strains showed a general inverse relationship in the proportion of NKT1 versus NKT2 cells. Notably, three strains (CBA, DBA/2 and BALB/c) with more NKT2 subset had a substantial population of memory-like $CD8^+$ thymocytes that expressed Eomes (Lee, Holzapfel et al. 2013).

4.3. Functional roles of innate-like $CD8^+$ T cell in the immune system

Innate-like $CD8^+$ T cells phenotypically resemble conventional memory $CD8^+$ T cells, yet do not require antigen experience to obtain this status, as demonstrated by the fact that OT-I Rag $^{-/-}$ cells adopt a memory phenotype and function when present as bystander cells in KLF2-deficient mixed bone marrow chimeras (Weinreich, Odumade et al. 2010, Lee, Jameson et al. 2011). They have similarities to homeostatic (or virtual) memory T cells (Haluszczak, Akue et al. 2009), which are generated in peripheral lymphoid organs in lymphopenic animals, in response to IL-7, IL-15 and self MHC-peptide (Surh and Sprent 2008, Takada and Jameson 2009). By contrast, innate-like $CD8^+$ T cells develop in the thymus in an IL-4-dependent manner (and presumably in response to self MHC-peptide). These three subsets of memory cells are not functionally equivalent. Certainly, the fact that homeostatic and innate-like $CD8^+$ T cells do not require foreign antigen recognition for their generation means that they are unlikely to play an important role in the secondary infections, unlike conventional memory $CD8^+$ T cells that are clonally expanded during a primary response and play essential role during secondary infections. $CD8^+$ T cells have been shown to play important roles to provide innate immunity early in some primary infections like *Listeria monocytogenes* in the absence of cognate antigen, there is growing evidence for them as sensors of an inflammatory environment which has been shown to

be caused by the IL-12 and IL-18 produced by activated myeloid cells (Berg, Crossley et al. 2003, Berg, Crossley et al. 2005, Berg and Forman 2006). Therefore, both homeostatic and innate-like $CD8^+$ cells, which also produce IFN-γ in response to IL-12 and IL-18 (Weinreich, Takada et al. 2009), most likely could play roles early during infection, via production of IFN-γ. Particularly in the human immune system, these types of non-conventional or unprimed $CD8^+$ T cells could be important because they are able to participate in host defense during the neonatal and early childhood period before conventional memory networks are established (Lee, Jeon et al. 2010, Weinreich, Odumade et al. 2010, Lee, Jameson et al. 2011). BALB/c mice with highly abundant populations of innate-like $CD8^+$ T cell are good models to study the functional roles of these cells in the immune system. In a model of malarial infection, BALB/c mice have been shown to be more resistant than C57BL/6 mice, with a protective response dependent on receptors for IL-4 on $CD8^+$ T cells (Morrot, Hafalla et al. 2005). Furthermore, IL-4- or CD1d-deficiency made BALB/c mice more susceptible to parasite infection and to develop severe pathology (Hansen, Siomos et al. 2003). In another study also BALB/c mice were found to be much more effective than C57BL/6 strains at controlling malaria pathogens after immunization with radiation-inactivated forms of *Plasmodium berghei* or *P. yoelii sporozoites* (Schmidt, Butler et al. 2010), which might be related to the high frequency of innate-like $CD8^+$ T cells in the BALB/c mice compared to the other strain (Lee, Jameson et al. 2011).

5. Hypothesis and research objectives

TCR signal strength/duration determines specific gene expression and regulates different checkpoints during T cell development. Therefore, signaling molecules modulating TCR signal strength should play critical roles in the regulation of T cell development. The two members of the Dok family of adaptor proteins Dok-1 and Dok-2 are expressed in T cells. They are known to have a negative role downstream of TCR signaling. The current model proposes that Dok recruitment of RasGAP inhibits Ras activity and consequently Erk pathway downstream of TCR signaling. Erk activation is involved in pre-TCR signaling, positive selection, CD4/CD8 lineage commitment and γδ/αβ lineage choice. Therefore, given that TCR-mediated activation of Erk is inhibited by Dok, we proposed that this protein might play an important role in thymocyte development and maturation. To investigate the role of Dok in T cell development we generated transgenic mice overexpressing Dok-1 under the control of the CD2 promoter leading to the transgene expression in thymocytes and mature T cells. We performed a detailed analysis of T cell development in Dok-1 transgenic mice compared to WT controls with the following specific objectives (with few experiments performed on Dok-1/Dok-2-deficient mice):

1- To determine the role of Dok in the modulation of TCR signaling strength in developing thymocytes (looking at the activation level of the key effector molecules downstream of TCR signaling pathway).

2- To specify the role of Dok at different checkpoints during T cell development.

We showed that Dok controls the development of innate-like γδ NKT cells. This small subtype of innate-like T cells is poorly understood compared to αβ NKT cells. We pursue our study to

further characterize γδ NKT cells by studying the signaling pathways that might be important for their development and maturation.

CD28/B7 interaction has been shown to be essential for the development of innate-like αβ iNKT cells. Given the fact that along with their phenotypical and functional similarities innate-like αβ and γδ NKT cells share common developmental properties, we hypothesized that the coreceptor CD28 might also be important in the regulation of the development and maturation of γδ NKT cells. Furthermore, knowing that IL-4 producing NTK cells are responsible for the innate conversion of $CD8^+$ thymocytes, we proposed that the size of innate-like $CD8^+$ thymocytes might be affected by CD28 signaling as well. To test this hypothesis we used mice deficient in CD28 and we compared them with WT controls, pursuing these specific objectives:

1- To test whether the absence of CD28 correlates with any defects in the generation of both $PLZF^+$ αβ and γδ NKT cells

2- To characterize the mechanism of CD28 signaling in the regulation of NKT cell development and to determine what could be its functional consequence.

This study reveals previously unappreciated mechanisms by which Dok-1 and CD28 control NKT cell homeostasis and will help to understand the precise developmental requirements and mechanisms for the regulation of γδ NKT cells and more generally for shaping the T cell repertoire and innate-like T cell development.

CHAPTER 2

PUBLICATIONS

Publication no.1

Dok-1 overexpression promotes development of γδ natural killer T cells

Dok-1 overexpression promotes development of γδ natural killer T cells

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***These authors contributed equally to this work.**

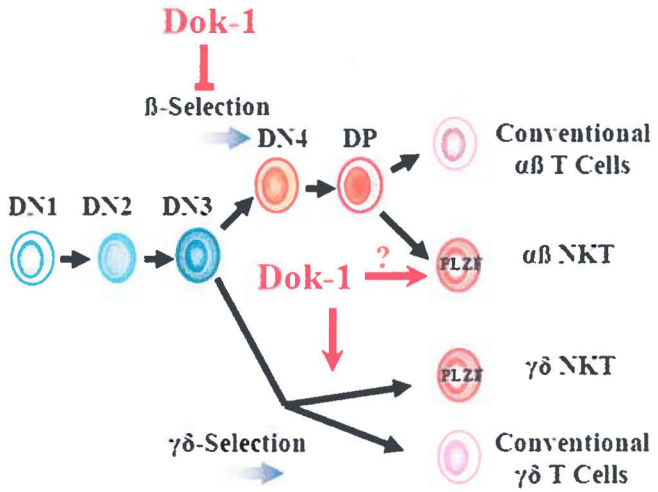
Contribution of the student

The article has been published in European Journal of Immunology, 2012 Sep; 42(9):2491-504. Gilles Besin started the project and it was continued by **Mitra Yousefi**. All the experimental works and data analysis were done by Gilles Besin and **Mitra Yousefi** contributing equally, under the supervision of Prof. Pascale Duplay. Dok-1 transgenic mice were generated by Ingrid Saba. Roscoe Klinck helped for the Q-RT-PCR experiments and Pier Paolo Pandolfi gave us the Dok-1/Dok-2-deficient mice. Manuscript was written by Prof. Pascale Duplay and **Mitra Yousefi.**

This article was selected by the editorial board of EJI to be highlighted in the "In this issue" section.

Dok-1: A new player in γδ NKT-cell development

γδ and αβ NKT cells share functional characteristics and homing properties that are distinct from conventional T cells. These subtypes likely utilize common molecular pathways during their differentiation. Thus far, the signaling pathways and transcriptional networks that regulate the developmental fates of NKT cells, in particular the γδ NKT-cell subtype remain poorly understood. In this issue, Besin et al. highlight a role for Dok-1 in the control of γδ NKT-cell development in mice overexpressing Dok-1 in thymocytes and peripheral T cells. In these Dok-1 transgenic mice, Dok-1 selectively promotes SAP-dependent expansion of γδ NKT cells, while simultaneously inhibiting β-selection. The authors propose that attenuation of the TCR signaling pathway by Dok-1 promotes the development of γδ NKT cells. These findings support the hypothesis that TCR signal strength influences the lineage fate of γδ T cells and reveal for the first time a major role for Dok-1 in the regulation of this unconventional population of lymphocytes.



SUMMARY

In T cells, two members of the Dok family, Dok-1 and Dok-2, are predominantly expressed. Recent evidence suggests that they play a negative role in T cell signaling. In order to define whether Dok proteins regulate T-cell development, we have generated transgenic mice overexpressing Dok-1 in thymocytes and peripheral T cells. We show that overexpression of Dok-1 retards the transition from the $CD4^-CD8^-$ to $CD4^+CD8^+$ stage. Moreover, there is a specific expansion of PLZF-expressing $V\gamma1.1^+$ $V\delta6.3^+$ T cells. This subset of γδ T cells acquires innate characteristics including rapid IL-4 production following stimulation and requiring SLAM-associated adaptor protein (SAP) for their development. Moreover, Dok-1 overexpression promotes the generation of an innate-like $CD8^+$ T-cell population that expresses Eomesodermin. Altogether, these findings identify a novel role for Dok-1 in the regulation of thymic differentiation and in particular, in the development of $PLZF^+$ γδ T cells.

Keywords: γδ T cell, Dok, PLZF, SAP, Thymocyte development

INTRODUCTION

αβ T-lymphocyte development is dependent on productive rearrangement of the TCRβ locus and intracellular signaling initiated through the pre-TCR at the $CD4^-CD8^-$ double-negative (DN) stage. Pre-TCR signaling leads to cellular proliferation and progression to the $CD4^+CD8^+$ double positive (DP) stage where TCRα locus rearrangement is induced. In DP thymocytes, initial TCR signal strength/duration will determine specific gene expression and as a consequence will regulate the outcome of thymocyte positive versus negative selection and CD4 versus CD8 lineage differentiation or conventional versus innate lymphocyte lineage choice (Liu and Bosselut 2004). γδ TCR lymphocytes also develop in the thymus and require productive rearrangement of the TCRγ and δ loci at the DN stage. The strength of TCR signaling is important in controlling αβ/γδ lineage choice. Strong TCR signaling is required for the commitment to the γδ lineage whereas weak TCR signals favor development of αβ lineage cells (Ciofani and Zuniga-Pflucker 2010, Hayes, Laird et al. 2010, Lee, Stadanlick et al. 2010). Moreover, TCR signal strength regulates also maturation and acquisition of effector functions in γδ and αβ T-cell development. Therefore, signaling molecules that modulate TCR signal strength play a pivotal role in the regulation of T-cell development and homeostasis. Development signals from the pre-TCR and from the TCR are controlled by the coordinated activity of tyrosine kinases such as Syk-family kinases (Cheng and Chan 1997), Src-family kinases (Salmond, Filby et al. 2009), and Tec-family kinases (Prince, Yin et al. 2009, Readinger, Mueller et al. 2009) and adaptor molecules such as SLP-76 (Clements 2003), LAT (Malissen, Aguado et al. 2005), Grb2 (Jang, Zhang et al. 2010), and Gads (Yoder, Pham et al. 2001). Dok are adaptor proteins that contain a pleckstrin homology domain, a phosphotyrosine binding domain, and a COOH-terminal region containing multiple tyrosine phosphorylation sites (Carpino, Wisniewski et al.

1997, Yamanashi and Baltimore 1997). Dok-1 and Dok-2, the two members of Dok family proteins expressed in T cells, are involved in signaling downstream of a variety of receptors in hematopoietic cells (Janssen and Zhang 2003, Mashima, Hishida et al. 2009). In T cells, studies performed initially in Jurkat cells demonstrated that Dok-1 and Dok-2 are tyrosine phosphorylated following CD28 or CD2 cross-linking (Nemorin and Duplay 2000, Michel, Attal-Bonnefoy et al. 2001). Overexpression of Dok-1 led to the specific inhibition of CD2-mediated Erk1/2 activation (Nemorin, Laporte et al. 2001). TCR stimulation induces Dok-1 and Dok-2 phosphorylation in primary murine or human T cells and in T-cell lines that express PTEN and SHIP-1 at levels comparable with primary T cells. More direct evidence that Dok-1 and Dok-2 are implicated as a transducer of TCR signals came from studies with primary human or murine Dok-deficient cells where deficiency of Dok-1 and Dok-2 expression results in increased TCR-mediated cytokine production and proliferation (Yasuda, Shirakata et al. 2004). In addition to their role in TCR and costimulatory receptor signaling, Dok proteins may be important downstream of the chemokine receptor CXCR4, SLAM, and IL-4 receptor (Latour, Gish et al. 2001, Latour, Roncagalli et al. 2003, Okabe, Fukuda et al. 2005, Inoue, Yasuda et al. 2007). Dok proteins are therefore likely to be a common component in several signaling pathways in T cells. How Dok proteins negatively regulate TCR signaling is not fully elucidated. In Dok-1- and Dok-2-deficient mature T cells, phosphorylation of ZAP-70, LAT, SLP-76, Akt, and Erk1/2 is increased compared to wild-type (WT) cells (Dong, Corre et al. 2006, Yasuda, Bundo et al. 2007). The negative regulation of ZAP-70 by Dok might be attributable to the competitive binding of Dok PTB domain to the ITAMs of TCRζ and CD3ε. In T cells, phosphorylated Dok-1 and/or Dok-2 form molecular complexes with RasGAP (Nemorin, Laporte et al. 2001). Dok-mediated RasGAP recruitment to the proximity of Ras is likely

responsible for the attenuation of Erk1/2 signaling. Moreover in T-cell lines, Dok-1 and/or Dok-2 have been shown to interact with SHIP-1 (Dong, Corre et al. 2006), Tec kinase (Gerard, Favre et al. 2004), Lck (Nemorin and Duplay 2000), SAP (Latour, Gish et al. 2001), Nck (Gugasyan, Quilici et al. 2002), Crk-L (Martelli, Boomer et al. 2001), and Csk (Schoenborn, Tan et al. 2011), but the mechanism of action of these interactions and whether they have functional relevance await further investigation. Erk activation is involved in pre-TCR signaling (Michie, Trop et al. 1999), positive selection (Pages, Guerin et al. 1999), CD4/CD8 lineage commitment (Sharp, Schwarz et al. 1997, Bommhardt, Basson et al. 1999, Sharp and Hedrick 1999), and γδ/αβ lineage choice (Lauritsen, Haks et al. 2006). Given that TCR-mediated activation of Erk is inhibited by Dok, Dok might play an important role in thymocyte development. Recent evidence supports this hypothesis. The overexpression of Dok-2 in bone marrow cells selectively inhibited early maturation of thymocytes from the DN to DP stage (Gugasyan, Quilici et al. 2002). In addition, Dok-1 has been shown to cooperate with SHIP in the regulation of T-cell development since the combined loss of Dok-1 and SHIP expression led to a severe reduction in thymocyte numbers (Kashiwada, Cattoretti et al. 2006). In this study, we investigated the involvement of Dok in the regulation of T-cell development. To this end, we generated transgenic mice overexpressing Dok-1 under the control of the CD2 promoter leading to transgene expression in thymocytes and mature T cells. We performed a detailed analysis of T-cell development in Dok-1 transgenic mice. We show that overexpression of Dok-1 leads to a partial block in DN to DP transition and to the specific accumulation of PLZF-expressing $V\gamma1.1^+$ $V\delta6.3^+$ T cells (referred to also as γδ NKT cells).

RESULTS

Dok-1 overexpression interferes with T-cell development at the DN to DP transition

Dok-1 and Dok-2 mRNAs (Yasuda, Bundo et al. 2007) are expressed early during thymocyte maturation at the DN stage and throughout all stages of T-cell development. In order to determine the functional role of Dok-1 in T-cell development, we generated transgenic mice overexpressing Dok-1 under the control of the human CD2 promoter and locus control region (LCR). The CD2-LCR vector drives expression of the transgene in DN thymocyte and throughout thymocyte development (Zhumabekov, Corbella et al. 1995). Out of 10 transgenic founder lines, two lines (Tg82 and Tg104) expressing the highest levels of Dok-1 were chosen for further studies. The level of Dok-1 expression in line Tg104 was higher than that observed in line Tg82 as shown by immunoblot analysis with anti-Dok-1 antibodies (Fig. 1A). We also tested the expression of Dok-1 transgene in specific thymocyte subsets (defined by CD4 and CD8 expression) using affinity purified Dok-1 Abs (Fig. 1B). By cytometry analysis, endogenous expression of Dok-1 was detectable in all thymocyte subsets in WT mice using thymocytes deficient for Dok-1 and Dok-2 as specificity control. The level of Dok-1 expression in transgenic mice line 82 and 104 was dramatically increased in all thymocyte subsets compared to WT mice (Fig. 1B) and in γδ thymocytes (Fig. 1C).

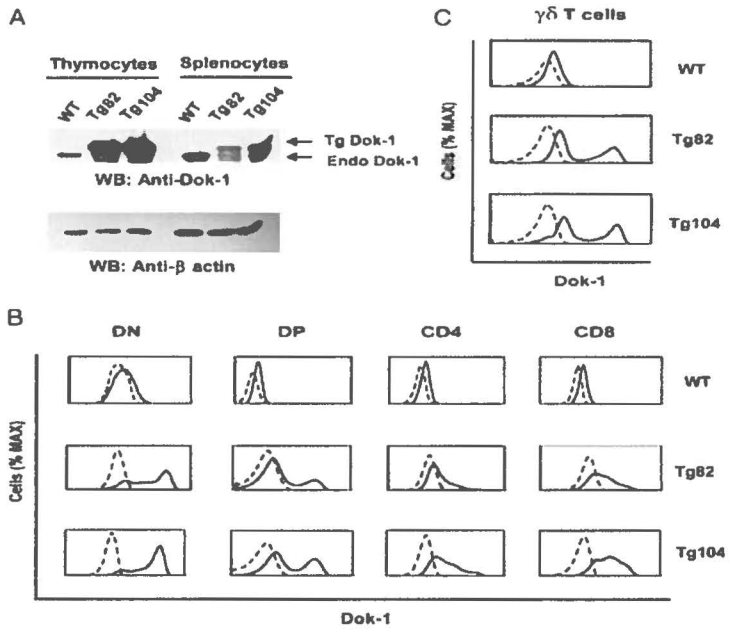


Figure 1. Expression profiles of Dok-1 in WT and Dok-1 transgenic mice. (A) Lysates from WT or Dok-1 transgenic mice (Tg82 and Tg104) thymus or spleen were prepared and equivalent cell numbers were loaded for western blot analysis using anti-Dok-1 antibodies raised against mouse-Dok-1. Ha-Tagged transgenic Dok-1 and endogenous Dok-1 are indicated by an arrow. Loading was verified by probing the membrane with anti-β actin antibodies. (B, C) Flow cytometric analysis of Dok-1 expression in thymocytes. Thymocytes from age-matched WT, transgenic Dok-1 Tg82, and Tg104 were stained with anti-CD4-FITC, anti-CD8–allophycocyanin (B), or with anti-CD3- allophycocyanin and TCRγ/δ-FITC (C) and after permeabilization were stained with anti-Dok-1- PE Abs. Thymocytes from Dok-1-/Dok-2-deficient mice were used as specificity control (dotted line). Intracellular expression of Dok-1 is shown for gated $CD4^-CD8^-$ DN (DN), $CD4^+CD8^+$ DP (DP), $CD4^+$ SP (CD4), and $CD8^+$ SP (CD8) thymocytes (B) and γ/δ thymocytes (C). The data shown are representative of three experiments.

The impact of the expression of Dok-1 transgene on T-cell development was first evaluated on the basis of CD4 and CD8 expression both in terms of percentage and cellularity (Fig. 2A and B). There was 8- to 20-fold increase in the percentage of DN thymocytes and 1.2- to 1.5-fold decrease in the percentage of DP thymocytes in Dok-1 transgenic mice compared to littermate controls. Importantly, in Dok-1 transgenic mice, the absolute number of DP thymocytes and consequently $CD4^+$ and $CD8^+$ single positive (SP) thymocytes decreased dramatically. By contrast, the number of thymocytes in the DN subset is unchanged or increased slightly (Fig. 2B). To determine whether the decreased DP number in Dok-1 transgenic mice resulted from a lack of survival due to increased cell death, we performed ex vivo apoptosis analysis on freshly isolated thymocytes using annexin V and 7-AAD staining (Fig. 3A). The percentage of thymocytes from transgenic mice undergoing either early or late apoptosis was comparable to control littermates. Moreover, the rate of cell death and the induction of Nur77 in DP thymocytes subjected to apoptotic signals in vitro (TCR and CD28 cross-linking) were identical between Dok-1 transgenic and WT littermate mice (Fig. 3A and B). These results indicated that the loss of DP in Dok-1 transgenic mice was likely due to a partial DN to DP transition block. To further define the stage at which thymocyte development was blocked in Dok-1 transgenic mice, we analyzed DN subpopulations with respect to CD44 and CD25 expression. There was a greater percentage of Dok-1 transgenic thymocytes at the DN3 stage ($CD44^-CD25^+$) and a lower percentage at the DN4 stage ($CD44^-CD25^-$) when compared with age-matched WT mice (Fig. 4A). Pre-TCR signals induce maturation of $CD27^{low}$ DN3a cells into $CD27^{high}$ DN3b blasts (Hoffman, Passoni et al. 1996, Taghon, Yui et al. 2006). To determine whether this transitional step was altered in transgenic mice, we analyzed the proliferation rate and the size of DN3 cells. As shown in Fig. 4B, the percentage of large cycling DN3 cells was dramatically reduced by

high levels of Dok-1 expression. These results indicated that Dok-1 overexpression affects the β-selection-associated processes and causes a decrease in subsequent proliferative expansion. Remarkably, the amplitude of the developmental defect at the DN to DP transition and consequently the reduction in thymic cellularity correlated with the level of Dok-1 overexpression (Fig. 2B and 4).

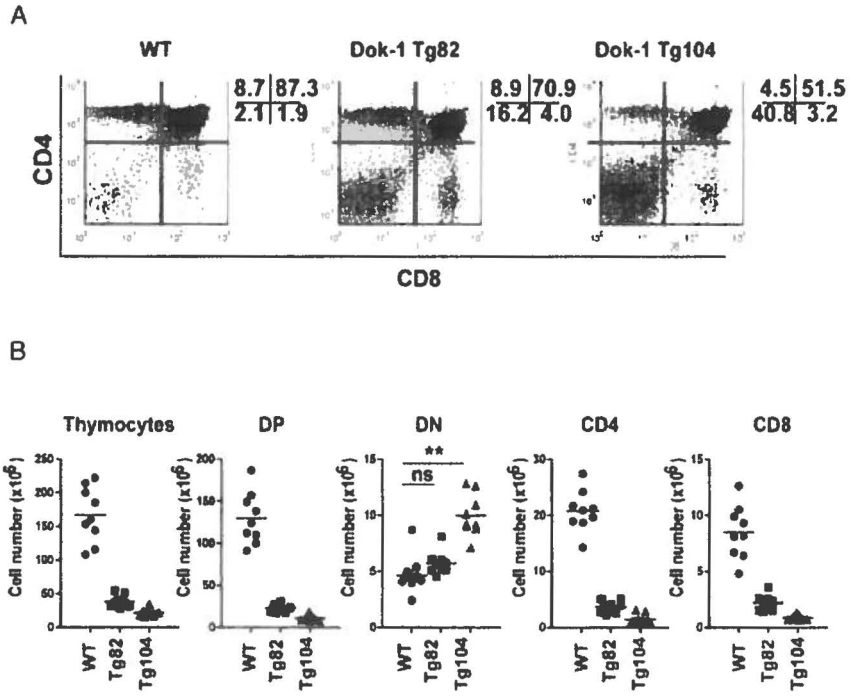


Figure 2. Dok-1 overexpression interferes with T-cell development. (A) A representative flow cytometry analysis of thymic CD4 and CD8 expression from littermate controls (WT) or Dok-1 transgenic mice (Tg82 and Tg104). Percentages of cells in each quadrant are indicated. Similar results were obtained in at least five experiments. Mice were 3–7 weeks old. (B) Absolute cell numbers were calculated for total thymocytes and thymocyte subsets based on their CD4 and CD8 expression. Each symbol represents a single mouse. The bars represent the mean from groups of mice. Statistical significance was determined by unpaired student's t-test (NS, $p > 0.05$; $^{**}p < 0.01$).

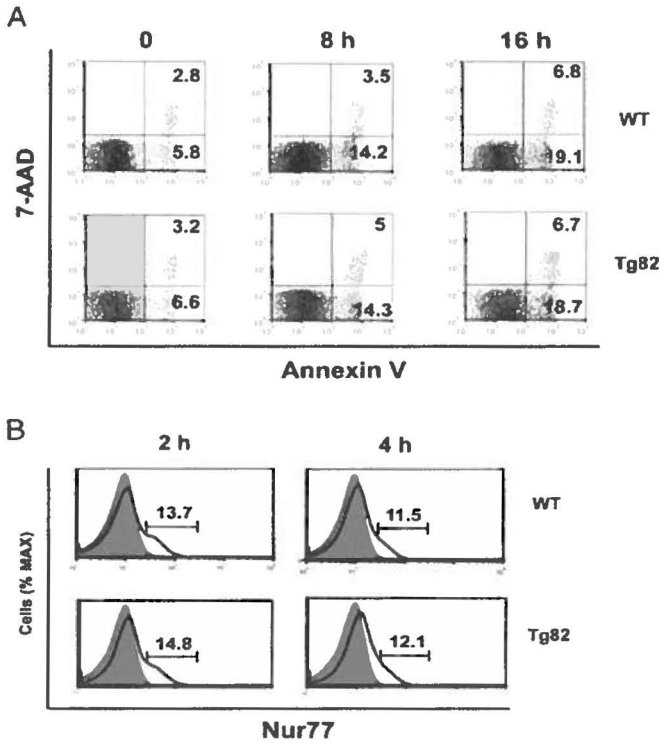


Figure 3. Transgenic expression of Dok-1 does not promote apoptosis of DP thymocytes. (A) Thymocytes from Dok-1 transgenic (Tg82) and littermate control (WT) mice were left unstimulated (0) or stimulated with plate-bound anti-TCR and soluble anti-CD28 Abs for 8 or 16 h and stained with annexin V and 7-AAD to identify apoptotic and dead thymocytes. Data are representative of two experiments. (B) Histograms show the expression of Nur77 on DP thymocytes following 2 and 4 h of incubation with media alone (shaded histograms) or with anti-TCR plus anti-CD28 antibodies (black line). Data are representative of two experiments.

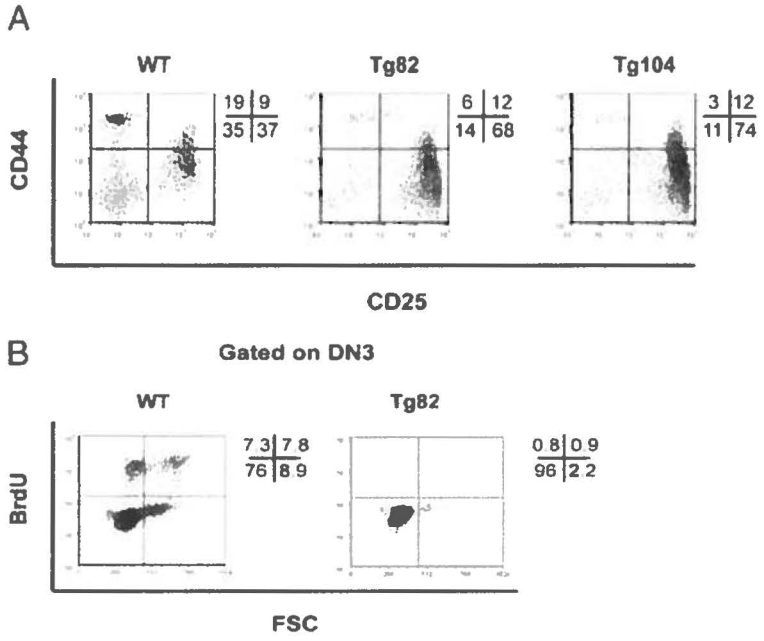
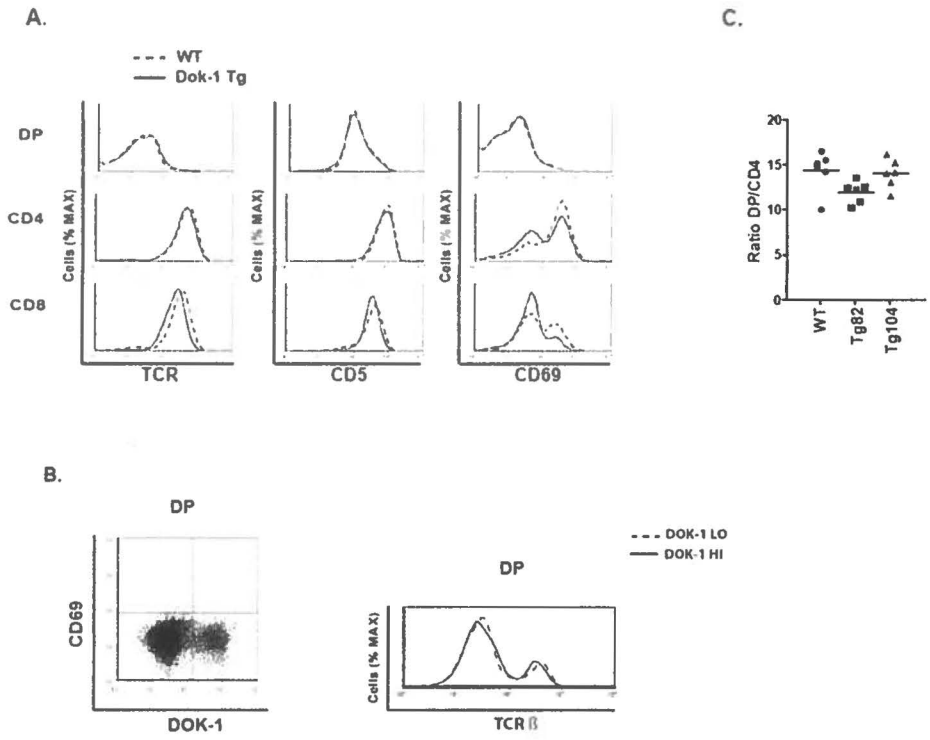


Figure 4. Transgenic expression of Dok-1 blocked T-cell development at DN3 stage. (A) Thymocytes were stained with PE-CD25, allophycocyanin-CD44, and a mixture of biotinylated Abs against CD4, CD8, TCRγδ, CD19, B220, CD11b, CD11c, Gr-1, CD49b (DX5), and Ter-119 followed with streptavidin-PerCP. Lineage-negative DN thymocytes were electronically gated and analyzed for CD44 and CD25 expression. Percentages of DN1–DN4 subsets are shown. Data are representative of three experiments. (B) Mice were injected i.p. with BrdU for 4 h. Lineage-negative thymocytes were analyzed for CD44 and CD25 expression as described in (A). BrdU staining of $CD44^{-}CD25^{+}$ (DN3) thymocytes is shown. Data are representative of two experiments.

Development of innate-like $CD8^+$ T cells in Dok-1 transgenic mice

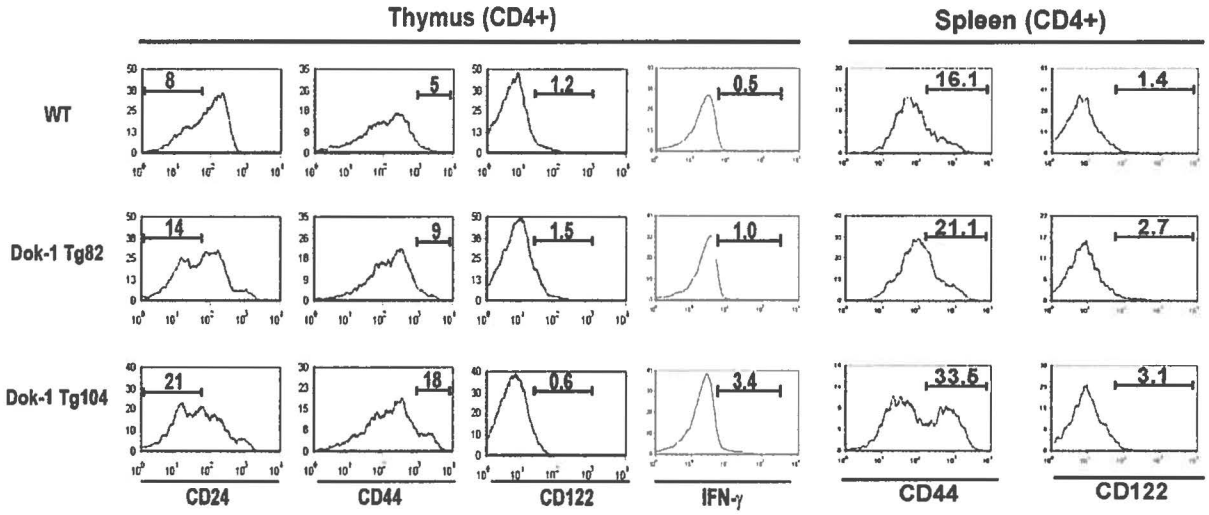
Despite the reduced percentage of DP, $CD4^+$, and $CD8^+$ thymocytes were selected in Dok-1 transgenic mice. Moreover, in a context of a polyclonal repertoire, signal generated during positive selection seemed to lead to normal expression of CD5, CD69, and TCR (Supporting Information Fig. 1A and B). Maturation of the $CD4^+$ thymocytes appeared to occur at roughly the same efficiency since similar ratios of CD4/DP thymocytes in Dok-1 transgenic and WT mice were observed (Supporting Information Fig. 1C). Dok-1 transgenic mice showed decreased CD4/CD8 ratios compared to WT littermates both in thymus and periphery (Fig. 5A). To analyze whether this altered lineage development corresponds to the accumulation of an aberrant $CD8^+$ population, we characterized the maturity and memory phenotype of the $CD4^+$ and $CD8^+$ thymocytes (Fig. 5B and Supporting Information Fig. 2). There was an increased fraction of $CD8^+$ with a mature, $CD24^{low}$ phenotype. In addition, many $CD8^+$ thymocytes showed increased expression of the memory/activation markers CD44 (61%, Tg82 or 82%, Tg104 versus 27% WT of $CD44^{high}$) and CD122 (27%, Tg82 or 42%, Tg104 versus 2% WT of $CD122^{high}$). In the periphery, most of the splenic $CD8^+$ T cells also expressed activation markers CD44 and CD122 (Fig. 5B). By contrast, the percentage of $CD44^{high}CD122^{high}$ $CD4^+$ thymocytes or splenocytes only slightly increased in Dok-1 transgenic mice compared to WT mice (Supporting Information Fig. 2). To determine whether the $CD8^+$ $CD44^{high}$ thymocytes could directly express cytokine *ex vivo*, we performed intracellular staining. More than 20% of the $CD8^+$ thymocytes from Dok-1 transgenic mice produced IFN-γ rapidly following PMA and ionomycin stimulation compared to less than 3% in WT mice (Fig. 5B). Interestingly and as shown in Itk and Rlk/Itk-deficient $CD8^+$ T cells (Atherly, Lucas et al. 2006, Broussard, Fleischacker et al. 2006), Eomesodermin mRNA expression was dramatically increased in Dok-1 transgenic $CD8^+$ thymocytes (Fig. 5C).

Expression of T-bet, another T-box transcription factor that also promotes the expression of IFN-γ and CD122 also increased but to a much lesser extent. Dok-1-derived signals also promoted the expression of Runx-3 whereas GATA3 and NFATc1 expression were comparable to that found in WT thymocytes (Fig. 5C). Therefore, these results demonstrate that overexpression of Dok-1 in DP thymocytes leads to the development of $CD8^+$ T cells with a $CD44^{high}$ $CD122^{high}$ memory phenotype and effector function ex vivo in response to stimulation.



Supplemental Figure 1: Surface expression of CD3, CD69 and CD5 in Dok-1 transgenic thymocytes is normal

(A) Thymocytes from littermate controls (dotted line) and Dok-1 transgenic mice Tg82 (solid line) were stained with anti-CD4-PE, anti-CD8–FITC and as indicated on the bottom of each panel with anti-CD3-biotin, anti-CD69-biotin or anti-CD5-biotin followed by streptavidin Red670. Cell surface expression of CD3, CD69 and CD5 is shown for gated $CD4^+$ SP (CD4), $CD8^+$ SP (CD8) and $CD4^+CD8^+$ DP (DP). (B) Thymocytes from Dok-1 transgenic Tg82 were stained for CD4, CD8, CD69 or CD4, CD8 and TCRb and, after fixation and permeabilization, with anti-Dok-1 Abs. Dot plots for CD69 and Dok-1 expression are shown for gated DP thymocytes (left). Histogram for TCRb on gated DP expressing low levels of Dok-1 ($Dok\text{-}1^{low}$, dotted line) and high levels of Dok-1 ($Dok\text{-}1^{high}$, solid line) is shown (right). (C) The ratio of total cell numbers of DP / $CD4^+$ thymocytes is indicated for 6 experiments.



Supplemental Figure 2: Cell surface expression of CD44 and CD122 in $CD4^+$ T cells in Dok-1 transgenic mice

Thymocytes or splenocytes from littermate WT controls and Dok-1 transgenic mice were stained with anti-CD4 anti-CD8 and as indicated on the bottom of each panel with anti-CD24, anti-CD122, or anti-CD44. Cell surface expressions of CD24, CD122, and CD44 are shown for $CD4^+$ SP thymocytes ($CD4^+$). For IFN-γ expression, thymocytes were stimulated ex vivo with a combination of PMA and ionomycin for 4 h at 37°C. The percentages of gated cells are indicated. Data are representative of 3 experiments. Mice were 4- to 6-wk-old.

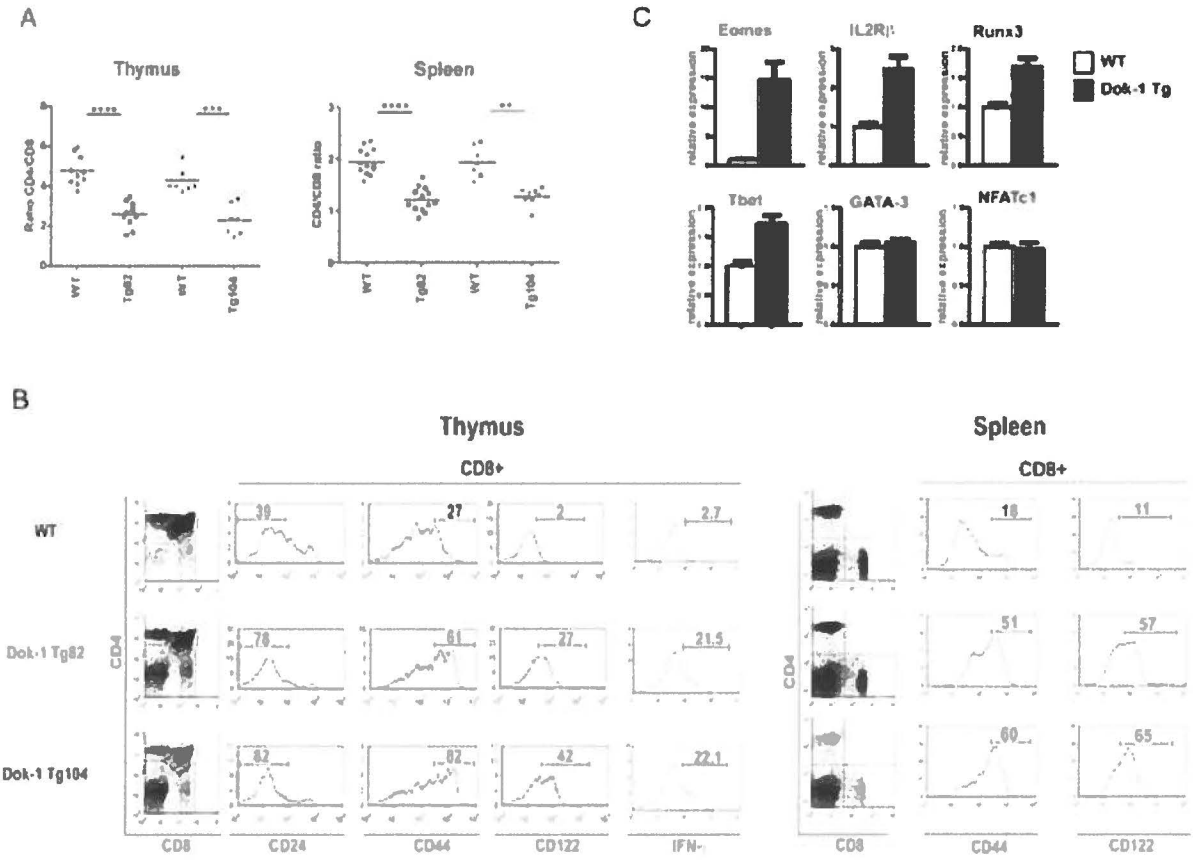


Figure 5. Innate-like CD8$^+$ T cells accumulate in Dok-1 transgenic thymus and spleen. (A) Thymocytes and spleen cells from 4- to 6-weeks-old WT and Dok-1 transgenic mice were analyzed by flow cytometry with anti-CD4 and anti-CD8 Abs. Ratios of CD4/CD8 cells in the spleen or thymus of transgenic mice (Tg82 and Tg104) and wild-type littermate controls (WT) are shown. Each symbol represents a single mouse. The bars represent the mean from groups of mice. Statistical significance was determined by paired student's t-test (**p < 0.01; ***p < 0.001, ****p < 0.0001). (B) Thymocytes or splenocytes from littermate WT controls and Dok-1 transgenic mice (Dok-1 Tg82 and Dok-1 Tg104) were stained with anti-CD4 anti-CD8 and as indicated on the bottom of each panel with anti-CD24, anti-CD122, or anti-CD44. Cell surface expressions of CD24, CD122, and CD44 are shown for gated CD8$^+$ thymocytes (CD8$^+$). For IFN-γ expression, thymocytes were stimulated ex vivo with a combination of PMA and

ionomycin for 4 h at 37°C. The percentages of gated cells are indicated. Data are representative of three experiments. Mice were 4- to 6-weeks-old. (C) RNA from purified $CD8^+$ SP thymocytes was prepared from WT and Dok-1 transgenic mice Tg82 (Dok-1 Tg) and quantitative RT-PCR was performed. Relative mRNA expression was compared by setting the WT expression to an arbitrary value of 1. Data are shown as mean ± SEM of one to two mice of each genotype and are representative of two independent experiments.

SAP is required for the development of the CD8$^+$ innate-like population

The phenotype of CD8$^+$ CD44^{high}CD122^{high} T cells in Dok-1 transgenic mice is very similar to that of innate-like CD8$^+$ thymocytes that accumulate in Itk-deficient mice (Atherly, Lucas et al. 2006, Broussard, Fleischacker et al. 2006). We crossed Dok-1 transgenic mice with SAP-deficient mice to evaluate whether, as shown for *Itk*$^{-/-}$ mice (Horai, Mueller et al. 2007), SAP was required for the development of these innate-like CD8$^+$ T cells. The percentage of innate-like CD8$^+$ SP thymocytes in Dok-1 transgenic mice, as determined by expression of CD44 and CD122 and secretion of IFN-γ upon ex vivo stimulation, was dramatically reduced in the absence of SAP (Fig. 6A). Remarkably, expression of Eomesodermin in CD8$^+$ thymocytes of Dok-1 transgenic mice correlated with the level of SAP expression (Fig. 6B). Together, these results indicate that SAP was required for the development of innate-like CD8$^+$ thymocytes in Dok-1 transgenic mice. Thus, overexpression of Dok-1 appears to mimic Itk deficiency insofar as the SAP requirement for the development of CD8 innate-like population is concerned.

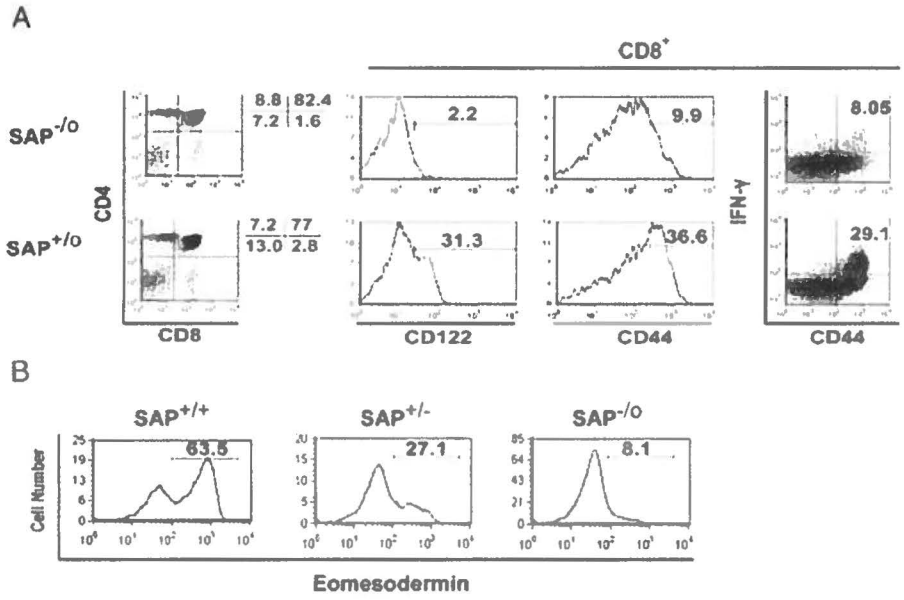


Figure 6. SAP is required for the development of a $CD8^+$ innate-like T-cell population in Dok-1 transgenic thymus. (A) Thymocytes from littermate Dok-1 transgenic mice Tg82, ($SAP^{+/0}$) or SAP-deficient Dok-1 transgenicmice Tg82, ($SAP^{-/0}$) were stained with anti-CD4, anti-CD8, anti CD122, and anti-CD44. Percentages of thymocytes in each quadrant are indicated. Cell surface expressions of CD122 and CD44 are shown for gated $CD8^+$ SP thymocytes ($CD8^+$). For IFN-γ expression, thymocytes were stimulated as described in Fig. 5 and intracellular staining for IFN-γ expression was performed. IFN-γ expression and CD44 expression are shown for gated $CD8^+$ SP thymocytes. Data are representative of four independent experiments and mice were 4–6 weeks old. (B) Intracellular staining for Eomesodermin expression in γδ T cells in thymus is shown for gated $CD8^+$ SP thymocytes from littermate Dok-1 transgenic mice Tg82 $SAP^{+/+}$ ($SAP^{+/+}$), Tg82 $SAP^{+/-}$ ($SAP^{+/-}$), or Tg 82 $SAP^{-/0}$ ($SAP^{-/0}$).

Dok-1 regulates the development of SAP-dependent Vγ1.1$^+$ Vδ6.3$^+$ NKT cells

Recently, it was demonstrated that the innate-like phenotype of CD8$^+$ thymocytes in *itk*$^{-/-}$, *id3*$^{-/-}$, and *klf2*$^{-/-}$ mice was due to a cell extrinsic effect and was dependent on the production in trans of IL-4 by PLZF$^+$ γδ NKT cells (Verykokakis, Boos et al. 2010, Weinreich, Odumade et al. 2010). Therefore, we tested whether there was an increase in PLZF$^+$ γδ NKT cells in Dok-1 transgenic mice. As shown in Fig. 7A and B, Dok-1 transgenic mice develop a larger population of γδ T cells in the thymus, spleen, and liver compared to WT mice. Remarkably, the increase in total γδ T-cell numbers in these organs correlated with the level of Dok-1 expression and was mainly due to the specific increase in percentage and absolute numbers of the Vγ1.1$^+$ Vδ6.3$^+$ subset of γδ T cells (Fig. 7A and B). PLZF expression in γδ T cells is largely restricted to the Vγ1.1$^+$ Vδ6.3$^+$ subset (Kreslavsky, Savage et al. 2009, Alonzo, Gottschalk et al. 2010). Intracellular staining of PLZF showed that the percentage of PLZF-expressing γδ T cells was increased in Dok-1 transgenic mice compared to WT mice (Fig. 7C). It should be noted that the minority of PLZF$^+$ thymocytes were γδ T cells in WT mice (17%, Fig. 7C) whereas in Dok-1 transgenic mice, most of PLZF$^+$ thymocytes were γδ (58% Tg82 and 93% Tg104, Fig. 7C). The non-γδ PLZF$^+$ thymocytes likely correspond to the αβ invariant NKT cells. This increase in PLZF expression was specific to the Vγ1.1$^+$ Vδ6.3$^+$ subset since more than 80% of this subset expressed high levels of PLZF in the thymus (Fig. 7C). PLZF expression has been shown to correlate with the ability of cells to secrete IL-4. Accordingly, there was a significant increase in the percentage of γδ thymocytes that express IL-4 in Dok-1 transgenic mice (5.6% Dok Tg82 versus 0.4% WT, Fig. 7D). In addition, the percentage of PLZF$^+$ γδ T cells that express IL-4 increased slightly in thymocytes from transgenic mice (30% Dok Tg82 versus 18% WT, Fig. 7D). SAP has been shown to play an important role in the development of PLZF$^+$

$V\gamma1.1^+$ $V\delta6.3^+$ T cells. The $V\gamma1.1^+$ $V\delta6.3^+$ cell numbers in thymus and spleen in SAP $^{-/-}$ Dok-1 transgenic mice were reduced several fold compared to Dok-1 transgenic mice (Fig. 8A and B). Importantly, there was no PLZF expression in the remaining $V\gamma1.1^+$ $V\delta6.3^+$ T cells from the thymus of $SAP^{-/-}$ Dok-1 transgenic mice (Fig. 8A). It should be noted that in $SAP^{-/-}$ mice, there was a dramatic reduction but not a total absence of PLZF expression, in $V\gamma1.1^+$ $V\delta6.3^+$ T cells (Fig. 8A). To determine whether the specific accumulation of $V\gamma1.1^+$ $V\delta6.3^+$ thymocytes in Dok-1 transgenic mice is due to increased proliferation, we performed cell cycle and BrdU labeling experiments. $V\delta6.3^+$ thymocytes from Dok-1 transgenic mice proliferated at a lower rate than WT $V\delta6.3^+$ thymocytes (Fig. 9). This was not due to a general inhibitory role of Dok-1 on thymocyte proliferation since DP thymocytes from Dok-1 transgenic mice show only minor differences in their proliferation rate compared to DP thymocytes from WT mice (Supporting Information Fig. 3). Moreover, overexpression of Dok-1 does not seem to affect apoptosis of $V\delta6.3^+$ cells as represented by cells in sub-G0/G1 (Fig. 9). Taken together, our data show that Dok-1 overexpression favors the SAP-dependent development of $PLZF^+$ $V\gamma1.1^+$ $V\delta6.3^+$ T cells.

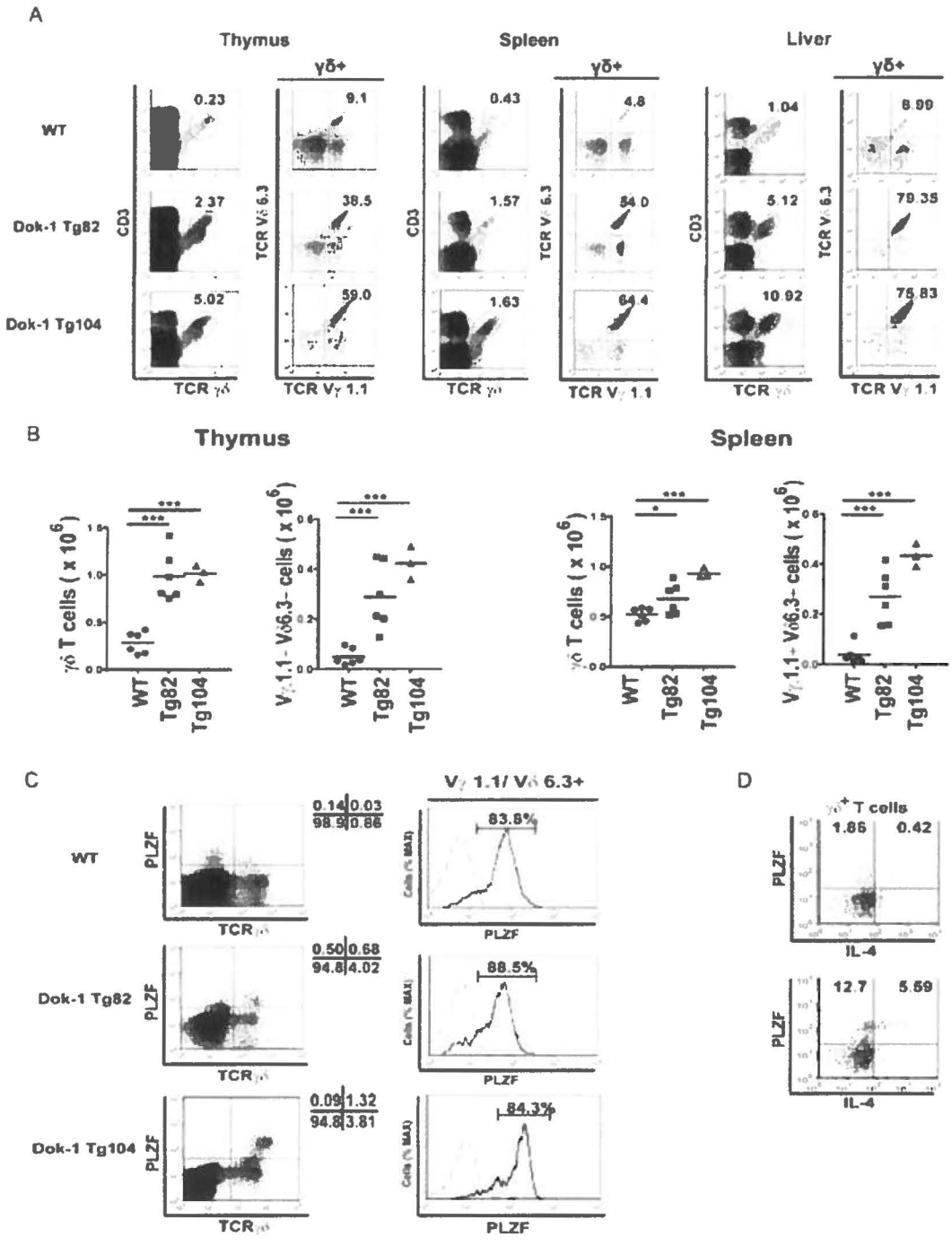


Figure 7. Dok-1 transgenic mice have an increased population of Vγ1.1$^+$ Vδ6.3$^+$ T cells. (A) Thymocytes, splenocytes, and liver cells from WT, Dok-1 transgenic (Tg82), and (Tg104) mice were stained with antibodies against TCRγδ, CD3, Vγ1.1, and Vδ6.3. A representative experiment is shown with the percentage of total γδ T cells indicated adjacent to outlined areas.

The percentage of $V\gamma1.1^+$ $V\delta6.3^+$ T cells on gated TCR γδ T cells is also indicated. (B) Absolute numbers of total γδ cells and of $V\gamma1.1^+$ $V\delta6.3^+$ cells in thymus and spleen of WT and Dok-1 transgenic are indicated. Each symbol represents an individual mouse. The bars represent the mean from groups of mice of each genotype. Statistical significance was determined by unpaired student's t-test (*$p < 0.05$; **$p < 0.01$; ****; $p < 0.0001$). (C) Intracellular staining for PLZF expression in γδ T cells from thymus of WT and Dok-1 transgenic mice (Tg82 and Tg104) is shown. Percentages of cells in each quadrant are indicated. Expression of PLZF in $V\gamma1.1^+$ $V\delta6.3^+$ cells (solid line) and $V\gamma1.1^-$ $V\delta6.3^-$ γδ T cells (dotted line) is indicated. Bracketed lines next to the graphs indicate the percentages of $PLZF^+$ cells. Data are representative of more than three independent experiments. (D) IL-4 and PLZF expression in gated $\gamma\delta^+$ thymocytes from WT (top) and Dok-1 transgenic (bottom) mice. Thymocytes were stimulated ex vivo with PMA and ionomycin for 4 h and intracellular staining for IL-4 and PLZF expression was performed. γδ thymocytes were identified with anti-CD3 and anti-γδ staining. The percentage of cells in each quadrant is indicated. Data are representative of two experiments.

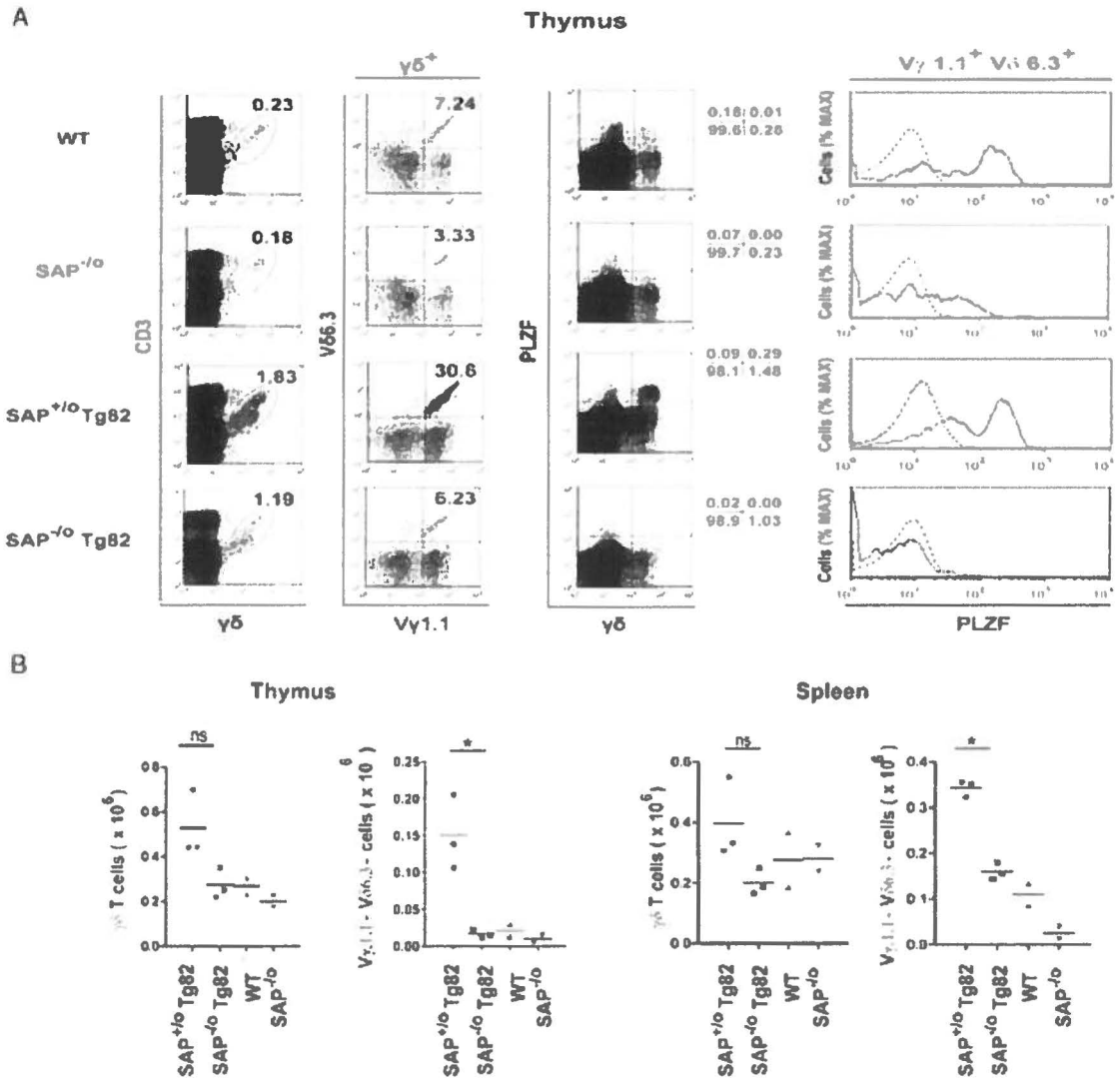


Figure 8. Accumulation of Vγ1.1$^+$ Vδ6.3$^+$ cells in Dok-1 transgenic mice required SAP. (A) Thymocytes from WT, SAP$^{-/0}$, Dok-1 transgenic Tg82 SAP$^{+/0}$, and Tg82 SAP$^{-/0}$ littermates were stained with antibodies to TCRγδ, CD3, and Vγ1.1 and Vδ6.3. A representative experiment is shown with the percentage of total γδ T cells indicated adjacent to outlined areas. The percentage of Vγ1.1$^+$ Vδ6.3$^+$ T cells on gated TCR γδ T cells is also indicated. Intracellular staining for PLZF expression in γδ T cells in thymus is shown. Percentages of cells in each quadrant are indicated. Expression of PLZF in Vγ1.1$^+$ Vδ6.3$^+$ cells (solid line) and Vγ1.1$^-$ Vδ6.3$^-$ γδ T cells (dotted line) is indicated. Data are representative of more than three independent experiments.

(B) Absolute numbers of total γδ cells and of $V\gamma1.1^+$ $V\delta6.3^+$ cells in thymus and spleen of WT, $SAP^{-/0}$, Dok-1 transgenic Tg82 $SAP^{+/0}$, and Tg82 $SAP^{-/0}$ littermates are indicated for at least two experiments. Each symbol represents an individual mouse. The bars represent the mean from groups of mice. Statistical significance was determined by paired student's t-test (NS, $p > 0.05$; $*p < 0.05$).

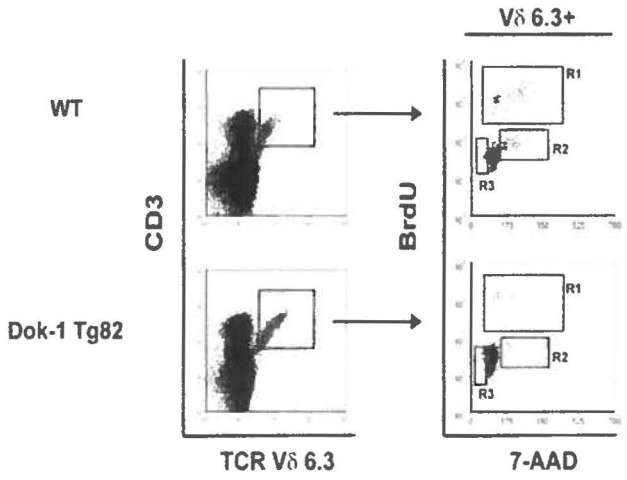
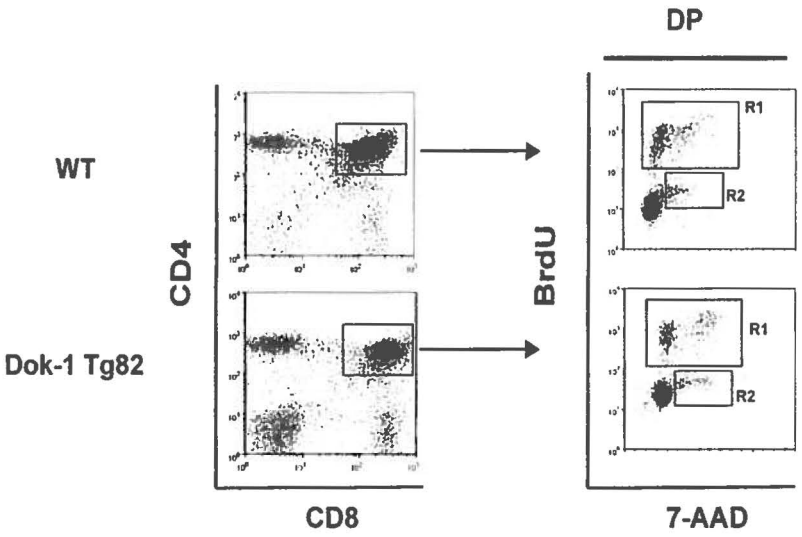


Figure 9. Proliferation rate of $V\delta6.3^+$ thymocytes. Mice were injected i.p. with BrdU. Four hours postinjection-enriched TCRγδ thymocytes from WT mice and total thymocytes from transgenic mice 82 were stained for Vδ6.3 and CD3ε followed by BrdU and 7-AAD staining. BrdU incorporation and total DNA content (7-AAD) is shown for gated $V\delta6.3^+$ thymocytes. Data are representative of two independent experiments. Gates, representing different phases of cell cycle are shown: R1, S WT: 14.9%, Tg82: 3.3%; R2, $G2^+M$, WT: 7.13, Tg82: 3.51 and R3, sub-G0/G1 WT: 2%, Tg82: 2%.



Supplemental Figure 3: Proliferation rate of DP thymocytes from WT and Dok-1 transgenic mice

Mice were injected i.p. with BrdU. 4 h post-injection, thymocytes from WT and transgenic mice 82 were stained for CD4 and CD8 followed by BrdU and 7-AAD staining. BrdU incorporation and total DNA content (7-AAD) is shown for gated DP thymocytes (n=2). Gates, representing different phases of cell cycle are shown: R1, S WT: 12.3%, Tg82: 10.8% and R2, G2+M, WT: 4.5, Tg82: 3.5

Dok-1 negatively modulates TCR signaling in DP thymocytes

In primary human T cells, CD3 stimulation has been shown to induce Dok phosphorylation (Dong, Corre et al. 2006). To examine whether the same stimulus induced Dok-1 phosphorylation in mouse T cells, purified thymocytes were stimulated with anti-CD3 Abs alone or in combination with anti-CD4 Abs. Upon CD3 stimulation, phosphorylation of Dok-1 was increased and CD3-induced Dok-1 phosphorylation was further increased by CD4 co-ligation (Fig. 10A). This result confirms the involvement of Dok-1 in TCR-induced signaling events in thymocytes. To examine the impact of Dok-1 overexpression on TCR signaling in thymocytes, we analyzed the extent of phosphorylation of several key molecules involved in the signaling cascade induced upon stimulation of thymocytes with anti-CD3 and anti-CD4 mAbs, the stimulation condition that induces the maximum phosphorylation of Dok-1 (Fig. 10A). To avoid a potential bias caused by the difference in the relative numbers of the different thymocyte populations in WT and Dok-1 transgenic mice, we used purified DP thymocytes in this assay. The extent of phosphorylation of specific proteins was affected by the level of Dok expression (Fig. 10B). Immunoblotting with phosphospecific mAbs showed that in comparison to WT control thymocytes, Dok-1 transgenic thymocytes exhibited a decrease in CD3-induced phosphorylation of ZAP-70, LAT, and PLC-γ1 (Fig. 10B). Together, these results support the idea that overexpression of Dok in thymocytes inhibits TCR signaling.

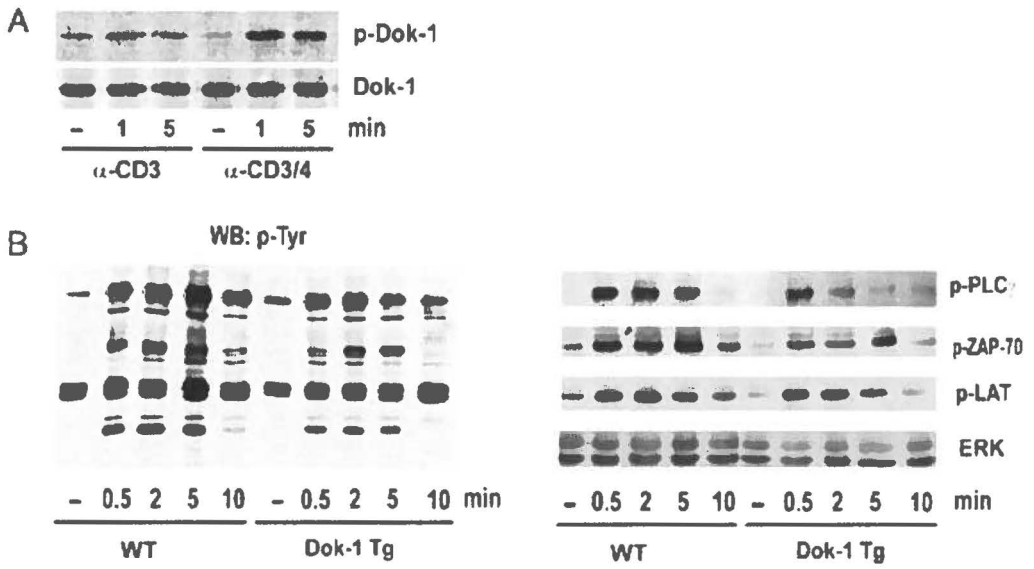


Figure 10. Dok modulates the strength of TCR signaling. (A) Dok-1 is tyrosine phosphorylated following TCR stimulation. Thymocytes were left unstimulated (−) or stimulated with anti-CD3 alone or with anti-CD4 for the indicated times in minutes. Cells were harvested and analyzed by western blotting for Dok-1 phosphorylation indicated as pDok-1. Antibodies against Dok-1 and Dok-2 were used as loading controls. Data are representative of two independent experiments. (B) DP thymocytes WT (WT) or Dok-1 Tg82 (Dok-1 Tg) mice were left unstimulated (−) or stimulated with anti-CD3 and anti-CD4 for the indicated times in minutes. Thymocytes lysates were analyzed by western blotting (WB) with the indicated antibodies. Antibodies against Erk1/2 were used as loading controls. Data are representative of at least three independent experiments.

DISCUSSION

In this study, we evaluated the role of Dok-1 in the development of thymocytes. We showed that transgenic expression of Dok-1 induced a partial block at the DN to DP transition and a specific expansion of PLZF-expressing SAP-dependent γδ NKT cells. Several components or effectors of the pre-TCR signaling pathway such as ZAP-70, LAT, PLC-γ1, and Erk1/2 are negatively regulated by Dok proteins (Fig. 10) (Mashima, Hishida et al. 2009). Therefore, the inhibition of pre-TCR signaling by Dok-1 is likely the mechanism of Dok-1-mediated inhibition of the DN to DP transition step. Constitutive expression of Dok-2 in bone marrow cells has been shown to inhibit the transition from DN to DP of thymocytes in reconstituted mice (Gugasyan, Quilici et al. 2002). This finding indicates that there is potential functional redundancy of Dok-1 and Dok-2 in thymic maturation. The intensity of the Erk1/2 signal is also critical for the development of DP to SP thymocytes (McNeil, Starr et al. 2005). Therefore, expression level of Dok-1 might alter the threshold for positive selection; its overexpression might potentially convert a weak positive signal to death by neglect; whereas absence of Dok expression might allow selection of cells that would have been neglected. Conversely, Dok expression may have the opposite effect on deletion of self-reactive thymocytes by negative selection. Surprisingly, in the context of a polyclonal repertoire, positive selection of $CD4^+$ thymocytes seems to occur normally in mice overexpressing Dok-1. The effect of Dok on thymic positive selection might become visible in TCR transgenic mouse model with low affinity ligand where selecting self-peptides would be limited. In support of a role of Dok in positive selection, and in agreement with previous reports (Yasuda, Bundo et al. 2007, Mashima, Hishida et al. 2009), we found that there is an increase in the absolute number of mature SP thymocytes in Dok-1-/Dok-2-deficient mice compared to WT mice (data not shown). Although other possibilities such as alteration of

export of mature thymocytes to the periphery cannot be excluded, one possible explanation of these data is that in absence of Dok, positive selection of thymocytes is more efficient. We showed that the level of Dok-1 expression correlated with the specific increase in the number of $V\gamma1.1^+V\delta6.3^+$ $PLZF^+$ T cells. These γδ NKT cells have innate properties and are able to secrete IL-4 ex vivo as shown in WT mice (Kreslavsky, Savage et al. 2009). These data are reminiscent of TCR signaling mutant such as in $Itk^{-/-}$ (Alonzo, Gottschalk et al. 2010), $CD3\zeta^{-/-}$ (Arase, Ono et al. 1995), and SLP-76 (Alonzo, Gottschalk et al. 2010) or LAT (Nunez-Cruz, Aguado et al. 2003) mutant mice where the development of γδ NKT cells have been shown to be favored. Therefore, as proposed for TCR signaling mutant or Id3-deficient thymocytes, overexpression of Dok-1 by attenuation of TCR signaling might rescue these cells from deletion, allowing their specific survival (Lauritsen, Wong et al. 2009, Alonzo and Sant'Angelo 2011). Accumulation of DN3 thymocytes in Dok-1 transgenic mice might have favored the expansion of γδ NKT cell subset among γδ thymocytes. However, in TCRβ-deficient mice although the total number of γδ thymocytes increased, the proportion of $NK1.1^+$ cells among γδ thymocytes is identical to that of WT mice (Vicari, Mocci et al. 1996). Therefore, the specific development of γδ NKT cells over non-NKT γδ thymocytes does not seem to be influenced by a block in DN to DP transition. Interestingly, the development of $PLZF^+$ $V\gamma1.1^+V\delta6.3^+$ cells is strictly dependent on the SLAM/SAP signaling pathway in Dok-1 transgenic mice, whereas in WT mice, the loss of SAP only partially affects the frequency and level of PLZF expression in $V\gamma1.1^+V\delta6.3^+$ cells (Alonzo, Gottschalk et al. 2010). Defective TCR signaling may make $PLZF^+$ cell expansion more susceptible to signals from SLAM family members. We failed to detect any alteration in the development of $V\gamma1.1^+V\delta6.3^+$ or in the level of PLZF expression in Dok-1-/Dok-2-deficient mice (data not shown). Therefore, the generation of $V\gamma1.1^+V\delta6.3^+$ $PLZF^+$ T cells is not affected

when TCR signal strength is increased. This result might suggest that positive selection of this subset of γδ T cells is already operating at a maximum rate or that increased TCR signal in Dok-1-/Dok-2- deficient thymocytes results in both increased positive and negative selection. We found that Dok-1 overexpression induces the SAP-dependent accumulation of $CD8^+$ innate-like T cells. CD8 innate-like lymphocytes that develop in Dok-1 transgenic mice are very similar to those that develop in the Tec kinase (Itk and Rlk)-deficient mice (i.e. $CD44^{high}$ and $CD122^{high}$) express high levels of Eomesodermin, and high levels of IFN-γ upon stimulation. There is a remarkable correlation between the percentage of Eomesodermin-expressing $CD8^+$ cells and the number of γδ NKT cells (Fig. 6B and data not shown). Therefore, as demonstrated for *Itk*$^{-/-}$ mice, the development of these innate-like $CD8^+$cells is likely a consequence of elevated IL-4 produced by the expanded $PLZF^+$ γδ T cells in Dok-1 transgenic mice (Verykokakis, Boos et al. 2010, Weinreich, Odumade et al. 2010). What is the target of Dok downstream of TCR that is important for generation of $V\gamma1.1^+V\delta6.3^+$ $PLZF^+$ T cells? Id3 is a target of the TCR-triggered signal that is important in both γδ and αβ T-cell development (Hayes, Li et al. 2005, Lauritsen, Wong et al. 2009, Ueda-Hayakawa, Mahlios et al. 2009, Lee, Stadanlick et al. 2010, Miyazaki, Rivera et al. 2011) and was shown to control the frequency of $PLZF^+$ γδ T cells (Alonzo, Gottschalk et al. 2010, Verykokakis, Boos et al. 2010). This pathway is dependent on early growth response 2/3 (Egr2/3) induction (Bain, Cravatt et al. 2001) and is regulated by Itk (Miller and Berg 2002). Interaction of Dok-1 and Dok-2 proteins with Tec, a member of the Tec kinase family, has been proposed to exert a negative feedback regulation on Tec kinase activity (Gerard, Favre et al. 2004). Therefore, it is possible that Dok by negatively regulating Itk activity downmodulates the Egr/Id3 pathway. However, we were unable to demonstrate functional relationship between Itk and Dok in primary murine T cells. Although Dok-mediated inhibition

of TCR signaling likely plays an important role in the development of γδ NKT cells, other Dok-mediated signaling pathways might be involved in this process. Dok proteins could play a role in signaling pathways required for the development of this lineage, such as those downstream of SLAM family receptors or other unidentified receptors important for the expansion of this particular subset of γδ T cells. Further analysis will provide clues to Dok-mediated regulation of this γδ T-cell lineage that share characteristics with the αβ NKT lineage and appear to be at the boundary of innate and adaptive immune responses.

MATHERIALS AND METHODS

DNA constructs

The 1.9 kbp Kpn I-Not I fragment containing human Dok-1 cDNA was excised from pUD and inserted intoMT073 plasmid harboring a hemagglutinin (HA) tag. The HA-Dok-1 sequence was excised from MT073-HA Dok-1 by Sma I-Hinc II digestion and inserted into the Sma I site of human CD2 VA-expressing vector kindly provided by Dr D. Kioussis (Zhumabekov, Corbella et al. 1995). The resulting construct was named pCD2-HA Dok-1.

Mice

C56BL/6 mice and 129/Sv mice were obtained from the Jackson Laboratories. Transgenic mice overexpressing Dok-1 protein were generated at the McIntyre Transgenic Core Facility (McGill University, Canada). Prokaryotic DNA sequence pBS SK from pCD2-HADok-1 plasmid was removed by a Sac II-Cla I digestion prior to microinjection. The purified fragment was microinjected into fertilized eggs of (CBA × C57BL/6) F1 mice. Dok-1 transgenic mice were backcrossed to C57BL/6 for at least ten generations to be on a C57BL/6 genetic background. 129/Sv Dok-$1^{-/-}$Dok-$2^{-/-}$ mice were previously described (Niki, Di Cristofano et al. 2004). C56BL/6 $SAP^{-}/^{+}$ mice were kindly provided by André Veillette (IRCM, Montréal, Canada) and Luo Yin (International Agency for Research on Cancer, Lyon, France). Male ($SAP^{-/0}$ and $SAP^{+/0}$) and female ($SAP^{+/+}$ and $SAP^{+}/^{-}$) littermates were used for experiments. All mice were maintained in our pathogen-free animal facilities (Institut Armand-Frappier, Laval, Canada).

Identification of transgenic founders

Transgenic founders were identified by PCR performed on genomic DNA from mice tails using primers specific for a 5' and a 3' sequence of human Dok-1 coding sequence. A set of primers specific for FasL was used as an internal control of the PCR reaction. Transgene expression was evaluated by immunoblotting total lysates from thymocytes and splenocytes with anti-Dok-1 Abs. Immunoblotting was performed as previously described (Nemorin, Laporte et al. 2001). Transgenic mice were backcrossed to C57BL/6 and transmission rate was analyzed by PCR.

Antibodies and reagents

Anti-CD28 mAbs (clone 37.51) and anti-TCRβ mAbs (clone H57–597) were kindly provided by J. Allison (Memorial Sloan-Kettering Cancer Center, New York, NY, USA) and were purified from hybridoma culture on protein G Hi-Trap affinity columns (Pharmacia Amersham). PE-anti-CD4 (clone RM4–5), FITC-anti-CD8α or PE-anti-CD8α (clone 53–6.7), PE-anti-CD44 (clone IM7), biotinylated anti-CD3ε (clone 145–2C11), PE-anti-CD122 (clone 5H4), PE-Nur77 (clone 12.14), allophycocyanin-anti-TCRβ (clone H57–597), allophycocyanin-anti-CD4 (clone L3T4), alexa647-anti-Eomesodermin (clone dan11mag), FITC-anti-TCRγδ (clone GL3), biotinylated anti-CD5 (clone 53–7.3), FITC-anti-CD69 (clone H1.2F3), PE-anti-IL-4 (clone 11B11), FITC-anti-IFN-γ (clone XMG1.2), and FITC-anti-CD24 (clone 30-F1) were purchased from eBioscience. PerCP-anti-CD8 (clone 53–6.7), PE-anti-IFN-γ (clone XMG1.2), PE-Vδ6.3/2 (clone 8F4H7B7), and Streptavidin PerCP were purchased from BD Biosciences. Biotinylated anti-Vγ1.1 TCR was kindly provided by Lynn Puddington (Farmington, CT, USA). Biotinylated anti-CD4 was obtained from purified hybridoma cultureMT4 on protein G column and biotinylated with EZ-Link Sulfo-NHS-LC-biotin (Pierce Biotechnology) according to the

manufacturer's instructions. Anti-phosphoPLC-γ1 (Tyr783), anti-phosphoLAT (Tyr171), anti-phosphoZAP-70 (Tyr319), and anti-total ERK1/2 were purchased from Cell Signaling Technology. Anti-phosphoDok-1 (Y362) was from ECM Biosciences. Alexa 647- anti-PLZF (clone 9E12) Abswere generated at the memorial Sloan-Kettering Cancer Center (MSKCC) antibody facility. Anti-mouse Dok-1 antibodies directed against the C-terminal region of murine Dok-1 were produced in rabbits using a glutathione S-transferase fusion protein encompassing amino acids 252–482 of murine Dok-1 (a gift from Dr. J. Nunes, Institut Paoli-Calmettes, Marseille, France). Mouse anti-Dok-1 Abs were adsorbed on GST-sepharose 4B beads and anti-Dok-1 Abs were immunoaffinity purified on GST/Dok-1 fusion protein coupled to sepharose beads as described (Beattie, Volsen et al. 1997). Immunoffinity purified Dok-1 Abs were labeled with R-PE using an R-PE-Antibody conjugation kit (Solulink) according to the manufacturer's instructions.

Cell isolation, magnetic beads enrichment, flow cytometry analysis, and intracellular cytokine detection

Cell suspensions from thymus and spleen were prepared as previously described (Dupuis, De Jesus Ibarra-Sanchez et al. 2003). Red blood cells were removed using ACK lysis buffer (150 mM NH_4Cl, 1 mM $KHCO_3$, and 0.1 mM Na_2EDTA, pH 7.2). Liver samples were pressed through a cell strainer with a 10-mL syringe piston in the presence of FACS buffer (PBS + 1% FCS + 0.01% NaN3). Cells were homogenized in 50 mL FACS buffer and centrifuged for 5 min at 1500 rpm. The pellet was resuspended and lymphocytes were separated by density gradient centrifugation over Ficoll-plaque plus solution (GE healthcare). After centrifugation, cells were washed twice with FACS buffer and stained with the appropriate antibodies as described before. To enrich TCRγδ thymocytes from WT mice, cells were labeled with biotinylated anti-TCRγδ

antibodies and purified with anti-biotin-coated magnetic beads from Miltenyi Biotec (Bergish Gladbach, Germany) according to the manufacturer's protocols. For intracellular IFN-γ or IL-4 detection, cells were stimulated with PMA (50 ng/mL) and ionomycin (500 ng/mL) in the presence of GolgiPlug (BD Pharmingen) for 4 h at 37°C. Surface and intracellular antigens were stained sequentially using a fixation and permeabilization kit from BD Biosciences according to the manufacturer's instructions. Viable cells were acquired on a FACSCalibur cytometer (Becton Dickinson) and FACS analysis was performed using CellQuest software or FCS Express software (De Novo software). For labeling of thymocyte precursors, cells were stained with PE-CD25, allophycocyanin-CD44, and a mixture of biotinylated Abs against CD4, CD8, TCRγδ, CD19, B220, CD11b, CD11c, Gr-1, CD49b (DX5), and Ter-119 followed with streptavidin-PerCP. Lineage-negative DN thymocytes, were electronically gated and analyzed for CD44 and CD25 expression.

DP and $CD8^+$ thymocyte purification

$CD4^+CD8^+$ thymocytes were purified from thymus of newborn mice by positive panning on Petri dishes coated with 1 μg/mL anti-CD8 (clone 83–12-5) for 60 min at 37°C followed by 3–5 washes to remove unbound cells. Adherent cells were recovered and plated on 2 μg/mL PNA-coated Petri dishes. Plates were incubated for 60 min at 37°C and washed three times to remove unbound cells. Adherent cells were recovered by washing at 37°C with 10 mL/plate of medium supplemented with 0.5 M Galactose. More than 90% of the recovered adherent cells were $CD4^+CD8^+$. $CD8^+$ thymocytes were purified from thymi of newborn mice by negative panning on Petri dishes coated with 5 μg/mL anti-CD4 (clone GK1.5) at 4°C. After 1 h 30 min, unbound cells were collected and washed twice with HBSS supplemented with 5% FCS. Cells were then plated on 2 μg/mL PNA-coated Petri dishes. Plates were incubated for 60 min at 37°C and

unbound cells were collected. $CD8^+$ SP thymocytes were further purified using CD8α antibody-coated magnetic beads from Miltenyi Biotec according to the manufacturer's protocols.

Q-RT-PCR

Total RNA extractions were performed on pelleted $CD8^+$-purified thymocytes with the Absolutely RNA Microprep Kit (Stratagene) as recommended by the manufacturer, except that DNAse treatments were done at 37°C. RNA quality and presence of contaminating genomic DNA was verified as previously described (Brosseau, Lucier et al. 2010). RNA integrity was assessed with an Agilent 2100 Bioanalyzer (Agilent Technologies). Reverse transcription was performed on 200 ng–2 μg total RNA with Transcriptor reverse transcriptase, random hexamers, dNTPs (Roche Diagnostics), and ten units of RNAseOUT (Invitrogen) following the manufacturer's protocol in a total volume of 20 μL. All forward and reverse primers were individually resuspended to 20–100 μM stock solution in Tris-EDTA buffer (Integrated DNA Technologies, Coralville, IA, USA) and diluted as a primer pair to 1 μM in RNase/DNase free water. Quantitative PCR (qPCR) reactions were performed in 10 μL in 96-well plates on a Realplex2 thermocycler (Eppendorf) with 5 μL of 2X FastStart Universal SYBR Green Master mix (Roche Diagnostics), 10 ng (3 μL) cDNA, and 200 nM final (2 μL) primer pair solutions. Reactions were performed in triplicate using the following cycling conditions: 10 min at 95°C; 50 cycles: 15 s at 95°C, 30 s at 60°C, 30 s at 72°C. Relative expression levels were calculated using the qBASE framework (Hellemans, Mortier et al. 2007) and the geometric average of three reference genes, UBC, HPRT1, and GAPDH. Primer design and validation was evaluated as described elsewhere (Brosseau, Lucier et al. 2010). Primer sequences are available upon request.

BrdU incorporation and cell cycle analysis

Mice were injected i.p. with 1 mg BrdU (in PBS). After 4 h, thymocytes were stained for cell surface markers, followed by fixation, permabilization, and intracellular staining for BrdU and 7-AAD following manufacturer's instructions (BD Biosciences).

Cell stimulation and detection of apoptosis

Cells (10^6) were left unstimulated or stimulated with plate-bound anti-TCRβ mAbs in the presence of soluble anti-CD28 mAbs at 10 μg/mL. mAbs were coated on plastic at 10 μg/mL in PBS at $4^{\circ C}$ overnight. After 8 and 16 h, cells were harvested and the percentage of thymocytes undergoing apoptosis was determined by staining cells with annexin V-PE plus 7-amino-actinomycin D (7-AAD) according to the manufacturer's instructions (BD PharMingen).

Immunoblot analysis

DP-purified thymocytes were washed in RPMI 1640 and resuspended at 4×10^7 cells/mL in RPMI-1640. Cells were left unlabeled or labeled with biotinylated anti-CD3 (10μg/mL) alone or in combination with biotinylated anti-CD4 (10μg/mL) for 30 min on ice and then washed once with RPMI 1640. Ig was cross-linked at $37^{\circ C}$ with avidin (20μg/mL, Jackson ImmunoResearch Laboratories, Inc). Cells were harvested and lysed in 1% NP-40 containing 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM $MgCl_2$, and 1 mM EGTA in the presence of protease and phosphatase inhibitors. Immunoblotting was performed as previously described (Nemorin, Laporte et al. 2001)

Statistical analysis

All statistical analysis was performed with prism software (Graphpad). Two groups were compared using two-tailed unpaired or paired Student's t test when appropriated. Differences were considered to be statistically significant when $p < 0.05$. The p values were defined as following: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$, $****p < 0.0001$ and ns, non-significant. Graph results are shown as the mean ± SEM.

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Conflicts of interest: The authors declare no financial or commercial conflict of interest.

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Publication no.2

CD28 controls the development of innate-like $CD8^+$ T cells by promoting the functional maturation of NKT cells

CD28 controls the development of innate-like $CD8^+$ T cells by promoting the functional maturation of NKT cells

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SUMMARY

NK T cells (NKT cells) share functional characteristics and homing properties that are distinct from conventional T cells. In this study, we investigated the contribution of CD28 in the functional development of γδ NKT and αβ NKT cells in mice. We show that CD28 promotes the thymic maturation of promyelocytic leukemia zinc finger^{+} IL-4^{+} NKT cells and upregulation of LFA-1 expression on NKT cells. We demonstrate that the developmental defect of γδ NKT cells in CD28-deficient mice is cell autonomous. Moreover, we show in both wild-type C57BL/6 mice and in downstream of tyrosine kinase-1 transgenic mice, a mouse model with increased numbers of γδ NKT cells, that CD28-mediated regulation of thymic IL-4^{+} NKT cells promotes the differentiation of eomesodermin^{+} CD44^{high} innate-like CD8^{+} T cells. These findings reveal a previously unappreciated mechanism by which CD28 controls NKT-cell homeostasis and the size of the innate-like CD8^{+} T-cell pool.

Keywords: CD28, Innate-like CD8 T cells, NKT cells, PLZF, Thymocyte development

INTRODUCTION

NK T cells (NKT cells) have unique phenotypic and functional characteristics when compared to conventional T cells. Within the NKT-cell population, there are distinct subsets including CD1d-restricted TCRαβ NKT cells (hereafter referred to as αβ NKT cells) and TCRγδ NKT cells (hereafter referred to as γδ NKT cells). These two subsets share common properties. They exhibit an "activated" phenotype; secrete cytokines upon primary stimulation, and most of them express highly restricted repertoires. The development of αβ NKT cells has been extensively studied and is well characterized (Das, Sant'Angelo et al. 2010). In contrast, the development of γδ NKT cells is poorly defined. αβ NKT cells develop from $CD4^{+}CD8^{+}$ double positive thymocytes and type I αβ NKT cells use a TCR composed of an invariant Vα14-Jα18 TCR-α chain that recognizes glycolipids presented by CD1d (Lantz and Bendelac 1994, Gapin, Matsuda et al. 2001). Although not formally demonstrated by fate-mapping experiments, γδ NKT cells likely arise from thymocytes at the $CD4^{-}CD8^{-}$ double-negative stage similar to conventional γδ T cells. In C57BL/6 mice, most of the γδ NKT cells express Vγ1.1 and Vδ6.3 (Kreslavsky, Savage et al. 2009). The selecting ligands of γδ NKT cells are unknown, but are likely expressed on other thymocytes rather than stromal cells since γδ NKT-cell development is dependent on the signaling lymphocytic activation molecule-associated protein pathway (Kreslavsky, Savage et al. 2009). Moreover, our previous results (Besin, Yousefi et al. 2012) together with work from other groups (Alonzo and Sant'Angelo 2011), have clearly established that attenuated TCR signaling promotes the development of γδ NKT-cell subsets. Maturation of αβ NKT cells progresses through well-defined stages (Godfrey and Berzins 2007). Recently, the phenotypic, functional, and transcriptional changes occurring during γδ NKT-cell maturation were characterized (Narayan, Sylvia et al. 2012, Pereira and Boucontet 2012, Yin, Cho et al.

2013). The functional developmental intermediates of αβ and γδ NKT cells in the thymus can be distinguished by expression of promyelocytic leukemia zinc finger (PLZF) and NK1.1 (Pereira and Boucontet 2012). PLZF is a transcription factor that controls the development and the acquisition of effector properties of αβ and γδ NKT cells and is absent in conventional T cells (Kovalovsky, Uche et al. 2008, Savage, Constantinides et al. 2008, Kreslavsky, Savage et al. 2009, Alonzo and Sant'Angelo 2011). More importantly, high expression of PLZF confers the capacity of NKT cells to produce IL-4 (Pereira and Boucontet 2012). In different mouse models, high level of IL-4 production by $PLZF^+$ thymic NKT cells has been shown to drive innate-like $CD8^+$ development through induction of Eomesodemin (Eomes) expression (Lee, Jameson et al. 2011). These Ag-naive $CD8^+$ T cells have a memory-like phenotype and by their capacity to rapidly produce IFN-γ may act as first responders during an immune response. Therefore, regulation of the size of PLZF-expressing cell population might have important functional consequences. The level of PLZF expression is linked to the maturation stage of NKT cells (Kovalovsky, Uche et al. 2008, Savage, Constantinides et al. 2008, Narayan, Sylvia et al. 2012, Pereira and Boucontet 2012). Although, high levels of Egr2 induced by TCR- and SLAMF6-mediated signaling is clearly important for PLZF induction (Seiler, Mathew et al. 2012, Dutta, Kraus et al. 2013), the signals controlling the development of PLZF-expressing cells are incompletely defined (Alonzo and Sant'Angelo 2011). Several factors and receptors selectively control the early maturation stages of postselected NKT cells (Matsuda and Gapin 2005, Godfrey and Berzins 2007, Sintes, Cuenca et al. 2013). In particular, the CD28/B7 interaction was shown to be essential for the development of αβ NKT cells at an early stage of maturation (Chung, Nurieva et al. 2008, Williams, Lumsden et al. 2008, Zheng, Zhang et al. 2008). In this study, we analyze whether the absence of CD28 correlates with defects in the generation of both PLZF–

expressing αβ and γδ NKT cells. Our results demonstrate that CD28 controls the number of $PLZF^+$ cells and thereby modulates the functional capacity of NKT cells to produce IL-4 during thymic development. These findings describe a mechanism by which CD28 regulates the generation of $Eomes^+$ memory-like $CD8^+$ T cells.

RESULTS

γδ NKT-cell development depends on CD28

CD28/B7 interaction regulates intrathymic expansion and differentiation of postselected αβ NKT cells (Chung, Nurieva et al. 2008, Williams, Lumsden et al. 2008). We predicted that the development of γδ NKT cells would also be affected by CD28 deficiency given the shared properties and developmental program of αβ and γδ NKT cells. To investigate whether CD28 was involved in γδ NKT-cell development, we compared the percentage and absolute numbers of γδ NKT cells in WT and *Cd28*$^{-/-}$ C57BL/6 mice using the expression of Vδ6.3 as a marker for γδ NKT cells. In the thymus, liver, and spleen, there was a significant decrease in both the number and frequency of Vδ6.3^{+} cells in *Cd28*$^{-/-}$ mice (Fig. 1A and B). As expected, CD28 deficiency did not affect the numbers of Vδ6.3^{-} γδ thymocytes (Fig. 1A). PLZF, which is mainly expressed on NKT cells in mice, is a key factor for the development and effector function of these cells (Alonzo and Sant'Angelo 2011). In the γδ lineage, PLZF is mainly expressed in Vδ6.3^{+} cells but is also detected in a small percentage of Vδ6.3^{-} T cells (Kreslavsky, Savage et al. 2009, Alonzo, Gottschalk et al. 2010). We compared the number of PLZF^{+} bona fide γδ NKT cells in WT and *Cd28*$^{-/-}$ mice. As shown in Fig. 2A, the number of PLZF^{+} γδ NKT cells is dramatically reduced in *Cd28*$^{-/-}$ mice. Moreover, as previously reported (Kreslavsky, Savage et al. 2009, Alonzo, Gottschalk et al. 2010), the vast majority of Vδ6.3^{+} cells expressed PLZF in WT C57BL/6 (Fig 2B). In contrast, in absence of CD28, only 52% of Vδ6.3^{+} cells are PLZF^{+} (Fig. 2B). Importantly, the number of PLZF^{+} Vδ6.3^{+} NKT cells is dramatically reduced in *Cd28*$^{-/-}$ mice whereas the number of PLZF^{-} Vδ6.3^{+} cells is less affected (Fig. 2C). Altogether these results demonstrate that CD28 specifically regulates the development of the PLZF-expressing γδ T-cell lineage.

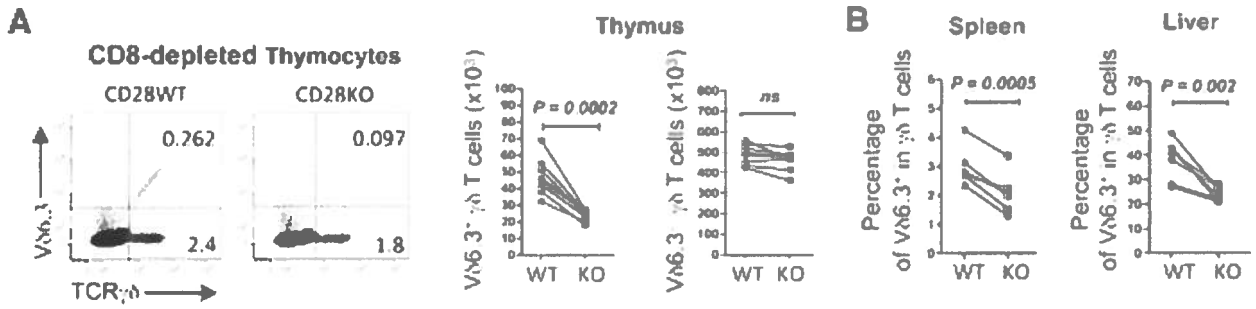


Figure 1. CD28 controls the number of γδ NKT cells in thymus, spleen, and liver. (A) $CD8^+$-depleted thymocytes from 4–5-week-old age-matched or littermate WT and *Cd28*$^{-/-}$(KO) mice were stained with antibodies against TCRγδ and Vδ6.3. Representative dot plots of eight pairs of mice are shown (left). Absolute numbers of Vδ6.3$^+$ NKT cells and Vδ6.3$^-$ γδ T cells in individual thymi are indicated (right). (B) Splenocytes and liver cells from 4-5-week-old age-matched or littermate WT and CD28 KO were stained with antibodies against TCRγδ and Vδ6.3. The percentage of Vδ6.3$^+$ T cells on gated TCRγδ T cells is indicated for six and seven pairs of mice. Each pair corresponds to an independent experiment. Statistical significance was determined with a two-tailed paired Student's t-test. ns: not significant.

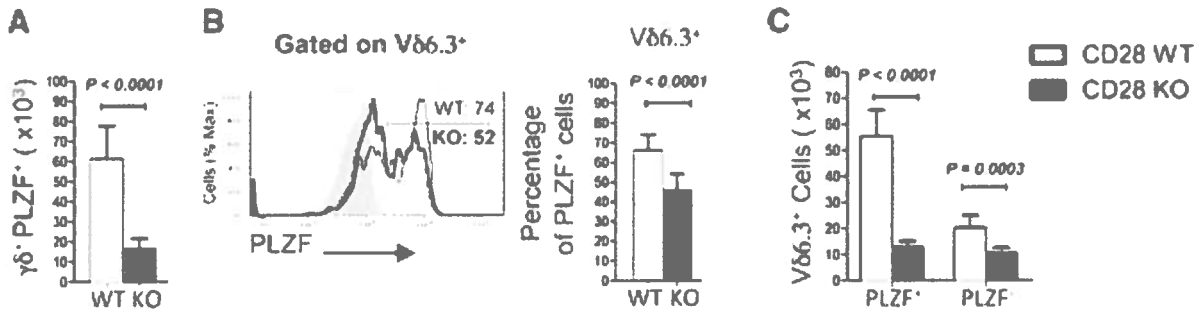


Figure 2. CD28 controls the pool size of PLZF$^+$ γδNKT cells. (A) Thymocytes from 4–6-week-old C57BL/6 WT and *Cd28*$^{-/-}$ (KO) mice were depleted of CD8$^+$ cells and stained for cell surface expression of TCRγδ and Vδ6.3 followed by intracellular staining for PLZF expression. Quantification of absolute numbers of PLZF$^+$ γδ T cells is indicated. (B) Representative histograms show the PLZF expression of Vδ6.3$^+$ γδ T cells in WT (thin line histogram), in *Cd28*$^{-/-}$ (thick line histogram) and as a negative control in WT Vδ6.3$^-$ γδ T cells (filled histogram). Bracketed lines next to the graphs indicate the percentages of PLZF$^+$ within Vδ6.3$^+$ thymocytes. The percentages of PLZF$^+$ in Vδ6.3$^+$ WT and *Cd28*$^{-/-}$ (KO) thymocytes for eight pairs of mice are shown (right). (C) Absolute numbers of PLZF$^+$ and PLZF$^-$ Vδ6.3$^+$ WT and *Cd28*$^{-/-}$ (KO) thymocytes are indicated. Data are representative of eight pairs of mice pooled from five independent experiments. The data are shown as the mean + SD of all mice tested. Statistical significance was determined with a two-tailed paired Student's t-test.

CD28 controls the maturation of γδ NKT cells after CD24 downregulation

CD44, NK1.1, and CD24 are useful cell surface markers to distinguish the maturation steps of thymic αβ NKT cells, from stage 0 ($CD24^{high}$ $CD44^{low}NK1.1^-$), to stage 1 ($CD24^{low}CD44^{low}NK1.1^-$), stage 2 ($CD24^{low}CD44^{high}$ $NK1.1^-$), and stage 3 ($CD24^{low}CD44^{high}NK1.1^+$) (Matsuda and Gapin 2005). Recent studies indicate that γδ NKT cells likely progress through similar developmental stages as αβ NKT cells in the thymus (Narayan, Sylvia et al. 2012, Pereira and Boucontet 2012, Yin, Cho et al. 2013). We performed CD24, CD44, and NK1.1 cell surface staining on $V\delta6.3^+$ cells. $V\delta6.3^+$ cells are largely $CD24^{low}$ in the thymus of WT mice (Fig. 3A). There was a significant increase in the fraction of $CD24^{high}$ γδ NKT cells in *Cd28*$^{-/-}$ mice compared with that in WT mice, mainly in the $CD44^{low}$ $NK1.1^-$ cell population (stage 0) (Fig. 3A and Supporting Information Fig. 1). The increased proportion of $CD24^{high}$ $CD44^{low}NK1.1^-$ $V\delta6.3^+$ cells (stage 0) most likely reflected a partial block in the transition to the later maturation stages of *Cd28*$^{-/-}$ thymic γδ NKT cells. Analysis of $CD24^{low}$ $V\delta6.3^+$ cells revealed an increase in the percentage of the $CD44^{low}NK1.1^-$ cell subset (stage 1) with a concomitant decrease in the percentage of the $CD44^{high}$ cell subsets (stages 2 and 3) in *Cd28*$^{-/-}$ mice compared to WT mice (Fig. 3B). These results indicate that the upregulation of CD44 in thymic $V\delta6.3^+$cells is greatly dependent on CD28. In αβ NKT cells, PLZF expression increases after positive selection, is highest in $NK1.1^-CD44^{low}$ cells, and decreases as differentiation proceeds to stage 3 (Kovalovsky, Uche et al. 2008, Savage, Constantinides et al. 2008). We analyzed the expression of PLZF at different stages of γδ NKT cell maturation (Fig. 3C). PLZF is induced at stage 0 is maximum at stages 1 and 2, and decreased at stage 3 (Fig. 3C). The developmental regulation of PLZF expression of WT γδ NKT cells is therefore similar to that of αβ NKT cells. In the absence of CD28, the percentage of $V\delta6.3^+$ cells that are $PLZF^+$

decreased at stages 1 and 2. Similar to WT cells, all the NK1.1^{+} Vδ6.3^{+} cells are PLZFdim at stage 3 (Fig. 3C). The absolute number of stage 0 (CD24^{high} CD44^{low}NK1.1^{-}) PLZF^{+} Vδ6.3^{+} cells was comparable in *Cd28*$^{-/-}$ and *Cd28*$^{+/+}$ mice (Fig. 3C and D). Because PLZF^{+} CD24^{high} CD44^{low} thymocytes mainly correspond to cells that have just undergone positive selection, we can conclude that positive selection of γδ NKT cells seems to occur normally in *Cd28*$^{-/-}$ mice. In the absence of CD28, there was a clear decrease in the number of Vδ6.3^{+} PLZF^{+} cells after stage 0 (Fig. 3D). Altogether, our data suggest that CD28 controls the maturation of γδ NKT cells. PLZF high γδ NKT cells do not express NK1.1 and constitute the actively proliferating population (Pereira and Boucontet 2012). The reduced number of PLZF^{+} γδ NKT cells in *Cd28*$^{-/-}$ mice was not a direct consequence of altered proliferative capacity in the thymus as a comparable percentage of PLZF^{+} γδ NKT cells incorporated BrdU after a 4 h pulse in vivo in WT and *Cd28*$^{-/-}$ mice (Fig. 3E). Altogether, these results clearly indicate that CD28 is critical for further maturation and/or survival of CD24^{low} positively selected γδ NKT cells and further substantiate the important role of CD28 in the control of NKT-cell thymic maturation. The high level of CD28 expression in PLZF^{+} γδ NKT cells is consistent with this hypothesis (Supporting Information Fig. 2).

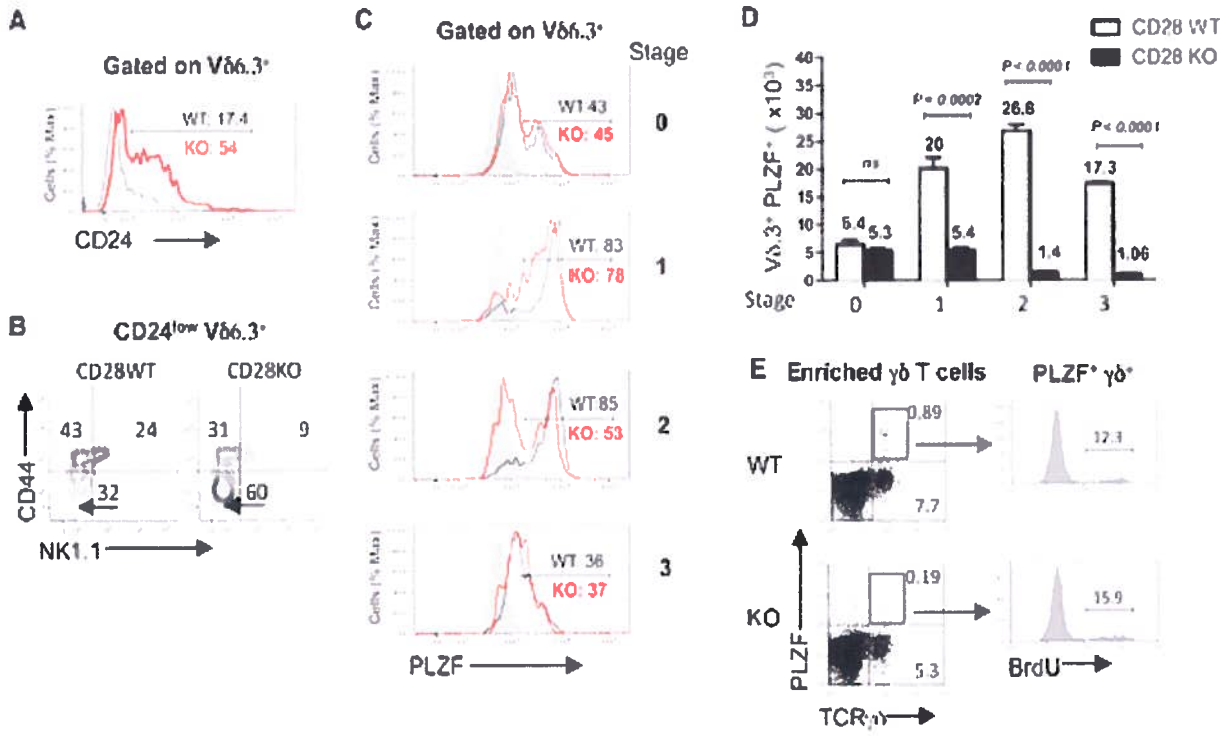
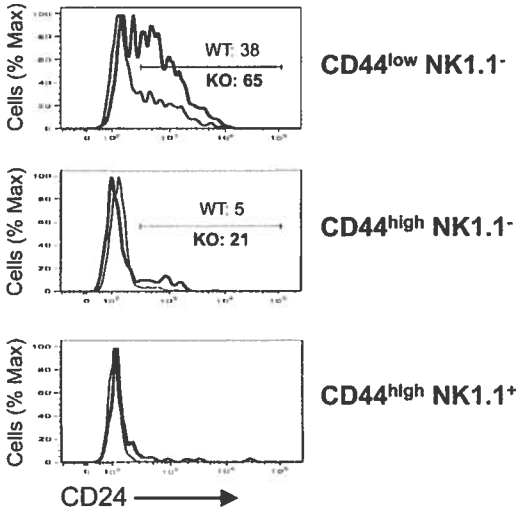
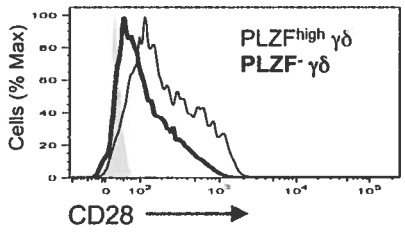


Figure 3. CD28 regulates thymic maturation of γδ NKT cells. $CD8^+$-depleted thymocytes from 4-week-old age-matched WT and *Cd28*$^{-/-}$ (KO) mice were stained for cell surface expression of TCRγδ, Vδ6.3, CD24, CD44, and NK1.1 followed by intracellular staining for PLZF expression. (A) Representative histograms show CD24 expression of $V\delta6.3^+$ γδ T cells in WT (black line) and *Cd28*$^{-/-}$ (red line). Bracketed lines next to the graphs indicate the percentages of $CD24^{high}$ in WT and CD28KO $V\delta6.3^+$cells. (B) Representative contour plots of CD44 and NK1.1 expression on gated $CD24^{low}TCR\gamma\delta^+V\delta6.3^+$ thymocytes are shown for WT and *Cd28*$^{-/-}$ (KO) mice. Percentages of cells in each quadrant are indicated. (C) Representative histograms show PLZF expression of WT (black line) and *Cd28*$^{-/-}$ (red line) $V\delta6.3^+$ γδ T cells at stage 0 ($CD24^{high}$ $CD44^{low}NK1.1^-$), stage 1 ($CD24^{low}CD44^{low}NK1.1^-$), stage 2 ($CD24^{low}CD44^{high}$ $NK1.1^-$), and stage 3 ($CD24^{low}CD44^{high}$ $NK1.1^+$). Bracketed lines next to the graphs indicate the percentages of $PLZF^+V\delta6.3^+$cells. (D) Quantification of the absolute number of WT and *Cd28*$^{-/-}$ (KO) $PLZF^+$ $V\delta6.3^+$cells in stages 0–3 of maturation. Data are shown as mean + SD of three

pairs of mice, each pair examined in an independent experiment. Statistical significance was determined with an unpaired Student's t-test. (E) Cells from 3-week-old littermate WT and *Cd28*$^{-/-}$ (KO) mice were labeled with BrdU. Enriched-TCRγδ thymocytes were stained for TCRγδ, followed by PLZF and BrdU staining. BrdU incorporation is shown for gated PLZF^{+} TCRγδ^{+} thymocytes. Data are representative of two pairs of mice analyzed.



Supplemental Figure 1: CD24 expression in different maturation stages of gd NKT cells. Thymocytes from C57BL/6 mice were depleted of CD8^{+} cells and stained for cell surface expression of Vδ6.3, CD24, CD44 and NK1.1. Representative histograms show the expression of CD24 in gated WT (thin line) or *Cd28*$^{-/-}$ (thick line) Vδ6.3^{+} cells at different stages of maturation based on the expression of CD44 and NK1.1. Data are representative of 3 pairs of 4-wk-old age matched WT and *Cd28*$^{-/-}$ mice. Bracketed lines show the percentage of CD24high in WT and CD28KO γδ NKT cells in each stage.



Supplemental Figure 2: CD28 expression in γδ NKT cells. Thymocytes were depleted of $CD8^+$ cells and stained for cell surface expression of TCRγδ, CD3 and CD28 followed by intracellular staining for PLZF expression. Representative histograms show the expression of CD28 in gated $PLZF^{high}$ (thin line) and $PLZF^-$ (thick line) γδ T cells from thymi of 5 wks-old WT mice and in gated γδ $PLZF^{high}$ T cells from CD28KO control mice (filled histogram). Data are representative of 4 mice in two independent experiments.

CD28 controls LFA-1 upregulation in NKT cells

Recently, PLZF was shown to control the induction of high levels of LFA-1 on tissue-resident αβ NKT cells (Thomas, Scanlon et al. 2011). We, therefore, analyzed whether CD28 regulates LFA-1 cell surface expression on NKT cells. Remarkably, in the thymus and liver, CD28-deficient γδ NKT cells exhibited reduced levels of LFA-1 cell surface expression (Fig. 4). This reduction was specific to NKT cells as similar levels of LFA-1 were detected on CD28-deficient and WT conventional γδ T cells (Fig. 4). Similarly, we observed a reduced LFA-1 expression on CD1d -tetramer^{+} αβ NKT cells in the liver of CD28-deficient compared to WT mice (Supporting Information Fig. 3). Thus, CD28 deficiency results in impaired upregulation of PLZF and LFA-1 expression in NKT cells.

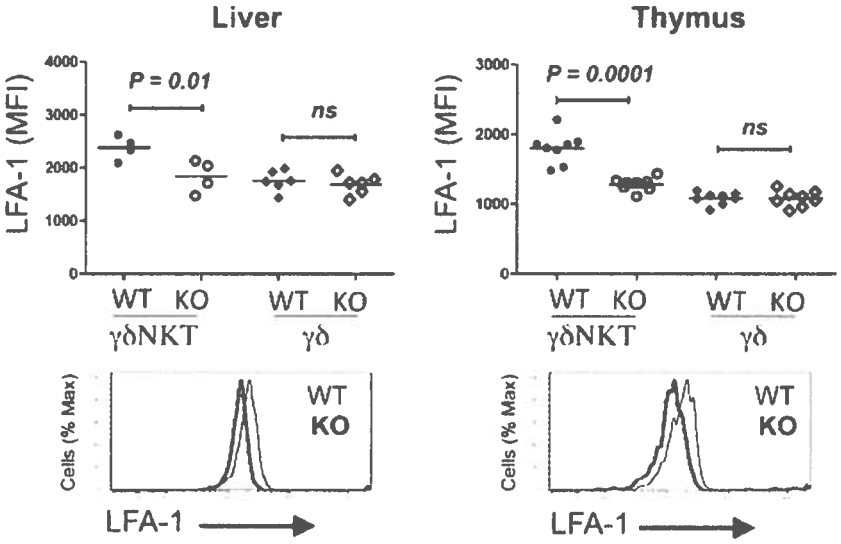
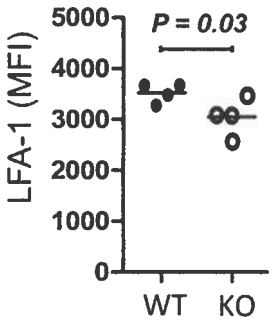


Figure 4. CD28 controls LFA-1 upregulation in γδ NKT cells. Quantification of LFA-1 median fluorescence intensity (MFI) of Vδ6.3$^+$ γδ T cells (γδ NKT), Vδ6.3$^-$ γδ T cells (γδ) from liver and thymus of WT and *Cd28*$^{-/-}$ (KO) mice is shown. Data are representative of four to eight pairs of mice pooled from three independent experiments. Horizontal bars indicate the mean. Representative LFA-1 histograms on gated Vδ6.3$^+$ γδ T cells from liver or thymus of WT (thin line histogram) and CD28 KO (thick line histogram) mice are shown (bottom). Statistical significance was determined with a two-tailed paired Student's t-test. ns: not significant.



Supplemental Figure 3: LFA-1 expression in αβ NKT cells in the liver. Quantification of LFA-1 median fluorescence intensity (MFI) of CD1d-Tetramer^{+} αβ NKT cells from liver of WT and *Cd28*$^{-/-}$ (KO) mice is shown. Data are representative of 4 pairs of mice 5-6 wk-old in 2 independent experiments Horizontal bars indicate the mean. Statistical significance was determined with an unpaired Student's *t* test.

CD28 is required for NKT-cell maturation by cell-intrinsic mechanisms

To determine whether the development of γδ NKT cells is dependent on CD28 in a cell autonomous manner, we generated mixed BM chimeras. After lethal irradiation, CD45.1/2 WT mice were reconstituted with a 1:1 ratio of CD45.1 BM cells from WT mice and CD45.2 BM cells from WT or *Cd28*$^{-/-}$ mice. After 6 weeks, γδ NKT thymocytes from the mixed BM chimeras were analyzed for expression of cell surface markers (Fig. 5). We observed a slight increase in the percentage of immature CD44^{low}NK1.1^{-} (stages 0–1) γδ NKT-cell subset, while the percentage of CD44^{high}NK1.1^{-} and CD44^{high}NK1.1^{+} stages 2–3, respectively, γδ NKT-cell subsets were significantly reduced in the *Cd28*$^{-/-}$ mice (Fig. 5A and B). More importantly, the proportion of PLZF^{+} αβ and γδ NKT thymocytes was significantly reduced in the absence of CD28 (Fig. 5C). These findings demonstrate that the impaired development of NKT cells observed in *Cd28*$^{-/-}$ mice could not be rescued with WT BM cells. Thus, the defect in the development of *Cd28*$^{-/-}$ PLZF^{+} NKT cells is cell-autonomous.

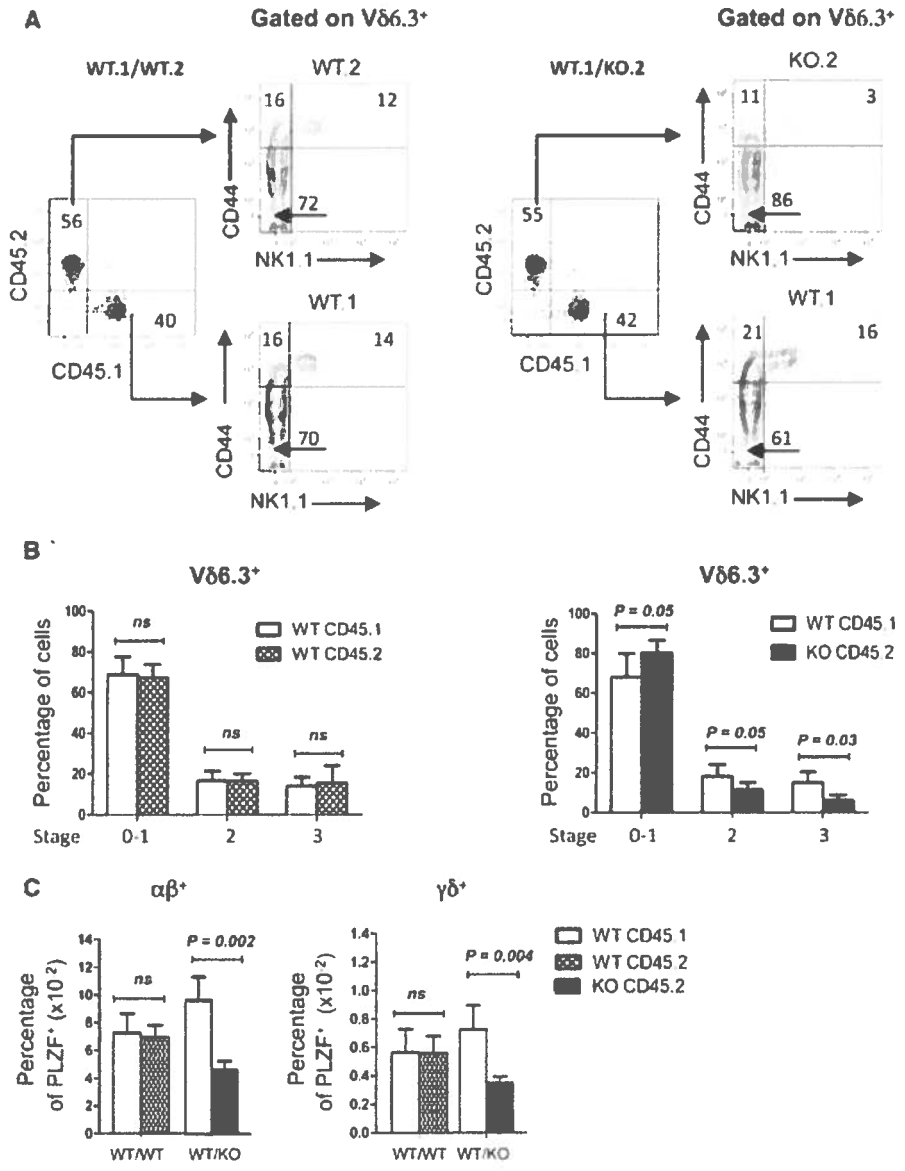


Figure 5. Cell-intrinsic NKT-cell defects in CD28 KO mice. CD45.1$^+$ WT (WT.1) and CD45.2$^+$ WT or *Cd28*$^{-/-}$ (WT.2 or KO.2) bone marrow cells were mixed at a 1:1 ratio and transferred into lethally irradiated CD45.1/2 hosts. (A) CD8$^+$-depleted thymocytes were stained for CD45.1, CD45.2, TCRγδ, Vδ6.3, CD44, and NK1.1. The relative proportions of CD45.1 and CD45.2 thymocytes in chimeras reconstituted with a mix of WT CD45.1 and WT CD45.2 BM (WT.1/WT.2; left) and a mix of WT CD45.1 and *Cd28*$^{-/-}$ CD45.2 BM are shown (WT.1/KO.2; right). Representative contour plots show CD44 and NK1.1 expression on gated Vδ6.3$^+$ CD45.1

and CD45.2 thymocytes. Data are representative of six individual chimeras. (B) The percentage of thymic Vδ6.3^{+}cells at stages 0–1 (CD44^{low}NK1.1^{-}), stage 2 (CD44^{high}NK1.1^{-}), and stage 3 (CD44^{high}NK1.1^{+}) is indicated for six chimeras WT.1/WT.2 and six chimeras WT.1/KO.2. Data are shown as mean + SD of six individual chimeras from two independent experiments. (C) Quantifications of the proportion of PLZF^{+} γδ and αβ T cells are indicated for six chimeras. Data are shown as mean + SD. Statistical significance was determined with a paired Student's t-test.

CD28-mediated regulation of IL-4$^+$ NKT cells promotes the differentiation of innate-like CD8$^+$ T cells

The ability of NKT cells to rapidly secrete large amounts of cytokines is dependent on their stage of maturation (Benlagha, Kyin et al. 2002, Gadue and Stein 2002). In particular, high PLZF expression confers the capacity of NKT cells to produce high levels of IL-4 (Pereira and Boucontet 2012). Since CD28 regulates the number of PLZF-expressing cells, we predicted that CD28 deficiency would interfere with the production of IL-4 by NKT cells. As previously reported (Pereira and Boucontet 2012), the majority of γδ NKT cells that produced high levels of IL-4 after stimulation with PMA and ionomycin were in the PLZF^{high} cell population (Supporting Information Fig. 4A). In *Cd28*$^{-/-}$ mice, we observed a 3- and 12-fold decrease in the proportion of αβ and γδ PLZF$^+$ IL-4$^+$ cells, respectively (Fig. 6A). The negative effect of CD28 on IL-4 production is due to the decreased frequency of PLZF$^+$ NKT cells (CD8−depleted thymocytes; 0.74%, *Cd28*$^{+/+}$ and 0.29%, *Cd28*$^{-/-}$ for αβ NKT cells; 0.10%, *Cd28*$^{+/+}$ and 0.02%, *Cd28*$^{-/-}$ for γδ NKT cells; Fig 6B) and to the decreased percentage of IL-4-producing cells among PLZF$^+$ NKT cells, especially for γδ NKT cells (35%, *Cd28*$^{+/+}$ and 15% *Cd28*$^{-/-}$, Fig. 6B). CD28 deficiency affected more IFN-γ/ IL-4 double-producing than IL-4 single-producing PLZF γδ NKT cells (Supporting Information Fig. 4A). Given that CD24^{high} produces less IL-4 than CD24^{low} PLZF$^+$ NKT cells (Supporting Information Fig. 4B), the reduced production of IL-4 among PLZF$^+$ γδ NKT cells in *Cd28*$^{-/-}$ C56BL/6 mice is due to the higher proportion of functionally immature CD24^{high} PLZF$^+$ γδ NKT cells compared to *Cd28*$^{+/+}$ mice (12% in *Cd28*$^{+/+}$ versus 31% in *Cd28*$^{-/-}$ mice, Fig. 3D and Supporting Information Fig. 4B). To further substantiate the CD28-mediated regulation of IL-4 production in NKT cells, we used downstream of tyrosine kinase-1 (Dok-1) transgenic mice. In these mice, there was an expanded γδ NKT cell population due to Dok-1

overproduction in thymocytes (Besin, Yousefi et al. 2012). In Dok-1 transgenic mice, the majority of PLZF$^+$ thymocytes were γδ T cells, whereas in C57BL/6 WT mice, the majority of thymic PLZF$^+$ NKT cells corresponded to αβ NKT cells, (Fig. 6B and D). In addition, there was a 79-fold increase in the percentage of PLZF$^+$ IL-4$^+$ γδ NKT cells compared with that in C57BL/6 WT mice, while the percentage of PLZF$^+$ IL-4$^+$ αβ NKT cells was comparable with that in C57BL/6 mice (Fig. 6A and C). Maturation of PLZF$^+$ NKT cells in Dok-1 transgenic mice occurred normally, except for the lower percentage of stage 0 (CD24^{high}) PLZF$^+$ Vδ6.3$^+$ cells compared to C57BL/6 mice (Supporting Information Fig. 4C and D). Moreover, CD28 regulated the same maturation steps in C57BL/6 and Dok-1 transgenic mice although with quantitative differences (Supporting Information Fig. 4C–E). Importantly, as shown for C57BL/6 mice, in the absence of CD28, there was a significant reduction in the frequency of PLZF$^+$ IL-4$^+$ NKT cells in Dok-1 transgenic mice which was mainly due to the decreased proportion of αβ and γδ PLZF$^+$ NKT cells (0.12%, *Cd28*$^{+/+}$ and 0.06%, *Cd28*$^{-/-}$ for αβ NKT cells; 0.65%, *Cd28*$^{+/+}$ and 0.18%, *Cd28*$^{-/-}$ for γδ NKT cells, Fig. 6 C and D). CD28 deficiency in Dok-1 transgenic mice did not alter significantly the ratio of immature CD24^{high}/mature CD24^{low} PLZF$^+$ γδ NKT cells and therefore did not change the overall capacity of PLZF$^+$ γδ NKT cells to produce IL-4 or IFN-γ (Fig. 6D and Supporting Information Fig. 4F). The generation of Eomes$^+$ CD44^{high} memory-like CD8$^+$ T cells has been shown to be dependent on IL-4 produced by PLZF$^+$ NKT cells in several mouse models (Verykokakis, Boos et al. 2010, Weinreich, Odumade et al. 2010, Lee, Jameson et al. 2011, Min, Lee et al. 2011, Sharma, Chen et al. 2012). The percentage of CD8$^+$ thymocytes that were Eomes$^+$ CD44^{high} was reproducibly lower in *Cd28*$^{-/-}$ than in *Cd28*$^{+/+}$; C57BL/6 mice (1.75%, *Cd28*$^{+/+}$; 0.46%, *Cd28*$^{-/-}$, Fig. 7A). Moreover, the percentage of CD44^{high} cells that produced IFN-γ following PMA plus ionomycin was reduced in *Cd28*$^{-/-}$ mice (Fig.

7A). However, the difference did not reach statistical significance most likely because of the small number of innate-like $CD8^+$ T cells in C57BL/6 mice. To confirm that CD28-mediated regulation of $PLZF^+IL\text{-}4^+$ NKT cells participates in the generation of innate-like $CD8^+$ T cells, we analyzed Dok-1 transgenic mice, a mouse strain that generates larger numbers of $PLZF^+$ NKT cells than C57BL/6 mice. In these mice, a high percentage of the $CD8^+$ thymocytes have an innate-like phenotype induced by an increased frequency of IL-4-producing γδ NKT cells (Fig. 7B and (Besin, Yousefi et al. 2012)). As shown in Figure 7B, 50% of the $CD8^+$ T cells from 4-week-old mice, expressed Eomes, a transcription factor upregulated in innate-like $CD8^+$ T cells (Lee, Jameson et al. 2011). A high percentage of these $Eomes^+$ $CD8^+$ cells expressed markers associated with activated/memory phenotype such as CD44, CXCR3, and CD122 (Fig. 7C). In addition, there was an increase in the expression levels of CD124 in the $CD8^+$ T cells from Dok-1 transgenic mice compared with those in C57BL/6 mice that corresponded to the specific upregulation of CD124 in $Eomes^+$ cells compared with that in $Eomes^-$ cells (Fig. 7D). Since IL-4 signaling leads to increased surface expression of CD124 (Ohara and Paul 1988), this result suggests that $CD8^+$ T cells have been stimulated with IL-4 during their development in the thymus of Dok-1 transgenic mice. Finally, the increased expression of Eomes correlated with enhanced production of IFN-γ after ex vivo stimulation (Fig. 7E). Remarkably, the reduced number of $PLZF^+$ $IL\text{-}4^+$ NKT cells in *Cd28*$^{-/-}$ Dok-1 transgenic mice compared to WT Dok-1 transgenic mice correlated with the reduced number of innate-like $CD8^+$ thymocytes (Fig. 7B–E). It should be noted that the remaining higher frequency of $IL\text{-}4^+$ NKT cells in Cd28$^{-/-}$ Dok-1 transgenic mice compared to C57BL/6 mice (8.9% *Cd28*$^{-/-}$ Dok-1 Tg and 4.1% WT, Fig. 6A and C) led to the development of a higher percentage of innate-like $CD8^+$ T cells. Collectively, these

results suggest that CD28 positively contributes to the generation of innate-like $CD8^+$ T cells through the control of IL-4^+ $PLZF^+$ NKT-cell development.

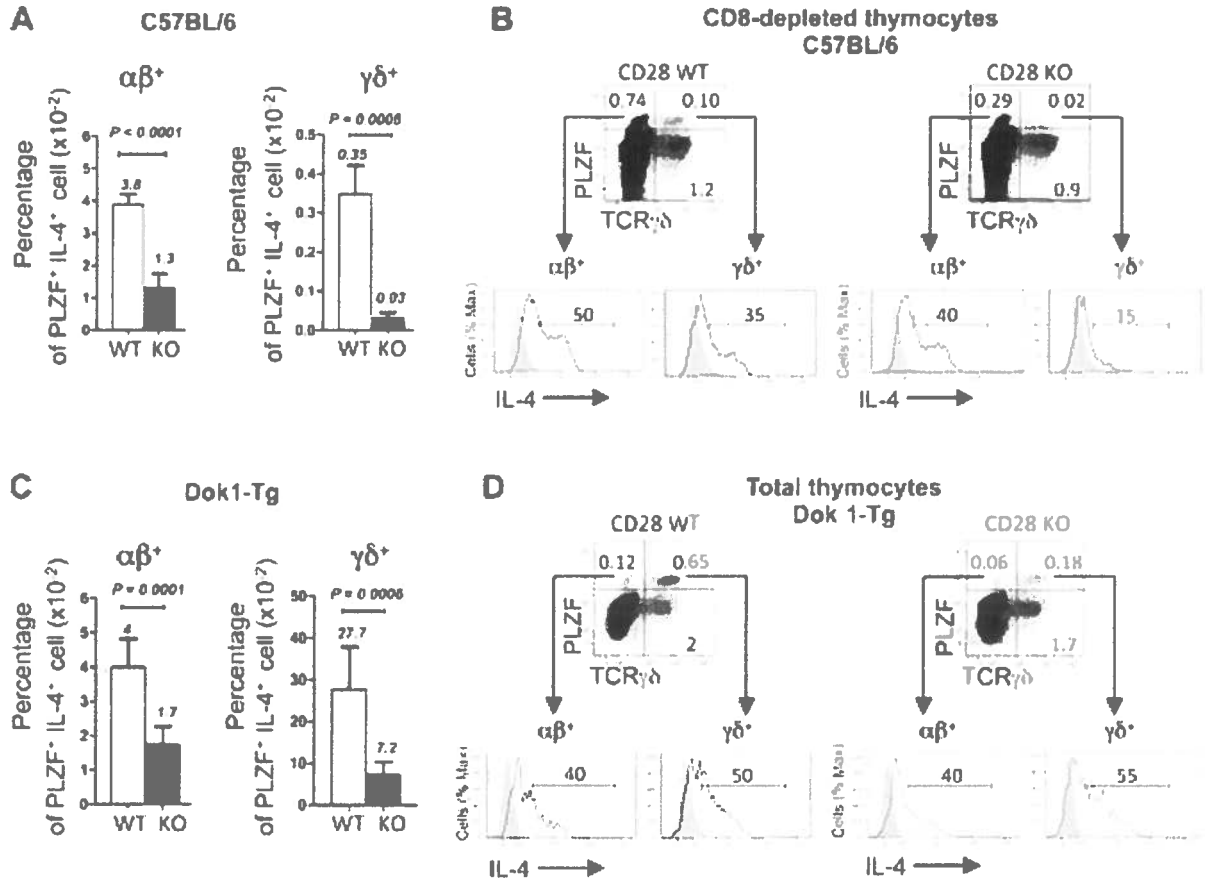
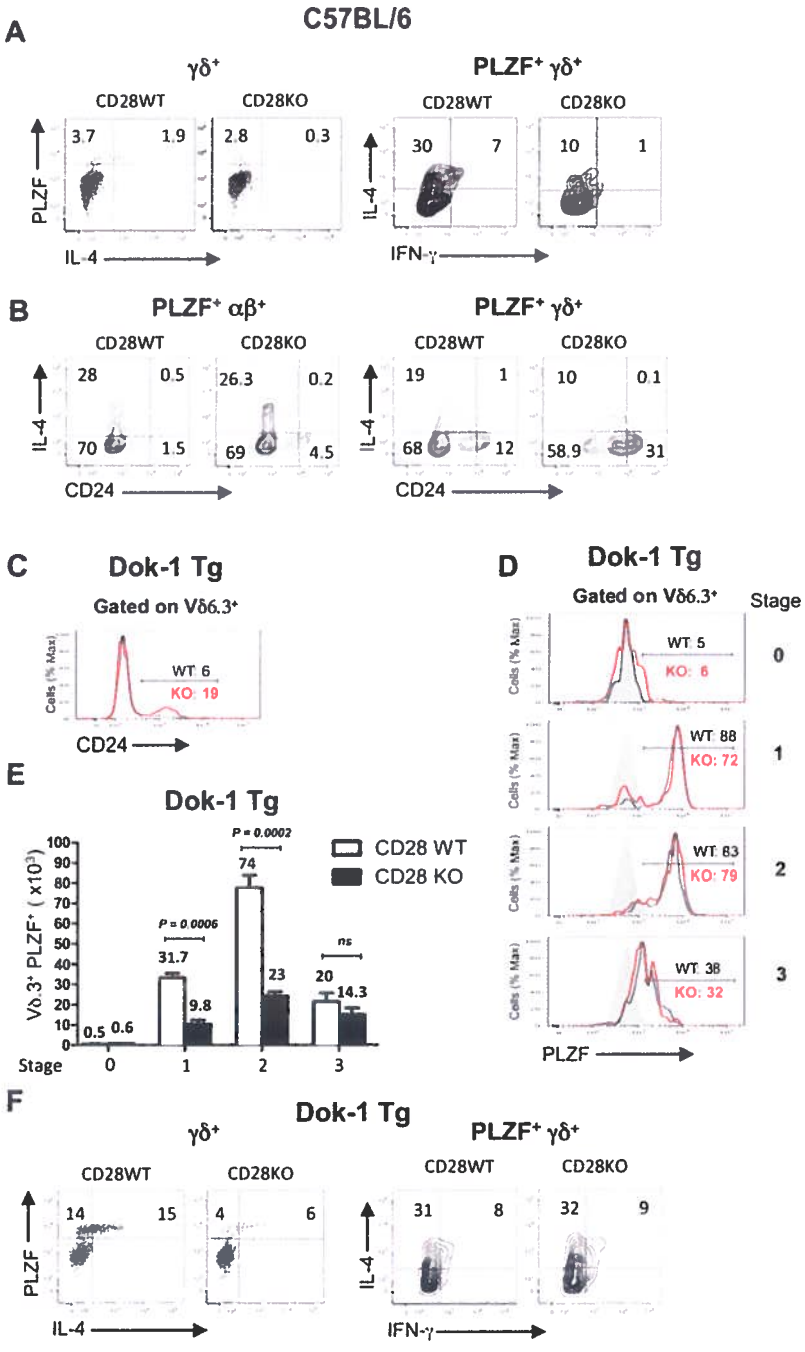


Figure 6. CD28 controls the number of $PLZF^+IL\text{-}4^+$ NKT cells. $CD8^+$-depleted thymocytes from WT and *Cd28*$^{-/-}$ (KO) C56BL/six mice or total thymocytes from WT and *Cd28*$^{-/-}$ (KO) Dok-1 transgenic mice were stimulated with PMA and ionomycin. After stimulation, cells were stained for surface expression of TCRγδ and stained for PLZF and IL-4 intracellular expression. (A) Quantification of the proportion of $PLZF^+$ IL-4^+ TCRγδ (γδ) or TCRαβ (αβ) thymocytes

from WT and *Cd28*$^{-/-}$ (KO) C56BL/six mice is shown. Values were obtained by multiplying the ratio of IL-4^{+}/PLZF^{+} by the percentage of PLZF^{+} αβ or γδ thymocytes divided by the cell-enrichment factor after CD8 depletion and are shown as mean $^{+}$ SD of five pairs of mice pooled from three independent experiments. (B) Representative dot plots show TCRγδ and PLZF expression of CD8^{+} cell-depleted thymocytes in WT and *Cd28*$^{-/-}$ (KO) C56BL/six mice. A minimum of 3×10^{6} events were acquired. Representative histograms below show the proportion of IL-4^{+}cells in PLZF^{+} αβ or γδ thymocytes (black line histogram) and as a negative control in WT PLZF^{-} αβ or γδ thymocytes (filled histogram). Data shown are representative of five pairs of mice pooled from three independent experiments. (C) Quantification of the proportion of PLZF^{+} IL-4^{+} γδ or αβ thymocytes from WT and *Cd28*$^{-/-}$ (KO) Dok-1 transgenic mice is shown. Values were obtained by multiplying the ratio of IL-4^{+}/PLZF^{+} by the percentage of PLZF^{+} αβ or γδ thymocytes and are shown as mean + SD of seven pairs of mice pooled from four independent experiments. (D) Representative dot plots show TCRγδ and PLZF expression of total thymocytes in WT and *Cd28*$^{-/-}$ (KO) Dok-1 Tg mice. Representative histograms below show the proportion of IL-4^{+}cells in PLZF^{+} αβ or γδ thymocytes (black line histogram) and as a negative control in WT PLZF^{-} αβ or γδ thymocytes (filled histogram). Data are representative of seven pairs of mice pooled from four independent experiments. Statistical significance was determined with a two-tailed paired Student's t-test. ns: not significant.


A
C57BL/6
γδ+
PLZF+ γδ+
CD28WT
CD28KO
3.7
1.9
2.8
0.3
30
7
10
1
PLZF
IL-4
IFN-γ
B
PLZF+ αβ+
PLZF+ γδ+
28
0.5
70
1.5
26.3
0.2
69
4.5
19
1
68
12
10
0.1
58.9
31
CD24
C
Dok-1 Tg
Gated on Vδ6.3+
WT 6
KO 19
D
Dok-1 Tg
Gated on Vδ6.3+
Stage
WT 5
KO 6
WT 88
KO 72
WT 83
KO 79
WT 38
KO 32
Cells (% Max)
E
Dok-1 Tg
CD28 WT
CD28 KO
P = 0.0002
P = 0.0006
ns
74
31.7
23
20
14.3
9.8
0.5
0.6
Vδ.3+ PLZF+ (x10³)
Stage
F
Dok-1 Tg
14
15
4
6
31
8
32
9

Supplemental Figure 4: CD28 regulates IL-4 production by PLZF-expressing NKT cells. A. Representative dot plots show IL-4 and PLZF expression in $CD8^+$-depleted TCRγδ thymocytes from CD28WT and CD28KO C56BL/6 mice (left) and total TCRγδ thymocytes from CD28WT and CD28KO Dok-1 Tg mice (right). Thymocytes were stimulated *in vitro* with PMA and ionomycin for 4 h and intracellularly stained for IL-4 and PLZF expression. The percentage of cells in each quadrant is indicated. Data are representative of 7 pairs of mice in 4 independent experiments. B. Representative dot plots show IL-4 and IFN-γ expression in CD28WT and CD28KO $PLZF^+$ TCRαβ thymocytes (left) and $PLZF^+$ TCRγδ thymocytes (right) in C56BL/6 mice. The percentage of cells in each quadrant is indicated. Bar graphs show the percentage of IL-4 single positive and IL-4/IFN-γ double positive CD28WT and CD28KO $PLZF^+$ TCRαβ thymocytes (left) and $PLZF^+$ TCRγδ thymocytes (right). Bar graphs show the value of the mean and SD. Data are representative of 6 pairs of mice in 3 independent experiments.

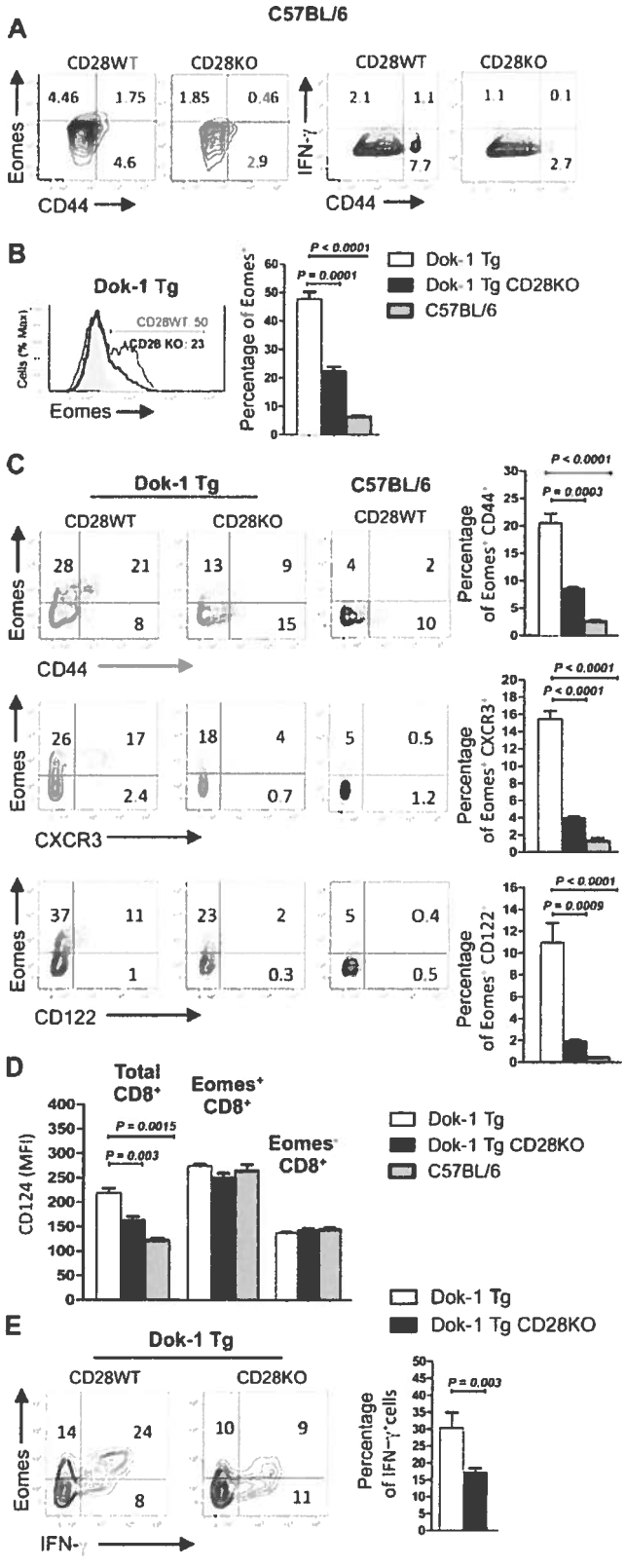

A
C57BL/6
CD28WT
CD28KO
CD28WT
CD28KO
4.46
1.75
4.6
1.85
0.46
2.9
2.1
1.1
7.7
1.1
0.1
2.7
Eomes
CD44
IFN-γ
CD44
B
Dok-1 Tg
CD28WT 50
CD28 KO: 23
Cells (% Max)
Eomes
Percentage of Eomes+
P < 0.0001
P = 0.0001
Dok-1 Tg
Dok-1 Tg CD28KO
C57BL/6
C
Dok-1 Tg
C57BL/6
CD28WT
CD28KO
CD28WT
28
21
8
13
9
15
4
2
10
Eomes
CD44
Percentage of Eomes+ CD44+
P < 0.0001
P = 0.0003
26
17
2.4
18
4
0.7
5
0.5
1.2
Eomes
CXCR3
Percentage of Eomes+ CXCR3+
P < 0.0001
P < 0.0001
37
11
1
23
2
0.3
5
0.4
0.5
Eomes
CD122
Percentage of Eomes+ CD122+
P < 0.0001
P = 0.0009
D
Total CD8+
Eomes+ CD8+
Eomes- CD8+
P = 0.0015
P = 0.003
CD124 (MFI)
Dok-1 Tg
Dok-1 Tg CD28KO
C57BL/6
Dok-1 Tg
Dok-1 Tg CD28KO
E
Dok-1 Tg
CD28WT
CD28KO
14
24
8
10
9
11
Eomes
IFN-γ
Percentage of IFN-γ+ cells
P = 0.003

Figure 7. CD28 controls the number innate-like $CD8^+$ T cells in the thymus. (A) Thymocytes from WT and CD28KO C56BL/6 mice were depleted for $CD4^+$ cells. Representative contour plots of the expression of CD44 and Eomes in $CD8^+$ thymocytes (left) and the expression of CD44 and IFN-γ in $CD8^+$ thymocytes after 4 h of stimulation with PMA and ionomycin (right) are shown. (B) Representative histograms show the expression of Eomes in $CD8^+$ thymocytes from Dok-1 Tg CD28WT (thin line histogram), Dok-1 Tg CD28KO (thick line histogram), and in CD28WT C56BL/6 mice (filled histogram). The percentages of $Eomes^+$ fraction of $CD8^+$ cells in Dok-1 Tg, Dok-1 Tg CD28KO, and C56BL/6 mice are shown as mean ± SD of three pairs of mice. (C) Representative contour plots of the expression of Eomes and CD44/CXCR3/CD122 in $CD8^+$ Dok-1 Tg, Dok-1 Tg CD28KO, and C56BL/6 thymocytes are shown. Percentages of $Eomes^+$ $CD44^+$ (top), $Eomes^+$ $CXCR3^+$ (middle), and $Eomes^+$ $CD122^+$ (bottom) in $CD8^+$ Dok-1 Tg, Dok-1 Tg CD28KO, and C56BL/6 thymocytes are shown. Data are also shown as mean ± SD of three pairs of mice. (D) The CD124 median fluorescence intensity (MFI) of total $CD8^+$, $Eomes^+$ and $Eomes^-$ Dok-1 Tg, Dok-1 Tg CD28KO, and C56BL/6 thymocytes is shown as mean ± SD of three pairs of mice. (E) Contour plots represent the expression of Eomes and IFN-γ in $CD8^+$ Dok-1 Tg, Dok-1 Tg CD28KO thymocytes after 4 h of stimulation with PMA and ionomycin. Percentages of IFN-γ^+ $CD8^+$ Dok-1 Tg and Dok-1 Tg CD28KO thymocytes are also depicted as mean ± SD of three pairs of mice. All data shown are from one experiment. Statistical significance was determined with an unpaired Student's t-test. ns: not significant.

DISCUSSION

This study investigates the role of CD28 in the regulation of γδ NKT-cell development. We demonstrate for the first time a critical role of CD28 in governing the generation of PLZF- and LFA-1high-expressing NKT cells. These findings have important consequences on the homeostasis and function of NKT cells. Our results show that upregulation of LFA-1 within NKT cells in the thymus is dependent on CD28. LFA-1 is believed to play an essential role in tissue-specific cell migration and retention of NKT cells in the liver (Emoto, Mittrucker et al. 1999, Ohteki, Maki et al. 1999). Thus, it is possible that impaired LFA-1 expression contributes in part to the reduced NKT-cell number in the liver of *Cd28*$^{-/-}$ mice. Additional experiments will be required to directly test this hypothesis. The signal transduction pathway involved in the regulation of LFA-1 expression initiated after CD28 stimulation may be mediated in part by WASp. Indeed, WASp was shown to promote LFA-1 high expression on αβ NKT cells (Astrakhan, Ochs et al. 2009) and to regulate actin remodeling required for CD28 signaling (Badour, McGavin et al. 2007). We also demonstrate that CD28 controls the number of thymocytes that express high levels of PLZF and produce large amount of IL-4 following in vitro stimulation with PMA and ionomycin. Constitutive secretion of IL-4 by immature NK1.1$^-$ thymocytes has been recently demonstrated *in vivo* (Dickgreber, Farrand et al. 2012). It is tempting to speculate that developing thymocytes producing IL-4 in the steady state correspond to the PLZF$^+$ IL-4$^+$ thymocytes described in this study. Importantly, previous reports demonstrated that PLZF$^+$ NKT cells are the only cells in the thymus secreting IL-4 and have the capacity to promote the differentiation of innate-like CD8$^+$ T cells (Lee, Jameson et al. 2011). It has been proposed that the low abundance of PLZF$^+$ NKT-cell population in the thymus limits the production of IL-4 and thereby the number of innate-like CD8$^+$ T cells generated in the

thymus (Rafei, Rouette et al. 2013). A prediction for this hypothesis is that CD28 controls the generation of innate-like $CD8^+$ T cells by promoting the development of thymocytes that produce large amount of IL-4. In this study, we verified this prediction in C57BL/6 and Dok-1 transgenic mice where, in the absence of CD28, a decreased number of $PLZF^+$ $IL\text{-}4^+$ NKT cells correlates with a reduced number of innate-like $CD8^+$ T cells generated in the thymus. It should be emphasized that in Dok-1 transgenic mice, innate-like $CD8^+$ T-cell development is strictly dependent on the presence of $PLZF^+$ NKT cells since we have previously shown that signaling lymphocytic activation molecule-associated protein deficiency in these mice led to a complete absence of innate-like $CD8^+$ thymocytes (Besin, Yousefi et al. 2012). CD28 requirement for the generation of innate-like $CD8^+$ T cells was previously reported in $Itk^{-/-}$ mice (where Itk is IL-2 inducible T-cell kinase) (Horai, Mueller et al. 2007). Like Dok-1 transgenic mice, Itk-deficient mice have an expanded population of $PLZF^+$ cells that are mostly γδ NKT cells (Weinreich, Odumade et al. 2010). Moreover, the development of innate-like $CD8^+$ T cells in $Itk^{-/-}$ mice is attributable to a cell-extrinsic effect dependent on IL-4 (Weinreich, Odumade et al. 2010). Therefore, similar to our present findings in Dok-1 transgenic mice, we propose that the CD28-dependent development of innate-like $CD8^+$ T cells in $Itk^{-/-}$ mice is an indirect effect of CD28 on the maturation of γδ NKT cells. The fact that in $Itk^{-/-}$ mice, the requirement of CD28 for full development of innate cell characteristics occurred upon selection on hematopoietic cells and not on the thymic stroma is in agreement with this interpretation. However, CD28 signaling may also synergize with IL-4 to induce the innate-like phenotype in $CD8^+$ T cells by a cell-intrinsic mechanism. Mice with different relative proportions of αβ and γδ NKT-cell population have been previously characterized (Lee, Jameson et al. 2011). In several C57BL/6 mutant mice including Dok-1 transgenic, $Id3^{-/-}$ and $Itk^{-/-}$ mice, it is likely the expanded $PLZF^+$ γδ NKT-cell

population that promotes the IL-4-mediated generation of innate-like $CD8^+$ T cells (Alonzo, Gottschalk et al. 2010, Verykokakis, Boos et al. 2010, Weinreich, Odumade et al. 2010, Besin, Yousefi et al. 2012). By contrast, in BALB/c mice, the $PLZF^+$ NKT cells that mediate the memory conversion of thymic $CD8^+$ T cells are αβ CD1-d restricted NKT cells (Weinreich, Odumade et al. 2010). Additional experiments would be required to determine the role of CD28 in the relative contribution of αβ and γδ NKT cells to the development of thymic innate-like $CD8^+$ T cells. Reduced TCR signaling strength such as in *Itk*$^{-/-}$ or Dok-1 transgenic mice resulted in a dramatic expansion of γδ NKT cell subset (Felices and Berg 2008, Besin, Yousefi et al. 2012). In contrast, reduced CD28 signal led to a decreased number of γδ NKT cells. This suggests that CD28, in γδ NKT cells, initiates downstream signaling events different from amplifying TCR-initiated signals. CD28 does not appear to play a role in positive selection of γδ NKT cells as normal numbers of $CD24^{high}$ $PLZF^+$ γδ NKT cells were present in Cd28$^{-/-}$ mice. Although CD28 deficiency does not affect the proliferative capacity of NKT cells, it may lead to an increased susceptibility to apoptosis of immature NKT-cell populations in the thymus. Alternatively and not exclusively, CD28 may modulate the expression levels of selected genes that are important for further differentiation of immature NKT cells. Importantly, we clearly show that CD28 specifically affects the development of PLZF-expressing $V\delta6.3^+$ T cells and seems to be dispensable to direct cells into the non-PLZF-expressing $V\delta6.3^+$ T-cell lineage. Additional experiments will be required to determine the precise mechanisms by which CD28 regulates the survival and/or maturation of $PLZF^+$ γδ NKT cells. In summary, our findings highlight the critical role of CD28 in the maturation and function of NKT cells. We demonstrate that CD28 regulates the generation of $Eomes^+$ $CD44^{high}$ innate-like $CD8^+$ T cells in part by controlling the size of the $PLZF^+$ $IL\text{-}4^+$ pool in the thymus. Innate-like $CD8^+$ T cells appear to

contribute to early host defense against pathogens by rapidly producing IFN-γ in an Ag-independent (Lertmemongkolchai, Cai et al. 2001, Berg, Crossley et al. 2003, Hu, Sahu et al. 2007) and Ag-dependent manner (Huang, Hu et al. 2013). Hence, CD28 is among others an important regulator of the innate immune response by controlling the development of NKT cells and innate-like $CD8^+$ T cells.

MATERIALS AND METHODS

Mice

C56BL/6 (CD45.2) WT and *Cd28*$^{-/-}$ mice were obtained from the Jackson Laboratories. C56BL6.SJL (CD45.1) mice were kindly provided by A. Lamarre (INRS-Institut Armand-Frappier, Laval, Canada). Dok-1 transgenic mice on a C57BL/6 genetic background (Tg82) were previously described (Besin, Yousefi et al. 2012). All mice were maintained in our pathogen-free animal facilities (INRS-Institut Armand-Frappier, Laval, Canada). Mice were manipulated in strict accordance to protocols 0910–01 and 1201–02, approved by the institutional animal care committee of the INRS-Institut Armand- Frappier. This protocol respects guidelines on good animal practice provided by the Canadian Council on animal care.

Antibodies and reagents

PE-anti-CD4 or allophycocyanin-anti-CD4 (clone GK1.5), FITCanti- CD8α or PE-anti-CD8α (clone 53–6.7), PE-anti-CD44 or allophycocyanin-anti-CD44 (clone IM7), biotinylated anti-CD3ε or allophycocyanin-anti-CD3ε (clone 145–2C11), FITC-anti-TCRβ or allophycocyanin-anti-TCRβ (clone H57–597), biotinylated anti-TCRγδ or FITC-anti-TCRδγ (clone GL3), PerCp-Cy5.5-anti-CD45.1 (clone A20), PF-anti-CD28 (clone 37.51), Alexa647-anti-Eomesodermin

(clone Dan11mag), PE-anti-IL-4 (clone 11B11), FITC-anti-CD62L (clone MEL-14), and FITC-anti-CD24 (clone 30-F1) were purchased from eBioscience. PerCP-anti-CD8 (clone 53–6.7) PE-Vδ6.3/2 (clone 8F4H7B7) and Streptavidin PerCP were purchased from BD Biosciences. Brilliant violet 421TM anti-CD3ε (clone 145–2C11), allophycocyanin-anti-CD62L (clone MEL-14), allophycocyanin-anti-NK1.1 or allophycocyanin/Cy7-anti-NK1.1 (clone PK136), Streptavidin allophycocyanin or Brilliant violet 421TM, PerCp-Cy5.5-anti-LFA-1 (clone H155–78) and Pacific Blue™ anti-CD45.2 (clone 104) were purchased from Biolegend. Alexa647-anti-PLZF (clone 9E12) Abs were generated at the Memorial Sloan-Kettering Cancer Center (MSKCC) antibody facility. Allophycocyanin-CD1d tetramers unloaded or loaded with PBS-57 were supplied by the National Institutes of Health Tetramer facility.

Cell preparations, NKT-cell enrichments, and flow cytometry

Cell suspensions from thymus, spleen, and liver were prepared as previously described (Besin, Yousefi et al. 2012). Red blood cells were removed using ACK lysis buffer (150mMNH_4Cl, 1mM$KHCO_3$, and 0.1Mm Na_2EDTA, pH 7.2). For analysis of γδ NKT thymocytes from C57BL/6 mice, thymocytes were depleted of $CD8^+$ cells using anti-CD8α antibodycoated magnetic beads from Miltenyi Biotec (Bergish Gladbach, Germany) according to the manufacturer's protocols. Alternatively, cells were labeled with biotinylated anti-TCRγδ antibodies and purified with anti-biotin-coated magnetic beads from Miltenyi according to the manufacturer's protocols. Cells were stained as described before (Besin, Yousefi et al. 2012). Viable cells were acquired on a Fortessa Flow cytometer (Becton Dickinson) and analysis was performed using FlowJo software (Tree Star).

Intracellular IL-4 detection

For intracellular IL-4 detection, cells were stimulated with PMA (100 ng/mL) and ionomycin (1 μg/mL) in the presence of Golgi-Plug (BD Pharmingen) for 4 h at 37°C. Surface and intracellular antigens were stained sequentially using a fixation and permeabilization kit from BD Biosciences according to the manufacturer's instructions.

BrdU incorporation

Mice were injected i.p. with 1 mg BrdU (in PBS). After 4 h, thymocytes were stained for cell surface markers, followed by fixation, permeabilization, and intracellular staining for BrdU following the manufacturer's instructions (BD Biosciences).

Bone marrow chimeras

Chimeras were generated by reconstituting lethally irradiated (950 rad) C57BL/6 CD45.1/CD45.2 mice with 10^7 BM cells from CD45.2 WT or *Cd28*$^{-/-}$ mice mixed 1:1 with BM from CD45.1 WT mice. Mice were analyzed 6 weeks after reconstitution.

Statistical analysis

All statistical analyses were performed with prism software (Graphpad). Two groups were compared using unpaired or paired Student's t-test when appropriate. Differences were considered to be statistically significant when $p < 0.05$. Graph results are shown as the mean ± SD.

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CHAPTER 3

DISCUSSION

1. Dok proteins regulate T cell development

1.1. Developmental arrest mediated by Dok-1 determines thymic cellularity

Evaluating the role of Dok proteins in the development of thymocytes, we showed that the overexpression of Dok-1 in two different transgenic mouse lines induced a partial block at the DN to DP transition of thymocyte maturation. More precisely, the block occurred between the DN3a to DN3b stage of maturation where the process of β-selection occurs. There was a direct correlation between the levels of Dok-1 overexpression and the magnitude of this block in thymocyte maturation showing that it is related to Dok protein activity. Due to this block the absolute number of total, DP and SP thymocytes decreased dramatically and the number of thymocytes in the DN subset increased especially in the transgenic line with the highest overexpression of Dok-1 (Dok-1 Tg 104). Consistent with our results, it has been previously shown that transplantation of Dok-2-overexpressing bone marrow into lethally irradiated mice led to impairment in the DN to DP transition stage (Gugasyan, Quilici et al. 2002). In another study, Dok-1/Dok-2-deficient mice showed increased numbers of DP thymocytes indicating that the DN to DP transition was favored in the absence of Dok (Yasuda, Bundo et al. 2007). It is well known that thymic T cell production is characterized by differentiating waves of non-self-renewing, bone marrow-derived progenitors. The factors constraining new progenitor recruitment, intrathymic precursor expansion, and thymus size are believed to be controlled by a feedback loop responding to lymphoid cellularity and competition for stromal niches. The precise mechanism behind the developmental perturbation observed in the thymi of Dok-1 Tg mice is yet to be elucidated. It was previously shown that Dok-2 overexpression in BM cells transplanted into irradiated mice inhibits repopulation of the thymus in two ways; first by inhibiting the seeding of the thymus with T cell precursors, either by inhibiting their formation or

their migration to the thymus and, second, by inhibiting their maturation from DN to DP thymocytes (Gugasyan, Quilici et al. 2002). In contrast to Dok-2 overexpressing BM cells used in the previous study, in Dok-1 transgenic mouse lines the effect of Dok-1 in the formation or migration of early T cell precursors (ETPs) to the thymus is unlikely since the exogenous Dok-1 is under the control of CD2 promoter and thymocytes begin to express CD2 earliest at the DN2 stage of their maturation. In another study, reconstitution of non-irradiated RAG-2 deficient mice with wild type BM cells showed that there is a competition for stromal niches which is solely limited to DN3 precursors (Prockop and Petrie 2004). Therefore, the overall size of the thymus is determined not only by the expansion of progenitor cell transiting to the DP stage but also by the limitation on early precursor cells entry. One can speculate that, similar to RAG-2 deficient recipient mice, the accumulation of thymocytes at DN3 stage in the thymi of Dok-1 transgenic mice might limit the progenitor cell recruitment to the thymus due to limited available stromal niches. Therefore, it can be suggested that this limited precursor cell entry together with loss of DP thymocytes (caused by the partial DN to DP block) determine the total thymic cellularity in Dok-1 Tg mice.

1.2. Dok can regulate β-selection by inhibition of Erk pathway

As discussed earlier, our results show that Dok-1 overexpression partially blocks β-selection during thymocyte development. Consistent with previous studies in mature T cells (Yasuda, Bundo et al. 2007), our results also show that Dok proteins negatively modulate TCR signaling in DP thymocytes where several effector molecules like ZAP-70, LAT, PLC-γ and Erk show reduced levels of phosphorylation when Dok-1 is overexpressed (data in the first paper) while they show increased levels of phosphorylation in the absence of Dok-1 and Dok-2 (data

not shown). Since TCR and pre-TCR share common downstream effector molecules we can assume that Dok-1 and likely Dok-2 plays the same negative role in the regulation of pre-TCR signaling strength which is important for β-selection. Erk becomes activated upon pre-TCR stimulation and plays an important role during β-selection (Michie, Trop et al. 1999). It can be hypothesized that Dok-mediated Erk inactivation (Campbell, Khosravi-Far et al. 1998) might regulate β-selection. Now the question that remains to be answered is what can be the underlying mechanism?

It has been demonstrated that Erk signaling is an important determinant in TCR signaling and T cell development because it regulates the activity of E proteins E2A (E12, E47) and HEB, essential transcription factors in T cell development (Murre, McCaw et al. 1989). How can Erk signaling modulate E proteins activity? Signaling through the pre-TCR activates the Erk pathway, leading to the expression of Egr family members Egr1-4 (Carleton, Haks et al. 2002). Egr transcription factors are required for the development of DN3 cells across the β-selection checkpoint to the DP stage (Carleton, Haks et al. 2002). Egr expression is important at this stage because it serves to control thymocyte proliferation in response to pre-TCR signals (Xi, Schwartz et al. 2006) and also induces the expression of the transcription factor Id3 (Engel and Murre 2004) which has been shown to have elevated gene expression at the β-selection stage (Taghon, Yui et al. 2006). By inducing Id3 expression, the developmental arrest imposed by E2A function is removed as a result of the Id3/E2A heterodimerization (Rivera, Johns et al. 2000). The developmental impairment in thymocytes of Dok-1 Tg mice could be at least in part due to the inhibition of the Erk pathway and consequently inhibition of Egr and Id3 expression, which leads to a developmental arrest at DN3 stage of thymocyte maturation imposed by E2A function. Therefore, it will be interesting to test whether Egr and/or Id3 expression is controlled by the

levels of Dok expression at early stages of thymocyte maturation such as DN3. This could be feasible by tracking Id3 gene expression at DN stages using an Id3-GFP reporter mouse strain (Miyazaki, Rivera et al. 2011) in the presence or absence of Dok-1 and Dok-2 proteins.

1.3. Dok proteins might control thymocyte positive and negative selection

After becoming DP, thymocytes move deep into the thymic cortex where they can interact with the self-antigens presented by MHC-I or MHC-II molecules. As mentioned before, TCR signal strength play a critical role in the decision of a DP cell to continue maturation, which is called positive selection. Likewise, TCR signal strength is very important for negative selection, where thymocytes having high affinity interactions will be eliminated to avoid developing self-reactive T cells in the immune system (Starr, Jameson et al. 2003, Prince, Yin et al. 2009). It has been shown that the intensity and duration of the Erk signaling pathway is also critical for both positive and negative selection with more sustained Erk signaling pathway leading to increased positive selection and a rapid and robust Erk activation resulting in negative selection (McNeil, Starr et al. 2005). It has also been shown that the expression of Egr-1 and Id3 correlates with the kinetics of Erk activation during positive selection (McNeil, Starr et al. 2005). Therefore, the expression level of Dok-1 by regulating TCR strength and Erk activity may affect both positive and negative selection during thymocyte development. It can be predicted that in thymocytes with low affinity TCR-Ag interactions, Dok overexpression might decrease and its absence might increase positive selection. Surprisingly, in the context of a polyclonal repertoire, positive selection of $CD4^+$ thymocytes seemed to occur normally in mice overexpressing Dok-1. The effect of Dok on thymic positive selection might become visible in TCR transgenic mouse models with low affinity ligands (OT-I and OT-II) where selecting self-peptides would be limited. In support of a role of Dok in positive selection, and in agreement with previous reports

(Yasuda, Bundo et al. 2007), we found that there is an increase in the absolute number of mature SP thymocytes in Dok-1-/Dok-2-deficient mice compared to WT controls (data not shown). Studying the role of Dok proteins in thymocyte negative selection could also be performed in TCR transgenic mice. HY TCR transgenic mice which express a transgenic TCR specific for HY antigen (i.e. a peptide of the DBY protein) presented by class I D^b MHC molecules, are the best tools to study negative selection. In male transgenic mice, DP cells undergo negative selection by TCR agonist ligands and die by apoptosis (Kisielow, Teh et al. 1988). Using this model we can investigate if Dok expression level regulating TCR signaling strength/Erk activation might consequently modulate thymocyte negative selection.

1.4. Dok proteins regulate the development of γδ NKT cells

Our data show that Dok-1 overexpression promotes the accumulation of total γδ T cells in the thymus, spleen, and liver of the transgenic mice compared to their WT controls. This accumulation correlates with the level of Dok-1 overexpression and is mainly due to the specific expansion of the γδ NKT cells. These expanded γδ NKT cells expressed the transcription factor PLZF, were developmentally dependent on SLAM-SAP signaling and produced IL-4 *ex vivo* upon stimulation. It is possible that the accumulation of DN3 thymocytes in Dok-1 transgenic mice might have favored the expansion of γδ NKT cell subset among total γδ thymocytes. This hypothesis can be partly correct, however, in previous studies using TCRβ-deficient mice, the proportion of $NK1.1^+$ cells among γδ thymocytes was found to be identical to that of WT mice (Vicari, Mocci et al. 1996). This suggests that the specific development of γδ NKT cells over non-NKT γδ thymocytes in Dok-1 transgenic mice might not be exclusively due to the block in DN to DP transition. Interestingly, similar to Dok-1 transgenic mice, a specific expansion of PLZF-expressing γδ NKT cells has been previously observed in mouse models, such as Id3-

deficient mice (Lauritsen, Wong et al. 2009, Ueda-Hayakawa, Mahlios et al. 2009, Verykokakis, Boos et al. 2010) and also mice deficient in some components of TCR signaling pathway like Itk knockouts (Felices, Yin et al. 2009, Qi, Xia et al. 2009, Yin, Cho et al. 2013), as well as in mice with a mutated form of the adapter protein SLP-76 (SLP-76:Y145F), which cannot interact with and maintain Itk activity (Alonzo, Gottschalk et al. 2010). Similarly, it was previously shown that there is an accumulation of $NK1.1^+$ $TCR\gamma\delta^+$ thymocytes in CD3ζ-deficient mice (Arase, Ono et al. 1995) and there is an increased number of an IL-4 producing γδ T cell subtype in LAT mutant mice (Nunez-Cruz, Aguado et al. 2003). These latter cells likely belong to the recently defined γδ NKT cell subset. Different hypothesis have been proposed to explain this particular phenotype in both Id3 and TCR signaling mutant mice. It has been originally proposed that maybe some signaling pathways dowstream of TCR like those that require Itk inhibit the development of $PLZF^+$ γδ T cells, whereas other branches promote it (Kreslavsky, Gleimer et al. 2010). An alternative hypothesis suggested by Lauritsen et al. proposed that some of the $V\gamma1.1^+V\delta6.3^+$ T cells might be deleted by receiving a very strong TCR signal and they are rescued from negative selection when TCR signaling is somewhat attenuated. According to this hypothesis, in the WT animal these cells are rare since the immune system tends to avoid accumulating a highly auto-reactive T cell population (Lauritsen, Wong et al. 2009, Kreslavsky, Gleimer et al. 2010). There are several lines of evidence in support of this theory. In both αβ and γδ NKT cell subsets, PLZF expression is associated with a very restricted combination of TCR chains and both cell types are believed to become positively selected by their agonist ligands (agonist selection), an alternative way of selection which induces a very strong TCR signal that would normally be expected to induce T cell deletion (Baldwin, Hogquist et al. 2004). Moreover, it has been previously shown that *in vitro*, PLZF can be induced by strong TCR signal

(using antibody-mediated TCR crosslinking) in polyclonal immature γδ thymocytes, suggesting that PLZF may actually be induced *in vivo* by agonist selection and very strong TCR signaling (Kreslavsky, Savage et al. 2009). In this regard Dok-1 transgenic mice can be considered in the category of mutant mice with attenuated TCR signaling. Therefore, γδ NKT cells become expanded in Dok-1 transgenic mice because they get rescued from deletion since they receive a weaker TCR signal compared to their WT controls. Comparing WT and Dok-1-/Dok-2-deficient mice, we did not detect any alteration in the number of $V\gamma1.1^+V\delta6.3^+$ T cells or in the level of PLZF expression in this subset of γδ T cells in thymus and periphery (data not shown). The development of $V\gamma1.1^+V\delta6.3^+$ $PLZF^+$ T cells might not be influenced by a higher strength of TCR signal since both positive selection and PLZF expression might already be operating at a maximum rate. Alternatively, the increased TCR signaling strength in Dok-1-/Dok-2- deficient thymocytes might enhance both positive and negative selection leading to the similar numbers of PLZF-expressing γδ NKT cells in WT and Dok-1-/Dok-2- deficient mice.

1.5. Potential Target of Dok-mediated regulation of γδ NKT cell development

What might be the target of Dok downstream of TCR that is important for the generation of $V\gamma1.1^+V\delta6.3^+$ $PLZF^+$ T cells? Id3 is a unique transcription factor which is the antagonist of E2A and together they play important roles downstream of TCR signaling in different checkpoints during T cell development (Bain, Cravatt et al. 2001, Miyazaki, Rivera et al. 2011, Naito, Tanaka et al. 2011). It was demonstrated that this transcription factor plays dual roles for the development of different subtypes of γδ T cells. Using TCRγδ transgenic mouse models, it had been established that the axis of Erk-Egr-Id3 plays essential roles in αβ vs. γδ fate decision checkpoint favoring γδ T cell development (Haks, Lefebvre et al. 2005, Hayes, Li et al.

2005, Kreslavsky, Gleimer et al. 2010). However, it was found that among total γδ T cells the $V\gamma1.1^{+}V\delta6.3^{+}$ γδ NKT subset accumulated in Id3-deficient mice (Alonzo, Gottschalk et al. 2010, Verykokakis, Boos et al. 2010). It was suggested that Id3 might be restricting the development of γδ T cell subsets bearing high affinity γδ TCRs but favoring the development of the other subtypes (Kreslavsky, Gleimer et al. 2010). However a very recent study demonstrated that both Id3 and Id2, by controlling the levels of E protein activity during T cell development, regulate the size of γδ NKT cell population by affecting their survival and proliferation (Zhang, Lin et al. 2013) As mentioned earlier, it is well established that Id3 expression is induced by the Erk pathway which is mediated by Egr2/3 (Bain, Cravatt et al. 2001). Since Dok can negatively regulate Erk signaling, it could be a potential negative regulator of Id3 induction and consequently E protein activity downstream of TCR signaling (Figure 1). Interestingly, both Dok-1 Tg and Id3-deficient mice show the particular phenotype of γδ NKT cell expansion. Furthermore, Tec kinase activity which is required for maximal transcription of Egr2 and Egr3 upon TCR stimulation (Miller and Berg 2002) is negatively regulated by Dok-1 and Dok-2 (Gerard, Favre et al. 2004). One hypothesis is that there might be functional interaction between Itk, another member of the Tec kinase family, and Dok-1/Dok-2 proteins leading to negative regulation of Erk (downstream of Itk activity) and consequently downregulation of Egr2/3 transcription and Id3 expression.

Presuming Dok might negatively regulate Id3 induction raises the question whether Dok-mediated regulation of γδ NKT cell development is through inhibition of Id3 expression. This can be tested by measuring the level of Id3 induction (in an Id3-GFP mouse reporter strain (Miyazaki, Rivera et al. 2011)) in the DN thymocytes, at early stages of γδ NKT cell development and during their maturation in the presence or absence of Dok proteins. Comparing

WT and Dok-1-/Dok-2- deficient mice we didn't see any changes in the frequency and the number of γδ NKT cells (data not shown). It can be hypothesized that in normal conditions Id3 might be expressed at its maximum level and the absence of Dok as the negative regulator will not help to induce its expression to a higher level. The effect of Dok on Id3 expression and consequently γδ NKT cell development might be more visible in mice heterozygote for Id3 expression, $Id3^{+/-}$ where the absence of Dok might help to increase the level of Id3 expression.

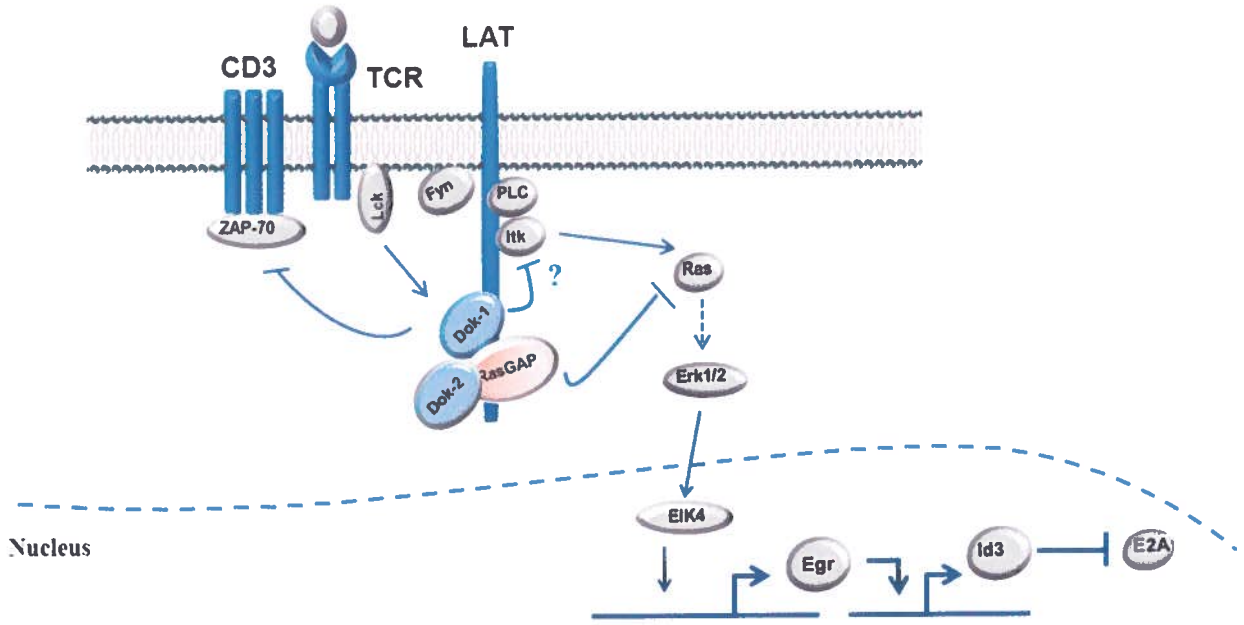

Figure 1) Dok can potentially regulate Id3 expression and E protein activity Upon TCR stimulation Dok proteins recruite RasGAP which inhibits Ras activity and will lead to the negative regulation of Erk pathway. Since Dok-1 and Dok-2 proteins are involved in negative regulation of Tec kinase activity and downstream signalling pathways including the Ras pathway (Gerard, Favre et al. 2004), Dok may also inhibit Ras and Erk pathway downstream of Itk activity (a member of the Tec family of tyrosine kinases). By inhibiting Erk pathway, Dok can potentially regulate Egr and Id3 expression and consequently E protein activity downstream of TCR signaling.

1.6. Dok might regulate γδ NKT cell development via SLAM-SAP signaling pathway

Besides TCR signaling pathway, Dok might be involved in the regulation of γδ NKT cells development by regulating SLAM-SAP signaling pathway. Interestingly, we show that the development of PLZF-expressing $V\gamma1.1^{+}V\delta6.3^{+}$ T cells is strictly dependent on SLAM-SAP signaling in Dok-1 transgenic mice, whereas in WT mice, the loss of SAP only partially affects the frequency and level of PLZF expression in $V\gamma1.1^{+}V\delta6.3^{+}$ cells (Alonzo, Gottschalk et al. 2010). We can assume that the defective TCR signaling in the presence of Dok overexpression might affect the induction of PLZF expression and make $PLZF^{+}$ cell expansion more susceptible to signals from SLAM family members. Moreover, the involvement of Dok in SLAM-SAP signaling might be important for the selection and development of γδ NKT cells. It can be hypothesized that the strength of the signal that γδ NKT cell precursors receive during positive selection or in the process of maturation is the result of both TCR and SLAM signaling. So that Dok might control γδ NKT cell development via regulation of both of these signaling pathways. Dok-1 and Dok-2 phosphorylation and RasGAP recruitment have been shown downstream of SLAMF1 ligation in DP thymocytes, the precursors of αβ iNKT cells, although the functional outcome of this phosphorylation has not yet been identified (Latour, Gish et al. 2001). Although the SLAM receptor(s) essential for positive selection and development of γδ NKT cells has not been identified yet, Dok phosphorylation and RasGAP recruitment is anticipated upon SLAM ligation in γδ NKT precursors as well. Further investigation is needed to identify the role of Dok downstream of SLAM receptor signaling in the development of NKT cells.

2. CD28 regulation of NKT cell development

2.1. CD28 regulate NKT cell development after positive selection

Our data clearly show that CD28 regulates the development of PLZF-expressing γδ NKT cells. Similarly, three independent studies showed the critical role of CD28-B7 interaction during intrathymic development, expansion and function of $CD1d^+$ iNKT cell population (Chung, Nurieva et al. 2008, Williams, Lumsden et al. 2008, Zheng, Zhang et al. 2008). It was demonstrated that in the absence of CD28 signaling, there is a defective intrathymic development of iNKT cells, which leads to a lower expansion of these cells in the thymus and periphery. Both phenotypical and functional maturation of these cells was impaired after their positive selection. Indeed both WT and CD28 deficient mice had the same frequency and numbers of $CD24^{high}$ CD1d-$tetramer^+$ T cells representing early selected iNKT cells. However, in the absence of CD28 signaling there were fewer iNKT cells that expressed NK1.1 and were able to produce simultaneously both Th1 and Th2 cytokines upon stimulation. These cells are considered to be the most mature stage of iNKT cells (Chung, Nurieva et al. 2008, Williams, Lumsden et al. 2008). Likewise, our results indicate that CD28 does not seem to play important roles in positive selection of γδ NKT cells as similar numbers of $CD24^{high}$ $PLZF^+$ γδ NKT cells was found in CD28-deficient mice compared to WT controls. CD28 was rather found to be essential for their maturation since the number of the more mature γδ NKT cells was less in the absence of CD28. Cells at these stages were defined by the expression of cell surface markers CD44 and NK1.1 and simultaneous Il-4 and IFN-γ production. We also show that unlike αβ NKT cells in which CD28-B7 interaction was found to be important in iNKT cell proliferation and apoptosis (Zheng, Gao et al. 2004), CD28 deficiency does not affect the proliferative capacity of γδ NKT cells although it might lead to an increased susceptibility to apoptosis of immature γδ NKT cell

populations in the thymus. Alternatively but not exclusively, CD28 may modulate the expression levels of selected genes that are important for further differentiation of immature γδ NKT cells. Additional experiments are required to identify the precise mechanism and developmental stage(s) in which CD28 promotes the maturation and/or survival of γδ NKT cells. Dok proteins might be involved in the CD28-mediated regulation of the survival and/or maturation of γδ NKT cells. It has been previously shown in cell lines that upon CD28 ligation (using antibody or ligand) Dok-1 becomes phosphorylated (Yang, Ghiotto et al. 1999, Michel, Attal-Bonnefoy et al. 2001). Dok-2 phosphorylation upon CD28 stimulation has also been demonstrated in primary T cells (Dong, Corre et al. 2006). It can be hypothesized that Dok can negatively regulate CD28 signaling via inhibiting Tce kinase activity. Further investigation is needed to identify the role of Dok proteins downstream of CD28 signaling in the regulation of γδ NKT cell development.

2.2. CD28 regulates γδ NKT cell maturation by cell-intrinsic mechanisms

Data generated from our mixed BM chimeras showed that the proportion of $PLZF^+$ αβ and γδ NKT thymocytes was significantly reduced in the absence of CD28 suggesting that this developmental impairment could not be rescued with WT BM cells. Therefore the defect in the development of CD28-deficient $PLZF^+$ NKT cells is cell-autonomous. Similar studies have been done previously to identify whether CD28 regulates iNKT cell development by cell-extrinsic or cell-intrinsic mechanisms and the results showed that when the ratio of the chimerism is not equal and WT cells are in the majority they can exert cell-extrinsic effects on bystander CD28-deficient cells (Williams, Lumsden et al. 2008). In our study however, we used equal ratios of BM cells from WT and CD28-deficient donors to generate the mixed BM chimeras and this ratio remained equal after reconstitution. It might be interesting to test the effect of WT donors on

CD28-deficient bystander cells in an unequal ratio of chimerism on the development of γδ NKT cells.

2.3. CD28-dependent LFA-1 upregulation might control NKT cell homeostasis

We also show that the upregulation of LFA-1 within both αβ and γδ NKT cells in the thymus and periphery is dependent on CD28 signaling. The adhesion molecule, lymphocyte function-associated antigen (LFA)-1 was previously shown to play an essential role in tissue-specific cell migration and retention of NKT cells. It was shown that LFA-1-deficient mice have markedly decreased numbers of $CD4^+$ iNKT cells in the liver while ICAM-1-deficient mice showed only minor reduction in NKT cell number suggesting a crucial role for LFA-1 in the accumulation and homeostasis of iNKT cells in the liver (Emoto, Mittrucker et al. 1999, Ohteki, Maki et al. 1999). However, LFA-1 expression has not been shown as a requirement for the retention of γδ NKT cells in the liver or any peripheral organ yet. It is possible that impaired LFA-1 expression contributes in part to the reduced αβ and γδ NKT cell number in the liver of CD28-deficient mice although additional experiments will be required to directly test this hypothesis. There is one hypothetical candidate which could be the potential link between CD28 signaling and LFA-1 expression. The Wiskott-Aldrich syndrome protein (WASp) serves as a crucial link between cellular stimuli and cytoskeletal rearrangements. Similar to its role in the homeostasis of regulatory T and marginal zone B cells, WASp was shown to play important roles in the homeostasis of iNKT cells with decreased homing and/or retention of iNKT cells within peripheral tissues in WASp-deficient mice (Astrakhan, Ochs et al. 2009). On the other hand WASp was found to play a major role in coupling TCR stimulation to induction of actin cytoskeletal changes required for T cell activation. It has been shown that in T cells, the complex of WASp/SNX9 (a protein containing a phosphoinositide binding domain involved in

intracellular trafficking)/p85 (PI3K regulatory subunit)/CD28, enables a unique interface of endocytic, actin polymerizing, and signal transduction pathways required for CD28-mediated T cell costimulation (Badour, McGavin et al. 2007). Therefore the signal transduction pathway involved in the regulation of LFA-1 expression initiated after CD28 stimulation might be mediated in part by WASp which was shown to promote elevated expression of LFA-1 on αβ NKT cells and to regulate actin remodeling required for CD28 signaling.

3. Relative contribution of αβ and γδ NKT cells in the innate conversion of $CD8^+$ T cells in the thymus

Our data show that Dok-1 overexpression induces a SAP-dependent accumulation of $CD8^+$ innate-like T cells which is very similar to what has been reported in the Tec kinase (Itk and Rlk)-deficient mice, where $CD8^+$ T cells showed an upregulation of surface memory markers like CD44 and CD122 and the transcription factor Eomesodermin. These cells were also shown to produce high levels of IFN-γ upon stimulation (Atherly, Lucas et al. 2006, Broussard, Fleischacker et al. 2006). We demonstrated that there is a remarkable correlation between the percentage of Eomesodermin-expressing $CD8^+$ cells and the number of γδ NKT cells, comparing the two lines of Dok-1 transgenic mice and their WT controls. Consistently, in transgenic lines there was a fine correlation between the levels of SAP expression and frequency of $Eomes^{high}$ $CD8^+$ thymocytes. Like Itk and other gene-deficient models the development of these innate-like $CD8^+$ T cells is likely a consequence of elevated IL-4 produced by the expanded $PLZF^+$ γδ T cells in Dok-1 transgenic mice (Verykokakis, Boos et al. 2010, Weinreich, Odumade et al. 2010, Lee, Jameson et al. 2011). We also demonstrated that CD28 controls the number of αβ and γδ thymocytes that express high levels of PLZF and produce large amounts of IL-4 following *in*

vitro stimulation with PMA and ionomycin. In the thymus, these PLZF$^+$ IL-4$^+$ αβ and γδ thymocytes are those NKT cells that produce IL-4 in the steady state since they are constantly exposed to their own ligand and are able to promote the innate conversion of CD8$^+$ thymocytes. Therefore we hypothesized that CD28-mediated regulation of αβ and γδ IL-4$^+$ NKT cells might promote the innate conversion of CD8$^+$ T cells. But what is the relative contribution of αβ and γδ NKT cells to the development of thymic innate-like CD8$^+$ T cells?

In several C57BL/6 mutant mice including Id3 and Itk knockouts, it is likely that the expanded PLZF$^+$ γδ NKT cell population promotes the IL-4-mediated generation of innate-like CD8$^+$ T cells (Alonzo, Gottschalk et al. 2010, Verykokakis, Boos et al. 2010, Weinreich, Odumade et al. 2010), while in KLF2 deficient mice both αβ and γδ NKT cell populations are expanded and able to produce IL-4 in the thymus, which can mediate the innate conversion of CD8$^+$ T cells (Weinreich, Takada et al. 2009, Weinreich, Odumade et al. 2010). By contrast, in BALB/c mice, the PLZF$^+$ NKT cells that mediate the generation of innate-like CD8$^+$ T cells are αβ CD1-d restricted iNKT cells (Hammond, Pellicci et al. 2001, Rymarchyk, Lowenstein et al. 2008, Weinreich, Odumade et al. 2010). Since the frequency of PLZF$^+$ IL-4$^+$ αβ T cells is equal in both WT C57BL/6 and Dok-1 Tg mice, a/b T cells are unlikely to be responsible for the accumulation of innate-like CD8$^+$ T cells. Instead, similar to Itk and Id3 knockouts, γδ NKT cells seem to be the main effector cell population providing extrinsic IL-4 in Dok-1 Tg mice. Moreover, in CD28-deficient Dok-1 Tg thymi where the frequency of PLZF$^+$ IL-4$^+$ αβ thymocytes are about 2 times less (Figure 6 – publication no.2) than in C57BL/6 mice, there is still a much larger population of innate-like CD8$^+$ T cells because of higher γδ NKT cells population. Using TCR γδ- or CD1d-deficient mice will help to define the role of γδ or αβ NKT cells in the innate conversion of CD8$^+$ T cells in the thymus.

4. Conclusion: new roles for Dok and CD28 in T cell development

The data presented in this thesis illustrate the role of Dok in different checkpoints of T cell development. This study clearly shows that Dok protein blocks thymocytes β-selection while its precise role in positive or negative selection should be investigated. We also show that Dok positively regulates the development of innate-like γδ NKT cells. Role of Dok in the development of αβ NKT cells should be studied in Dok-1/Dok-2-deficient mice since in Dok-1 Tg mice this population is dramatically reduced because of the developmental block in the transition of DN to DP thymocytes (Figure 2). The transcription factor Id3 is proposed as a potential target of Dok for the regulation of γδ NKT cell development. Id3 has been found to limit the maturation and survival of γδ NKT cells. We also show that similar to αβ iNKT cells the development of γδ NKT cells is dependent on CD28. Our results demonstrate that in the absence of CD28 there is a block in γδ NKT cells development that occurs after positive selection and during maturation of these cells. Moreover, modulating the NKT cell population, we show that both Dok and CD28 indirectly control the pool size of innate-like $CD8^+$ T cells. These cells beside NKT cells play important roles at the interface of the innate and adaptive immune responses. Further work needs to be undertaken to elucidate the precise role of Dok downstream of each of these signaling pathways (TCR, SLAM or CD28) which are all important for the development of γδ NKT cells. These studies will help to understand the precise developmental requirements and mechanisms for the regulation of NKT cells and more generally for shaping the T cell repertoire and innate-like T cell development.

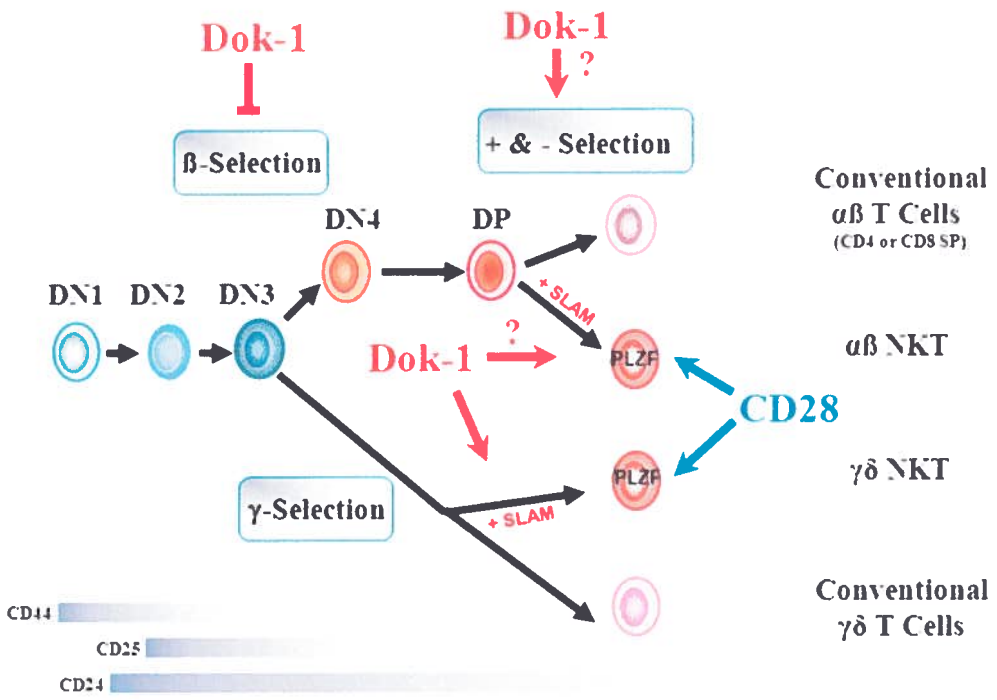

Figure 2) Role of Dok-1 and CD28 at different check points of T cell development. Dok-1 is negatively involved in thymocytes β-selection and positively involved in γδ NKT cell development. Role of Dok proteins in the development of αβ NKT cells and in thymocyte positive and negative selection is not identified yet. CD28 positively regulates both αβ and γδ NKT cell development after selection and during maturation.

CHAPTER 4

Résumé en français

1. Introduction

1.1. Lymphocytes T non conventionnels ou « de type inné »

Des études récentes ont prouvé que la distinction entre l'immunité innée et acquise est moins nette qu'on ne le pensait. Une étude a notamment démontré l'existence de catégories distinctes de lymphocytes T présentant des caractéristiques spécifiques aux deux branches du système immunitaire. Ces lymphocytes T spécifiques sont qualifiés de non conventionnels ou d'« de type inné » (en contraste avec les cellules T conventionnelles). Ce sont des lymphocytes T natural killer (NK) TCR$\alpha\beta^+$ ou TCR$\gamma\delta^+$ appelés $\alpha\beta$ NKT (type I et type II) et $\gamma\delta$ NKT, qui sont les prototypes de la population de lymphocytes T « de type inné ». Il existe également quelques autres sous-types de lymphocytes T « de type inné » comme les cellules T $CD8^+$ « de type inné » (Prince, Yin et al. 2009, Das, Sant'Angelo et al. 2010). Ces lymphocytes T non conventionnels présentent des caractéristiques particulières qui les distinguent de leurs homologues conventionnels. Par exemple, ils possèdent un répertoire TCR restreint et présentent un phénotype de type mémoire (Berg 2007, Veillette, Dong et al. 2007, Prince, Yin et al. 2009, Das, Sant'Angelo et al. 2010). Ces différences de phénotype et de fonction entre les lymphocytes T conventionnels et « de type inné» pourraient être le résultat de différentes exigences en matière de signalisation reçue lors de la sélection et du développement.

1.2. Exigences spécifiques au développement des lymphocytes T « de type inné »

Bien qu'elles soient issus de précurseurs communs, le développement des cellules αβ et γδ NKT est différent de celui des lymphocytes T conventionnels (Coles and Raulet 1994, Bendelac, Lantz et al. 1995). Elles expriment un facteur de transcription signature nommé PLZF qui leur confère leurs propriétés innées (Benlagha, Kyin et al. 2002, Pellicci, Hammond et al. 2002, Dao, Guo et al. 2004, Kreslavsky, Savage et al. 2009, Das, Sant'Angelo et al. 2010). Contrairement à ce qui est observé chez les lymphocytes T conventionnels, les récepteurs SLAM et leurs protéines adaptatrices associées SAP jouent un rôle crucial dans le développement des cellules αβ et γδ NKT (Chung, Aoukaty et al. 2005, Nichols, Hom et al. 2005, Alonzo, Gottschalk et al. 2010).

1.3. Composantes de la voie de signalisation pré-TCR et TCR

Les signaux de développement du pré-TCR et du TCR sont sous le contrôle de l'activité coordonnée de tyrosines kinases telles que les kinases des familles Syk (Cheng and Chan 1997), Src (Salmond, Filby et al. 2009) et Tec (Prince, Yin et al. 2009, Readinger, Mueller et al. 2009), ainsi que de molécules adaptatrices telles que SLP-76 (Clements 2003), LAT (Malissen, Aguado et al. 2005), Grb2 (Jang, Zhang et al. 2010), Gads (Yoder, Pham et al. 2001).

1.3.1. Rôle de l'intensité du signal TCR dans le développement des cellules T

Plusieurs études ont montré que la qualité du signal TCR (intensité/durée) est un important déterminant pour le choix du destin des cellules entre αβ et γδ, la sélection positive ou négative et la différenciation vers les lignées CD4 ou CD8, ainsi que le choix de se tourner vers

des lignées de lymphocytes T conventionnels ou « de type inné» (Liu and Bosselut 2004, Hayes, Li et al. 2005).

– Choix entre αβ vs γδ

Parmi les quelques modèles décrivant le rôle du signal TCR dans la prise de décision entre αβ et γδ, le modèle prenant en compte l'intensité du signal semble être le plus juste. Selon ce modèle, l'intensité du signal délivré par le récepteur d'antigène définit le choix de la lignée. Les thymocytes DN immatures qui reçoivent un signal fort deviennent des lymphocytes TCRγδ, tandis que ceux qui reçoivent un signal faible deviennent des lymphocytes TCRαβ (Hayes, Shores et al. 2003, Haks, Lefebvre et al. 2005).

- Sélection positive vs sélection négative

La sélection permettant à la cellule de continuer sa maturation de DN à DP, qui correspond à la sélection positive, est déterminée par l'intensité du signal TCR. À cette étape, le signal doit être assez fort pour que les cellules soient sélectionnées positivement, faute de quoi elles mourront par négligence (Starr, Jameson et al. 2003). Plus tard, un processus appelé la sélection négative élimine les thymocytes capables de se lier fortement aux complexes CMH/antigènes du soi. Ces lymphocytes qui reçoivent une signalisation TCR très forte finissent par subir la mort cellulaire, sinon ces cellules autoréactives pourraient se rendre dans les organes périphériques et provoquer des maladies auto-immunes chez l'hôte (Starr, Jameson et al. 2003, Prince, Yin et al. 2009).

- L'engagement des lignées CD4/CD8

Au cours des deux dernières décennies, différentes hypothèses ont été avancées pour expliquer la coordination des phénotypes SP CD4/CD8 avec la reconnaissance du CMH. Le

modèle « instructionnel », modifié ultérieurement pour former le modèle « instructionnel quantitatif » (Itano, Salmon et al. 1996, Matechak, Killeen et al. 1996), propose qu'un signal relativement fort, souvent accompagné d'interactions entre CD4 et le CMH de classe II, ordonne aux thymocytes DP de se différencier en lymphocytes T $CD4^+$ et qu'un signal plus faible, souvent accompagné d'interactions entre CD8 et le CMH de classe I, ordonne une différenciation en lymphocytes T $CD8^+$.

- Choix entre lignées de lymphocytes T conventionnel vs « de type inné »

Les lymphocytes T « de type inné » présentent une hétérogénéité en ce qui concerne le rôle de la force du signal TCR dans leur sélection et leur développement. Par exemple, dans les cellules αβ NKT, un TCR invariant peut se lier aux complexes ligand/CMH (αGalCer-CD1d) avec une affinité relativement élevée et une demi-vie particulièrement longue, de l'ordre de quelques minutes plutôt que de quelques secondes, ce qui est caractéristique aux TCR conventionnels réactifs au peptide-CMH I/II (Sidobre, Naidenko et al. 2002, Cantu, Benlagha et al. 2003, Sim, Holmberg et al. 2003, Sidobre, Hammond et al. 2004). D'un autre côté, les choses fonctionnent de façon inverse pour les lymphocytes T « H2-M3-restricted de type inné ». Des études peptidiques ont en effet démontré que seuls les agonistes les plus faibles pouvaient entraîner une sélection des cellules T « H2-M3-restricted », alors que les agonistes forts induisent la mort cellulaire (Chiu, Wang et al. 1999, Berg, Irion et al. 2000).

1.4. CD28

CD28 est exprimé de manière constitutive dans les lymphocytes T. Ce co-récepteur est impliqué dans la prolifération des lymphocytes T, la production d'IL-2, la lutte contre l'anergie des lymphocytes T, l'induction du facteur anti-apoptotique Bcl-xL et la différenciation des

lymphocytes T naïfs en lymphocytes T « helper » de type Th1/Th2 (Lenschow, Walunas et al. 1996, Chambers and Allison 1997, Harris and Ronchese 1999, Alegre, Frauwirth et al. 2001, Bour-Jordan and Blueston 2002). Tous les composants de la voie CD28 forment une petite partie des facteurs impliqués dans la voie de signalisation TCR. Les principaux facteurs intervenant en aval de la voie CD28 sont PI3K (Rudd 1996), 2 membres de la famille de Tec PTKs, Tec (Yang, Ghiotto et al. 1999) et Itk (August, Gibson et al. 1994), le facteur d'échange guanine nucléotide Vav1 (Klasen, Pages et al. 1998, Michel, Grimaud et al. 1998) et la sérine/ thréonine kinase Akt (Parry, Reif et al. 1997, Michel, Attal-Bonnefoy et al. 2001).

1.4.1 CD28 et développement des lymphocytes T

Alors que le rôle de CD28 dans le développement des thymocytes T αβ conventionnels est sujet à controverse, il a un fort impact sur le développement des lymphocytes T régulateurs non conventionnels $CD25^{+}CD4^{+}$ (Tregs). La déficience de CD28 mène à un nombre considérablement réduit de Tregs à la fois dans le thymus et dans la périphérie (Salomon, Lenschow et al. 2000, Bour-Jordan and Blueston 2002, Lohr, Knoechel et al. 2003). Il a également été montré que la voie de signalisation CD28 régule positivement le développement de lymphocytes T « natural killer » (iNKT) (Chung, Nurieva et al. 2008, Williams, Lumsden et al. 2008, Zheng, Zhang et al. 2008).

1.5 La voie de signalisation SLAM

La famille des récepteurs SLAM appartient à la superfamille des récepteurs d'immunoglobulines (Ig). Cette famille est composée de six membres, à savoir SLAM (CD150), 2B4 (CD244), NTB-A (Ly108 dans la souris), CRACC (CS1, CD319), CD84, and Ly-9 (CD229) qui sont exprimés et régissent divers processus dans les cellules hématopoïétiques. (Veillette and

Latour 2003, Veillette 2006, Veillette, Dong et al. 2007, Claus, Meinke et al. 2008). Ces récepteurs peuvent se lier à une famille de protéines adaptatrices qui orchestrent la signalisation SLAM incluant SAP, EAT-2 et ERT (Engel, Eck et al. 2003, Latour and Veillette 2004, Roncagalli, Taylor et al. 2005, Veillette 2006, Ma, Nichols et al. 2007). L'importance de la fonction de SAP et SLAM dans l'immunité a été mise en évidence de la découverte que le syndrome immunitaire sévère de la maladie lymphoproliférative liée à l'X (XLP « X-linked lymphoproliferative ») est causé par l'absence ou le dysfonctionnement de SAP (Coffey, Brooksbank et al. 1998, Nichols, Harkin et al. 1998, Sayos, Wu et al. 1998). Le nombre de cellules NK, de cellules T et de cellules B est normal chez les patients atteints de XLP, seul le sous-groupe de cellules NKT ne se développe pas correctement (Nichols, Hom et al. 2005, Pasquier, Yin et al. 2005).

1.5.1 La voie de signalisation SLAM-SAP et le développement des thymocytes

Il est établit que Ly108 ainsi que SLAM, deux membres de la famille de récepteurs SLAM exprimés sur les thymocytes DP, sont essentiels pour le développement des cellules iNKT (Griewank, Borowski et al. 2007). Hormis les cellules αβ iNKT conventionnelles, il y a un autre groupe de cellules T « de type inné », appelées cellules γδNKT. Dans ces cellules, il a été également rapporté que la perte de la protéine adaptatrice SAP affecte négativement leur développement (Alonzo, Gottschalk et al. 2010), suggérant que SAP pourrait réguler le développement des cellules NKT exprimant le TCRαβ ou le TCRγδ.

1.6. Les protéines adaptatrices : la famille des protéines Dok

Les protéines Dok sont des protéines adaptatrices qui contiennent un domaine PH, un domaine PTB et une région terminale contenant de multiples sites de phosphorylation et des régions riches en résidus proline (Carpino, Wisniewski et al. 1997, Yamanashi and Baltimore 1997). Dok-1 et Dok-2, deux membres de la famille de protéines Dok qui sont exprimés dans les cellules T, sont impliquées dans la signalisation d'une variété de récepteurs dans les cellules hématopoïétiques (Janssen and Zhang 2003, Mashima, Hishida et al. 2009). Les études réalisées initialement dans les cellules Jurkat ont montré que Dok-1 et Dok-2 sont phosphorylées au niveau des tyrosines suite à la liaison de CD28 ou CD2 au niveau des cellules T (Nemorin and Duplay 2000, Michel, Attal-Bonnefoy et al. 2001). La surexpression de Dok-1 conduit à une inhibition spécifique de l'activation d'ERK1/2 dépendante de CD2 (Nemorin, Laporte et al. 2001). L'évidence que Dok-1 et Dok-2 sont impliquées en tant que transducteur de signaux TCR provient des études utilisant des cellules humaines ou murines déficientes en Dok, où la déficience de l'expression de Dok-1 et Dok-2 conduit à une augmentation de la prolifération et de la production de cytokine dépendante du TCR (Yasuda, Shirakata et al. 2004). En plus de leur rôle dans la signalisation du TCR et des récepteurs de costimulation, les protéines Dok pourraient être importantes dans les voies de signalisation des récepteurs de chimiokines CXCR4 et SLAM ainsi que du récepteur IL-4 (Latour, Gish et al. 2001, Latour, Roncagalli et al. 2003, Okabe, Fukuda et al. 2005, Inoue, Yasuda et al. 2007). Les protéines Dok sont donc probablement un élément commun dans plusieurs voies de signalisation au niveau des cellules T. Par contre, la façon dont les protéines Dok régulent négativement la signalisation du TCR n'est pas entièrement élucidée. Dans les cellules T matures déficientes en Dok-1 et Dok-2, la phosphorylation de ZAP-70, LAT, SLP-76, Akt, et Erk1/2 est augmentée par rapport aux cellules

WT (Dong, Corre et al. 2006, Yasuda, Bundo et al. 2007). La régulation négative de ZAP-70 par Dok pourrait être attribuée à la liaison compétitive du domaine PTB de Dok aux motifs ITAM du TCRζ et du CD3ε. Dans les cellules T, Dok-1 et/ou Dok-2 phosphorylées forment des complexes moléculaires avec RasGAP (Nemorin, Laporte et al. 2001). Le recrutement de RasGAP dépendant de Dok à proximité de Ras est probablement responsable de l'atténuation de la signalisation ERK1/2.

1.7. Les cellules αβ NKT

Pour la première fois, les cellules T TCRα$^+$ Vα14–Jα18 dans les souris C57BL/6 ont été présentées comme un sous-groupe distinct qui exprime des niveaux intermédiaires de TCR plutôt que de hauts niveaux de TCR, avec une fréquence de deux à trois fois plus élevée d'expression de Vβ8 comparée aux cellules T conventionnelles, et une perte de l'expression de CD4 et de CD8 (Budd, Miescher et al. 1987, Ceredig, Lynch et al. 1987, Fowlkes, Kruisbeek et al. 1987). L'intérêt général porté aux lymphocytes DN TCRαβ$^+$ a redoublé lors de la découverte que ces cellules sont une source considérable de cytokines immunorégulatrices incluant IL-4, IFN-γ et TNF (Zlotnik, Godfrey et al. 1992). Il a été également rapporté que ces cellules expriment le marqueur NK1.1, précédemment considéré comme seulement exprimé par les cellules NK (Sykes 1990, Levitsky, Golumbek et al. 1991). D'autres études ont révélé que les cellules exprimant la chaîne invariante TCRα Vα14–Jα18 étaient restreintes au CD1d, une molécule semblable au CMH de classe I qui présente des antigènes lipidique aux cellules T (Beckman, Porcelli et al. 1994, Bendelac, Lantz et al. 1995). Ultérieurement, il a été constaté qu'elles reconnaissent les Glycosphingolipides (GSL) et les α-galactosylcéramides (α-GalCer) dérivés d'une éponge marine (Kawano, Cui et al. 1997). Au même moment, une autre population de

cellules TCR$\alpha\beta^+$ restreinte au CD1d a été découverte exprimant des chaines α et chaines β du TCR variables (nommé NKT de type II), au lieu du TCRα Vα14–Jα18 invariant et un nombre limité de chaine β. NKT type I qui avait été reporté auparavant (Cardell, Tangri et al. 1995, Kadri, Blomqvist et al. 2008). Il a été rapporté que les cellules NKT de type II sont capables de reconnaître des antigènes composés de sulfatide (Jahng, Maricic et al. 2004, Van Rhijn, Young et al. 2004).

1.7.1. Maturation des cellules iNKT

Au stade DP, les cellules NKT sont sélectionnées positivement par d'autres thymocytes DP exprimant des complexes formés de lipides et de CD1d. La sélection des cellules NKT par CD1d associé aux lipides du soi induit un phénotype mémoire/activé ($CD69^{high}$) dès le « stade 0 » de développement. Le processus de la sélection positive, par le complexe formé du CD1d-lipide avec le TCR, nécessite la liaison du TCR, de SLAM et la signalisation de la kinase Src (Fyn) (Nichols, Hom et al. 2005, Pasquier, Yin et al. 2005). À cette étape, l'induction de différents facteurs de transcription tels que PLZF, Runx1, c-Myc et Egr2 est primordiale (Das, Sant'Angelo et al. 2010). Suite à la liaison établie entre le TCR et le CD1d, les cellules NKT entrent au « stade 0 », souligné par l'expression de CD24 et CD69, et l'absence d'expression du marqueur de mémoire CD44 ainsi que de NK1.1 ($CD24^{high}$, $CD69^{high}$, $CD44^-$ and $NK1.1^-$). La prochaine étape de maturation se caractérise par une régulation à la baisse de CD24 pour atteindre le « stade 1 » (cellule NKT $CD4^+$ avec une faible expression de CD44). Les cellules NKT « stade 1 » restent dans le thymus où ils poursuivent leur programme de développement jusqu'au « stade 2 » déterminé par la régulation à la hausse du CD44 et du récepteur IL-2 (CD122) permettant l'induction d'une faible transcription basale des cytokines Th2 suivie des cytokines Th1. En quittant le thymus, les cellules NKT commencent à exprimer des récepteurs

de la lignée NK tel que NK1.1 et elles entrent en « stade 3 ».Le facteur de transcription T-bet est essentiel pour la transition du « stade 2 » au « stade 3 ». Quelques cellules NKT au « stade 3 » résident dans le thymus, toutefois la fonction de ces cellules dans le thymus n'a pas encore été identifiée (Benlagha, Kyin et al. 2002, Godfrey, Stankovic et al. 2010). De récentes études ont montré que, tout comme les cellules CD4 Th, les cellules iNKT peuvent se différencier en cellules NKT1 (T-bet^{high}) (Benlagha, Kyin et al. 2002, McNab, Berzins et al. 2005), en cellules NKT2 (GATA3^{high}) (Terashima, Watarai et al. 2008, Watarai, Sekine-Kondo et al. 2012) et en cellules NKT17 (RORγt^{high}) (Michel, Keller et al. 2007, Doisne, Becourt et al. 2009, Watarai, Sekine-Kondo et al. 2012) qui produisent respectivement de l'IFN-γ, de l' IL-4 et de l'IL-17. Les cellules NKT2 and NKT17 sont rares dans les souris C57BL/6, mais elles peuvent être dominantes dans d'autres souches de souris ou dans différentes souris mutantes.

1.7.2. Fonction des cellules NKT dans le système immunitaire

Les cellules NKT ont un phénotype activé/mémoire avec des niveaux d'expression élevé de CD44 et de CD122, contrairement au phénotype naïf des cellules T conventionnelles. Durant une réponse immunitaire, les cellules NKT sont rapidement activées, et peuvent secréter rapidement de fortes quantités de cytokines pouvant inclure IFN-γ, IL-4, IL-10, IL-13, IL-17, IL-21 et le facteur de nécrose tumorale (TNF) (Gumperz, Miyake et al. 2002, Bendelac, Savage et al. 2007, Coquet, Kyparissoudis et al. 2007, Michel, Keller et al. 2007, Sakuishi, Oki et al. 2007). Ainsi dans cet état d'activation, les cellules NKT peuvent favoriser ou inhiber le système immunitaire (Smyth and Godfrey 2000, Matsuda, Mallevaey et al. 2008). Il a aussi été décrit qu'elles sont impliquées dans les infections contre plusieurs pathogènes tels que les virus, les bactéries à gram positif et à gram négatif, les champignons, les parasites et les helminthes (Tupin, Kinjo et al. 2007), dans l'immunité tumorale orientée par l'IL-12 (Cui, Shin et al. 1997)

et dans les maladies auto-immune comme MS (Jahng, Maricic et al. 2001, Singh, Wilson et al. 2001, Furlan, Bergami et al. 2003).

1.8. Cellules γδ NKT

Chez la souris, le sous-groupe de cellule γδ T ayant des caractéristiques du système immunitaire inné a été défini historiquement comme étant des cellules Thy-1^{dull} qui représentent 5% et 30% des cellules γδ T totales chez les souris adultes C57BL/6 et DBA/2 respectivement. Elles sont capables de sécréter simultanément de l'IFN-γ, de l'IL-4, de l'IL-10, et de l'IL-3, tandis que les thymocytes Thy-1^{+} γδ sont seulement capable de sécréter de l'IFN-γ. Comme pour les cellules αβ NKT, il a été rapporté qu'elles résident dans le thymus, la rate, et le foie (Vicari, Mocci et al. 1996, Azuara, Levraud et al. 1997, Azuara, Lembezat et al. 1998). Il a été démontré que la majorité des thymocytes Thy-1^{dull} γδ exprime un TCR codé par le gène Vγ1, associé à Vδ6, originalement appelé Vδ6.4 chez la souris DBA/2 et Vδ6.3 chez la souris C57BL/6 (Azuara, Levraud et al. 1997, Azuara, Lembezat et al. 1998, Gerber, Azuara et al. 1999, Azuara, Grigoriadou et al. 2001). En 2009, pour la première fois, il a été reporté que le facteur de transcription PLZF est aussi exprimé chez les cellules γδ T Vγ1.1^{+}Vδ6.3^{+}, et comme chez les cellules αβ NKT, ce facteur est responsable des propriétés innées du sous-groupe de cellule γδ T (Kreslavsky, Savage et al. 2009). Un autre membre de la famille BTB-POZ, ThPOK, a aussi révélé être exprimé dans le sous-groupe Vγ1.1^{+}Vδ6.3^{+} des cellules γδ T, et il a été démontré que les niveaux d'expression de ThPOK augmentent lors de la maturation des cellules γδ T Vγ1.1^{+}Vδ6.3 (Alonzo, Gottschalk et al. 2010, Park, He et al. 2010). Comme pour les cellules αβ NKT, la sélection et le développement des cellules γδ NKT est dépendant de la voie de signalisation SLAM-SAP. Deux études différentes ont révélé que le développement du sous-

groupe de cellule γδ T ayant des caractéristiques du système immunitaire innée est aussi dépendant de SAP, puisque le nombre de cellules γδ T Vγ1.1^{+}Vδ6.3^{+} était réduit chez les souris déficientes en SAP, mais pas aussi sévèrement que le nombre de cellules αβ NKT (Kreslavsky, Savage et al. 2009, Alonzo, Gottschalk et al. 2010).

1.8.1. Rôle de la force de la signalisation du TCR dans le développement des cellules γδ NKT

Il a été reporté que le nombre de cellules γδ T Vγ1.1^{+}Vδ6.3^{+} est augmenté déchez des souris mutantes dans la voie de signalisation du TCR tels que les souris déficientes en Itk (Felices, Yin et al. 2009, Qi, Xia et al. 2009, Yin, Cho et al. 2013), les souris possédant une version mutante de la protéine adaptatrice SLP-76 (SLP-76:Y145F) (Alonzo, Gottschalk et al. 2010), les souris déficientes en CD3ζ, dont il a été précédemment démontré qu'elles ont un plus grand nombre de thymocytes NK1.1^{+} TCRγδ $^{+}$ (Arase, Ono et al. 1995) et les souris déficientes en LAT. L'accumulation de cellules γδ NKT a aussi été observée chez les souris n'exprimant pas Id3, un facteur de transcription induit par la signalisation du TCR (Lauritsen, Wong et al. 2009, Ueda-Hayakawa, Mahlios et al. 2009, Verykokakis, Boos et al. 2010). Il a été suggéré que chez ces mutants de signalisation du TCR, les cellules γδ T Vγ1.1^{+}Vδ6.3^{+} qui devraient normalement être supprimées (en recevant un signal suffisamment fort du TCR) seraient sauvées et pourraient ainsi éviter la sélection négative grâce à l'atténuation de la signalisation du TCR (Lauritsen, Wong et al. 2009).

1.8.2. Rôle des protéines E et Id dans le développement des cellules γδ NKT

Tandis que l'expression d'Id3 a précédemment été démontrée comme essentielle au développement de la lignée γδ des cellules T (Acuto and Michel 2003, Haks, Lefebvre et al. 2005, Hayes, Li et al. 2005, Lauritsen, Wong et al. 2009), le nombre total de cellules γδ T chez les souris TCR non-transgénique Id3-déficient était augmenté dû à une surcroissance de la population de cellules γδ NKT (Lauritsen, Wong et al. 2009, Ueda-Hayakawa, Mahlios et al. 2009, Verykokakis, Boos et al. 2010). Il a ultérieurement été démontré qu'Id3 et Id2, en contrôlant les niveaux d'activités de la protéine E, contrôlent le développement des cellules γδ NKT de manière spécifiquement dépendante du stade de développement (Zhang, Lin et al. 2013).

1.8.3. Rôles fonctionnels des cellules γδ NKT au sein du système immunitaire

Contrairement aux cellules αβ NKT qui sont bien étudiées quant à leur rôle fonctionnel au sein du système immunitaire, le rôle des cellules γδ NKT nécessite d'être étudié davantage. Cependant, de nombreux rôle, protecteur ainsi que pathogénique, ont déjà été identifiés pour ces cellules selon différents contextes tels qu'en auto-immunité, immunité anti-tumorale et lors d'une infection, ce qui reflète leurs propriétés innées et leur habileté à produire simultanément des cytokines pro- et anti-inflammatoires. Les cellules T Vδ6.3$^+$ et Vδ6.4$^+$ ont été démontrées comme étant dominantes dans la réponse contre la bactérie intracellulaire *Listeria monocytogenes* chez les souris Balb/c (Belles, Kuhl et al. 1996). Dans une autre étude, un rôle régulateur des cellules γδ T Vγ1$^+$ a été démontré pour l'immunité anti-tumorale en inhibant les cellules T Vγ4$^+$ (Hao, Dong et al. 2011). De plus, il a été reporté que les souris déficientes en Id3 développent spontanément une maladie auto-immunitaire similaire au syndrome de Sjögren

(Li, Dai et al. 2004). La grande population de cellules γδ T $V\gamma1.1^{+}V\delta6.3^{+}$ chez ces souris pourrait potentiellement être impliquée dans la pathogenèse de cette maladie.

1.9. Les cellules T $CD8^{+}$ «de type inné »

Les cellules T $CD8^{+}$ « de type inné » ont été décrites pour la première fois chez les souris déficientes en Itk (Atherly, Lucas et al. 2006, Broussard, Fleischacker et al. 2006). Chez ces souris, en absence d'Itk, les thymocytes SP CD8 présente un phénotype unique quant aux marqueurs de surfaces et à leurs fonctions, tel qu'une expression très basse de CD24 et une expression très élevée des marqueurs mémoires CD44, CD122, et CXCR3 ($CD24^{lo}CD44^{hi}CD122^{hi}$ $CXCR3^{hi}$). Ces cellules sont aussi capables de produire une grande quantité d'IFN-γ suite à la stimulation du TCR, agissant ainsi comme des cellules T activées (Atherly, Lucas et al. 2006, Broussard, Fleischacker et al. 2006). Des études approfondies ont révélés que, contrairement aux cellules T mémoires conventionnelles qui expriment le facteur de transcription de la T-Box, T-bet, les cellules T CD8 « de type inné » expriment un autre facteur de transcription de la T-Box appelé Eomesodermin, ce qui leur confèrent leur propriétés innées (Atherly, Lucas et al. 2006, Broussard, Fleischacker et al. 2006, Berg 2007, Prince, Yin et al. 2009). L'expansion de cette population a été reporté chez d'autres souris ayant un gène déficient, incluant les facteurs de transcription « Kruppel-like factor 2 » (KLF2) (Weinreich, Takada et al. 2009, Weinreich, Odumade et al. 2010), la protéine de liaison à CREB (CBP) (Fukuyama, Kasper et al. 2009), et Id3 (Verykokakis, Boos et al. 2010). De plus, les souris ayant une forme mutée de SLP-76 (SLP76:Y145F), qui ne peut interagir avec Itk et maintenir sa forme active (Jordan, Smith et al. 2008), ont un phénotype identique aux souris $Itk^{-/-}$ en ce qui attrait à l'accumulation thymique de cellules T $CD8^{+}$ « de type inné ». Les premiers indices de la

découverte du mécanisme complexe menant au développement des cellules T $CD8^+$ « de type inné » proviennent d'études chez les souris déficientes en KLF2, un modèle démontrant une accumulation de cellules αβ and γδ NKT (Odumade, Weinreich et al. 2010, Lee, Jameson et al. 2011). Ce mécanisme s'est avéré être en lien avec des effets extrinsèques à l'IL-4 (Weinreich, Takada et al. 2009, Weinreich, Odumade et al. 2010).

1.9.1. Le rôle fonctionnel des cellules T $CD8^+$ « de type inné » au sein du système immunitaire

Les souris BALB/c possédant une population abondante de cellules T $CD8^+$ « de type inné » sont un bon modèle pour étudier les rôles fonctionnels de ces cellules dans la réponse immunitaire. Dans un modèle d'infection pour étudier le paludisme, les souris BALB/c se sont révélées plus résistantes que les souris C57BL/6, avec une réponse protectrice dépendante des récepteurs de l'IL-4 sur les cellules T $CD8^+$ (Morrot, Hafalla et al. 2005). De plus, une déficience en IL-4 ou CD1d rend les souris BALB/c plus susceptible à l'infection parasitaire et elles développent une pathologie beaucoup plus sévère (Hansen, Siomos et al. 2003). Une autre étude a révélé que les souris BALB/c sont plus efficaces que les souris C57BL/6 pour contrôler les pathogène du paludisme après une immunisation avec une forme inactivée par radiation de *Plasmodium berghei* ou *P. yoelii sporozoites* (Schmidt, Butler et al. 2010). Ceci pourrait être en lien avec la grande quantité de cellules T $CD8^+$ « de type inné » chez les souris BALB/c en comparaison avec d'autres souches de souris (Lee, Jameson et al. 2011).

1.10. Hypothèse et objectifs de recherche

L'intensité et la durée du signal du TCR déterminent l'expression de gène spécifique et régulent les différentes étapes durant le développement des cellules T. Par conséquent, les molécules de signalisation modulant l'intensité du signal du TCR joueraient un rôle dans la régulation du développement des cellules T. Les deux membres de la famille des protéines adaptatrice Dok, Dok-1 et Dok-2, qui sont exprimés dans les cellules T, ont un rôle négatif dans la voie de signalisation du TCR. Le modèle actuel propose que le recrutement de RasGAP par Dok inhibe l'activité de Ras et conséquemment la voie d'Erk impliquée dans la signalisation du TCR. L'activation d'Erk est impliquée dans la signalisation du pré-TCR, la sélection positive et les choix de lignée entre CD4/CD8 et entre γδ/αβ. Donc, puisque l'activation d'Erk médiée par le TCR est inhibée par Dok, nous proposons que cette protéine puisse jouer un rôle important dans le développement et la maturation des thymocytes. Afin d'étudier le rôle de Dok dans le développement des lymphocytes T, nous avons généré des souris transgéniques surexprimant Dok-1 sous le contrôle du promoteur CD2, menant à l'expression transgénique dans les thymocytes et les cellules T matures. Nous avons effectué une analyse détaillée du développement des cellules T dans les souris transgéniques Dok-1 en les comparant aux souris WT témoins selon les objectifs spécifiques suivants :

1- Déterminer le rôle de Dok dans la modulation du l'intensité de la signalisation du TCR dans les thymocytes en développement (niveau d'action des molécules effectrices clés dans la voie de signalisation du TCR)

2- Spécifier le rôle de Dok aux différentes étapes durant le développement des cellules T.

Nous avons montré que Dok contrôle le développement des cellules γδ NKT « de type inné ». Cette petite population de cellules T de type « de type inné » est mal comprise comparativement

aux cellules αβ NKT . Nous avons poursuivi notre étude afin de mieux caractériser les cellules γδ NKT en étudiant les voies de signalisation qui pourrait potentiellement être importante pour leur développement et leur maturation. Il a été démontré que l'interaction entre CD28 et B7 est essentielle pour le développement des cellules αβ iNKT. En plus de leurs similarités phénotypiques et fonctionnelles, les cellules « de type inné » αβ et γδ NKT partagent des propriétés communes de développement. C'est pourquoi nous avons émis l'hypothèse que le corécepteur CD28 serait important dans la régulation du développement et dans la maturation des cellules γδ NKT. De plus, sachant que les cellules NKT, qui produisent de l'IL-4, sont responsables de la conversion des thymocytes $CD8^+$ en cellules T $CD8^+$ « de type inné », nous proposons que la taille des thymocytes $CD8^+$ « de type inné » puisse être également affecté par la signalisation de CD28. Pour tester cette hypothèse, nous avons utilisé des souris déficientes en CD28 et nous les avons comparés aux souris sauvages, en suivant les objectifs suivants :

1- Déterminer s'il y a une corrélation entre l'absence de CD28 et des anomalies dans la génération de cellules αβ et γδ NKT $PLZF^+$

2- Caractériser le mode d'action de CD28 dans la régulation du développement des cellules NKT

2. Résultats et discussion

2.1. Les protéines Dok régulent la β-sélection

En évaluant le rôle de Dok dans le développement des thymocytes, nous avons démontré que la surexpression de Dok-1, dans deux lignées de souris transgéniques, induit un blocage partiel à l'étape de transition de DN à DP dans la maturation des thymocytes. Plus précisément, le blocage a lieu entre les étapes de maturation DN3a et DN3b, où le processus de la β–sélection a lieu. Il y a une corrélation directe entre les niveaux de surexpression de Dok-1 et

l'ampleur de ce blocage dans la maturation des thymocytes, démontrant une relation directe avec Dok. En accord avec des études précédentes sur les cellules T matures (Yasuda, Bundo et al. 2007), nos résultats démontrent aussi que les protéines Dok modulent négativement la signalisation du TCR dans les thymocytes DP, où plusieurs molécules effectrices telles que ZAP-70, LAT, PLC-γ et Erk ont des niveaux de phosphorylation réduits lorsque Dok est surexprimé (Besin et al 2012) tandis qu'elles ont des niveaux de phosphorylation élevés en absence de Dok (données non montrées). Puisque les voies de signalisation du TCR et du pré-TCR ont des molécules effectrices communes, nous pouvons présumer que Dok joue le même rôle négatif dans la régulation de l'intensité de la signalisation du pré-TCR, lequel est important dans la β–sélection. Erk devient activé suite à la stimulation du pré-TCR et joue un rôle important dans la sélection β (Michie, Trop et al. 1999). Nous proposons donc que l'inactivation d'Erk médiée par Dok (Campbell, Khosravi-Far et al. 1998) régulerait la sélection β. La déficience du développement chez les souris Dok-1 transgéniques pourrait partiellement être due à l'inhibition de la voie Erk et par conséquent l'inhibition de l'expression d'Egr et Id3, résultant en un blocage des thymocytes à l'étape DN3, imposé par la présence d'E2A fonctionnel.

2.2. Les protéines Dok régulent le développement des cellules γδ NKT

Nos données démontrent que la surexpression de Dok-1 promeut le développement des cellules γδ T dans le thymus, la rate et le foie des souris transgéniques en comparaison au WT. Cette promotion du développement corrèle avec le niveau de surexpression de Dok-1 et est principalement due à l'expansion spécifique des cellules γδ NKT. En effet, ces cellules similaires aux cellules γδ NKT de souris WT, puisqu'elles expriment le facteur de transcription PLZF, sont dépendantes de la signalisation SLAM-SAP pour leur développement et produisent

de l'IL-4 *ex vivo* après la première stimulation. Les souris transgénique Dok-1 peuvent être considérées dans la catégorie des souris mutantes de la signalisation du TCR, avec une signalisation de TCR atténuée. Les cellules γδ NKT seraient sauvées de la sélection négative en recevant un signal de TCR plus faible, en comparaison avec les souris WT contrôles. En comparant les souris sauvages aux souris déficientes en Dok-1 et Dok-2, nous n'avons détecté aucune modification dans le nombre de cellules T $V\gamma1.1^+V\delta6.3^+$ ou bien dans le niveau d'expression de PLZF dans ce sous-groupe de cellules γδ T, dans le thymus et la périphérie (donnée non montrée). Le développement des cellules T $V\gamma1.1^+V\delta6.3^+$ $PLZF^+$ pourrait ne pas être influencé par une intensité plus forte de la signalisation du TCR, puisque la sélection positive et l'expression de PLZF pourrait déjà fonctionner à un niveau maximal. Alternativement, l'augmentation de l'intensité de la signalisation du TCR chez les thymocytes déficients en Dok-1 et Dok-2 pourrait augmenter la sélection négative et positive, ce qui ramènerait un nombre de cellules γδ NKT exprimant PLZF similaire entre les souris WT et Dok-1/Dok-2 déficientes.

2.3. Dok pourrait réguler le développement des cellules γδ NKT via la voie de signalisation SLAM-SAP

En plus de la voie de signalisation du TCR, Dok pourrait être impliqué dans la régulation du développement des cellules γδ NKT en médiant la voie de signalisation de SLAM-SAP. De façon intéressante, nous avons montré que le développement des cellules T $V\gamma1.1^+V\delta6.3^+$ $PLZF^+$ est strictement dépendant de la voie de signalisation SLAM-SAP chez les souris Dok-1 transgéniques, tandis que chez les souris sauvages, la perte de SAP n'affecte que partiellement la fréquence et le niveau d'expression de PLZF des cellules $V\gamma1.1^+V\delta6.3^+$ (Alonzo,

Gottschalk et al. 2010). En présumant que l'induction de PLZF a besoin d'une forte intensité lors de la signalisation du TCR, la déficience dans la signalisation du TCR en présence d'une surexpression de Dok pourrait affecter l'induction de l'expression de PLZF et par le fait même rendre les cellules $PLZF^+$ plus susceptibles aux signaux provenant des membres de la famille SLAM. En plus, l'implication de Dok dans la signalisation de SLAM-SAP pourrait être importante pour la sélection et le développement des cellules γδ NKT. Nous pouvons supposer que l'intensité du signal que les précurseurs des cellules γδ NKT reçoivent durant la sélection positive ou durant le processus de maturation est la résultante de la signalisation du TCR et de SLAM. Dok pourrait donc contrôler le développement des cellules γδ NKT via la régulation des voies de signalisation du TCR et de SLAM. La phosphorylation de Dok-1 et Dok-2, ainsi que le recrutement de RasGAP, ont été démontrés dans la signalisation impliquant la liaison de SLAMF1 dans les thymocytes DP, les précurseurs des cellules αβ iNKT, malgré que le résultat fonctionnel de cette phosphorylation n'a pas encore été identifié (Latour, Gish et al. 2001). Bien que les récepteurs de SLAM, essentiels pour la sélection positive et le développement des cellules γδ NKT, n'aient toujours pas été identifiés, nous supposons que la phosphorylation de Dok et le recrutement de RasGAP se font dans les cellules γδ NKT suite à la liaison de SLAM. Davantage d'études seront nécessaires pour identifier le rôle de Dok dans la voie de signalisation de SLAM durant le développement des cellules NKT.

2.4 Cible potentielle médiée par Dok dans la régulation du développement des cellules γδ NKT

Quelle est la cible de Dok dans la génération des cellules γδ NKT? Id3 est un facteur de transcription unique qui est l'antagoniste d'E2A. Ces deux molécules ensemble jouent d'importants rôles dans la voie de signalisation du TCR à différents moments durant le développement des cellules T (Bain, Cravatt et al. 2001, Miyazaki, Rivera et al. 2011, Naito, Tanaka et al. 2011). Il a été suggéré qu'Id3 puisse limiter le développement des groupes de cellules γδ T ayant des TCRγδ de haute affinité, mais favoriser le développement des autres sous-types (Kreslavsky, Gleimer et al. 2010). Cependant, une étude très récente a démontré que les protéines Id3 et Id2 régulent la taille des populations de cellules γδ NKT en affectant leur survie et leur prolifération, par le contrôle des niveaux d'activité de protéine E durant le développement des cellules T (Zhang, Lin et al. 2013). Tel que mentionné précédemment, il est établi que l'expression d'Id3 est induite par la voie Erk, qui elle-même est médiée par Egr2/3 (Bain, Cravatt et al. 2001). Comme Dok peut réguler négativement la signalisation Erk, il est un régulateur négatif potentiel de l'induction d'Id3 et par conséquent, de l'activité de la protéine E dans la voie de signalisation TCR. Il est pertinent de savoir que les souris Dok-1 Tg et Id3-déficientes montre un phénotype particulier et comparable au niveau de l'expansion des cellules γδ NKT. L'hypothèse que la régulation du développement des cellules γδ NKT, médiée par Dok, se fait par l'inhibition d'Id3, qui par sa fonction module l'activité de la protéine E, peut ainsi être formulée.

2.5. CD28 régule le développement des cellules NKT après la sélection positive

Nos données montrent clairement que CD28 régule le développement des cellules γδ NKT qui expriment PLZF. Nous montrons que CD28 est critique pour la maturation et/ou la survie ultérieure des cellules γδ NKT sélectionnées positivement pour $CD24^{low}$. De plus, trois études indépendantes ont montré le rôle essentiel de l'interaction CD28-B7 durant le développement intra-thymique, l'expansion et la fonction de la population cellulaire iNKT $CD1d^{+}$ (Chung, Nurieva et al. 2008, Williams, Lumsden et al. 2008, Zheng, Zhang et al. 2008). En absence de CD28, la sélection positive des cellules iNKT n'est pas affectée. Par contre, en absence de signalisation CD28 il y a moins de cellules iNKT exprimant NK1.1, capables de produire simultanément les cytokines des Th1 et Th2 suite à la stimulation, qui sont considérés comme la lignée la plus mature des cellules iNKT (Chung, Nurieva et al. 2008, Williams, Lumsden et al. 2008). Nous avons aussi montré que contrairement aux cellules αβ NKT, dont l'interaction CD28-B7 est importante dans la prolifération et l'apoptose des cellules iNKT (Zheng, Gao et al. 2004), la déficience de CD28 n'affecte pas la capacité de prolifération des cellules γδ NKT, malgré qu'elle puisse mener à une augmentation de la susceptibilité à l'apoptose des populations de cellules γδ NKT immatures dans le thymus. Alternativement, mais non exclusivement, CD28 pourrait moduler les niveaux d'expression de gènes sélectionnés qui sont important pour la différenciation ultérieure des cellules γδ NKT immatures. Des expériences additionnelles seront nécessaire pour identifier le mécanisme précis et les étapes de maturation dans lesquels CD28 promouvoit la maturation et/ou la survie des cellules γδ NKT. Étant donné que Dok peut être phosphorylé suite à la stimulation par CD28 (Yang, Ghiotto et al. 1999, Michel, Attal-Bonnefoy et al. 2001 ces protéines pourraient réguler négativement la signalisation CD28 en inhibant par exemple l'activité de la kinase Tec. D'autres études seront nécessaires

pour identifier le rôle des protéines Dok dans la voie de signalisation CD28 au niveau de la régulation du développement des cellules γδ NKT.

2.6. CD28 régule la maturation des cellules γδ NKT par des mécanismes cellulaires intrinsèques

Les résultats obtenus avec des souris chimériques obtenues par transfert de moelle osseuse ont montré que la proportion de thymocytes αβ et γδ NKT $PLZF^+$ est significativement réduite en absence de CD28 même en présence de cellules NKT WT. Le défaut dans le développement des cellules NKT $PLZF^+$ CD28-déficientes est donc intrinsèque à ces cellules. Des études similaires ont été faites précédemment pour identifier si CD28 régule le développement des cellules iNKT par des mécanismes cellulaires extrinsèques ou intrinsèques. Les résultats montrent clairement que lorsque le ratio de « chimerisme » n'est pas égal et que les cellules WT sont en majorité, elles peuvent exercer des effets cellulaires extrinsèques sur les cellules témoins CD28-déficientes (Williams, Lumsden et al. 2008). Par contre, dans nos études, les cellules WT et CD28-déficientes étaient dans des ratios égaux avant et après la reconstitution. Il serait important donc de vérifier l'effet de cellules donneurs WT lorsque présentes en majorité sur des cellules CD28-déficientes sur le développement des cellules γδ NKT.

2.7. La régulation positive CD28-dépendante de LFA-1 pourrait contrôler l'homéostasie des cellules NKT

Nous avons aussi montré que la régulation positive de LFA-1, dans les cellules αβ et γδ NKT dans le thymus et la périphérie, est dépendante de la signalisation par CD28. LFA-1 a été préalablement identifié comme molécule jouant un rôle essentiel dans la migration cellulaire spécifique aux tissus et la rétention des cellules iNKT. Il est possible que l'altération de l'expression de LFA-1 contribue partiellement à réduire le nombre de cellules αβ et γδ NKT dans le foie des souris CD28-déficientes, mais des expériences supplémentaires seront nécessaires pour vérifier directement cette hypothèse. Il existe un candidat hypothétique qui pourrait potentiellement se trouver entre la signalisation CD28 et l'expression de LFA-1. La protéine du syndrome Wiskott-Aldrich (WASp) est un lien crucial entre les stimuli cellulaires et les réarrangements du cytosquelette. Il a été démontré que WASp joue un rôle important dans l'homéostasie des cellules iNKT puisqu'il y a une diminution du « homing » et/ou de la rétention des cellules iNKT dans les tissus périphériques chez les souris WASp-déficientes (Astrakhans, Ochs et al. 2009). D'autre part, il a été démontré que WASp joue un rôle majeur dans le lien entre la stimulation TCR et l'induction des changements du cytosquelette d'actine requis pour l'activation des cellules T. Il a aussi été démontré dans les cellules T, que le complexe WASp/SNX9/p85/CD28 permet une interface unique pour les voies endocytiques, de polymérisation d'actine et de transduction de signal requises pour la costimulation des cellules T médié par CD28 (Badour, McGavin et al. 2007). La voie de transduction du signal, impliqué dans la régulation de l'expression de LFA-1, initiée après la stimulation de CD28 pourrait donc être médiée partiellement par WASp, sachant que WASp promouvoit la forte expression de

LFA-1 dans les cellules αβ NKT et régule le remodelage de l'actine nécessaire pour la signalisation par CD28.

2.8. Dok et CD28 régulent la conversion des cellules T $CD8^+$ en cellules T $CD8^+$ innées dans le thymus

Nos résultats montrent que la surexpression de Dok-1 induit l'accumulation, de manière SAP-dépendante, de cellules T $CD8^+$ « de type inné ». Ces thymocytes $CD8^+$ « de type inné » accumulés qui se développent chez les souris Dok-1 transgénique sont très similaires à ceux qui ont été reportés comme se développant chez les souris Tec kinase (ITL et Rlk)-déficientes. Ces cellules T $CD8^+$ affichent une régulation positive des marqueurs de surface des cellules mémoires, tels que CD44 et CD122, ainsi que du facteur de transcription Eomesodermin et ils produisent des hauts taux d'INF-γ après stimulation (Atherly, Lucas et al. 2006, Broussard, Fleischacker et al. 2006). Nous avons aussi montré qu'il y a une corrélation remarquable entre le pourcentage de cellules T $CD8^+$ exprimant Eomesodermin et le nombre de cellules γδ NKT, en comparant les deux lignées de Dok-1 transgénique et leur contrôle WT. Conséquemment, dans les lignées transgéniques, il existe une bonne corrélation entre les niveaux d'expression de SAP et les thymocytes $CD8^+$ $Eomes^{high}$. Comme dans les modèles déficients en Itk et d'autres gènes, le développement de ces cellules T $CD8^+$ « de type inné » est probablement la conséquence d'une production élevée d'IL-4 par le grand nombre de cellules γδ T $PLZF^+$ chez les souris Dok-1 transgénique (Verykokakis, Boos et al. 2010, Weinreich, Odumade et al. 2010, Lee, Jameson et al. 2011). Nous avons aussi montré que CD28 contrôle le nombre de thymocytes αβ et γδ qui expriment des hauts taux de PLZF et produisent une grande quantité d'IL-4, suite à une stimulation *in vitro* avec PMA et ionomycine. Dans le thymus, les cellules T $PLZF^+$ $IL\text{-}4^+$ αβ et

γδ sont les cellules NKT, qui produisent de l'IL-4 de façon constitutive puisque qu'elles sont constamment exposées à leur propre ligand et sont capable de promouvoir la conversion des cellules T $CD8^+$ en T $CD8^+$ « de type inné ». C'est pourquoi nous émettons l'hypothèse que la régulation des cellules NKT $IL\text{-}4^+$ médiée par CD28 serait indispensable pour promouvoir la conversion en cellules T $CD8^+$ « de type inné ». Quelle est la contribution relative des cellules αβ et γδ NKT dans le développement des cellules thymiques T $CD8^+$ « de type inné »? Comme chez les souris $Itk^{-/-}$ et $Id3^{-/-}$, les cellules γδ NKT chez les souris Dok-1 Tg semblent être la principale population de cellules effectrices qui fournit extrinsèquement l'IL-4 pour la génération de cellules T $CD8^+$ « de type inné ». De plus, dans les thymus de souris Dok-1 Tg CD28-déficiente, la fréquence des thymocytes αβ $PLZF^+$ $IL\text{-}4^+$ est environ deux fois inférieure à celle de C57BL/6, mais il y a toujours une plus grande population de cellules T $CD8^+$ « de type inné » probablement car les cellules γδ NKT sont toujours présentes en grand nombre. L'utilisation de souris TCRγδ- ou CD1d-déficiente va aider à définir le rôle des cellules αβ et γδ NKT dans la conversion des cellules T $CD8^+$ en T $CD8^+$ « de type inné » dans le thymus.

3. Conclusion: nouveaux rôles de Dok et CD28 dans le développement des cellules T

Les données présentées dans cette thèse illustrent le rôle de Dok à différents moments dans le développement des cellules T. Cette étude montre clairement que la protéine Dok bloque la sélection β des thymocytes. Nous montrons aussi que Dok régule positivement le développement des cellules γδ NKT « de type inné ». Le rôle de Dok dans le développement des cellules αβ NKT devra être étudié chez les souris Dok-1/Dok-2-déficiente puisque chez les souris Dok-1 Tg cette population est dramatiquement réduite à cause d'un blocage dans le développement au niveau du passage de DN à DP. Le facteur de transcription Id3 est proposé

comme une cible potentielle de Dok pour la régulation du développement des cellules γδ NKT. Id3 a été identifié comme un facteur limitant au niveau de la maturation et de la survie des cellules γδ NKT. Nous avons aussi montré que similairement aux cellules αβ iNKT, le développement des cellules γδ NKT est dépendant de CD28. Nos résultats démontrent qu'en absence de CD28, il y a un blocage dans le développement des cellules γδ NKT après la sélection positive et la maturation de ces cellules. De plus, en modulant la population de cellules NKT, nous avons montré que Dok et CD28 contrôlent indirectement la taille du groupe de cellules T $CD8^+$ « de type inné ». Ces cellules avec les cellules NKT jouent d'importants rôles à l'interface des réponses immunitaires innée et adaptative. Des études ultérieures devront être faites pour élucider le rôle précis de Dok dans chaque voie de signalisation (TCR, SLAM ou CD28), qui sont toutes importantes pour le développement des cellules γδ NKT. Ces études vont aider à comprendre les mécanismes précis qui régulent le développement des cellules NKT, et plus généralement, qui vont générer l'ensemble du répertoire des cellules T y compris les cellules T « de type inné ».

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Annex

List of Communications

Meetings and Conferences

Oral Presentations

1- Mitra Yousefi, Gilles Besin, Ingrid Saba, Pier Paolo Pandolfi and Pascale Duplay
Les protéines adaptatrices Dok-1 et Dok-2 régulent la force du signal initié par le TCR dans les thymocytes. Congrès Armand- frappier 2011:17 -19 novembre 2011, Esterel, Canada.

2- Mitra Yousefi, Gilles Besin and Pascale Duplay. The adaptor proteins Dok-1 and Dok-2 regulate Th17 differentiation. 6th Leukocyte Signal Transduction Workshop: June 5-10 2011, Chania, Crete, Greece.

Poster Presentations

1- Mitra Yousefi and Pascale Duplay. CD28 signaling controls the development of innate-like CD8+ T cells by promoting the functional maturation of NKT cells. 2013 Annual Symposium of Immuno Montreal: December 6, 2013, Montreal, Canada

2- Gilles Besin, **Mitra Yousefi**, Ingrid Saba, Roscoe Klink, Pier Paolo Pandolfi and Pascale Duplay. Dok proteins regulate strength of TCR signaling in thymocytes. 9th Conference on signaling in normal and cancer cells: March 6-10, Banff, Alberta, Canada

3- Gilles Besin , **Mitra Yousefi**, Ingrid Saba, Roscoe Klink, Pier Paolo Pandolfi and Pascale Duplay. Dok proteins regulate strength of TCR signaling in thymocytes.
6th leukocyte signal transduction workshop. Aegean conference. June-6-10 2011 Crete, Greece