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Faculty of Biology

Modulation of the NFAT signaling pathway by protein kinase B (PKB); a perspective study in the context of thymocyte development and T cell function

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(Amiya Kumar Patra)

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Dedicated to my Teachers

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Contents

Summary

To analyze protein kinase B's (PKB) effects on developmental and functional aspects of T cells, we have generated transgenic (tg) mouse lines expressing a constitutively active form of PKB (myrPKB) in early stages of T cell development. Peripheral CD4+ T cells from myrPKB tg mice are hyper-reactive. They exhibit markedly higher proliferation upon suboptimal TCR ligation and are mostly independent of CD28-induced costimulatory signals. This becomes also evident in both, Th1 and Th2 cytokine production and cell cycle progression.

Interestingly, proliferation of myrPKB tg T cells is significantly resistant to the immunosuppressive agent cyclosporine A (CsA). CsA inhibits calcineurin activity and thereby negatively regulates NFAT signaling events by inhibiting its nuclear translocation. However, in the presence of CsA, myrPKB tg CD4+ T cells not only proliferate well, but are also able to produce cytokines shown to be dependent on NFAT activation. Thus, proliferation/cell division and cytokine production of myrPKB tg CD4+ T cells seems to be relatively independent of NFAT activity.

Analysis of nuclear and cytoplasmic extracts from unstimulated and TCR/CD3-stimulated myrPKB tg CD4+ T cells shows drastically reduced nuclear translocation of NFATc1 and NFATc2 proteins compared to wild type cells. A negative regulation, i.e. reduced nuclear accumulation, was also detected for NF-Bp65 and RelB, two members of the NF-B transcription factor family. The negative regulation of nuclear accumulation of NFATs by myrPKB seems to result from a direct interaction of PKB with NFAT, as we could co-precipitate both molecules in immunoprecipitation assays. Thus, myrPKB could be involved in direct phosphorylation of NFATs and thereby inhibit its nuclear translocation. Inhibition of various known NFAT kinases, like JNK, ERK and p38 MAPK by pharmacological inhibitors did not reverse the negative regulation of NFAT in myrPKB tg CD4+ T cells, indicating that these kinases are not involved in this process.

To study whether the negative regulation of NFATs by myrPKB affects T cell development, we analyzed double transgenic mice expressing both, a constitutively active version of calcineurin (ΔCam) (which leads to strong NFAT activation) and myrPKB. ΔCam tg mice have a severe block in thymocyte development at the DN3 stage. Compared to littermate controls ΔCam tg mice show an almost complete absence of the DP population and on average a twenty five-fold reduction in thymic cellularity. Strikingly, in the ΔCam/PKB

double tg mice this developmental block is significantly rescued with concomitant restoration of the DP population and cell numbers. ΔCam DN3 cells lack icTCR chain expression, which seems to be due to strongly diminished Rag1 protein expression. Expression of myrPKB in ΔCam DN cells rescues Rag1 and icTCR chain expression and restores 'normal' thymocyte development. However, expression of a pre-rearranged /TCR fails to overcome the severe block of differentiation in ΔCam cells, although a partial rescue is obvious. This suggests that there are additional defects in ΔCam DN cells, which are overcome by myrPKB.

CsA treatment of neonatal thymic lobe cultures from ΔCam mice restores normal thymocyte development, indicating involvement of NFATs as critical factors in the severe block in ΔCam thymocyte development. Confocal studies clearly established that compared to ΔCam DN cells there is a significant reduction in the nuclear levels of NFATc1 as well as NFATc3 in ΔCam/PKB cells. Downregulation of nuclear NFAT levels by myr PKB thus seems to be an essential parameter in ΔCam cells to proceed with normal differentiation.

In summary, the data from myrPKB tg peripheral CD4+ T cells and ΔCam/PKB double tg thymocytes clearly establish PKB as an important modulator of T cell development and T cell function and PKB as a novel negative regulator of calcineurin/NFAT activation. This is further supported by the nuclear absence of NFAT proteins in myrPKB-induced T cell lymphoma. Indeed, shut-down of NFAT signalling by PKB may be an essential mechanism involved in PKB-induced tumorigenesis in the immune system in particular and in other systems in general.

Zusammenfassung

Um die physiologische Rolle der PKB während der T-Zell-Reifung und –Aktivierung untersuchen zu können, analysierten wir transgene Mausstämme, die eine konstitutiv-aktive Form der PKB (myrPKB) T-Zell-spezifisch exprimieren (myrPKB-tg Mäuse). Periphere CD4+ T-Zellen von myrPKB-tg Mäusen verhalten sich hyperreaktiv. Sie zeigten bereits sehr starke Proliferation nach minimaler TZR-Stimulation und waren weitgehend unabhängig von Kostimulation durch CD28, und zwar nicht nur in Bezug auf Zellprolifertation und Zellzyklusprogression, sondern auch hinsichtlich der Polarisation in Th1 und Th2 Zellen.

Interessanterweise wiesen myrPKB-tg T-Zellen eine deutliche Resistenz gegenüber dem Immunsuppressivum Cyclosporin A (CsA) auf. CsA inhibiert die Aktivierung von Calcineurin und verhindert dadurch die Translokation von NFAT in den Zellkern. Myr PKB-tg CD4+ T-Zellen proliferierten in Gegenwart von CsA und produzierten Zytokine, deren Synthese von der Aktivierung von NFAT abhängt. Proliferation, Zellteilung und Zytokinproduktion myr PKB-tg CD4+ T-Zellen sind also relativ unabhängig von der NFAT-Aktivierung.

Die Analyse von nukleären und zytoplasmatischen Extrakten aus unstimulierten und über den TZR-stimulierten myrPKB-tg CD4+ T-Zellen zeigte eine drastisch reduzierte nukleäre Translokation von NFATc1 und NFATc2 im Vergleich zu wildtypischen Zellen. Diese Form der negative Regulation fand sich nicht nur bei NFAT–Transkriptionsfaktoren, sondern auch bei NF-B p65 und Rel B, wohingegen die Aktivierung von NF-B p50 normal war. Die Inhibierung verschiedener Kinasen, die für die zelluläre Lokalisation von NFAT verantwortlich sind, wie JNK, ERK, p38 MAPK etc., durch pharmakologische Inhibitoren führte nicht zur Blockade der negativen Regulation von NFAT in myrPKB-tg CD4+ T-Zellen. Durchgeführte Ko-Immunpräzipitationen legen nahe, daß die negative Regulation von NFAT durch PKB wahrscheinlich durch direkte Interaktion der beiden Moleküle geschieht.

Um einen möglichen Einfluß der beschriebenen negativen Regulation der NFATs duch die aktivierte PKB auf die T-Zell-Entwicklung untersuchen zu können, analysierten wir doppelt tansgene Mäuse, die neben myr PKB eine konstitutiv aktive Form von Calcineurin (ΔCam) exprimieren. ΔCam-tg Mäuse weisen eine schwere Störung der Thymozytendifferenzierung im Stadium der DN3-Zellen auf, was zu einem nahezu vollständigen Fehlen der DP-Population und einer ca. 25-fach reduzierten Zellzahl im Thymus führt. Zu unserer Überraschung, war diese Differenzierungsblockade in ΔCam/PKB doppelt-tg Mäuse signifikant schwächer ausgeprägt, was sich auch in einem Wiederauftreten

der DP-Population manifestierte. Wir konnten zeigen, daß ΔCam-DN3-Zellen keinen TZR exprimieren, was wahrscheinlich Folge einer nahezu vollständig fehlenden Expression des rag1 Gens ist. MyrPKB-Expression stellte die Expression von RAG1 und der TZR-Kette in ΔCam-DN3-Zellen wieder her und erlaubte die weitere Differenzierung von DN3-Zellen zu DP-Zellen. Die Behandlung von thymischen Lobi neonataler ΔCam Mäuse mit CsA in vitro hatte eine normale Thymozytendifferenzierung und normale Zellzahlen zur Folge. Somit scheinen NFAT-Proteine kritische Faktoren für die durch ΔCam-induzierte Blockade der Thymozytendifferenzierung zu sein.

Mittels konfokaler Mikroskopie konnten wir eine signifikante Reduktion in der nukleären Lokalisation von NFATc1 und NFATc3 in ΔCam/PKB-DN-Zellen im Vergleich zu ΔCam-DN-Zellen beobachten. Unsere Ergebnisse, die wir an myrPKB-tg peripheren CD4+ T-Zellen und an ΔCam/PKB doppelt-tg Thymozyten gewonnen haben, legen nahe, daß die aktive Form der PKB die Anreicherung von NFAT Proteinen im Kern negativ beeinflussen kann. Dieses Konzept wird ferner durch das Fehlen von NFATc1 und c2 in myrPKB induzierten T-Zell-Lymphomen und durch Kinase-Aktivitäts-Asssays gestützt, die deutlich zeigten, daß NFAT von PKB in vitro phophoryliert werden kann.

Unsere Daten deuten auf eine Schlüsselstellung der PKB bei der Modulation der T-Zell-Aktivierung, der Proliferation und der Modulation von T-Zell-Effektorfunktionen hin. Insbesondere zeigen wir erstmals, daß NFAT ein Substrat der PKB ist. Diese Arbeit eröffnet somit neue Perspektiven für das bessere Verständnis derjenigen Mechanismen, die der PKB-induzierten Tumorigenese im Immunsystem und in anderen Organsystemen zugrunde liegen.

Chapter 1.
Introduction

1. Introduction

1.1 The Immune System: An Overview

The immune system is an organism's inherent remarkable defense mechanism against all kind of infections. It provides the means to make rapid, specific, and protective responses against the myriad potentially pathogenic microorganisms that inhabit in our surrounding. Though, with evolution the complexity of the immune system has increased many fold in the higher vertebrates, the basic purpose of it remains unaltered i.e., to provide protection against any kind of pathogenic agent, both internal and external.

1.1.1 Cells of the immune system

The immune system consists of a wide range of distinct cell types. These include lymphocytes, the central players in determining the specificity of immune response, dendritic cells (DCs), Langerhans cells, monocytes/macrophages, natural killer (NK) cells, neutrophils, mast cells, basophils and eosinophils.

Lymphocytes differ from each other, both in their epitope specificity and functions. Functionally lymphocytes are divided into two broad classes: the B-lymphocytes, the antibody secreting cells and the T-lymphocytes. T-lymphocytes are involved in important helper functions and direct effector functions. Specialized T-lymphocytes (regulatory T cells) have the capacity to suppress specific immune responses.

T-Lymphocytes

Development and differentiation of T lymphocytes take place in the thymus. Based on their cell surface receptors T cells can be divided into two categories, the □□ T cell receptor (TCR) bearing T cells and the □□ TCR bearing T cells. □□ T cells comprise of two important sub-lineages: cells that express the CD4 co-receptor (CD4+ T cells) and those that express CD8 (CD8+ T cells). Upon priming with antigen, naive CD4+ T cells differentiate into cells that primarily secrete the cytokines IL-4, IL-13, IL-5, IL6 and IL-10 (Th2 cells) or into cells that mainly produce IL2, IFN-□ and lymphotoxin (Th1 cells). Th2 cells are very effective in helping B cells to develop into antibody-producing cells, whereas Th1 cells are effective inducers of cellular immune responses, involving enhancement in the microbicidal activity of macrophages and consequent increased efficiency in lysing microorganisms. CD8+ T cells, upon antigen encounter develop into cytotoxic T-lymphocytes (CTLs) capable of efficiently lysing target cells that express their specific antigens.

Antigen recognition by the TCR requires processing of the antigen and presentation of the processed antigen in the context of major histocompatibility (MHC) molecules. CD4+ T cells recognize antigens/peptides in the context of MHC II, whereas CD8+ T cells recognize antigen presented by MHC I molecules (1,2). MHC II molecules bind peptides derived from proteins that have been taken up by the APC through an endocytic process. Thus, CD4+ T cells are specialized to mainly recognize antigens derived from exogenous sources. In contrast, MHC I molecules are mainly loaded with peptides derived from internally synthesized proteins, including viral gene products. Thus, CD8+ T cells are specialized in recognizing antigens derived from endogenous sources.

1.1.2 T cell activation

The TCR is associated with a set of transmembrane proteins, collectively referred to as the CD3 complex, that play a critical role in TCR downstream signal transduction. The CD3 complex consists of γ, δ and ε chains and is associated with a homodimer of two ζ chains or a heterodimer of ζ and η chains (3,4). The cytosolic domains of CD3 γ, δ and ε and of ζ and η, contain one or more copies of a signaling motif, the immunoreceptor tyrosine-based activation motif (ITAM) (5,6). These motifs are targets for tyrosine phosphorylation by protein tyrosine kinases, which are the key players in propagating the downstream signaling events.

Interaction of the TCR/CD3 complex with its specific peptide leads to the activation of a T cell. Among the earliest signaling events are, the activation of various tyrosine kinases (7-9). Prominent among these early tyrosine kinases are the Src-family tyrosine kinases $p56^{lck}$ and $p59^{fyn}$ (10-17), which phosphorylate the ITAM motifs of the CD3 complex and ZAP-70, a Syk family tyrosine kinase (18-20), that binds to the phosphorylated ITAMs of the TCR ζ chains. The activation of these tyrosine kinases initiates a cascade of tyrosine phosphorylation of a series of substrates. These include (i) numbers of adaptor proteins that link the TCR to the Ras signaling pathway: (ii) Phospholipase Cγ1, upon tyrosine phosphorylation its catalytic activity increases and thereby it enhanced ionositol phospholipids metabolism, leading to elevation of intracellular free calcium concentration and the activation of Protein kinase C (PKC); and (iii) an array of other important enzymes that control cellular growth and differentiation. The phosphorylation of LAT, an adaptor molecule, is of particular importance as it acts as an organizing scaffold to which a series of signaling intermediates bind and get activated to control the downstream signaling (21,22).

The TCR-ligand interactions on a T cell and an APC lead to structural rearrangements of various cell surface and cytosolic molecules on both cells to a complex structure known as the *immunological synapse*. Formation of the immunological synapse concentrates the key

signaling molecules to a very small but specialized region of the membrane, where it has been shown to strengthen and intensify the signaling events (23-25). Even though a T cell gets activated upon TCR/CD3 ligation, very often this leads to a response in which the cells become anergic, a state of unresponsiveness to a subsequent competent stimulus. For optimal T cell activation, in extra to TCR/CD3 ligation, the engagement of an accessory co-stimulatory molecule is required. The engagement of CD28 on the T cell by CD80 (B7.1) and/or CD86 (B7.2) on the APC provides a potent co-stimulatory activity. Inhibition of this interaction both *in vivo* and *in vitro* shows diminished antigen-specific T cell-activation, indicating the physiological importance of CD80/CD86-CD28 interaction in T-cell activation (26,27).

1.1.2 T cell functions

T cells play important functions both in humoral and cell-mediated immunity. T cells provide help for B cells to develop into antibody producing cells, enhance the microbicidal capacity of monocytes and macrophages, inhibit certain types of immune responses, can directly kill target cells, and can mobilize inflammatory response. These effects significantly depend on their expression of specific cell-surface molecules and the secretion of cytokines.

T cell mediated antibody response

T helper cells can induce B cells to make antibody responses to proteins and other T cell dependent antigens. Optimal B cell activation requires in addition to B cell receptor (BCR) ligation the engagement of CD40 with CD154 (CD40 L) on the T cell surface (28,29). This interaction is similar to that of CD28 co-stimulation in case of T cells.

B-cell proliferation and immunoglobulin (Ig) secretion are enhanced by several type I cytokines including IL-2 and IL-4. Ig class switching is dependent on CD154/CD40 interaction and is very often determined by T cell derived cytokines (30). The well studied example of this is the role of IL-4 in determining switching to IgG1 and IgE in mouse and IgG4 and IgE in human.

Induction of cellular immunity

T cells also can enhance the pathogen clearance capabilities of monocytes and macrophages. IFN-γ produced by T cells modulates the activity of these cells in generating nitric oxide and production of tumor necrosis factor (TNF), which these cells utilize to destroy intracellular bacteria and parasites. Th1-type cells are particularly effective in enhancing microbicidal action as they produce IFN-γ. In contrast two major cytokines of Th2 cells, IL-4 and IL-10 block these activities. Thus, Th2 cells often oppose the action of Th1 cells in inducing cellular immunity.

Cytotoxic T cells

T cells, mainly CD8+ T cells can act as potent effector cells by directly killing target cells expressing specific antigens via cell-mediated cytotoxicity. Cytotoxicity is effected via two major mechanisms. One, via production of perforin and granzymes by the CTLs and the other by Fas and Fas ligand (Fas L) interaction (31,32).

Cytokines

Cytokines are a set of small molecular weight proteins, which mediate many important functions in the immune system. Cytokines can be divided into several classes: type I cytokines, includes hematopoietins (i.e., IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-11, IL-12, IL-13, IL-15, IL-21 and IL-23) as well as several hematopoietic growth factors. Type II cytokines include the interferons and IL-10, TNF related molecules including TNF, lymphotoxin and Fas ligand, Ig superfamily members comprising IL-1 and IL-18 and the chemokines, a growing family of molecules playing critical roles in a wide variety of immune and inflammatory functions.

1.2 T Cell Development

Development of T cells is initiated when common lymphoid progenitor cells (CLPs) from the fetal liver or bone marrow populate the thymic rudiment. Interactions with the thymic stroma induce these precursor cells to execute a highly ordered and precisely regulated T cell differentiation program. Along this developmental pathway T cell precursors are marked by sequential changes in expression of various differentiation antigens, such as CD4, CD8, CD44, and CD25, on the basis of which thymocytes can be classified into distinct subsets schematized in ascending order of maturity in Figure 1(A & B). The most immature T cell precursors are the CD4-CD8- double negative (DN) cells. The first major checkpoint that takes place during thymocyte differentiation ensures that only thymocytes that have generated a functional T cell receptor □ (TCR□) chain, a process known as □-selection, become selected to differentiate to the CD4+CD8+ double positive (DP) stage (33,34). The second checkpoint occurs at the DP stage when thymocytes rearrange the TCR□ chain and undergo positive and negative selection, as well as lineage commitment resulting in the differentiation of MHC restricted and self-tolerant CD4+ or CD8+ single positive (SP) □□-T cells (35).

Murine DN thymocytes can be further subdivided into four distinct stages, depending on the gain or loss of expression of two surface molecules, CD44 and CD25 (36). The most immature DN subset is marked by CD44 expression (CD44+ CD25-, DN1), which has the potential to differentiate into multiple lineage cells like T-lymphocytes, B-lymphocytes, NK cells, and dendritic cells. This multi-potent T cell precursor upregulates surface expression of

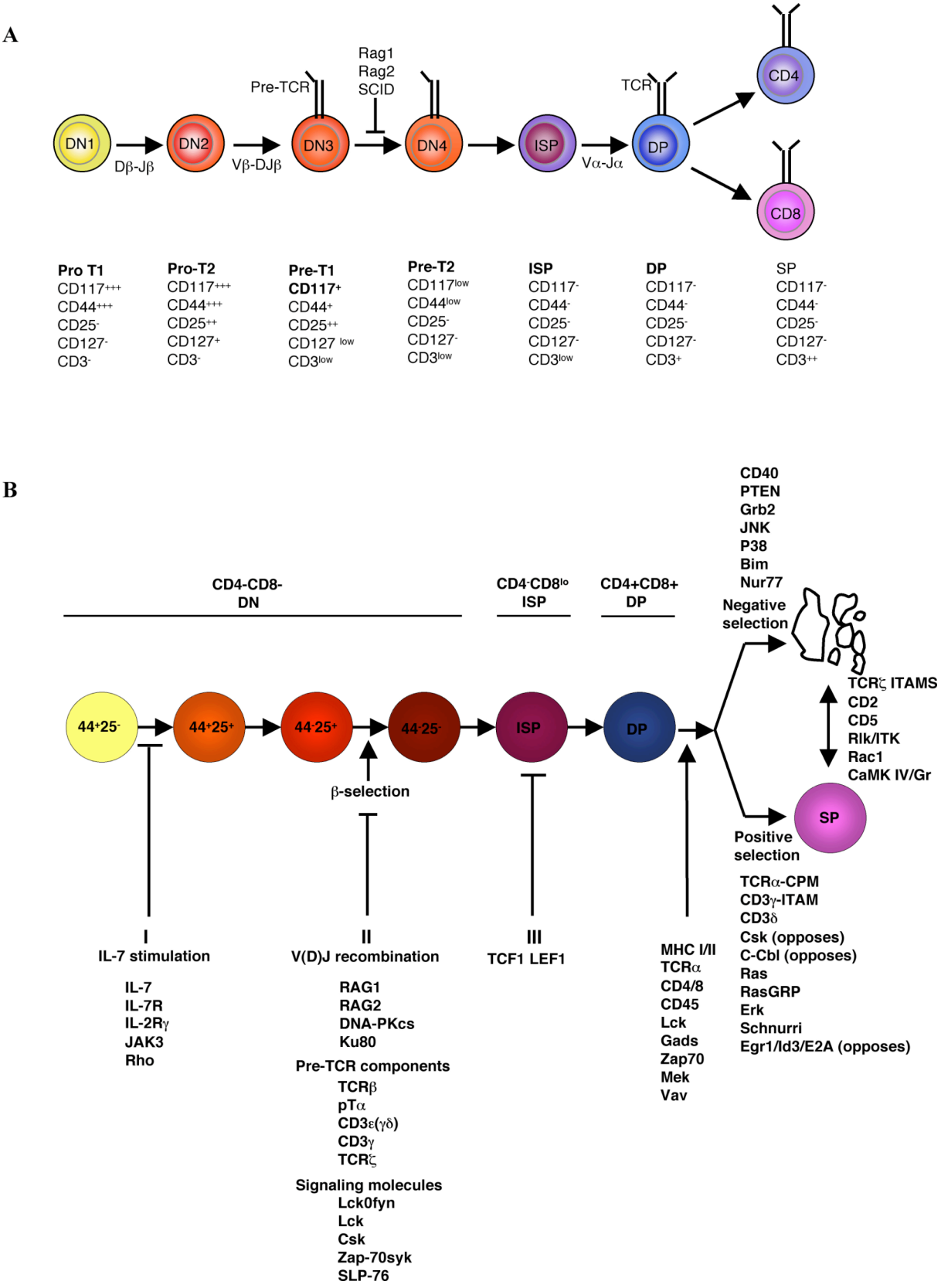

Figure 1. Scheme of early T cell development (A) Cell development from less mature to more mature cells proceeds from left to right. Cell surface phenotype of each subpopulation is shown below it. Fluorescence intensity, as measured by flow cytometry on an arbitrary scale from negative (-) through low to very bright (+++), is indicated for each marker. (B) Selected molecules identified as critical regulators of selection checkpoints in T cell development. The function of most of these molecules was established by analysis of gene-deficient mice. The molecules required for the DN3 to DN4 transition, may also be involved in positive and negative selection, but their function at the DP stage has not been evaluated. Molecules listed before the fork are

required for both positive and negative selection. Molecules listed on far right adjust the threshold of thymic selection. (Figures partially adapted from Nat Rev Immunol. 2002;2:2-10 & Annu Rev Immunol. 2003;21:139-76)

CD25 and progresses to the CD44+CD25+ (DN2) stage. Progression of DN2 cells to the CD44-CD25+ (DN3) stage is dependent upon the stromally produced growth factor IL-7 and is accompanied by several events that are important in preparing the DN3 precursors for β-selection. First, as CD25 expression is upregulated, the DN2 subpopulation undergoes proliferative expansion, second, accompanying down-modulation of CD44, DN3 cells lose their multipotency and become committed to the T lineage only. Third, c-kit (CD117) a receptor tyrosine kinase, whose expression closely parallels that of CD44, is down-regulated as the DN2 cells exit cell cycle, an event closely associated with initiation of gene rearrangement at the TCRβ locus. DN3 cells down-regulate CD25 and progress to CD44-CD25- (DN4) cells before transitioning into DP cells, where they undergo TCRα gene rearrangement and subsequent positive and negative selection to give rise to single positive T cells.

1.2.1 V(D)J recombination

The genetic rearrangement at the TCRβ locus i.e., the fusion of three widely dispersed gene segments (V-variable, D- diversity, and J- joining) into a single coding unit takes place by a process termed Vβ(Dβ)Jβ recombination. This recombination at the TCRβ locus is initiated at the CD44-CD25+, DN3 stage when the *cis*-acting TCRβ enhancer opens the locus, making it accessible to the recombination machinery (37,38). During the recombination process double stranded DNA break is initiated at the boarders of the D and J gene elements by two site specific recombinases, Rag1 and Rag 2, following which the D and J elements are processed by the DNA repair enzyme DNA-dependent protein kinase (DNA-PK) and then fused together. This order of events is repeated in the joining of the Vβ element to the newly formed DβJβ element (39-41).

1.2.2 β-selection and pre-TCR: the critical factors in early thymocyte development

β-selection is a developmental checkpoint characterized by the formation of pre-TCR (pTCR) complex, which is a forerunner of the αβ TCR complex (42). β-selected thymocytes successfully rearrange their TCRβ gene segments and express a functional TCRβ protein. The TCRβ protein, in association with a 33 kD glycoprotein known as pre Tα chain forms the pTCR.

Though structurally different, pTCR and mature TCR have functionally similar and dissimilar properties. Whereas signaling from both can induce proliferation, rescue from apoptosis, and expression of differentiation/activation genes, fundamental differences exist between the functional outcomes mediated by the two receptors in the induction of T cell effector functions, such as cytokine production and cell mediated cytotoxicity (43). Both these activities are the result of induction by the mature TCR only. In contrast pTCR signals result in allelic exclusion at the TCRβ locus, and the initiation of TCRα locus rearrangement (44-46). These differences raise the points whether pTCR and mature TCR have distinct signaling because of the difference in the cell population they operate or the functional differences are the reflection of their structural difference (i.e., the pTα chain in pTCR).

The pTα chain contains a longer cytoplasmic tail compared to the TCRα chain (~ 30aa in mouse pTα, ~110aa in human pTα vs. 6-8 aa in TCRα chain), indicating it might contribute to the unique role of pTα (47,48). Thymocytes from pTα-deficient mice show an impaired ability to transit from the DN to DP stage of T cell development (49). In addition, thymic cellularity was greatly reduced and the enforcement of allelic exclusion was impaired at the TCRβ locus, emphasizing the important role played by pTα chain in early thymocyte development (49,50). Surprisingly, replacement of the pTα with a tail-less pTα chain restored T cell development almost to normal levels, indicating that the dispensability of the cytoplasmic tail for pTCR functioning (51). However, expression of the tail-less pTα chain in mice could not result in a complete rescue of cellularity in the thymus.

There exists a lot of incongruity regarding the ligand-dependent or ligand-independent signaling by the pTCR complex. Studies on truncated expression of pTCR show, pre-T cells could progress through the β-selection checkpoint by expressing a pTCR that lacked extracellular portions, suggesting that specific ligand recognition was not required (52,53). Other reports indicate the ligand independent signaling of pTCR could be due to its spontaneous localization and aggregation in the lipid rafts because of a palmitoylation signal in the pTα chain (54).

As both pTCR and mature TCR share significant structural similarity and both transmit signals via the CD3 complex it was assumed that the signaling properties of both receptors might be similar. Studies on gene deficient or gene mutated mouse models and from manipulated T cell precursors in fetal thymic organ culture (FTOC) indicate that β-selection plays three important roles in early thymocyte development: (i) it induces proliferation; (ii) it promotes differentiation and survival; and (iii) it enforces allelic exclusion at the TCRβ gene locus.

Proliferation, differentiation and survival

β-selection is initiated on a small subpopulation of post-mitotic CD44-CD25+, DN3 thymocytes termed as 'E-cells' for expected size (55). Failure of productive TCRβ gene rearrangement results in E-cells undergoing apoptosis. Those that succeed express a 'functional' TCRβ protein and the pTCR complex triggers proliferative expansion (estimated 8-10 cell divisions) and maturation to the CD4+CD8+ DP stage (43,56). One of the earliest detectable effects of β-selection is entry into the cell cycle and increased cell size, for which β-selected cells were termed 'L-cells' for larger than expected (55). Unlike E cells, L cells are enriched for in-frame TCRβ rearrangements and have up-regulated CD5 expression, suggesting that they have received an activation signal (55,57). L cells, by down-modulating CD25 transit to the most mature DN subset, CD44-CD25- cells, where the expression of Rag1, Rag2, and pTα are downregulated. By first upregulating CD8 to become immature CD8 single positive (CD8 ISP) and then CD4, CD44-CD25- DN4 cells develop to the DP stage. During transition from CD8 ISP to the DP stage, sterile transcripts are generated from the TCRβ locus, indicating that the accessibility of TCRβ locus for rearrangement (58,59).

Allelic exclusion

Besides facilitating the proliferative expansion of DN4 cells eligible to attempt TCRβ rearrangements, β-selection also plays a critical role in regulating the activity of the site-specific V(D)J recombination machinery. Once the successful rearragement at one of the TCRβ gene locus is acheived the rearrangement at the remaining TCR β allele is prevented; a process otherwise known as allelic exclusion (60). This might be the result of induction of cell cycle during transition from the E to L stages. The correlation between cell cycle and prevention of rearrangement could be due to the degradation of Rag proteins once the cells enter cell cycle. Phosphorylation of Rag2 by the cdc2 mitotic kinase complex in vitro decreases Rag protein stability by 20 fold (61). Moreover, Rag2 protein level is decreased approximately 20 fold in cycling L and DN4 cells, but is re-expressed in post-mitotic DP cells. But in addition to the deficiency of Rag2 protein there could be other mechanisms involved in allelic exclusion at the second TCRβ locus, as after re-expression of Rag proteins in the DP stage, the TCRβ locus still remains inaccessible. Further, proliferation induced by β-selection can be attenuated by eliminating the activity of Rho GTPase; yet, allelic exclusion at the TCRβ locus could not be reversed (62). The long-term control of gene-rearrangement is most likely controlled through locus accessibility, which, in turn is controlled by enhancer function. Indeed, both the β and α enhancers are required to initiate rearrangement at the β and α loci, respectively.

Thus, pTCR mediated β-selection induces thymocyte proliferation, differentiation to the DP stage, and redirects the V(D)J recombination machinery from the TCRβ locus to the TCRα locus.

1.2.3 Proximal signaling molecules activated downstream of pTCR complex

Even though the exact molecular nature of pTCR signaling is yet to be clarified significant information have been derived from the studies involving various gene targeted mice models. Although a number of molecules have been characterized to play a critical role in pTCR signaling, the effect of their impairment can vary substantially.

Tyrosine kinases

Studies from mice doubly deficient for Lck and Fyn show these two Src kinases play an important role in pTCR signaling as thymocytes from these mice are arrested at the CD44-CD25+ DN3 stage (Fig. 1B) (63,64). Lck appears to be the more important of the two because (i) β-selection and allelic exclusion are severely compromised in Lck single deficient mice and in mice overexpressing a kinase dead Lck mutant (65,66), (ii) a constitutively active Lck transgene restores development of pTCR deficient thymocytes to the DP stage (67), and (iii) neither Fyn deficiency nor over-expression of a kinase inactive form of Fyn disrupts pTCR function (11,13). Surprisingly, constitutively active Fyn can substitute for Lck in pTCR expressing but not pTCR deficient thymocytes (63). In conformity with Lck's importance in pTCR signaling, deletion of Csk, a kinase which phosphorylates and inactivates Lck, obviates the need for the pTCR by deregulating Lck activity (68). Conversely, CD45, a phosphatase which dephosphorylates and activates Lck, interferes with pTCR signaling (69). An important target for Lck activity is the ζ-associated protein of 70kD or ZAP-70, a member of the ZAP-70/Syk tyrosine kinase family, whose recruitment to the TCR complex and activation by Lck is necessary for TCR signaling (70). However, in ZAP-70 deficient mice there is a complete block in the αβ TCR-induced maturation of DP thymocytes to CD4+ or CD8+ single positive stage (Fig. 1B), but the pTCR driven maturation of DN thymocytes to the DP stage is relatively normal (71,72). This could most possibly be the outcome from compensation by the other ZAP-70/Syk kinase family member, Syk, as mice doubly deficient in ZAP-70 and Syk exhibit a complete block in development at the CD44-CD25+ DN3 stage (Fig. 1B), similar to the observation from overexpression of a dominant negative ZAP-70 mutant.

Adaptor proteins

The SH2 domain containing leukocyte phosphoprotein of 76 kD or SLP-76 is a downstream target of ZAP-70, which is rapidly phosphorylated following TCR ligation. SLP-76 as an adaptor molecule plays an essential role in both TCR signaling, as well as in the functioning

of the pTCR (73,74). SLP-76 deficient mice show a block in β-selection (Fig. 1B) (75,76). Adaptor molecules function by linking tyrosine kinase activation to downstream effector molecules. Indeed, phosphoSLP-76 links ZAP-70 kinase activity to GTP binding protein activation in two ways (i) by recruiting the guanine nucleotide exchange factor (GNEF) Vav, which can activate Rac-1, a Rho family GTPase and (ii) through association with Grb-2, another adaptor protein which recruits Sos, a GNEF capable of activating the GTPase Ras (77). The role of Ras in pTCR function remains unclear because a dominant negative Ras transgene has no effect on pTCR function, yet, a constitutively active Ras transgene can restore development of pTCR deficient DN thymic precursors to the DP stage, thus, compensating for pTCR function (78,79). Many other adaptor molecules have been identified as tyrosine kinase substrates in T cells and one of these, LAT or linker for activation of T cells, has been reported to be essential for both TCR and pTCR signaling (80).

Ser/Thr kinases

Activation of the Rho and Ras GTPases lead to the activation of the JNK/SAPK and MAPK/ERK serine/threonine (Ser/Thr) kinase cascades (81). Alterations of these pathways have been demonstrated to have differential effects on TCR mediated positive and negative selection at the DP stage. However, their role in early thymocyte development and pTCR function is still not clear (81,82). Mutations of kinases in the SAPK pathway have either no effect on the DN to DP transition or have yielded conflicting results. Moreover, unlike tyrosine kinases the role of Ser/Thr kinases in thymocyte development is relatively less clear.

1.2.4 Involvement of transcription factors in controlling β-selection

Recent studies have characterized a number of transcription factors, which either facilitate or inhibit the β-selection process. Overexpression or deletion of different transcription factors during early thymocyte developmental stages indicates how differential gene expression could affect normal T cell development. The involvement of transcription factors belonging to different groups points to the complex nature of signaling involved in the β-selection process (Fig.2).

E-proteins and Id-proteins

E-proteins belong to the basic helix-loop-helix (bHLH) transcription activators that bind to E-box motifs in the regulatory sequences of a number of genes as homo-or heterodimers with other HLH proteins. In thymocytes bHLH-DNA binding complexes are mostly consist of heterodimers of E47 and HEB (83). T cell development in mice lacking HEB is blocked at the ISP stage, with 10 fold fewer thymocytes compared with wild type controls (84). Targeted deletions of E47 in wt type mice reduced the cellularity of the thymus, severely affecting

thymocyte development at a stage prior to $\gamma\delta$-T cell lineage commitment, before the initiation of TCRβ rearrangement (85). These studies indicate that HEB and E2A have unique roles in transcription control during early thymocyte development.

Id proteins act as antagonists of E2A and HEB DNA binding activity. Over-expression of Id3 in lymphoid precursor populations was shown to block T cell development in favor of NK cells (86). Recent studies have indicated that upon mimicking a pTCR signal, Id3 is upregulated through the ERK-MAPK signaling cascade. Thus upon formation of the pTCR E2A activity could be inhibited through the formation of E2A-Id3 heterodimers.

SCL-LMO

Stem cell leukemia (SCL) factor also belongs to the bHLH transcription factor family. E2A-SCL heterodimers in conjunction with non-DNA binding zinc finger-like proteins, LMO1 or LMO2, can act as transcriptional activators or repressors. During thymocyte development, the expression of SCL, LMO1, and LMO2 decreases as immature thymocytes differentiate. Enforced expression of SCL and LMO1 at later DN thymocyte stages blocks thymocyte differentiation by inhibiting E2A-HEB function (87). The phenotype of the developmental block resembles to that of the $HEB^{-/-}$ mouse, with a block at the ISP stage (84,87).

Notch signaling

Notch is a transmembrane protein that is cleaved during biosynthesis by a furin-like activity to produce a noncovalently associated extracellular fragment and a cytoplasmic fragment. Mammals have four different notch proteins (Notch1-4). The interaction of notch with one of its ligands (Jagged 1 (JAG1), JAG2 or δ-like 1 (DLK1) in mammals) induces a further cleavage of the molecule just inside the plasma membrane, which is mediated by presenilin/γ-secretase. The released intracellular fragment (Notch-IC) regulates gene expression by its effects on RBP-Jκ and Deltex (88).

Notch signaling plays an important role during thymocyte development from DN thymocytes to SP cells. Notch 1 gene expression is high in early DN thymocytes, low in DP cells and intermediate in CD4+ and CD8+ SP cells whereas, Notch 3 expression levels are significantly higher in DN and DP thymocytes (89). The presence of multiple Notch receptors and their differential expression along the different developmental steps indicates different receptors play distinct roles during thymocyte differentiation. Notch signaling has been linked to pTCR signaling and also Notch 1 has recently been shown to be essential for correct VDJβ rearrangement (276).

Wnt signaling

Binding of Wnt proteins with their ligand at the cell surface transduce a signal to several intracellular proteins that include Dishevelled (Dsh), GSK-3β, Axin, Adenomatous Polyposis Coli (APC) and the transcriptional regulator, β-catenin. β-catenin is a key player in the Wnt signaling cascade and is responsible for activating the transcription factors T cell factor-1 (TCF-1) and lymphocyte enhancer factor-1 (LEF-1) (90). An important role for β-catenin in the regulation of β-selection has been proposed as expression of soluble mutant receptors in

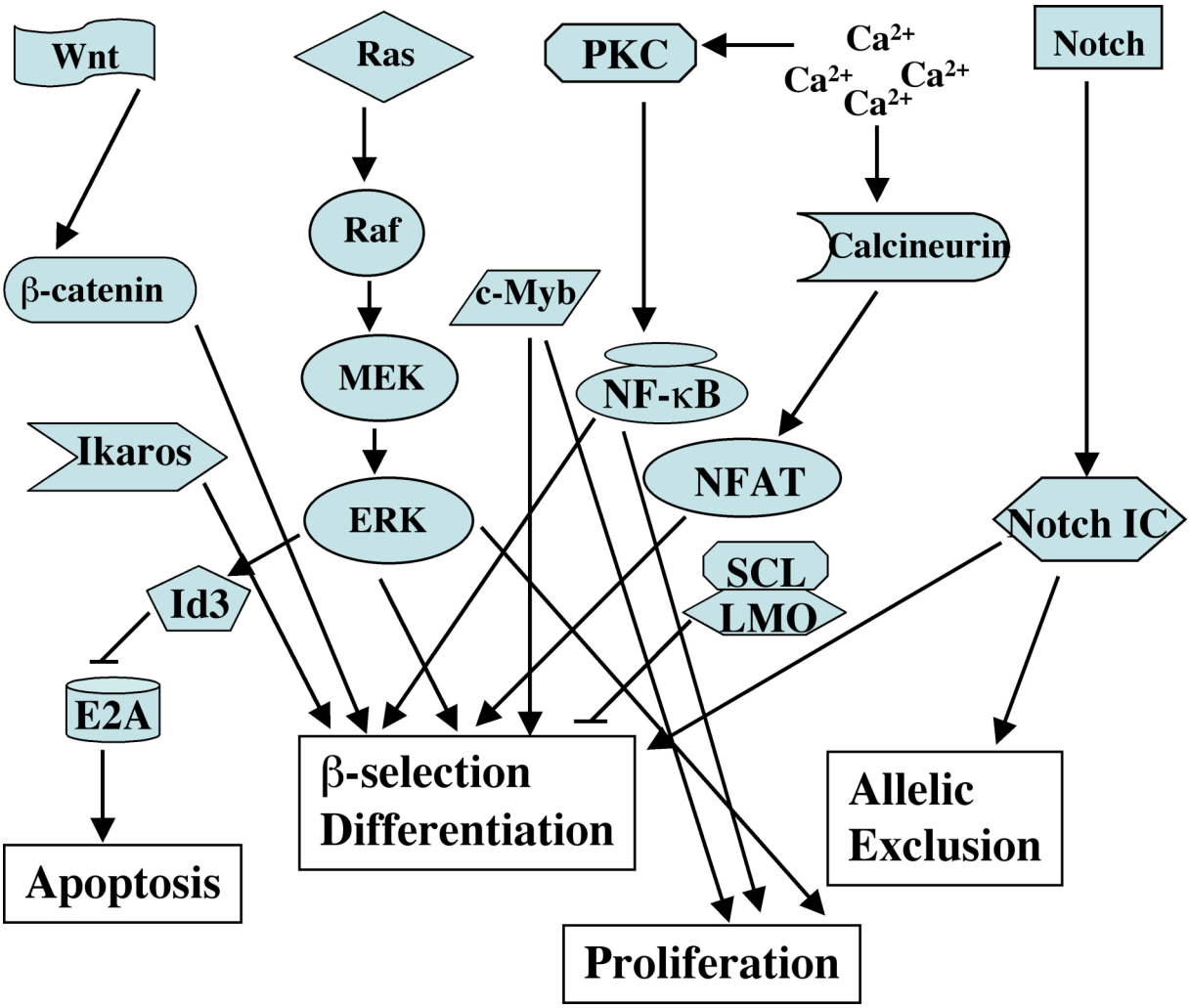

Figure 2. Schematic diagram of the transcription factors that are implicated in the control of β-selection.

FTOC lead to a block in T cell development at the DN to DP transition (91). Moreover, disruption of the TCF-1 DNA binding domain results in a delay in the transition from the ISP to the DP stage of T cell development (92). This partial block appears to be compensated for by LEF-1 as TCF-1 mutant plus LEF-$1^{-/-}$ deficiency results in an arrest at the ISP stage.

β-catenin is degraded in absence of Wnt signaling. However, stabilization of β-catenin expression results in the differentiation of thymocytes to the SP stage in the absence of β-selection, i.e., in the absence of a pTCR signal (93). A similar phenotype is observed in Ikaros-deficient thymocytes, where it was noted that thymocytes differentiated to the DP stage in the absence of a pTCR (94). Therefore there may be a connection between Ikaros preventing Wnt-dependent differentiation in the absence of TCR signals.

NFAT and NF-κB

Pre TCR signaling induces activation of nuclear factor of activated T cell (NFAT) and nuclear factor κB (NF-κB) (95,96). While NF-κB does appear to be constitutively active in pTCR deficient thymocytes, NF-κB activity is upregulated either by expression of the pTCR in DN4 thymocytes or by mimicking pTCR signals (by anti-CD3 treatment of $Rag^{-/-}$ mice). Additionally, NFAT activity is activated downstream of the pTCR, and this activity is indispensable for the transition of thymocytes through β-selection (96). NF-κB and NFAT activation both occur in response to increases in intracellular Ca^{2+} levels.

Transgenic mice encoding either IκB kinase γ (IKKγ) or IκBα, both regulators of the NF-κB signaling cascade, has established that activation of NF-κB results in a selective survival signal for thymocytes that have generated a functional TCR β chain (96). Moreover, IKKγ overexpression in $Rag^{-/-}$ mice resulted in a slight increase in thymic cellularity but DN cells differentiated into DP thymocytes, indicating that activation of NF-κB could be involved in differentiation process. Results from these studies have provided evidence for the importance of NFAT and NF-κB transcription factors in β-selection and pTCR mediated signaling.

c-Myb

The Myb protooncogene encodes a nuclear, DNA-binding protein (c-Myb) that functions as both a transcription activator and repressor. During T cell development c-Myb expression is independent of any particular stage or population. Tissue specific deletion of c-Myb during T cell development shows, c-Myb activity is essential for DN3 to DN4 transition, survival of DP cells and differentiation of CD4+ thymocytes (97).

1.2.5 Positive and negative selection

At the DP stage, developing thymocytes undergo extensive selection processes to ensure that the mature T cells coming out of the thymus are functional and tolerant to self-antigens. These processes are termed positive and negative selection and are dependent on lymphostromal interactions within the thymus (98,99). Positive selection is a consequence of whether the

TCR on the developing thymocytes is able to recognize (self) peptide within the context of self-MHC (100). Those that can not recognize self-MHC-peptide complex die by neglect. In contrast, thymocytes with very high affinity for self-MHC-peptide are deleted by negative selection, an active apoptotic process to remove self-reactive T cells (101). Thymocytes with intermediate affinity are selected to develop into CD4+CD8- or CD4-CD8+ single-positive (SP) thymocytes, with concomitant downregulation of the CD8+ or CD4+ coreceptor molecules respectively. Development into CD4+T-helper cells requires interaction with MHC II-expressing thymic stromal cells, while development into CD8+ cytotoxic T-lymphocytes (CTLs) requires interaction with MHC I-expressing thymic stromal cells (102,103).

Results from reaggregated thymic organ culture (RTOC) have established that MHC I+ and MHC II+ cortical thymic epithelial cells (cTEC) are both necessary and sufficient for positive selection of DP thymocytes and development of SP thymocytes. In addition, the concentration of peptide presented and the accessory molecules involved are also important in determining the strength of the signal that determines whether the thymocyte undergoes positive or negative selection (104,105).

Due to the TCR diversity of the developing thymocytes, a large array of self-peptides is required for efficient positive selection (99). The difference in antigen processing between TECs and hemopoietically derived APCs plays a significant role in the thymic selection process. Importantly, TECs, but not APCs, have been shown to express cathepsin L (106), which may potentially influence the peptides presented for positive selection compared to negative selection. DP thymocytes undergo many phenotypic changes before the generation of functional CD4+ and CD8+ SP thymocytes. Sustained interaction between the thymocytes and thymic stromal cells is essential for transition through these stages (107). Induction of positive selection correlates with the transient upregulation of the early activation marker CD69 (108), which discriminates MHC-dependent and –independent stages of thymocyte positive selection (109). Other phenotypic changes include downregulation of CD4/CD8 coreceptor molecules and Rag1/Rag2, and upregulation of □□TCR and CD5 (110,111).

Self-nonself discrimination results in the elimination of autoreactive T cells during differentiation in the thymus. This process termed negative selection leads to establishement of central tolerance by an apoptotic mechanism that ensures the positively selected thymocytes do not have too strong an affinity for their specific peptides. The requirements for negative selection differ from those for positive selection in both, the cell types that mediate selection and the thymic niche where it occurs. Negative selection of thymocytes has been reported in various experimental systems to occur either at the cortico-medulary junction or

within the thymic medulla, induced mainly by bone marrow-derived thymic stromal cells (112-114).

1.3 Protein Kinase B (PKB/Akt): forms and functions

1.3.1 Origins of PKB

Staal and co-workers are credited with initiating the PKB/Akt research in 1977 with their discovery of a transforming murine leukaemia virus from the AKR strain of mice with a high incidence of spontaneous lymphoma (115). A retrovirus termed Akt8 was isolated from one of these lines derived from a spontaneous thymoma. The non-viral DNA component transduced from the mouse genome was subsequently identified, and two human homologues, PKBα and PKBβ, cloned (116). The chromosomal location of the human PKB was mapped to chromosome 14q32 (117), proximal to the immunoglobulin-heavy-chain locus (118), a region frequently affected by translocations and inversions in human T-cell leukaemia/lymphoma, mixed lineage childhood leukaemia and clonal T cell proliferations in ataxia talengiectasia, supporting a role for this oncogene in development of a variety of tumors (119). Analysis of a number of human tumors revealed a 20-fold amplification of PKBα in a primary gastric adenocarcinoma. PKBα was mapped to chromosome region 19q13.1-q13.2 and shown to be amplified and overexpressed in several ovarian cancers and pancreatic cancer cell lines (120,121). Moreover, amplification of PKBα was especially frequent in undifferentiated tumors, suggesting that PKBα alterations may be associated with tumor aggressiveness.

1.3.2 Cloning of Protein Kinase B

In 1991, the cellular homologue of v-Akt was cloned independently by several groups and was characterized to be a 57 kD protein serine/threonine kinase (122-124). The kinase shows maximum similarity to protein kinase A (PKA) and protein kinase C (PKC), hence was named both protein kinase B (PKB) (122) or **R**elated to **A**- and **C**-kinase (RAC-PK) (123). In mammals three genes encode three different forms of PKB (termed as PKBα/Akt1, β/Akt2 and γ/Akt3 (Fig. 3). PKBα and PKBβ are approximately 82% identical with PKBγ, although PKBγ lacks 23 amino acids at the C-terminus compared with others.

Sequence analysis of PKB/Akt and v-Akt c-DNAs revealed that the viral gene is a fusion between a truncated tripartite viral group-specific antigen gag and PKBα/Akt1. This structure explains a mechanism for oncogenic activation of PKB. The viral gag protein is myristoylated at its N-terminus and normally targeted to the plasma membrane. PKB is normally a cytoplasmic protein (90%), whereas v-Akt is myristoylated and dispersed among various cellular compartments, with 40% being localized to the plasma membrane, 30%

nuclear and 30% cytosolic (124). This differential cellular localization probably has important consequences for regulation and function of PKB.

1.3.3 PKB expression

PKB is an ubiquitously expressed serine/threonine kinase with all tissues contain at least one form of PKB. PKBα and PKBβ have highest expression in brain, thymus, heart and lung (122,123). Expression of the γ isoform is more restricted with high expression in brain and testis and lower in heart, spleen, lung and skeletal muscle. Currently, the regulation of expression of PKB is not clear; however, it is upregulated in terminally differentiated cells.

1.3.4 Targeted disruption of PKB

Two of the three PKB/Akt genes have so far been disrupted in the mouse germ line. PKBα-null mice are viable, but have defective insulin metabolism (125). Mice with disrupted PKBβ gene are also viable. However, these mice show defects in the induction of apoptosis and their growth is retarded. Thymocytes from PKB$\beta^{-/-}$ mice show higher sensitivity to apoptosis induced by γ-irradiation and dexamethasone treatment (126). The viability and relatively mild phenotypes of the individual knockout mice point to the compensatory effect of the three gene products for each other, although the phenotypes of these animals do indicate that the three gene products have unique functions. However, PKBα/PKBβ double-knockout (DKO) mice exhibit severe growth deficiency and die shortly after birth. These mice display impaired skin development, severe skeletal muscle atrophy, impaired bone development and impeded adipogenesis (127).

1.3.5 Domain structure of PKB

All three PKB 'isoforms' consist of a conserved domain structure: a N-terminus pleckstrin homology (PH) domain, a central kinase domain and a C-terminus regulatory domain that contains a hydrophobic motif, which is a characteristic of AGC kinases (Fig. 3). The PH domain interacts with membrane lipid products such as phosphatidyl-ionositol(3,4,5)trisphosphate [PtdIns(3,4,5)P3] produced by phosphatidylionositol 3-kinase (PI3-kinase). Biochemical analysis revealed that the PH domain of PKB binds to both PtdIns (3,4,5)P3 and PtdIns(3,4)P2 with similar affinity (128,129).

The kinase domain of PKB, located in the central region of the molecule, shares a high similarity with other AGC kinases such as PKA, PKC, p70S6K, p90RSK (130). All three PKB isoforms have a C-terminal extension of around 40 amino acids, which harbors the F-X-X-F/Y-S/T-Y/F hydrophobic motif (where X is any amino acid) that is characteristic of the

AGC kinase family (130). For all AGC family kinases, phosphorylation of the Ser or Thr residue in this hydrophobic motif is necessary for full activation of the kinase.

1.3.6 Regulation

Phosphatidylionositol 3-kinase (PI-3K) mediates PKB activation

PKB has been shown to be a downstream target of PI-3K, a pluripotent lipid kinase involved in many signaling pathways. PI-3K phosphorylates phosphoinositides (PtdIns) at the 3-position of the inositol ring, generating PtdIns3P, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3. PKB as a PI-3K target was evident from two major observations: (i) inhibition of PI-3K activity by wortmanin completely blocked PKB activation after growth-factor stimulation, and (ii) growth-factor-receptor point mutations that prevented the activation of PI-3K also inhibited PKB. PKB was found to be rapidly and transiently phosphorylated by platelet-derived growth factor (PDGF), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF) and

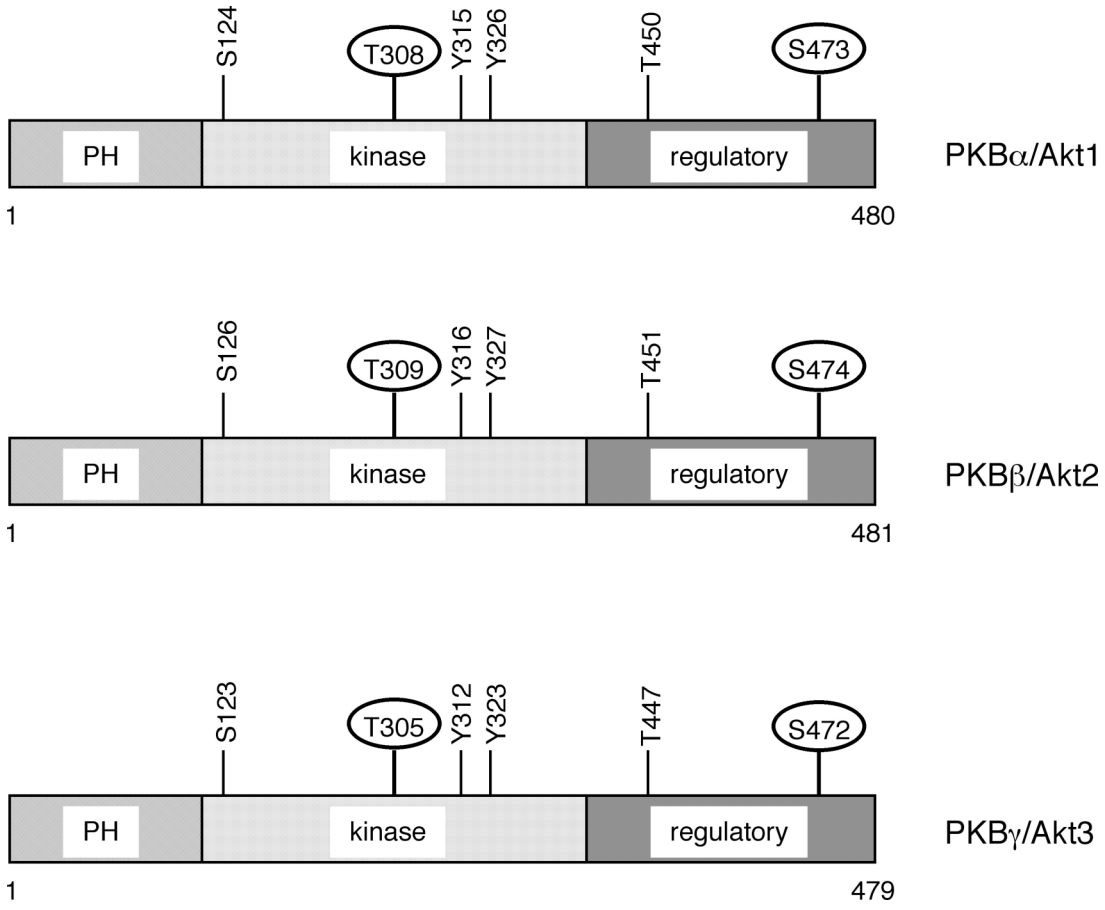

Figure 3. **Domain structure of the three PKB isoforms.** Each isoform consists of an N-terminal PH domain containing region for binding inositol phospholipids, a kinase domain, and a C-terminal regulatory domain. Residues contained within ovals are serine and threonine sites inducibly phosphorylated in response to various cell stimuli. Other residues in PKB/Akt1 known to be phosphorylated constitutively but not thought to regulate catalytic activity are also highlighted. Tyrosine residues Y315 and Y326 have recently been described as having a potential role in the regulation of PKB/Akt1 activity. The equivalent phosphorylation sites in PKB/Akt2 and PKB/Akt3 have been obtained from sequence databases.

insulin. Subsequently PKB has been shown to be activated by a wide variety of stimuli including haemopoietic cytokines such as IL-2, IL-3, IL-4, IL-5, chemokines (IL-8, RANTES), heat shock, hyperosmolarity, hypoxia, integrins, the T cell antigen receptor and nerve growth factor (131-135).

Mechanism of PKB activation

PKB as a direct target of PI-3K indicates that the 3'-phosphorylated lipid products of PI-3K could be involved in its activation (Fig. 4). These lipids, such as PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 , specifically associate with the PH domain and thus recruit PKB to the plasma membrane.

Activation of PI-3K leads to PKB phosphorylation at two residues, Thr^{308} within the kinase domain and Ser^{473} in the hydrophobic motif. Mutagenesis of each residue to alanine proved that both residues are essential for full activation (136). Furthermore, substitution with aspartic acid residues rendered PKB partially active and independent of agonists or inhibition by PI-3K antagonists such as wortmanin.

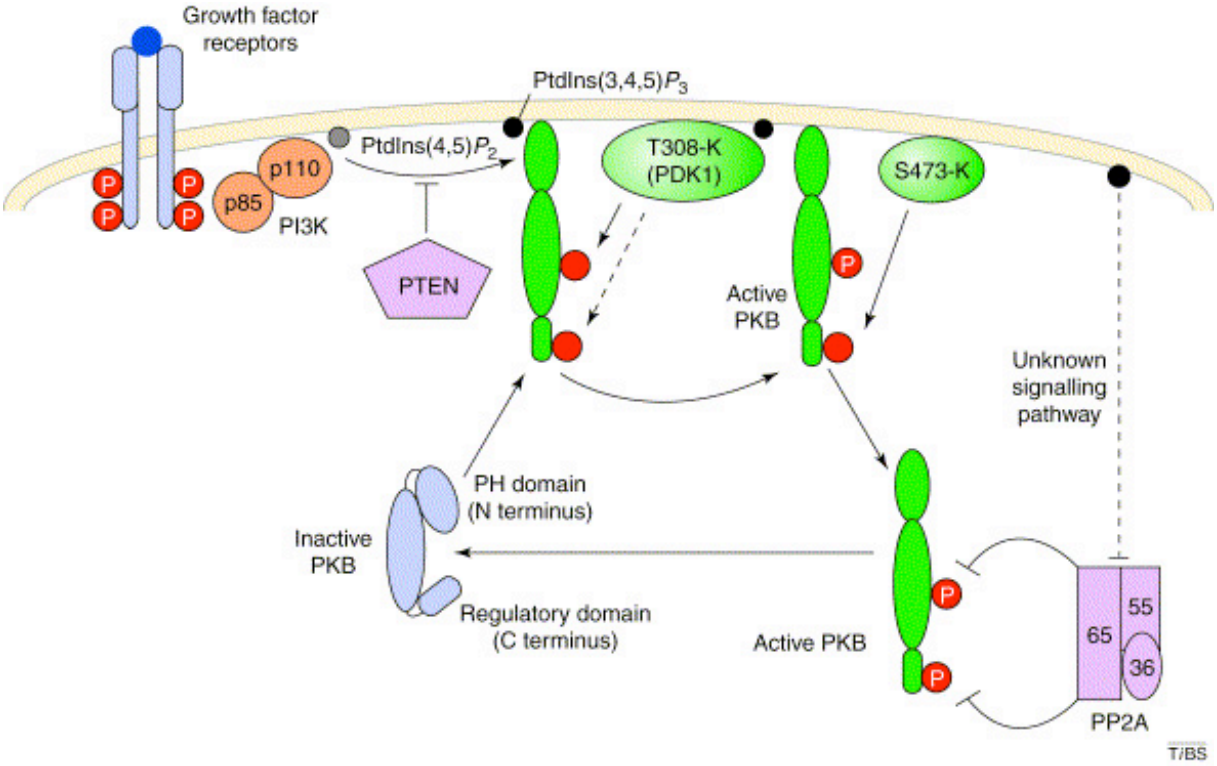

Figure 4. Proposed model for PKB/Akt regulation. Receptor ligand interaction leads to PI-3K activation which in turn activates PDK1. PKB/Akt is then phosphorylated on Thr^{308} and Ser^{473} by PDK1, and by an as yet unidentified Ser^{473} kinase. Abbreviations: PDK1, phosphoinositide-dependent kinase 1; PH, pleckstrin homology; S^{473}-K, kinase phosphorylating S^{473}; T^{308}-K, kinase phosphorylating T^{308}. (TIBS. 2001;26:657-664)

PDK1 has been shown to be the kinase responsible for Thr^{308} phosphorylation in PKB. The primary structure of this kinase is similar to other AGC kinase family members, with a PH domain at its carboxy-terminal. PKB and PDK1 both need to be translocated to the plasma membrane for full activation (137,138).

The role of PDK1 for Thr^{308} phosphorylation was confirmed by gene targeting in mouse embryonic stem (ES) cells. The deletion of PDK1 in ES cells resulted in inhibition of PKB, p70S6K and p90RSK activation, with complete inhibition of phosphorylation on the Thr residue in their activation loops. For PKB, co-localization with PDK1 to the plasma membrane is necessary for Thr^{308} phosphorylation, whereas, other AGC kinases are phosphorylated by PDK1 in the cytosol.

Compared to the Thr^{308} phosphorylation the kinase involved in phosphorylation of Ser^{473} is not yet clear. There are few reports describing PKB itself as a Ser^{473} kinase under certain conditions (139,140). Recently Hill et al. (141) reported to isolate a protein kinase activity that specifically phosphorylates Ser^{473}.

Activated PKB/Akt mediates its intracellular effects (Fig.4), and then becomes inactivated by the action of phosphatases such as PP2A, which dephosphorylate $pThr^{308}$ and $pSer^{473}$ and return PKB/Akt to its inactive conformation in the cytosol. PTEN (phosphatase and tensin homologue on chromosome ten) is a multifunctional phosphatase and acts to limit PKB/Akt activation by dephosphorylating PtdIns(3,4,5)P3' a lipid required for PKB/Akt activation.

1.3.7 Physiological functions of PKB

PKB 'isoforms' contribute to a variety of cellular responses, including cell growth, cell survival and metabolism. Up to now, more than 50 proteins have been identified as putative substrates for PKB. Peptide sequences that are preferentially phosphorylated by PKB were characterized by Alessi et al. (142) and Obata et al. (143). The minimal substrate consensus sequence for PKB is RXRXXS/T, where X is any amino acid and S/T is the phosphorylation site. Almost all reported PKB substrates have this minimal sequence.

Metabolism

GSK3 by phosphorylating inactivates glycogen synthase in response to insulin stimulation. Both isoforms of GSK3, GSK3□ and GSK3□, have the phosphorylation site in the amino-terminal region (Ser^{21} and Ser^{9}, respectively). They are phosphorylated and inactivated by PKB in a PI-3K-dependent manner. GSK3 is involved in signaling pathways other than insulin signaling, such as the wnt signaling pathway (see above).

Table 1. List of protein kinase B (PKB)/Akt substrates (TIBS. 2001;26:657-664)

*The amino acid phosphorylated by PKB is in bold font.

Protein	PKB phosphorylation sites	Species	Effect of phosphorylation
BAD	^{107}RSRHS**S**Y^{113}	Human	Blocks BAD-induced apoptosis
C-raf	^{130}RGRSR**S**A^{137}	Human	Inhibits C-raf activity
B-raf	^{254}RQRST**S**T^{260}	Human	Inhibits B-raf activity
	^{423}RERKS**S**S^{429}		
BRCA-1	^{504}KKRRP**T**S^{510}	Human	Interferes with nuclear localization of BRCA-1?
CREB	^{128}LSRRP**S**Y^{134}	Human	Increased association with CBP and p130
eNOS	^{1174}RIRTQSF**S**L^{1181}	Human	Activates eNOS and leads to NO production
FKHRL1	^{248}RRRAV**S**M^{254}	Human	Inhibits transcriptional activity of FKHRL1
GSK-3α	^{16}RARTS**S**F^{22}	Human	Inactivates GSK-3 activity
GSK-3β	^{4}RPRTS**S**F^{10}	Human	Inactivates GSK-3 activity
I-κB kinaseα	^{18}RERLG**T**G^{24}	Human	Activates transcriptional activity of NF-κB
IRS-1	^{297}RSRTE**S**I^{303}	Murine	Protects IRS-1from the action of PYPase
mTOR	^{2443}RTRTD**S**Y^{2449}	Murine	Inhibits mTOR activity
PDE-3B	^{268}RPRRR**S**S^{274}	Murine	Inactivates PDE-3B
PFK-2	^{460}RMRRN**S**F^{467}	Bovine	Activates PFK-2
	^{478}RPRNT**S**V^{484}		
Rac1	^{66}RIRPL**S**Y^{72}	Human	Inhibits Rac1-GTP binding
hTERT	^{819}RIRGK**S**Y^{825}	Human	Enhances telomerase activity
p21^{CIP1}	^{139}RKRRQ**T**S^{146}	Human	Causes cytoplasmic localization of p21^{CIP1}
Nur77	^{345}RGRLP**S**K^{351}	Human	Inhibits transcriptional activity of Nur77

The mammalian target of rapamycin (mTOR) is a regulator of mRNA translation in the signaling pathway controlled by nutrients such as amino acids. PKB phosphorylates mTOR on Ser^{2448} and activates this kinase (144). However, other report shows that this phosphorylation does not increase p70S6 kinase activity, which is a downstream target of mTOR (145).

Regulation of apoptosis

BAD (Bcl_2/Bcl_X antagonist) is a member of the Bcl_2 family of proteins that binds Bcl_2 and Bcl_X and inhibits their anti-apoptotic potential (146). Phosphorylation on Ser^{136} by PKB prevents BAD to execute its proapoptotic function by interfering in its interaction with Bcl_2 and Bcl_X. Caspase 9 acts as an initiator and effector of apoptosis (147,148). PKB can phosphorylate pro-caspase 9 on Ser^{196} in a Ras-dependent manner, which inhibits cytochrome C-induced cleavage of pro-caspase 9 essential for its enzymatic activity. Mutation of Ser^{196} to Ala reduces the apoptosis-inducing activity of pro-caspase 9 transfected into fibroblasts (148).

It has been reported that PKB can phosphorylate three kinases upstream of stress-activated protein kinase (SAPK). Apoptosis signal regulated kinase 1 (ASK 1) is one of the MAP kinase kinase kinase (MKKK) that interacts with and is phosphorylated by PKB on Ser^{83}, which results in the inhibition of SAPK pathway and subsequent apoptosis (149). PKB can phosphorylate another MKKK upstream of SAPK, the mixed lineage kinase 3 (MLK3). PKB phosphorylates MLK3 on Ser^{674} and this causes MLK3 inactivation and the promotion of cell survival (150). SEK1/MKKK4 is a direct upstream kinase for JNK, and p38 is also reported to be a PKB substrate. Similar to the two MKKKs mentioned above, SEK1 binds to and is phosphorylated on Ser^{78} by PKB, resulting in its inactivation and inhibition of ultraviolet (UV)-induced apoptosis (151).

Transcription factors

Transcription factors of the forkhead family (FH or FOXO factors) play a crucial role in cell cycle and apoptosis. To date, four isoforms of FH proteins (FKHR/FoxO1, FoxO2, FKHRL1/FoxO3 and AFX/FoxO4) have been shown to be directly phosphorylated by PKB (152,153). Phosphorylation of FH by PKB leads to it nuclear exclusion and thus decreased transcriptional activity resulting progression of cell cycle.

Members of the NF-κB/Rel family are key regulators of the immune response, and deregulation of its activity is implicated in the development of diseases such as autoimmune disease and cancer (154,155). In most cases, NF-κB activation is dependent on the phosphorylation and degradation of IκB, an inhibitor of NF-κB, by the IκB kinase (IKK) complex. PKB has been shown to regulate IKK activity in both direct and indirect manners.

Ozes et al. (155) reported that PKB interacts with and phosphorylates IKKα on Thr^{23} in a PI3-kinase dependent manner. Another study has shown that PKB phosphorylates the Ser/Thr kinase Tpl-2 (or Cot) on Ser^{400}, resulting in IKK complex activation (156). Activation of survival genes by NF-κB activation results in the inhibition of apoptosis.

Besides, PKB also phosphorylates a number of other transcription factors involved in various cellular functions. These include, cyclic AMP (cAMP)- response element binding protein (CREB) (157) and orphan nuclear receptor Nurr77 (158) in a PI3-kinase dependent manner.

Cell cycle regulators

One of the p53 target genes, $p21^{CIP1}$, is a cyclin/Cdk inhibitor which plays a crucial role in maintaining cell cycle progression, and its impaired function is one of the major abnormalities in many cancer cells (159). $p21^{CIP1}$ is reported to be a direct substrate for PKB, and its phosphorylation results in the inhibition of its potential to arrest cell cycle. Zhou et al. (160) first reported that phosphorylation of p21 on Thr^{145} by PKB inhibits nuclear localization of $p21^{CIP1}$, leading to activation of cyclin/cdk required for HER-2/neu-dependent tumor cell growth. Other groups however, have suggested different mechanisms for the regulation of $p21^{CIP1}$ by phosphorylation (161).

$p27^{KIP1}$, another major cyclin/cdk inhibitor, is also regulated by PKB dependent phosphorylation. Simultaneously, three groups demonstrated direct phosphorylation of $p27^{KIP1}$ by PKB in breast cancer cells (162-164). PKB phosphorylates $p27^{KIP1}$ on Thr^{157}, located in its nuclear localization signal (NLS), which excludes $p27^{KIP1}$ from the nucleus and results in the activation of cyclin/cdk and cell cycle progression.

p53 is one of the most important regulators for cell cycle progression and apoptosis in response to genotoxic stresses (165). Murine double minute 2 (MDM2) is an oncogene product induced by p53 (166). This ubiquitin E3 ligase directly binds to p53 and targets it for ubiquitination. In 2001, two groups reported that PKB-dependent phosphorylation might contribute to nuclear localization of MDM2 (167,168). Two putative phosphorylation sites, Ser^{166} and Ser^{186}, were determined by site-directed mutagenesis. However, the detailed mechanism of MDM2's regulation by PKB needs further investigation.

Besides, PKB also regulates a number of other substrates. These molecules are involved in a multitude of cellular functions and include endothelial nitric oxidase synthase (eNOS) (169,170), the Ser/Thr kinase Raf (171,172) and p47 phox NADPH oxidase (173).

1.3.8 PKB/Akt in the immune system

In T cells TCR ligation leads to the activation of PKB via induction of PI-3K. Expression of an activated PKB (gagPKB) allele in T cells resulted in decreased sensitivity to a variety of proapoptotic inducers, such as irradiation and exposure to Fas ligand (174). CD4+CD8+ DP thymocytes from these mice showed upregulation of the antiapoptotic protein, Bcl_{XL}, and enhanced NF-□B activity, which probably both contribute to the reduction in apoptosis. As the mice aged, evidence of autoimmunity was observed, along with increased populations of B cells and T cells and increased immunoglobulin levels (especially IgA) (175,176). These effects most probably reflect the increased longevity of activated cells as a consequence of reduced clearance by Fas induced apoptosis that normally occurs at the conclusion of an immune response. Abnormal activation of PKB could therefore contribute to inflammatory diseases in addition to cancer progression. Together, these animal models have begun to unravel the physiological consequences of PKB activation. However, as PI3K induces many other signaling pathways, with similar regulation of other protein kinases, like SGKs, and there is a growing evidence of the overlapping functions among the downstream kinases, it is difficult to ascribe any particular function to PKB specifically.

1.4 Nuclear Factor of Activated T cell (NFAT): forms and functions

The NFAT family of transcription factors comprises of five proteins that have similarity to the Rel/NF-□B family (177). Four of the NFAT proteins (NFAT1-NFAT4), also known as NFATc1-c4; (Table 2) are sensitive to Ca^{2+} dependent regulation and NFATc1, c2, and c3 are lymphoid specific NFATs, whereas, NFATc4 is expressed outside the lymphoid system.

The distinguishing feature of NFAT is its regulation by Ca^{2+} and the Ca^{2+}/calmodulin-dependent serine/threonine phosphatase calcineurin. NFAT proteins are phosphorylated and reside in the cytoplasm in resting cells; upon stimulation, they are dephosphorylated by calcineurin, translocate to the nucleus, and become transcriptionally active, thus providing a direct link between intracellular Ca^{2+} signaling and gene expression. NFAT activity is further modulated by additional inputs from diverse signaling pathways, which affect NFAT kinases and nuclear partner proteins.

It is clear that NFAT activates transcription of a large number of genes during an effective immune response (178-181). Besides, NFAT participates in the early phase of chromatin remodeling at specific genetic loci and plays a crucial role in the T cell

differentiation program. Also, there is evidence that NFAT regulates cell differentiation programs in cell types other than immune cells (182), such as, fibre-type specification in differentiated skeletal muscle, cardiac valve development, and osteoclast differentiation.

1.4.1 Functional domains in NFAT proteins

Three functional domains are critical for the activation and function of NFAT proteins (Fig. 5). These are, the rel similarity domain (RSD) or the DNA binding domain (DBD), responsible for DNA binding activity and interaction with AP-1 proteins; the NFAT homology region (NHR) or the regulatory domain, also known as calcineurin binding domain (CBD), regulates the intracellular localization; and the transcriptional activation domains (TADs).

Rel-similarity domain (RSD)

The most prominent feature of NFAT proteins is a highly conserved amino acid sequence (~ 65%) of about 290 amino acids. This domain has been referred to as the Rel-similarity domain because of its distant relationship (~20% sequence identity) to the DNA-binding domain of the Rel/NF-κB family of transcription factors (183).

Consensus derived from the alignment of NFAT-binding sites of several promoters results in a core consensus sequence, 5'-GGAAA-3' for DNA binding, for all NFAT family members. Therefore, a certain amount of redundancy or compensation by co-expressed NFAT proteins is likely.

NFAT homology region (NHR)

In all NFAT proteins, the RSD is preceded by an amino terminus sequence of about 400 amino acids (Fig. 5). This sequence can be divided into an amino-terminal region of 100 residues of little conservation and a serine/proline-rich region of about 300 residues of moderate similarity (~35% identity), termed the NFAT homology region. The main function of the NHR appears to be the control of intracellular localization of NFAT proteins (184-187). Deletion of NHR in NFATc2 or NFATc3 resulted in NFAT nuclear activity in absence of calcium mobilization (184,188). With regard to intracellular localization, the NHR seems to control at least four mechanistically different steps: nuclear import, nuclear localization, nuclear export and cytoplasmic localization.

Alignment of the NFAT NHR sequences revealed eight motifs of conserved residues (Fig. 5) (186,189). One of these motifs encodes a nuclear localization signal (NLS), which is essential for NFAT nuclear translocation. Mutations in the highly basic NLS motif in NFATc2 or NFATc1 resulted in their reduced translocation to the nucleus (185,186). An additional NLS sequence is also present in the RSD.

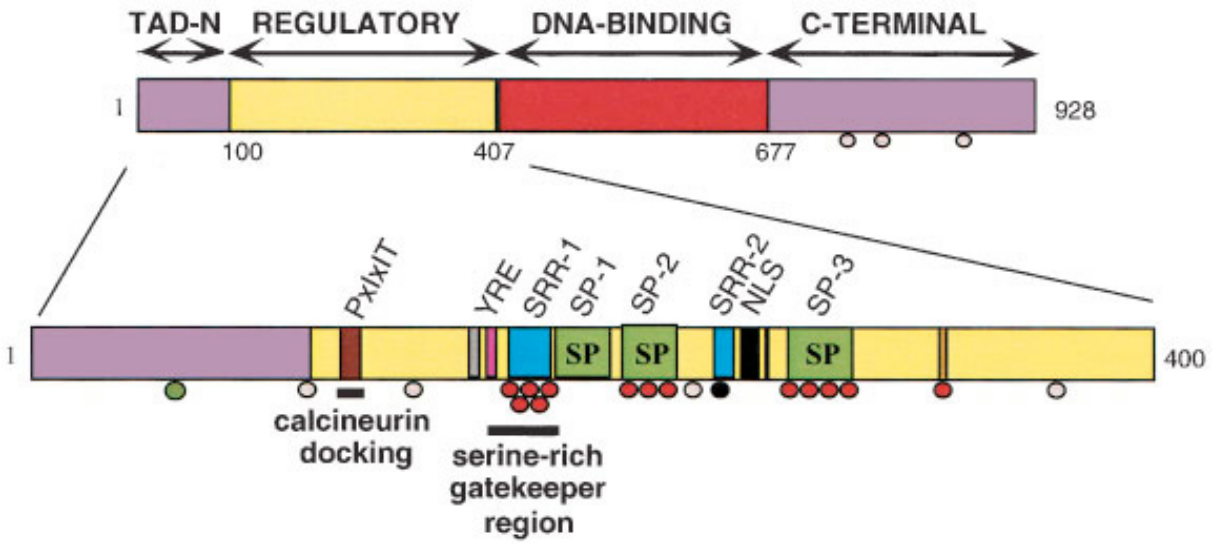

Figure 5. Domain structure and phosphorylation of NFAT proteins. The N-terminal transactivation domain (TAD-N), regulatory domain, DNA binding-domain and C-terminal domain of NFAT proteins are indicated. Some forms of the proteins have altered N-termini or are truncated at their C-termini due to alternative splicing. The regulatory domain is enlarged to show conserved sequence motifs as colored boxes: two serine-rich regions (SRR-1 and SRR-2), three SPxx repeat motifs (SP-1, -2, and -3), a major calcineurin docking site (PxIxIT), a nuclear localization sequence (NLS), and a conserved YRE/D sequence that forms part of the gatekeeper region (YRE). Phosphorylated residues are shown as filled circles based on data from NFAT1 (190): conserved phosphoserines that become dephosphorylated upon activation are shown in red, non-conserved phosphoserines in grey, the conserved phosphoserine situated N-terminal to the NLS in black; and the inducible phosphorylation site in the N-terminal transactivation domain in green. (Oncogene. 2001;20:2476-2489)

Conserved motifs in the NHR, in addition to the NLS, include a serine rich region (SRR, also called calcineurin-regulated-inhibitory (CRI) sequence), three copies of the serine-proline (SP) box of sequence SPxxSPxxSPxxxxx, a nuclear export sequence (NES) and other uncharacterized motifs (Fig. 5) (184,185,189). Interestingly, deletion of the SRR motif in NFATc3 or mutation of serines in the SRR motif in NFATc1 resulted in constitutively nuclear proteins, indicating that SRR motif masks the NLS sequence and thereby negatively regulates NFAT nuclear translocation (184,185).

Transcriptional-activation domains (TADs)

In NFATc2, two different TADs have been identified (188), one at the first 100 amino acids in the amino-terminus, and the other, within the last 200 amino acids in the carboxy terminus of NFATc2. Inducible phosphorylation of the TADs has been observed for NFATc2, and mutations of the modified serines eliminate transcriptional activity (190). Two TADs have also been found in the same regions of NFATc3.

1.4.2 Cellular inputs that affect NFAT

Ca^{2+} and calcineurin

In T cells NFAT is activated via increase in intracellular Ca^{2+} upon TCR ligation. Activation is initiated by dephosphorylation of the NFAT regulatory domain which is heavily phosphorylated in resting cells. Calcineurin dephosphorylates NFATs at this domain in SRR1, SPxx repeat, and SRR2 motifs, thus triggering NFAT nuclear accumulation and increasing the affinity of NFAT for its target sites in DNA (191).

Efficient dephosphorylation requires a docking interaction between NFAT and calcineurin (192,193). The major docking site for calcineurin is located at the N-terminus of the NFAT regulatory domain, and has the consensus sequence PxIxIT (SPRIET in NFATc2). When Ca^{2+} entry is prevented or calcineurin activity is inhibited, NFAT is rephosphorylated by various NFAT kinases and rapidly leaves the nucleus, and NFAT dependent gene expression is terminated (194).

NFAT kinases

NFAT kinases are an important component of the regulatory mechanism responsible for NFAT activation. NFAT kinases located either in the cytoplasm or inside the nucleus phosphorylate the serine residues present in various motifs in the calcineurin binding regulatory domain of NFATs. GSK3 has been shown to be a NFAT kinase (195). Many studies have indicated various other serine kinases like ERK, JNK and casein kinase (CK) as possible NFAT kinases. However a clear picture of various NFAT kinases is still to emerge.

The many serine residues that control NFATc2 nuclear localization are located in diverse sequence contexts that are unlikely to be recognized by a single kinase. Thus it could be possible that several constitutively active kinases co-operate to maintain the inactive, phosphorylated state of NFAT in resting cells, also in a similar way several inducible and/or constitutive kinases may act to rephosphorylate NFAT that has been dephosphorylated during cell activation. Sequence comparisons show that the SRR-1 region is extended in NFAT2-4, whereas the SP-2 and SP-3 motifs are truncated in NFAT3 (190); thus different NFAT proteins may be regulated by overlapping but distinct sets of constitutive and inducible kinases.

Although several NFAT kinases have been identified, an integrated picture of NFAT phosphorylation has not yet emerged. CK1 and GSK3 are constitutive NFAT kinases that promote NFAT nuclear export (195,196). The MAP kinases p38 and JNK are inducible kinases. JNK1 phosphorylates NFATc1 and NFATc3, whereas p38 selectively targets NFATc2 and NFATc4 (197-199).

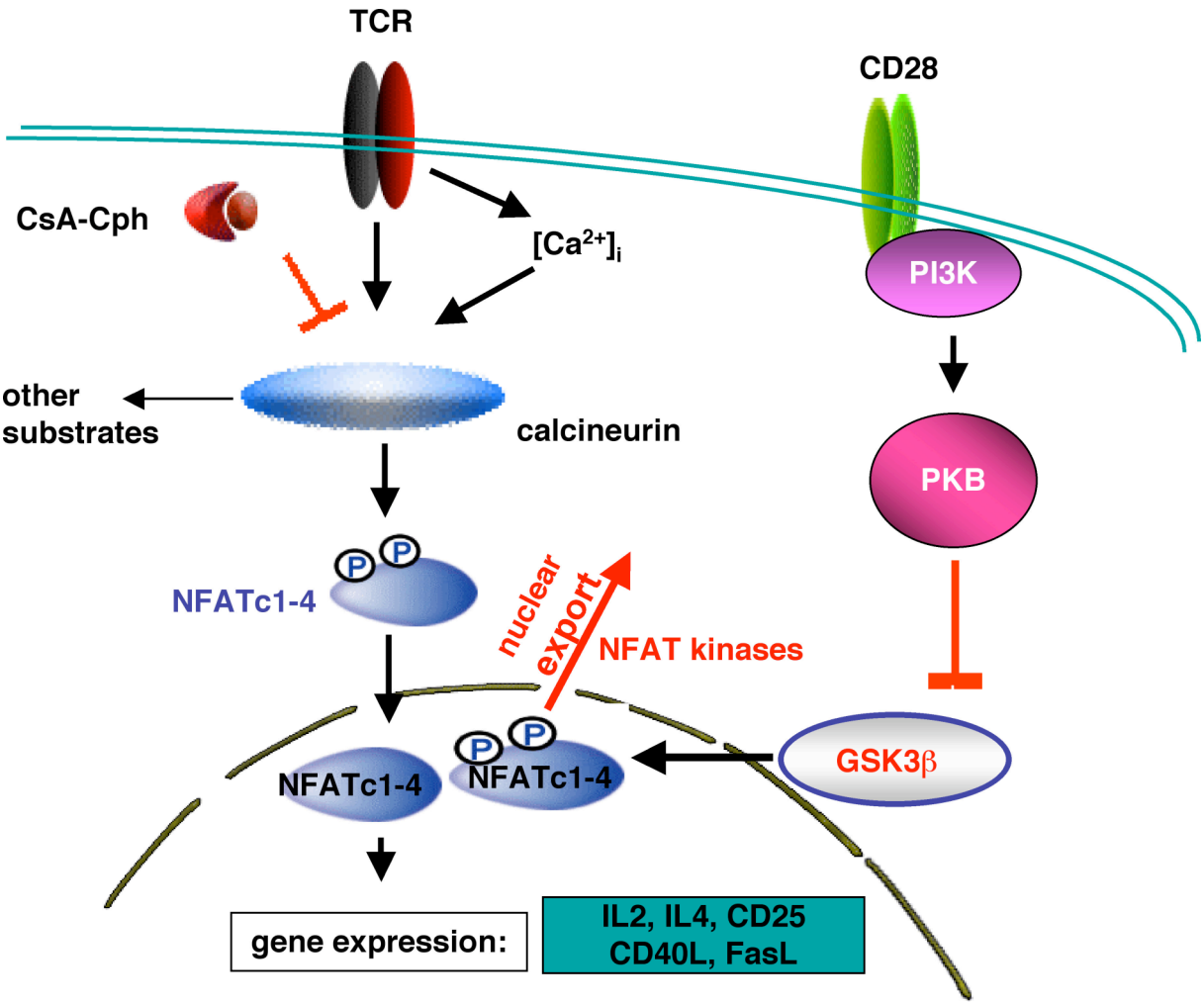

Figure 6. Schematic model of NFAT activation in T cells. TCR ligation induces intracellular increase in Ca^{2+} which then activates calcineurin. Activated calcineurin dephosphorylates NFAT and facilitates its nuclear localization. GSK3 phosphorylates NFAT and promotes NFAT nuclear exclusion. PKB upon activation via TCR/CD28 ligation inactivates GSK3 and thereby regulates NFAT activation.

In T cells, T-cell receptor stimulation is coupled to Ca^{2+}/calcineurin signaling and NFAT nuclear import, whereas stimulation through the costimulatory receptor CD28 potentiates activation of the PI-3 kinase pathway and hence the kinase Akt/PKB (200). PKB phosphorylates GSK3 and renders it inactive. Thus the net effect is that CD28 costimulation, by activating PKB, inhibits GSK3 and therefore NFAT nuclear export (201).

In T and B cells NFATs interact with AP-1, GATA proteins and many other transcription factors, co-activators and repressors to execute their target gene expression or repression. NFAT induced gene expression plays a crucial role in cytokine production, Th1 and Th2 lineage decision and regulation of apoptosis and cell cycle (263, 264, 283, 284).

Table 2. NFAT family members and Phenotype of NFAT knock out mice (Mol Cells. 2004;18:1-9)

NFAT Protein	Presence in immune cells	Regulation	Chromosomal location* (human/mice)	Phenotype of knockout mice
NFAT1 (NFATp, NFATc2)	Yes	Ca^{2+}/ calcineurin	20q13.2-q13.3/ 2 95.5 cM	Enhanced B and T cell response.Th2 bias with increased Th2 cytokines
NFAT2 (NFATc, NFATc1)	Yes	Ca^{2+}/ calcineurin	18q23/ 18 54.0 cM	Embryonic lethal (cardiac valve defect), reduced B and T cell proliferative response, impaired Th2 response with decreased IL-4production.
NFAT3 (NFATc4)	No	Ca^{2+}/ calcineurin	14q11.2/ 14 C1	Viable and fertile with defects in axon outgrowth.
NFAT4 (NFATx, NFATc3)	Yes	Ca^{2+}/ calcineurin	16q22.2/ 8 51.0 cM	Mildly impaired developm-ent of CD4 and CD8 SP thymocytes, (increased apo-ptosis of DP thymocytes), mild hyperactivation of peripheral T cells.
Double KO (NFAT1 + NFAT2)				Impaired effector T cell function (reduced Th1 and Th2 cytokines), but hyper-reactive B cell function
Double KO (NFAT1 + NFAT4)				Lymphoproliferative disor-der (hyper-reactive TCR, defect in T cell apoptosis)

(Continued……..)

				allergic blepharitis and intestinal pneumonitis (increased Th2 response).
Double KO (NFATc3 + NFAT4)				Embryonic lethal (E11) with generalized defect in vessel assembly, excessive and disorganized growth of vessels into the neural tube and somites.
NFAT5 (TonEBP)	Yes	Osmotic stress integrin (□6□4)	16q22.1/ 8 53.0 cM	Renal atrophy and lack of tonicity-responsive gene expression (aldose reductase, $Na^+/Cl2^-$ coupled betaine/ GABA transporter, and the Na^+/myo-ionositol cotransporter)

1.5 Calcineurin: forms and functions

1.5.1 Calcineurin properties

Calcineurin, the Ca^{2+}/calmodulin dependent serine/threonine phosphatase, is a heterodimer consisting of a 58 kD catalytic subunit, calcineurin A, and a 19 kD regulatory subunit, calcineurin B. Evidences suggest that all eukaryotic organisms possess one or more genes for each subunit.

Calcineurin A

In addition to calcineurin (PP2B), the serine/threonine protein phosphatase family members include protein phosphatase 1 (PP1), 2A (PP2A), and 2C (PP2C), phosphatases essential for a number of signal transduction pathways in eukaryotic cells. PP2A was originally described as having no requirement for divalent metal ion, calcineurin is regulated by Ca^{2+}/calmodulin, and PP2C is Mg^{2+} dependent. Differences among these phosphatases are also found with regard to sensitivity to inhibition by macrolide inhibitors. PP2A and PP1 are inhibited by okadaic acid, whereas, calcineurin is specifically inhibited by the immunosuppressive drugs CsA and

FK506 in the presence of cyclophilin and FK506 binding protein (FKBP), respectively (202, 203).

The active site of calcineurin is located on the A subunit which, in mammals, is 57-59 kD depending on the isoform. All calcineurin A genes encode for a polypeptide consisting of a catalytic domain homologous to other serine/threonine protein phosphatases and three regulatory domains at the carboxy terminus that distinguish calcineurin from other family members (Fig. 7). These domains have been identified as the calcineurin B binding domain (204-206), the calmodulin-binding domain (207), and the "autoinhibitory" domain (208,209), which binds in the active site cleft in the absence of Ca^{2+}/calmodulin (210) and inhibits the enzyme, acting in concert with the calmodulin binding domain to confer calmodulin regulation.

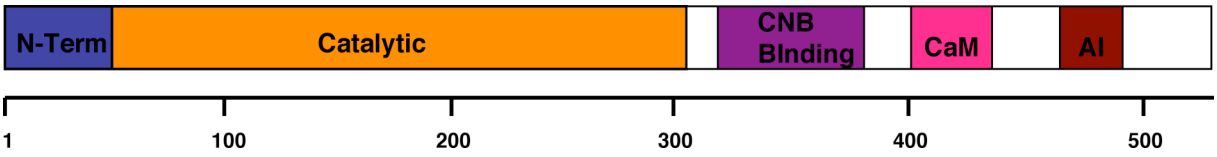

Figure 7. Schematic representation of domain structure of calcineurin A. CaM, calmodulin: CNB, calcineurin B: AI, autoinhibitory.

Calcineurin B

The gene for mammalian calcineurin B encodes a protein of 170 amino acids containing four Ca^{2+}-binding EF-hand motifs. The mature calcineurin B protein is myristoylated at the □-amino group of glycine at position 2. At present, the contribution of myristoylation towards calcineurin function is unknown.

1.5.2 Physiological roles of calcineurin

Subcellular distribution

Calcineurin is widely distributed in mammalian tissues, with the highest levels found in brain. Various studies show calcineurin is predominantly present in the cytoplasm and synaptosomal cytosol. There is overwhelming evidence for calcineurin in the nucleus along with other calmodulin-binding proteins such as casein kinase 2 and myosin light-chain kinase. In the context of signaling pathways that activate NFAT, Shibasaki et al. (187) have shown that calcineurin colocalizes to the nucleus along with dephosphorylated NFAT. Calcineurin has also been shown to be associated with the cytoskeleton (211).

1.5.3 Calcineurin functions in the immune system

In the immune system calcineurin plays a crucial role in thymocyte development, T lymphocyte activation, T cell anergy induction, cell cycle progression and T and B lymphocyte apoptosis.

Calcineurin in thymocyte development

Expression of a constitutively active version of calcineurin in tg mice induced a block in early thymocyte development and an enhancing effect on selection of CD4 lineage T cells (212). Deficiency of calcineurin A the predominant calcineurin isoform expressed in lymphocytes shows impaired T cell development in terms of fewer CD3 positive cells and reduced number of CD4+ and CD8+ SP cells (213) i.e., positive selection was impaired. The duration and intensity of calcineurin activity in combination with the ERK pathway has been shown to regulate thymocyte lineage commitment (214). Also, by using FK506 as an effective inhibitor of calcineurin activity, Nakayama et al. showed that calcineurin activation plays a role in thymic positive selection but not in negative selection (215). However, deficiency of calcineurin A (CNA) on a P14 TCR tg background showed no defect in thymic positive and negative selection, although mature T cell function was impaired (216). T lineage specific deletion of the calcineurin B1 subunit confirmed that calcineurin activity is essential in positive but not in negative selection (217).

Calcineurin in T cell activationand anergy induction

TCR ligation by appropriate antigen-MHC complexes induces intracellular Ca^{2+}, which in turn activates calcineurin and NFAT. Nuclear NFAT plays a critical role in the coordinate gene expression of several genes, including various cytokines like IL-2, IL-3, IL-4, IL-5, GM-CSF, IFN-and TNF-, and cell surface receptors such as CD40L, CTLA-4 and Fas L.

Sustained calcium signaling induces a state of anergy or antigen unresponsiveness in T cells, mediated through calcineurin and NFAT. Activation of NFATc2 in the absence of AP-1 appears to be required and sufficient for the induction of functional anergy in T cells (218). Ca^{2+}-induced anergy seems to be a multistep process, implemented through proteolytic degradation of specific signaling proteins (219).

Calcineurin in Cell cycle regulation

Cyclin dependent kinase 4 (cdk4) is a crucial regulator of cell cycle progression at the G0/G1 checkpoint. It has been shown that in Jurkat cells calcineurin specifically binds to cdk4 and inactivates the kinase by dephosphorylation, thus blocking cell cycle progression at the G0/G1 checkpoint. Inhibition of calcineurin phosphatase activity by FK506 and CsA resulted in an

overall increase in cdk4 kinase activity, suggesting that the phosphatase activity of calcineurin was inhibitory to the kinase activity of cdk4 (220).

Calcineurin and apoptosis

Calcineurin has been implicated in the programmed cell death of T and B-lymphocytes (221,222). In T cell hybridomas apoptosis can be induced by ligation of the TCR/CD3 complex. Both CsA and FK506 inhibit this process, implicating the involvement of calcineurin in the signaling pathway of apoptosis. Similarly, in B-cell lymphoma cell-lines apoptosis induced by cross-linking of surface immunoglobulin receptors was inhibited by these immunosuppressive drugs (223).

In lymphocytes, calcineurin and NFAT appear to participate in apoptosis, in part by mediating the induction of Fas and Fas L, which then interact and transduce the apoptotic signal after TCR ligation. Further, calcineurin forms a complex with Bcl_2, which targets it to the cytoplasmic membrane (224). Although still catalytically active, calcineurin bound to Bcl_2 is unable to promote nuclear translocation of NFAT. Also, BAD, a proapoptotic member of the Bcl_2 family, is a substrate of calcineurin. Dephosphorylation of BAD by calcineurin enhances BAD heterodimerization with Bcl-x_L and apoptosis (225).

Chapter 2.
Objective of the study

2. Objective of the study

2.1 Concept

Though PKB's role in various cellular functions has been well studied in different cell systems, its precise role in the development and functioning of the immune system is relatively unknown. Numbers of studies though have implicated many kinases, mostly belonging to the tyrosine kinase family in early T cell development and function the role of Ser/Thr kinases in these events has just started to be unraveled. Keeping in view, the broad range of signaling pathways it regulates, it can be assumed that definitely it could play a vital role in T cell development and function.

PI-3K has been implicated in regulating the nuclear factor of activated T cell (NFAT), via activation of PKB (226). Also protein kinase A (PKA) has been shown to regulate NFAT nuclear accumulation (227). PKA and PKB share similar consensus sequence for phosphorylation and both have sequence homology, being the members of AGC kinase family. Thus, probabilistically PKB, in fact could control NFAT signaling pathway in a way similar or different to that of PKA.

Like PKB, calcineurin is also ubiquitous in expression and controls a range of cellular events. In principle by its sheer nature of being a Ser/Thr phosphatase it could be expected to play an opposite role to that of the ser/thr kinase PKB. In a physiological setup, a cross talk, leading to counteraction of each other's function in modulating downstream events is most likely.

In T cell development and function calcineurin exerts its positive or negative effects primarily through activation of NFATs. Various knockout models have proved its essentiality for thymocyte development and normal T cell function (213,216,217). Over-expression studies in calcineurin tg mice show dramatic effect in the form of severe block in thymocyte development (212). Thus in both the situations, (i) NFAT deficiency, in case of calcineurin knockout model and (ii) NFAT over-activity in case of calcineurin tg model, it is well established that calcineurin/NFAT signaling pathway is vital for normal T cell development and function.

2.2 Hypotheses

1. PKB can regulate NFAT signaling in T cells

GSK-3 is a well-characterized substrate of PKB and has been shown to act as an NFAT kinase (195). Phosphorylated GSK-3 is functionally inactive. Thus in the Ca^{2+}/calmodulin-calcineurin-NFAT signaling pathway, PKB's role is well evident via regulation of GSK-3.

Theoretically, by inactivating GSK-3, it should enhance NFAT signaling, by prolonging nuclear duration of NFAT. Over expressed PKB (PKB tg mice), should in fact lead to stronger NFAT signaling upon T cell activation.

2. PKB can itself act as an effective NFAT kinase

PKB is a Ser/Thr kinase and NFAT activation depends on the balance between phosphorylation and dephosphorylation of its many serine residues. One possibility is that, PKB itself could act on NFAT and phosphorylate the serine residues and thereby keep it in an inactive state, irrespective of its effect on GSK-3. In this situation, how the T-cell functions are modulated is of enormous interest. Thus in principle PKB itself can act as an NFAT kinase.

3. Possible crosstalk between PKB and calcineurin signaling at the level of NFAT

Assuming PKB, as a NFAT kinase, it is of great interest to study how it will affect T- cell development in a setup where there is (i) an insufficiency of NFAT (NFAT k.o. model) and (ii) sufficiency of NFAT (calcineurin tg model). In calcineurin tg mice model, NFAT is constitutively nuclear and active, and in this model thymocyte development is severely blocked at a very early stage. Over expression of PKB in this model, in principle, should restore normal thymic development by acting on the overactive NFAT signaling, which is the main effector molecule in calcineurin induced block. We hypothesize PKB and calcineurin crosstalk with each other at the level of NFAT, to influence early thymocyte development.

2.3 Experimental system

To study the precise role PKB plays in T cell development and function, we have used a tg mouse model (myrPKB) where PKB is overexpressed and is constitutively active. This is to simulate an in vivo model for lymphomagenesis and how PKB modulates events that lead to development and progression of lymphomas and thymomas.

To delineate PKB's regulation of NFAT activation, in T cell development and function, we have used a NFAT sufficiency model; i.e., calcineurin tg mouse model where, independent of Ca^{2+} signaling, NFAT is constitutively nuclear. Crossing calcineurin single tg mice with PKB tg mice we have the mice where both, Ser/Thr kinase PKB and Ser/Thr phosphatase calcineurin are overexpressed. This is an interesting model to study and prove our hypothesis concerning crosstalk between PKB and calcineurin, in modulating NFAT signaling during early thymocyte development.

Chapter 3.
Materials and Methods

3. Materials and Methods

3.1 Materials

3.1.1 Mice

C57BL/6

PKB2 tg

PKB5 tg

OT1 tg (228)

ΔCam tg (Prof. E. Serfling, Institute of Pathology, Univ. of Würzburg)

Rag1 $^{-/-}$ (Prof. M. Klein, Institute of Virology and Immunology, Univ. of Würzburg)

PKB2/OT1 double tg

ΔCam/PKB2 double tg

ΔCam/PKB5 double tg

ΔCam/OT1 double tg

All mice used in this study were either one day or 6 to 8 weeks old unless mentioned otherwise. Mice were bred in the animal facility at the Institute of Virology and Immunology under pathogen free conditions. Institutional guidelines were followed in handling mice throughout the course of the study.

3.1.2 Chemicals and reagents

for proliferation assay :

Reagents	**Source**
CFSE (5-(and-6)-Carboxyfluoresceine diacetate succinimidyl ester)	Molecular Probe
CsA (Cyclosporin A)	Sigma
PD98059	Calbiochem
PP1	Calbiochem
LY294002	Calbiochem
SB202190	Calbiochem
UO	Calbiochem
Staurosporin	Sigma
Forskolin	Sigma
PMA	Calbiochem

Ionomycine	Calbiochem
for cell cycle analysis:	
Propidium Iodide	Sigma
7-Amino actinomycin D (7-AAD)	Sigma
Colcemid	Sigma

Miscellaneous:

Dimethyl Sulfoxoide (DMSO)	Sigma
HEPES	Sigma
Ethylene glycol tetraacetic acid (EGTA)	Sigma
Ethylene diamine tetraacetic acid (EDTA)	Sigma
Potassium chloride	Sigma
Sodium citrate	Sigma
Saponin	Sigma
Glycine	Roth
Tris	Roth
Boric acid	Roth
Magnesium chloride	Roth
Sodium chloride	Roth
Sodium azide	Sigma
Bovine serum albumin (BSA)	Sigma
Agarose	Applichem
Polyacrylamide	Applichem
Sodiumdodecyl sulfate (SDS)	Roth
Ammonium persulfate (APS)	Sigma
TEMED	Sigma
Ethydium bromide	Roth
Bromophenol blue	Roth
Xylene cyanol	Roth
Acetone	Roth
Methanol	Roth
Ethanol	Roth
Chloroform	Roth
Isopropanol	Roth
Formaldehyde	Roth

Formamide	Sigma
Cell strainers	Hartenstein
Trypan blue	Sigma
Proteinase K	SIGMA-Aldrich
Complete Protease inhibitor	Roche
Gene Ruler™ 100bp Ladder	MBI Fermentas
Cycloheximide	Sigma-Aldrich
ECL chemiluminescence kit	Pierce
CBA ELISA kit	BD Pharmingen
Goat anti-rat IgG Fc Magnetic beads	Quiagen
Protein G sepharose	Amersham/Biosciences
Glutathione sepharose	Amersham/Biosciences
Glutathione	Sigma
Filters for FCS	S&Smicroscience
Scintillation fluid (Betaplate Scint)	Wallac
Trizol	GIBCO/ Invitrogen
NP-40	Sigma
Bradford's Reagent	BioRad
Nitrocellulose membrane	Amersham/ Bioscience
Whatman filter paper	Hartenstein
Whatman membrane for NTOC	Herolab
96 well cell culture plates	Cellstar
96 well ELISA plates	Nunc
6 & 24 well cell culture plates	Cellstar
12 well cell culture plates	TPP, Switzerland
6 mm Petri dish	Cellstar
T-25, T-75 & T-250 tissue culture flasks	Nunc
Cuvettes	Hartenstein
FACS tubes	Greiner

3.1.3 Antibodies

for proliferation assay :

□CD3□(145.2C11)	BD Pharmingen
□CD28 (37.51)	BD Pharmingen

for isolation of DN thymocytes, CD4+ and CD8+ T cells

Hybridoma culture supernatants:

Rat-anti-mouse CD4 (GK1.5)

Rat-anti-mouse CD8 (YTS 169.4 & TiB 105)

Rat-anti-mouse CD19 (1D3)

Rat-anti-mouse NK1.1 (4D11)

Rat-anti-mouse MHC II (2G9)

for ELISA:

IL-2 ELISA BD Pharmingen

Capture antibody: (Anti-mouse IL-2 mAb)

Detection antibody: (Biotinylated anti-mouse IL-2 mAb)

Avidin-horseradish peroxidase conjugate

Recombinant mouse IL-2

CBA (Cytometric bead array)

Mouse Th1/Th2 cytokine CBA Kit BD Biosciences

Mouse IL-2, IL-4, IL-5, IFN-γ, TNF-α capture beads

Mouse Th1/Th2 PE detection reagent

Mouse Th1/Th2 cytokine standards

Cytometer setup beads

PE positive control detector

FITC positive control detector

Wash buffer

Assay diluent

for FACS staining:

All antibodies were purchased from BD Pharmingen unless otherwise mentioned.

Antibody	Clone
anti-CD4 PE	(GK1.5)
anti-CD4 FITC	(GK1.5)
anti-CD4 bio	(GK1.5)
anti-CD8 PE	(53-6.7)

anti-CD8 FITC	(53-6.7)
anti-CD8 bio	(self made)
anti-CD3 FITC	(145.2C11)
anti-CD25 PE	(PC61)
anti-CD25 FITC	(PC61)
anti-CD25 bio	(PC61)
anti-TCR FITC	(H57-597)
anti-TCR bio	(H57-597)
anti-CD44 FITC	(IM7)
anti-CD2 bio	(RPA-2.10)
anti-CD5 bio	(53-7.3)
anti-CD24(HSA) bio	(M1/69)
anti-CD69 bio	(H1.2F3)
anti-CD62L bio	(Mel 14)
anti-NK1.1 PE	(PK136)
anti-LFA bio	(HI111)
anti-CD49 bio	(9F10)
anti-CD18 bio	(6.7)
anti-V 2 bio	(B20.1)
anti-V 5 bio	(MR9-4)
anti-V 8 bio	(F23.1)
anti-V 11bio	(RR3-15)
anti-Fc receptor	(24G2)
anti-Rat IgG2a	(R35-95)
anti-Mouse Bcl_2 FITC	Bcl_2 Antibody Sampler Kit

for western blot analysis:

Rabbit anti-mouse pPKB (ser-473)	NEB
Rabbit anti-mouse PKB	NEB
Rabbit anti-mouse pERK	NEB
Rabbit anti-mouse ERK	NEB
Rabbit anti-mouse pP38	NEB
Rabbit anti-mouse pJNK	NEB
Rabbit anti-mouse pGSK3	NEB

Rabbit anti-mouse Lamin A	NEB
Mouse anti-mouse NFATc1	Alexis
Rabbit anti-mouse NFATc2	Gift from Dr. A. Rao (CBR, Harvard Medical School, Boston)
Rabbit anti-mouse NF-κB p65	Santa Cruz
Rabbit anti-mouse NF-κB p50	Santa Cruz
Rabbit anti-mouse Rel B	Santa Cruz
Rabbit anti-mouse Rag1	Santa Cruz
Goat anti-mouse Actin	Santa Cruz
Rabbit anti-mouse IκBα	Santa Cruz
Rabbit anti-mouse pIκBα	Santa Cruz
Goat anti-rabbit HRP	Jackson Laboratories
Goat anti-mouse HRP	Jackson Laboratories
Rabbit anti-goat HRP	Jackson Laboratories
Rabbit anti-human CRM1	Gift from Dr. G. Grosveld (St. Jude Children's Research Hospital, Memphis, TN)
for confocal microscopy:	
Mouse anti-mouse CD4 Alexa 647 conjugate	Caltag
Mouse anti-mouse CD8α Alexa 488 conjugate	Caltag
anti-Fc receptor (24G2)	purified from hybridoma supernatant
Rabbit anti-mouse NFATc1 & NFATc2	Prof. E. Serfling (Inst. of Pathology, Univ. of Würzburg)
Rabbit anti-mouse NFATc3	Santa Cruz
Rabbit anti-mouse pPKB (ser-473)	NEB
Donkey anti-Rabbit Cy3	Molecular Probe
DAPI	Molecular Probe
for apoptosis analysis:	
Annexin V FITC	BD Pharmingen

3.1.4 PCR and RT-PCR Primers

PCR:

PKB transgene:

F: 5’ –AGATTTCCTGTCCCCTCTCAGG-3’

R: 5’ –TGTTGGACCCAGCTTTGCAG-3’

Calcineurin (ΔCam) transgene:

F: 5’ –CCGAGCCCAAGGCGATTGATCC-3’

R: 5’ –CCCGGTTTCTGATGACTTCCTTCC-3’

RT-PCR:

Calcineurin

F: 5’ –AAGGAGGGAAGGCTGGAAGA-3’

R: 5’ –CCTGACTGTGTTGTGAGTGAA-3’

Rag1

F: 5’ –TGCAGACATTCTAGCACTCTGG-3’

R: 5’ –ACATCTGCCTTCACGTCGAT-3’

-Actin

F: 5’ –CCAGGTCATCACTATTGGCAAGGA-3’

R: 5’ –GAGCAGTAATCTCCTTCTGCATCC-3’

PCR reagents:

dNTPs	Roth
$MgCl_2$	Amersham
10x Buffer	Amersham
Taq Polymerase	Amersham

RT-PCR reagents:

Primer $p(DT)_{15}$	Roche
Superscript™ II RNase H Reverse Transcriptase	Invitrogen
5X RT-PCR Buffer	Invitrogen
0.1 M DTT	Invitrogen

3.1.5 Hybridomas:

All hybridomas (rat-anti-mouse) were cultured in c-RPMI 1640 medium supplemented with 5% FCS

anti-CD4	GK1.5
anti-CD8	YTS 169.4 & TiB 105
anti-CD19	1D3
anti-NK1.1	4D11
anti-MHC II	2G9

3.1.6 Mediums and buffers

for cell culture, proliferation assay and FACS staining:

BSS (Balanced Salt Solution):

1.07 g BES (N,Nbis[2-hydroxy-ethyl]-2-aminoethanesulfonic acid)

1.6 g NaCl

0.027 g Na_2HPO_4

in 100 ml ddH_20 pH 6.96

+ 0.1% BSA (Bovine Serum Albumin)

PBS (Phosphate buffered saline):

8 g	NaCl
0.2 g	KCl
1.44 g	Na_2HPO_4
0.24 g	KH_2PO_4

Dissolve in 800 ml dH_2O, adjust pH to 7.4 with HCl

Volume adjust to 1 L, autoclave and store at RT

FACS-Buffer:

PBS + 0.1% BSA + 0.01% Azide

$RPMI^+$ 1640 Medium:	Invitrogen, Karlsruhe
+L-Glutamin (0.07%)	GIBCO
+Na-Pyruvat (1 mM)	GIBCO
+□-Mercaptoethanol (0.05 mM)	GIBCO
+MEM (non essential amino acids) (1%)	GIBCO
+ Penicillin (100 U/ml),	Grunthal
+ Streptomycin (100 U/ml)	FatolGmbH
+ FCS (Foetal calf serum) (1-10%)	GIBCO

(heat inactivated at 56°C for 45 min)

Gey's Solution:

Solution A:	Solution B:	Solution C:
35 g NH_4Cl	0.42 g $MgCl_2$,6 H_2O	2.25 g $NaHCO_3$
1.85 g KCl	0.14 g $MgSO_4$,7 H_2O	add 100 ml H_2O
1.5 g Na_2HPO_4 ,2 H_2O	0.34 g $CaCl_2$	
0.12 g KH_2PO_4	add 100 ml H_2O	
5 g Glucose		
adjust to 1 L with dH_2O		

Final solution (100ml):

A+ B+ C+ ddH_2O = 20 ml+ 5 ml+ 5 ml+ 70 ml

for genomic DNA isolation and agarose gel electrophoresis:

Tail Lysis Buffer:

50 mM Tris pH 8.0

100 mM EDTA

100 mM NaCl

1% SDS

TBE Buffer (1x):

10.8 g Tris

5.5 g Boric acid

0.37 g EDTA

add 1 L H_2O

DNA Loading Buffer:

0.25% Bromophenol blue

0.25% Xylene Cyanol FF

30% Glycerol in water

store at 4°C

1% Agarose Gel: (150 ml)

1.5 g agarose

150 ml 1x TBE buffer

boil

add 4 µl Ethidium bromide (10 mg/ml)

for cell cycle analysis:

Citrate buffer:

0.1% Sodium Citrate

0.1% Triton X-100

for apoptosis assay

AnnexinV Buffer: (10x)

0,1 M HEPES pH 7.4

1,4 M NaCl

25 mM $CaCl_2$

for IL-2 ELISA

Coating buffer (0.1 M Sodium Carbonate, pH 9.5)

8.40 g $NaHCO_3$

3.56 g Na_2CO_3

Volume adjusted to 1L with dH_2O

Assay diluent

PBS with 10% Foetal Bovine Serum, pH 7.0

Wash buffer

PBS with 0.05% Tween-20

Substrate solution

Tetramethylbenzidine (TMB)

Hydrogen peroxide (H_2O_2)

Stop solution

2 N H_2SO_4

for intracellular staining

Saponin buffer:

PBA (1x PBS, 5% BSA, 10 mM HEPES, pH 7.4 and

0.02% NaN_3)

0.5% Saponin

for cell counting

Trypan Blue Solution:

0.4% (w/v) Trypan Blue in PBS

Dilute 1:10 in PBS for live cell counting

for cell extract preparation

Whole Cell Extract (WCE) Buffer:

20 mM HEPES (pH 7.4)

2 mM EGTA (pH 7.9)

50 mM β-Glycerophosphate

50 mM Sodium fluoride

1% Triton X-100

10% Glycerol

0.04% Sodium azide

adjust volume with ddH_2O to 50.0 ml

* Protease inhibitors to be added at appropriate concentration immediately before preparation of extract

1 mM DTT (Dithiothreitol)

1 mM Orthovanadate

2 mM Leupeptin

0.4 mM PMSF (Phenyl Methyl Sulfonyl Fluoride)

Nuclear and Cytoplasmic Extract (NE & CE) Buffer:

Buffer A (CE buffer):	Buffer C (NE buffer):
10 mM KCl	420 mM NaCl
10 mM HEPES pH 7.9	20 mM HEPES pH 7.9
0.1 mM EGTA pH 7.9	1 mM EGTA pH 7.9
0.1 mM EDTA pH 7.9	1 mM EDTA pH 7.9

*Protease inhibitors to be added immediately before preparation of extract

20 µl 50x Protease inhibitor/ml buffer

1 mM DTT

1 mM Orthovanadate

SDS-PAGE

Composition of SDS-Polyacrylamide gels

Resolving Gel:

	Component volumes (ml) per 12 ml gel mix	
	8%	12%
H_2O	5.52 ml	3.96 ml
30% acrylamide mix	3.24 ml	4.8 ml
1.5 M Tris (pH 8.8)	3.0 ml	3.0 ml
10% SDS	0.12 ml	0.12 ml
10% ammonium persulfate	0.12 ml	0.12 ml
TEMED	6 µl	4.8 µl

Stacking Gel:

	Component volumes (ml) per 3 ml gel mix
H_2O	2.1 ml
30% acrylamide mix	0.5 ml
1.0 M Tris (pH 6.8)	0.38 ml
10% SDS	0.03 ml
10% ammonium persulfate	0.03 ml
TEMED	3 µl

Protein Loading Buffer (1X SDS Gel Loading Buffer):

50 mM Tris.Cl (pH 6.8)
100 mM Dithiothreitol
2% SDS
0.1% Bromophenol Blue
10% Glycerol
store at -20°C

Gel Running Buffer: (10X)

144.13 g Glycine
30.3 g Tris
100 ml 10% SDS
volume adjusted to 1L with dH_2O
store at RT

Protein Transfer Buffer: (10X)

145 g Glycine
29 g Tris
volume adjusted to 1L with dH_2O
store at RT
dilute 10X transfer buffer: methanol: dH_2O in 1:2:7 ratio before use

Ponceau S Solution:

2 g Ponceau S
30 g Trichloroacetic acid
30 g Sulfosalicylic acid
dH_2O to 100 ml
store at RT

Dilute 1:10 in dH_2O for working solution

Blocking Solution:

5% (w/v) nonfat dried milk in PBS/0.1% Tween

RNA analysis:

DEPC H_2O

0.1% DEPC in dH_2O

stirr until DEPC is completely mixed in H_2O

Autoclave and store at RT

MOPS Buffer: (10X)

0.4 M MOPS

0.1 M NaOAc

0.01 M EDTA

pH 7, autoclave and store in dark at RT

Formaldehyde Buffer:

15% Formaldehyde

50% Formamide

10% 10X MOPS buffer

RNA Loading Buffer:

0.5% Xylene cyanol

0.5% Bromophenol blue

10 mM EDTA

50% Glycerol

30% Formamide

Fomaldehyde-Agarose gel electrophoresis:

for 150 ml of 1.2% gel

1.8 g Agarose

15 ml 10X MOPS buffer

98 ml dH_2O

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boil

add 27 ml formaldehyde in the fume hood

3.1.7 Radioactive material

^{3}[H]-Thymidin ICN Biomedicals

3.1.8 Instruments and accessories

Agarose gel reader	Mitsubishi
Microscope	Leica
Multichannel pipette	Micronic
Heat block	Hartenstein
pH meter	Hanna Instruments
Harvester “MACH 3”	Tomtec
-Counter “Micro Beta Trilux”	Wallac
Thermo-Cycler “Biometra”	Biotron
Cytospin Centrifuge	Shandon
Centrifuge “Megafuge 1,0R” and “Biofuge 13”	Heraeus Instruments
FACScan	Beckton Dickinson
FACSCalibur	Beckton Dickinson
FACSSorter vantage SE	Beckton Dickinson
ELISA-Reader “-max”	Molecular Devices
Ultraspec 2000, UV/Visible Spectrophotometer	Pharmacia Biotech
CO_2 incubator	Heraeus Instruments
Leica Confocal Microscope	Leica TCS SP II
Dynal Magnet	Dynal A.S.

3.2 Methods

3.2.1 Generation of transgenic mice

Myr PKB tg mice were generated in Dr. U. Bommhardt's lab in the Institute of Virology and Immunology, University of Würzburg. The calcineurin tg (ΔCam) mice were generated and kindly provided by Prof. E. Serfling, Institute of Pathology, University of Würzburg, Germany.

Construction of human PKB cDNA harboring the hemagglutinin (HA) epitope tag and the Lck myristoylation/palmitoylation signal (MGCWCSSNPEDD) for membrane targeting has been described previously (137). Myr-HA-*PKB* cDNA (myr PKB) was inserted into the *Eco*RI site of the human CD2 minigene cassette (229). The linearized hCD2-myr-HA-PKB fragment was microinjected into fertilized eggs from (CBA/J x C57BL/10) F1 animals. Transgenic founder mice were identified by DNA slot blots using a [^{32}P-] DNA probe specific for the human CD2 cassette. Stable PKB transgenic lines were established by crossing transgenic mice to C57BL/6 mice. Transgenic mice were identified by PCR of genomic tail DNA. Mice used throughout the study were offspring from two founder lines (PKB2 and PKB5), which were crossed to C57BL/6 mice for at least three generations.

The truncated murine calcineurin mutant was generously provided by Dr. R.L.Kincaid and described previously (230). The 1.2 kb fragment was cloned into the *Bam*H1 sites of the expression cassette harbouring the murine proximal Lck promoter, a 3' human growth hormone fragment and the human CD2 LCR. *Spe* I cut fragments were injected into oocytes from NMRI mice. Founder mice were crossed with C57/BL6 mice and offsprings were backcrossed to C57/BL6 mice for further analysis. Genomic integration of the ΔCam transgene was determined by PCR of genomic tail DNA with primers specific for the Lck promoter and the transgene F: 5'-CCG AGC CCA AGG CGA TTG ATC C-3' and R: 5'-CCC GGT TTC TGA TGA CTT CCT TCC-3'.

Mice heterozygous for ΔCam were crossed with PKB tg mice or OT1 TCR tg (228) mice (both on C57/BL6 background) to generate ΔCam/PKB and ΔCam/OT1 double transgenic mice. In all experiments, F1 littermate mice negative for both ΔCam and PKB, positive for ΔCam only and positive for PKB only were used as wild type control and control for ΔCam and PKB tg, respectively. Mice used were 1 day or 6 to 8 weeks old unless mentioned otherwise. Experiments with ΔCam mice crossed to the C57/BL6 background were also performed and showed identical results.

3.2.2 Genotyping of mice

Isolation of genomic tail DNA

Genomic DNA from PKB tg, ΔCam tg and ΔCam/PKB double tg mice was isolated from mouse tail fragments which were incubated o.n. in 500 µl tail lysis buffer (TLB) supplemented with 10 µl of Proteinase K (20 mg/ml) per sample at 56°C in a shaking waterbath. Samples were boiled for 5 min at 80°C and centrifuged at 10,000 rpm for 10 min. DNA was precipitated by addition of 500 µl isopropanol per sample. Isolated DNA was solubilized in dH_2O (56°C for 1hr) and was used in PCR for genotyping.

PCR:

PKB tg PCR

Composition of reaction mix for one sample:

Fwd primer (10 µmol)	1.0 µl
Rev primer (10 µmol)	1.0 µl
dNTP (10 mM)	0.4 µl
$MgCl_2$ (25 mM)	2.0 µl
Buffer (10X)	2.0 µl
dH_2O	12.5 µl
Taq polymerase (5U/µl)	0.1 µl
Genomic DNA	1.0 µl
	20.0 µl

PCR condition:

94°C	5 min	
94°C	1 min	31 cycles
55.5°C	1 min	
72°C	1 min	
72°C	5 min	
4°C	pause	

The size of the PCR product for detection of the myr PKB tg is 360 bp.

ΔCam tg PCR

Composition of reaction mix for one sample:

Fwd primer (10 µmol)	1.0 µl

Rev primer (10 µmol)	1.0 µl
dNTP (10 mM)	0.5 µl
$MgCl_2$ (25 mM)	0.8 µl
Buffer (10X)	2.5 µl
dH_2O	12.7 µl
Taq polymerase (5U/µl)	0.5 µl
Genomic DNA	1.0 µl
	20.0 µl

PCR condition:

95°C	1 min	
95°C	30 sec	40 cycles
60°C	40 sec	
72°C	1 min	
72°C	7 min	
4°C	pause	

The expected size of the PCR product for the ΔCam tg is 1.2 kb.

PCR products for the respective transgenes were resolved in 1% agarose gels in 1X TBE electrophoresis buffer.

3.2.3 Preparation of single cell suspension of thymocytes and LN cells

Mice were killed by asphyxiation with CO_2 and required organs were collected sterile in BSS/BSA on ice. Thymus and lymph nodes (LN) were mashed and passed through a nylon filter to obtain single cell suspensions. Cell suspensions were centrifuged at 1300 rpm for 5 min at 4°C and resuspended in BSS/BSA. Live cells were counted by trypan blue exclusion with a haemocytometer.

3.2.4 Isolation of DN thymocytes, CD4+ T cells, CD8+ T cells and FACS sorting

Negative selection of DN thymocytes, CD4+ and CD8+ T cells

For isolation of CD4-CD8- DN cells, total thymocytes were incubated with rat-anti-mouse CD4, rat-anti-mouse CD8, rat-anti-mouse NK1.1, rat-anti-mouse CD19 and rat-anti-mouse MHC II hybridoma supernatants for 25 min on ice with intermittent shaking. Cells were washed twice with PBS/0.1% BSA and were incubated with goat-anti-rat IgG coupled

magnetic beads (Biomag/Quiagen) (2 µl of 10X concentrated beads per 1 X 10^6 cells) for 25 min at 4°C with intermittent shaking. The tubes were filled with 5ml BSS/0.1% BSA and were placed on a magnet for 3 min for separation of labelled cells. Supernatant was collected, centrifuged at 1300 rpm for 5 min at 4°C and isolated cells were resuspended in cRPMI 1640 medium supplemented with 10% FCS.

CD4+ and CD8+ T cells from LNs were isolated as described above for DN thymocyte isolation and cells were suspended in 5% FCS cRPMI 1640 medium. Purity of isolated cells was controlled by flow cytometry for each experiment and routinely was 90 to 95%.

FACS sorting

Total thymocytes were stained for CD4, CD8, CD25 and CD44 surface molecules. Gating on DN thymocytes, CD4-CD8-CD25+CD44- DN3 and CD4-CD8-CD25-CD44- DN4 cells were electronically sorted on a FACSVantage (Beckton Dickinson).

3.2.5 Flow cytometry

For three-colour analysis, 8 x 10^5 thymocytes were immunostained with CD4 and CD8 and either CD5, CD24, CD25, CD44, CD2 or TCRβ, CD69, LFA1, CD18 and CD49d mAbs labelled with fluoresceine isothiocyanate (FITC), R-phycoerythrin (PE) or biotin in PBS containing 0.1% BSA and 0.1% sodium azide. Biotin-coupled Abs were revealed with streptavidin-cychrome. For further analysis of DN3 cells, thymocytes were gated on CD4-CD8- CD25+ cells. For analysis of CD4+ peripheral T cells isolated CD4+ T cells or total LN cells were surface stained for expression of CD4, CD62L (Mel14), CD25 and CD69.

3.2.6 Proliferation assay

2 x 10^5 purified CD4+ or CD8+ T cells were cultured in triplicate in complete RPMI 1640 medium supplemented with 5% FCS in 96 well plates coated with antiCD3ε mAb alone or in combination with antiCD28 mAb. PD98059, PP1, LY294002, SB202190, staurosporin and cyclosporinA (CsA) were added at the beginning of the culture, unless otherwise mentioned, at the concentrations indicated. After 48 hrs cells were pulsed with 1µCi ^{3}[H]-thymidine per well for 12 hrs. Cells were harvested in a Tomtec cell harvester and ^{3}[H]-thymidine incorporation was determined by a Perkin Elmer scintillation counter.

3.2.7 CFSE labeling

For analysis of cell division purified CD4+ or CD8+ T cells (1 x 10^7 cells/ml) were washed twice in PBS and labeled with CFSE at a final concentration of 2 µM in PBS for 5 min at RT. Cells were washed twice in cRPMI medium supplemented with 10% FCS and thereafter cultured in cRPMI 1640 mediun with 5% FCS at a density of 2 x 10^6 cells/ml in 96 well plates. Cells were activated with plate bound anti-CD3ε mAb alone or in combination with anti-CD28 mAb for 2 or 3 days and were analysed by FACS. For short term activation, CFSE labelled cells were stimulated with plate bound anti-CD3ε mAb for 12 or 18 hrs, harvested and recultured in fresh medium without further stimulus, until 24 or 48 hrs.

3.2.8 ELISA

2 x 10^5 purified CD4+ T cells were stimulated in triplicate in 96 well plates with plate bound anti-CD3ε mAb alone or in combination with antiCD28 mAb for 2 or 3 days and culture supernatants were collected for analysis of Th1 and Th2 cytokine production by ELISA and Cytokine Bead Array (CBA).

For interleukin 2 (IL-2) ELISA, 96 well plates were coated with 100 µl anti-mouse IL-2 mAb (capture Ab) in coating buffer o.n. at 4°C. Plates were washed thrice with 200 µl wash buffer per well and were blocked with 200 µl/well assay diluent for 1 hr at RT. 100 µl of undiluted or diluted culture supernatant from PKBtg or wt CD4+ T cells, IL-2 standard and control samples were added to respective wells and incubated for 2 hrs at 37°C. Plates were washed five times with the washing buffer. Plates were then incubated with 100 µl of detection solution (biotinylated anti-mouse IL-2 plus avidin-horse radish-peroxidase reagent) for 1 hr at RT. Plates were washed five times and incubated with 100 µl of substrate solution for 30 min at RT in the dark. Reaction was stopped by adding 50 µl of stop solution to each well and absorbance at 450 nm was measured in an ELISA reader.

IL-4, IL-5, IFN-γ and TNF-α were measured by mouse Th1/Th2 cytokine bead array according to the manufacturer's instructions. Briefly, mouse Th1/Th2 cytokine standards were reconstituted and serially diluted using the assay diluent. 10 µl of each cytokine capture bead suspension per sample were mixed and 50 µl of mixed beads were aliquoted to separate assay tubes. Cytokine standard dilutions and test samples (50 µl/ test) were added to appropriate sample tubes and 50 µl/test PE detection reagent was added to it. Samples were incubated for 2 hrs at RT in the dark. Samples were washed with 1 ml wash buffer and resuspended in 300

µl wash buffer and were analyzed immediately by a FACSCalibur using CellQuest and BD CBA software.

3.2.9 Cell cycle analysis: propidium iodide (PI) staining

For analysis of various cell cycle stages 2 x 10^5 purified DN thymocytes were stained with propidium iodide at a final concentration of 50 µg/ml in citrate buffer for 30 min at RT. Nuclei were analyzed on a FACSCalibur using Cell Quest software and a doublet discrimination program.

3.2.10 Apoptosis analysis: annexin V staining

For apoptosis analysis of CD4-CD8-CD25+ DN3 thymocytes, total thymocytes were first stained for CD4, CD8 and CD25 surface molecules. Cells were then stained with AnnexinV for 15 min at RT in AnnexinV binding buffer. AnnexinV positive apoptotic and AnnexinV negative DN3 cells were determined by gating on CD4-CD8-CD25+ cells using FACSCalibur and Cell Quest software.

3.2.11 Intracellular staining

For intracellular staining, 7 x 10^6 total thymocytes were first stained for cell surface CD4, CD8 and CD25 molecules. Cells were then fixed with 1% paraformaldehyde (10 min at RT) and permeabilized in PBA (1x PBS, 5% BSA, 10 mM HEPES, pH 7.4 and 0.02% NaN_3) containing 0.5% saponin (10 min at RT). Permeabilized cells were incubated with FITC-labelled mAbs against CD3ε, TCRβ and Bcl_2 diluted in PBA containing 0.5% saponin for 30 min at 4°C. Cells were washed twice in 1 ml of PBA/0.5% saponin, once with PBS/0.1%BSA/0.01% azide and analyzed by FACS.

3.2.12 NTOC (neonatal thymic organ culture)

Thymi from wt and ΔCam tg neonates (day 0-1) were collected aseptically and placed on nucleopore membranes and cultured in 2 ml complete RPMI 1640 medium containing 10% FCS in the absence or presence of 100 ng cyclosporin A (CsA) for 4 days. Subsequently single cell suspensions were made, immunostained for CD4 and CD8 surface molecules, and thymocyte subpopulations were determined by flow cytometry. Intracellular staining for TCRβ expression was analyzed gating on the CD4-CD8- population.

3.2.13 Preparation of whole cell protein extract (WCE)

$1x10^7$ unstimulated DN thymocytes or 3 x 10^6 stimulated or unstimulated CD4+ T cells from the indicated mice were harvested, washed once in PBS and were incubated in 50 µl WCE^+ (WCE + protease inhibitors) buffer for 30 min at 4°C. Samples were centrifuged at 14,000 rpm for 15 min at 4°C and cell lysate was collected. For IκB analysis, cells were pretreated with cycloheximide (50 µg/ml) for 15 min before stimulation of cells with soluble antiCD3ε mAb (1 µg/ml) in combination with antiCD28 mAb (5 µg/ml) for the indicated time periods. For longterm stimulation, cells were activated with plate-bound antiCD3ε mAb (5 µg/ml). Protein concentration was estimated with Bradford's reagent and lysates were stored at -20°C for further analysis.

3.2.14 Preparation of nuclear and cytoplasmic protein extract (NE & CE)

2 x 10^7 purified CD4+ T cells from wt and PKB2 tg mice were stimulated with anti-CD3ε Ab alone or in combination with anti-CD28 Ab in presence or absence of CsA for the time points indicated in 5% FCS/cRPMI 1640 medium. Kinase inhibitors PD98059 (100 µM), LY294002 (20 µM), SB202190 (40 µM), and staurosporine (10 nM) were added 1 hr before stimulation of cells. Cells were harvested, washed once in PBS and were incubated for 3 min on ice with 200 µl of buffer A^+ (buffer A + protease inhibitors), supplemented with 0.5% NP-40. Samples were centrifuged at 14,000 rpm for 3 min at 4°C and supernatants were collected as cytoplasmic extract (CE). Samples were washed thrice with bufferA+ and suspended in 75 µl buffer C+ (buffer C + protease inhibitors), vortexed and incubated for 2 hrs at 4°C with shaking. Samples were centrifuged at 14,000 rpm for 15 min and supernatants were collected as nuclear extract (NE). CE and NE protein concentrations were determined with Bradford's reagent and extracts were stored at -20°C for further analysis.

3.2.15 Isolation of RNA, cDNA synthesis and reverse transcriptase polymerase chain reaction (RT-PCR)

Total cellular RNA from purified DN thymocytes was prepared with Trizol reagent. 1 x 10^7 unstimulated DN cells were homogenized with 1 ml Trizol reagent, incubated for 5 min at RT and chloroform at 0.2 ml/ml Trizol reagent was added to the samples. Samples were further incubated for 3 min at RT with vigorous shaking and were centrifuged at 14,000 rpm for 15 min at 4°C. The aqueous phase was collected, and 500 µl isopropanol/ml trizol reagent was added and further incubated for 10 min at RT. Samples were centrifuged at 14,000 rpm for 10

min at 4°C and the RNA pellets were washed once with 75% ethanol. Pellets were briefly air dried and dissolved in DEPC water. RNA concentration was determined with a spectrophotometer.

cDNA was synthesized with SuperScript reverse transcriptase from 1 μg of RNA as described by the manufacturer's protocol. Each sample was normalized by PCR for β-actin expression. Serially diluted cDNA was analyzed for expression of calcineurin (detecting bp 301-909 of the N-terminus of calcineurin), RAG1 and β-actin using the following primers, calcineurin: F: 5'-AAG GAG GGA AGG CTG GAA GA-3' and R: 5'-GGC ATC CAT ACA GGC ATC AT-3'; RAG1: F: 5'-TGC AGA CAT TCT AGC ACT CTG-3' and R: 5'-ACA TCT GCC TTC ACG TCG AT-3', and β-actin: F: 5'-CCA GGT CAT CAC TAT TGG CAA GGA-3' and R: 5'-GAG CAG TAA TCT CCT TCT GCA TCC-3'.

RT-PCR:

Composition of the reaction mix for individual RT-PCRs was identical to that of PKB PCR

Calcineurin

94°C	5 min	
94°C	45 sec	31 cycles
54°C	45 sec	
72°C	45 sec	
72°C	10 min	
4°C	pause	

RAG1

94°C	5 min	
94°C	45 sec	30 cycles
55°C	45 sec	
72°C	45 sec	
72°C	10 min	
4°C	pause	

β-actin

94°C	2 min	
94°C	1 min	24 cycles
58°C	1 min	
72°C	1 min	

72°C	5 min
4°C	pause

3.2.16 Western blot analysis

For immunoblot analysis, 30 µg protein from whole cell lysates and 10 µg protein from NE & CE were denatured in 1X protein loading buffer and were fractionated on 8% SDS-PAGE. Proteins were transferred to nitrocellulose membranes o.n. at 4°C. WCE from DN thymocytes was analyzed for expression of pPKB (Ser 473), PKB, Rag1, and β-actin and WCE from CD4+ T cells was analysed for expression of cyclins B1 and D3, and IκBα. NE & CE were analysed for expression for PKB, phospho-PKB (Ser473), phospho-GSK3α/β (Ser21/9), phospho-FKHR (Ser256), phospho-JNK, and phospho-p38, NFATc1, NFATp, NF-κBp65, NF-κBp50 and RelB. Primary Abs were detected by goat-anti-rabbit, goat-anti-mouse or rabbit-anti-goat Abs coupled with horse radish-peroxidase and enhanced chemiluminescence (ECL). Blots were reprobed with anti-actin Ab to control protein loading in the case of CE. Controls for NE and CE, which are designated as n.s. in the figures are nonspecific proteins used as protein loading controls.

3.2.17 Immunoprecipitation (IP)

For immunoprecipitation experiments, 1 x 10^7 CD4+ T cells from myr PKB tg or wt mice were lysed in buffer A, and cytoplasmic protein extracts were incubated with anti-PKB, anti-phospho-PKB (pPKB) (Ser473), anti-Lamin A, or anti-NFATc1 Ab overnight at 4°C with shaking. After addition of 25 µl of 50% protein G-Sepharose for 1 hr at 4°C, extracts were centrifuged at 10,000 rpm for 1 min, and immunoprecipitates were washed four times with buffer A^+. After the final wash, pellets were boiled in loading buffer, and supernatants were resolved on 8% SDS-PAGE. Coprecipitation was analyzed for association of NFATc1 with PKB or pPKB.

3.2.18 Confocal microscopy

Single cell suspensions of thymocytes (1 x 10^7 cells) were fixed in 500 µl 0.5% formaldehyde for 10 min at RT. Fixed cells were cyto-spinned onto poly-lysine coated glass slides. Slides were incubated for 15 sec in chilled acetone (-20°C) and cells were permeabilized by incubating in chilled methanol (-20°C) for 3 min. Subsequently, cells were washed thrice in PBS and were incubated with 50 µl goat-anti-mouse Fc block for 15 min in a humid chamber.

Afterwards 40 µl of primary Ab solution for cell surface CD4 (Alexa-647) and CD8 (Alexa-488) and intracellular NFATc1 or NFATc3 in appropriate dilutions in PBS containing 1% BSA was added to the cells for 45 min. Cells were washed thrice in PBS and then incubated for 45 min with donkey-anti-rabbit Alexa-555 conjugated Abs in the dark. Cells were washed thrice in PBS and twice in dH_2O and then counter stained with DAPI for 5 min at RT for nuclear staining. Stained thymocyte preparations were analysed for NFAT expression in CD4-CD8- thymocytes using a Leica TCS SPII confocal microscope.

Chapter 4. Results

4. Results

Part I. Effect of PKB on T cell function

4.1 Generation of transgenic mice expressing myr PKB in lymphocytes

To investigate the role of PKB in T cell development, we established transgenic mouse lines expressing a human myr PKB cDNA (137) under the control of the human CD2 promoter and locus control region (Fig. 8A) (229). Of six transgenic founder lines, three lines (PKB 2, 5 and PKB 6) were crossed to the C57BL/6 background. Analysis of all three lines showed comparable results, with PKB 5 and PKB 2 being used throughout this study. Myr PKB expression in thymocytes and peripheral CD4+ and CD8+ T cells was determined by Western blots using anti-PKB and anti-phospho-PKB (Ser473) Abs (Fig. 8B & C). The kinase activity of myr PKB was verified for splenic CD4+ T cells in kinase assays as shown previously (231). All mice used in this study were heterozygous for myr PKB ($PKB^{+/-}$).

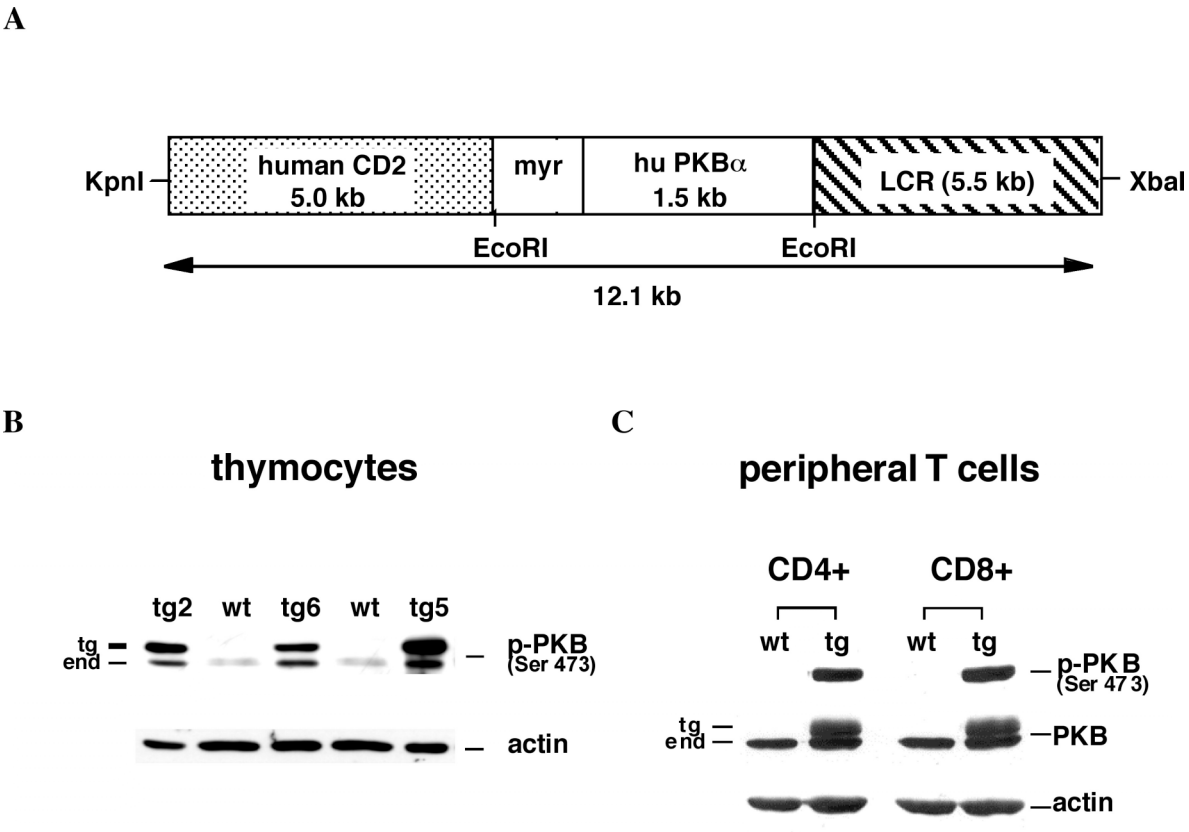


Figure 8. Generation of myr PKB transgenic mice. (*A*) Schematic representation of the myr PKB transgenic construct. (*B* and *C*) Expression of myr PKB in thymocytes and peripheral T cells. *B*, Thymocytes from wildtype (wt) and myr PKB transgenic lines (tg) were analyzed by Western blot using anti-PKB and anti-phospho-PKB (Ser473) Abs. Results are shown for three different transgenic lines, PKB 2, 6, and 5. Upper bands correspond to transgenic myr PKB, lower bands to endogenous (end) PKB. Anti-actin Ab was used to control equal protein loading. *C*, Western blot analysis for phospho-PKB (Ser473) expression in peripheral CD4+ and CD8+ T cells from wild-type and myr PKB transgenic mice (232).

4.2 Active PKB lowers the activation threshold of CD4+ and CD8+ T cells and confers resistance to CsA in proliferation

To examine the effects of constitutively active PKB (myr PKB) on activation and proliferation of peripheral T cells, CD4+ and CD8+ T cells from wt and myr PKB tg mice were activated with either high (10 μg/ml) or limiting (1 μg/ml) concentrations of anti-TCR/CD3 mAb alone or in the presence of CD28 costimulation. As shown in Fig. 9, strong TCR/CD3 stimuli led to comparable proliferation, whereas weak TCR/CD3 signals induced significant proliferation only in myr PKB CD4+ (Fig. 9A) and CD8+ (Fig. 9B) T cells. CD28 costimulation had only small enhancing effect in tg T cells, whereas in wt T cells, proliferation was increased 10- to 20-fold. In addition, in wt cells the response to TCR engagement alone or to CD28 costimulation in combination with weak TCR engagement was totally blocked in the presence of CsA, whereas myr PKB T cells showed significant proliferation under these conditions. Thus, myr PKB signaling lowers the threshold for activation by providing costimulatory signals similar to those induced by CD28 and confers partial resistance to CsA treatment with regard to proliferation.

4.3 Active PKB replaces CD28 costimulatory signals in cell cycle progression

Next we analyzed the effect of myr PKB on cell cycle by labeling T cells with CFSE, which allows tracing the number of cell divisions undergone at any particular time point. As shown in Fig. 10A, 2 days after anti-CD3 activation, 30% of wt and myr PKB CD4+ T cells had divided once; however, 3-fold more (26%) myr PKB CD4+ cells had progressed through a second cell division compared with wt cells (9%). After activation with anti-CD3 plus anti-CD28 mAbs, cell cycle in wt cells resembled that of myr PKB CD4+ T cells stimulated with anti-CD3 mAb only. In contrast, costimulation had only small enhancing effects on cell cycle progression in myr PKB CD4+ T cells. Comparable results were observed for day 3 cultures, in which the majority of myr PKB CD4+ T cells activated with either anti-CD3 mAb alone or in combination with anti-CD28 mAb had undergone four to six cell cycles, corresponding to the same pattern from CD28-costimulated wt cells. Wild type cells stimulated with anti-CD3 mAb only showed a less synchronous cell division profile, with 20% of cells each having divided one to three times and with only 14% of cells having completed five or six cell divisions compared with 54% of myr PKB CD4+ T cells. As evident from Fig. 10B, myr PKB shows the same enhancing effects on cell cycle progression in CD8+ T cells; the observed

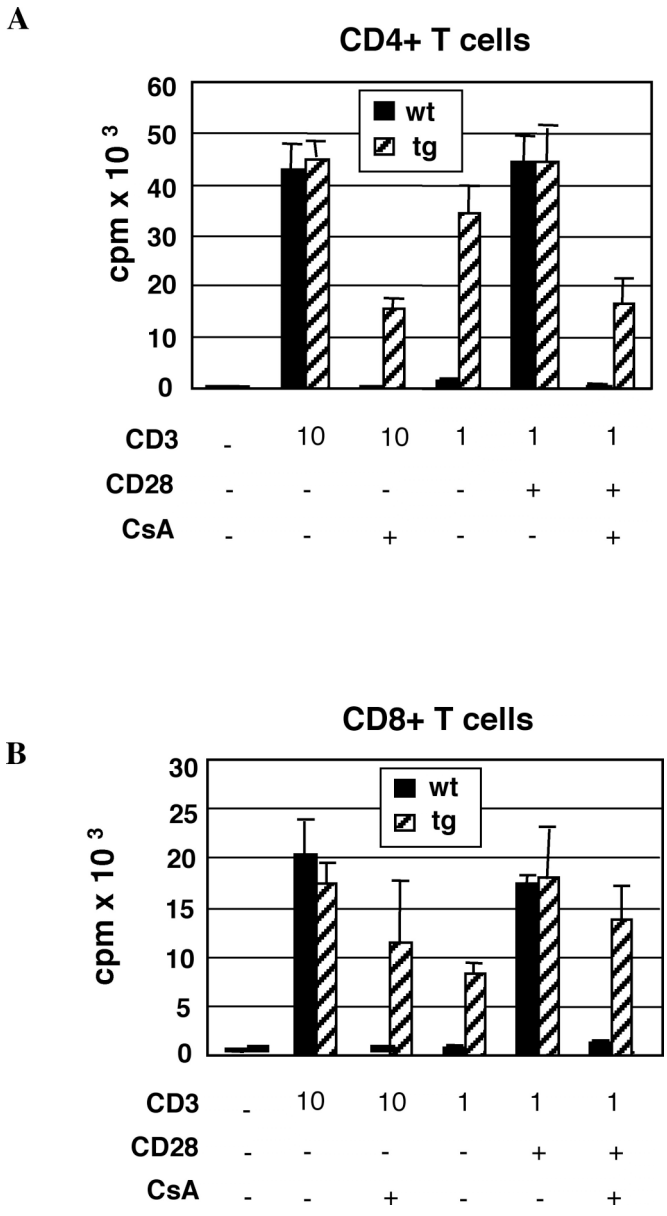


Figure 9. Active PKB lowers the threshold for T cell activation and confers significant CsA resistance in proliferation. Purified CD4+ (*A*) and CD8+ (*B*) T cells from wt and tg mice were cultured in medium only or stimulated with plate-bound anti-CD3 mAb (CD3) at a concentration of 10 or 1 μg/ml or with anti-CD3 (1 μg/ml) plus anti-CD28 (CD28; 5 μg/ml) mAbs in the presence or the absence of CsA (100 ng/ml). [^{3}H]thymidine incorporation was measured 48 hrs after initiation of cultures. Data show averages from triplicate cultures of two wt and tg mice each and are representative for three experiments (233).

advantage in the progression of the cell cycle in the case of CD8+ T cells is even more prominent than that in CD4+ T cells. These data attribute an augmentative role to PKB in cell cycle progression of CD4+ and CD8+ T cells by providing CD28-like costimulatory signals.

To further dissect the role of PKB in cell cycle progression, CD4+ T cells from tg and wt mice were activated with anti-CD3 mAb for 12 or 18 hrs and were re-cultured without further stimulus until 24 or 48 hrs. As shown in Fig.10C, a 12hrs stimulation pulse was sufficient for 16% of myr PKB CD4+ T cells to enter into the cell cycle and divide once

within 24 hrs; on day 2, 54% of tg cells had undergone one to three cell divisions. In stark contrast, stimulation of wt CD4+ T cells for 12 hrs induced only 5% of cells to divide once within 24 hrs, and 3-fold fewer cells had divided twice or three times on day 2 compared with tg cells. When the activating stimulus was extended to 18 hrs, 19% of wt cells showed one round of cell division on day 2, similar to what was observed for tg cells on day 1. Together, these data clearly show that myr PKB strongly enhances cell cycle entry and progression and also sustains cell division.

To define molecular mechanisms underlying the positive effect of myr PKB on the cell cycle, we analyzed the expression of cyclins D3 and B1, proteins regulating cell cycle entry and G2 phase transition, respectively. As shown in Fig. 10D, myr PKB accelerated and enhanced the induction of cyclin D3 and also increased cyclin B1 expression in activated T cells, thus providing a link between active PKB and the observed enhanced cell division. We also studied inactivation of the forkhead transcription factor FKHR, which, like other forkhead family members, is located in the nucleus in resting cells and is critically involved in cell cycle regulation. In various cell lines phosphorylation of these factors by PKB leads to their nuclear exclusion and thereby facilitates cell cycle entry and progression into M phase (234). In nuclear and cytoplasmic extracts of unstimulated wt cells (Fig. 10E, *upper left panels*), phosphorylated forms of FKHR could hardly be detected, whereas after TCR/CD3 engagement, phospho-FKHR was strongly present in both fractions. Interestingly, cytoplasmic phosphorylated FKHR was already prominent in unstimulated myr PKB CD4+ T cells, and only low levels of nuclear FKHR appeared in stimulated cells (Fig. 10E, *upper right panels*), suggesting that most FKHR proteins had been phosphorylated and shuttled out of the nucleus. This correlates with tg PKB also being located in the nucleus (Fig. 10E, *lower panels*). Thus, increased or constitutive inactivation and nuclear export of FKHR is one component that contributes to the enhanced cell cycle progression promoted by myr PKB.

4.4 Enhanced Th1 and Th2 cytokine production in myr PKB CD4+ T cells

T cell growth and apoptosis are highly dependent on various cytokines. To better understand why myr PKB T cells could proliferate after addition of CsA, we first surveyed the production of IL-2, which is an important growth factor in T cell activation and in an autocrine fashion regulates the expression of the high affinity IL-2R (235,236). We found that surface expression of the IL-2Rα -chain (CD25) in CD3-stimulated wt cells was downregulated to levels found in unstimulated cells when CsA was added to the cultures. In contrast, a higher

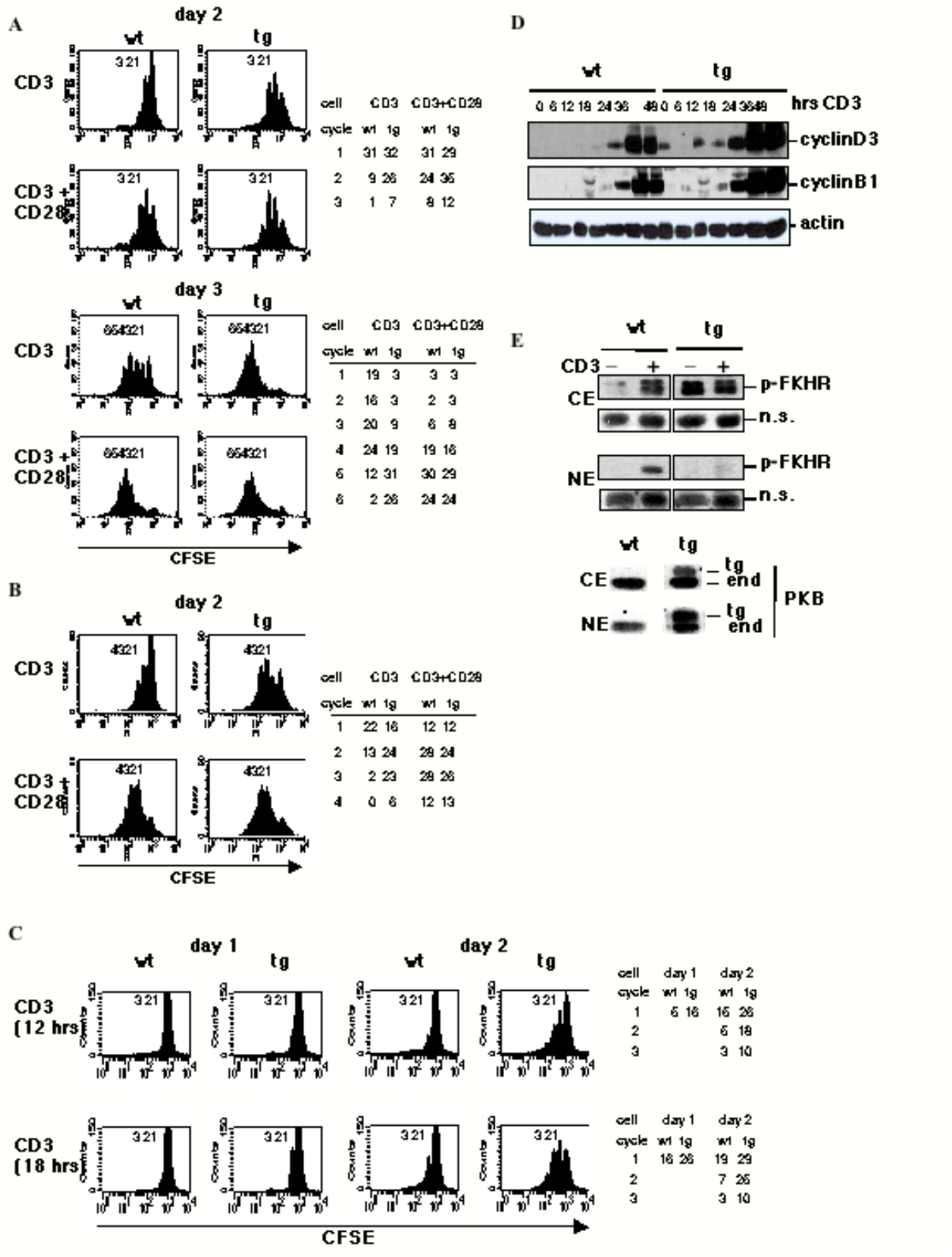


Figure 10. Active PKB enhances cell cycle progression. *A* and *B*, Wt and myr PKB CD4+ (*A*) and CD8+ (*B*) T cells labeled with CFSE were stimulated with anti-CD3 mAb only or with anti-CD3 plus anti-CD28 mAbs for 2 or 3 days as indicated. Cell division was analyzed by FACS gating on viable cells on the basis of forward and side scatter characteristics. Numbers at the *top* of histograms indicate the number of cell divisions undergone. Panels on the *right* give the percentage of wt and tg cells that have undergone the indicated numbers of cell divisions. *C*, Faster cell cycle entry of tg CD4+ T cells. CFSE-labeled CD4+ T cells from wt and tg mice were activated with plate-bound anti-CD3 mAb for 12 or 18 hrs and then cultured without further stimulus until 24 or 48 hrs. Cell division was determined as described above. *D*, Myr PKB positively regulates the expression of cell

cycle regulators. Wt and tg CD4+ T cells were stimulated by TCR/CD3 ligation for the time periods indicated, and the expression of cyclin D3 and B1 proteins in total cell extracts was analyzed by Western blot. *E*, Enhanced phosphorylation of FKHR in tg T cells. Western blot analysis of pFKHR levels in CE and NE from wt and tg CD4+ T cells 8 hrs after stimulation with anti-CD3 mAb (5 μg/ml; *upper panels*). n.s., Nonspecific loading control. The *lower panels* show expression of endogenous (end) and tg PKB in CE and NE from unstimulated wt and myr PKB tg CD4+ T cells (233).

percentage of myr PKB CD4+ T cells still expressed high levels of CD25 in the presence of CsA (data not shown). This suggested that myr PKB signaling allows sufficient production of IL-2 in the presence of CsA, thus maintaining proliferation. Indeed, as shown in Fig. 11, myr PKB CD4+ T cells produced significant amounts of IL-2 when only low TCR signals were provided and when CsA was administered. The amounts of IL-2 produced under these conditions, therefore, would be sufficient for myr PKB T cells to induce high affinity IL-2R and to sustain expansion. In contrast, IL-2 production of wt cells was only obvious when CD28 costimulatory signals were provided in addition to low TCR engagement, and under these conditions IL-2 production was completely ablated by CsA treatment.

Furthermore, under the same stimulatory conditions we determined the production of other cytokines. We found that besides IL-2, other Th1 cytokines, such as IFN-γ and TNF-β, and notably also Th2 cytokines such as IL-4 and IL-5 were increased in cultures from myr PKB CD4+ T cells, even in the presence of CsA (Fig. 11). As RNase protection assays revealed that mRNAs for other Th2 cytokines, including IL-10 and IL-13, were also enhanced after TCR/CD3 stimulation (data not shown), myr PKB has an overall positive regulatory effect on Th1 and Th2 cytokine production.

4.5 Active PKB impairs nuclear translocation of NFAT proteins

T cell activation involves induction of the transcription factors NFATc1 and NFATp, which play important roles in cytokine gene induction, Th cell differentiation, and apoptosis (180, 181). Sustained mobilization of intracellular Ca^{2+} ion triggered by TCR engagement activates calcineurin, which, in turn, dephosphorylates the phosphoserine residues in the NFAT homology region of NFATs and unmasks the NLS, resulting in their entry into the nucleus. In view of the effects of myr PKB on partial resistance to CsA treatment in proliferation and cytokine production, we examined whether this phenotype could be linked to increased or constitutive activation of NFAT proteins. As shown in Fig. 12A, NE and CE of CD4+ T cells from myr PKB and wt mice stimulated with anti-CD3 mAb alone or anti-CD3 plus anti-CD28

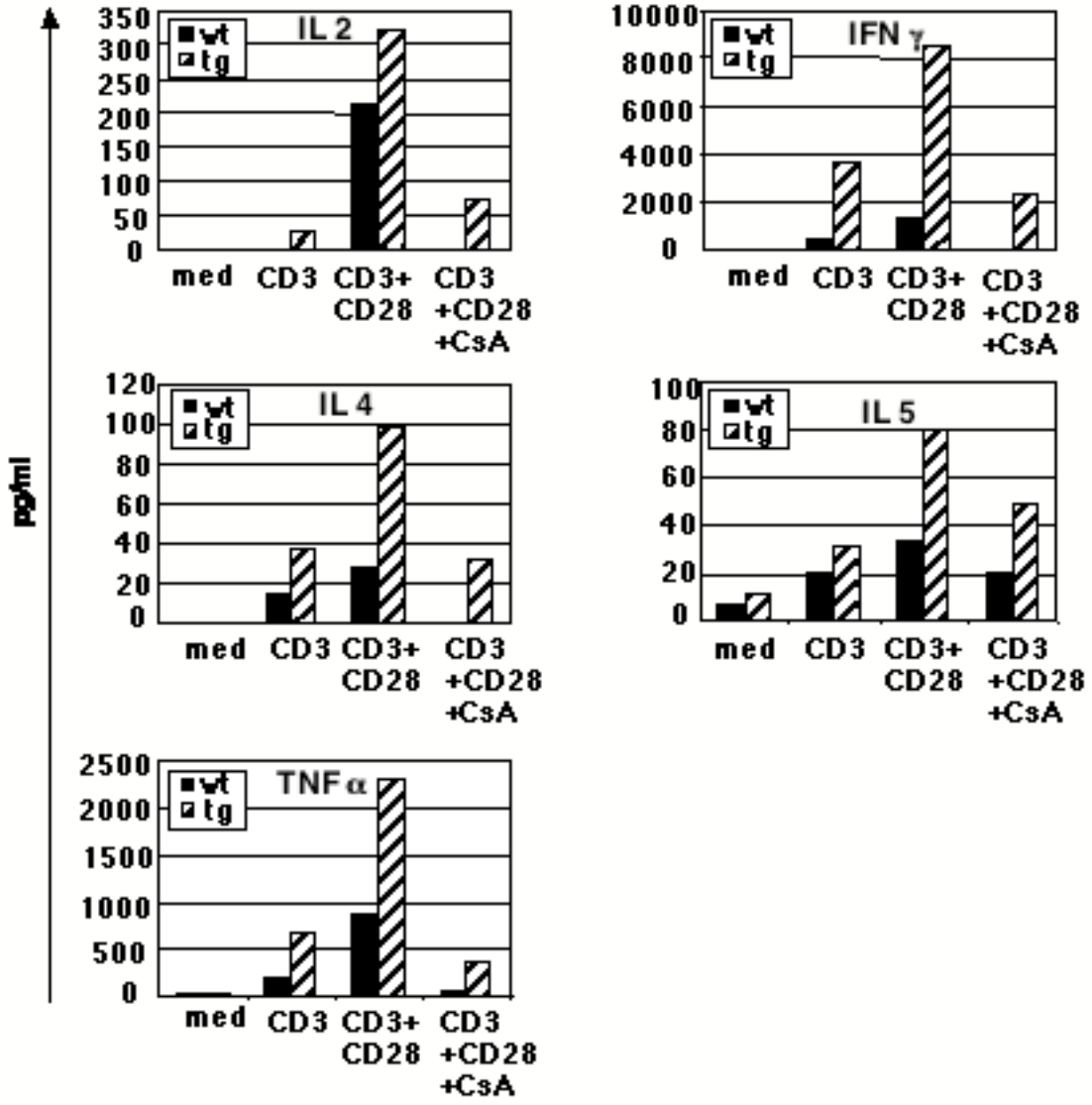


Figure 11. Myr PKB promotes the production of Th1 and Th2 cytokines. CD4+ T cells from wt and tg mice were left unstimulated or were treated with anti-CD3 mAb (1 □g/ml) without or with CD28 costimulation (5 □g/ml) in the presence or the absence of CsA (100 ng/ml). Secretion of the indicated cytokines was determined after 24 hrs of culture (233).

mAbs in the absence or the presence of CsA were analyzed by Western blot for nuclear translocation of NFATc1. Eight hours after activation, most induced NFATc1 protein in wt CD4+ T cells was translocated to the nucleus, and nuclear entry was clearly abolished in the presence of CsA. Strikingly, whereas in cytoplasmic extracts of myr PKB CD4+ T cells, NFATc1 induction after anti-CD3 and anti-CD3 plus anti-CD28 costimulation was obvious, nuclear translocation of NFATc1 was hardly detectable (Fig. 12A, *upper panel*). At earlier time points of activation, e.g., at 4 hrs (data not shown), nuclear translocation of NFATc1 could also not be detected and was drastically diminished even 16 hrs after activation (Fig. 12A lower panel).

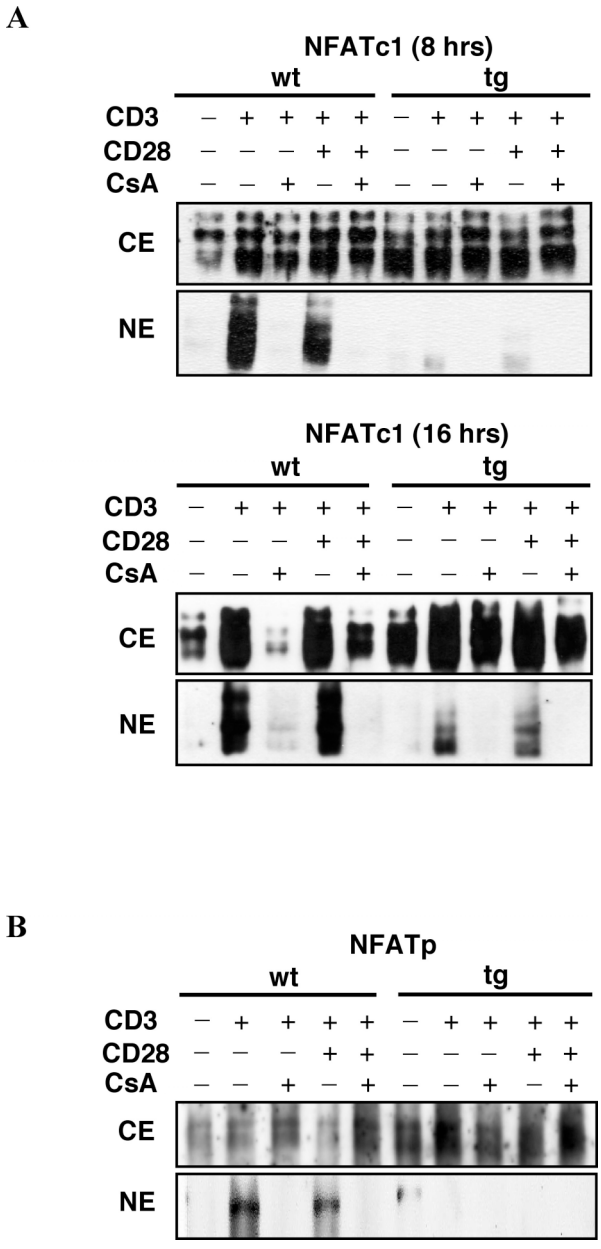


Figure 12. Myr PKB impairs nuclear accumulation of NFAT proteins. (*A*) Wt and tg CD4+ T cells were stimulated with anti-CD3 mAbs alone or in combination with anti-CD28 mAbs with or without CsA (100 ng/ml) for 8 hrs (*upper panel*) or 16 hrs (*lower panel*). NE and CE were analyzed for nuclear translocation of NFATc1 in Western blot. (*B*) Wt and tg CD4+ T cells were stimulated with anti-CD3 mAbs alone or in combination with anti-CD28 mAbs with or without CsA (100 ng/ml) for 4 hrs. CE and NE were probed for NFATp activation and nuclear localization (233).

To test whether this intriguing effect of myr PKB on nuclear shuttling was restricted to NFATc1, we examined NFATp activation (Fig. 12B). Similarly, although induction of NFATp in the cytoplasm was evident, nuclear translocation of NFATp was hardly detectable in myr PKB CD4+ T cells, similar to the blockade seen in CsA-treated wt cells. Thus, myr

PKB promotes proliferation and cytokine production in T cells despite immensely diminished nuclear levels of NFATc1 and NFATp.

4.6 Enhanced nuclear levels of p-p38, pJNK, and pGSK3 in myr PKB T cells

As phosphorylation and dephosphorylation are the basis of NFAT translocation and subsequent gene expression, we examined the activities of JNK, p38, and GSK3, kinases that have been implicated in the regulation of cytoplasmic-nuclear shuttling of NFAT proteins. In particular, overexpression of GSK3 has been shown to significantly reduce nuclear import, whereas inhibition of GSK3 slows nuclear export of NFATc1 (195). As shown in Fig. 13A, 8 hrs after TCR/CD3 activation, phosphorylated GSK3 is clearly detectable in cytoplasmic and

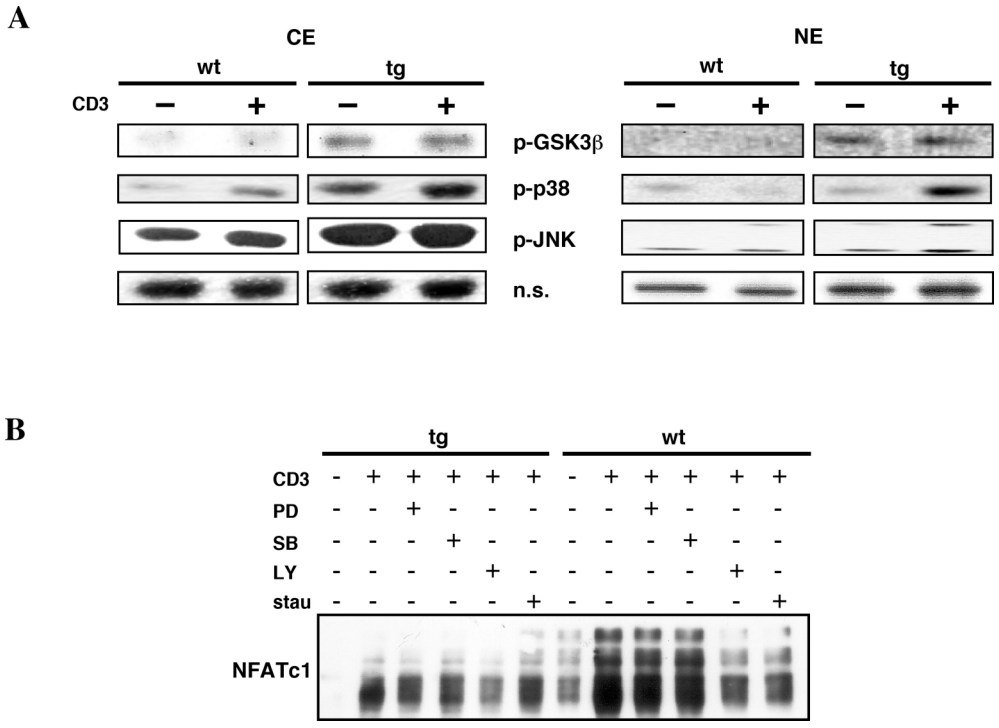


Figure 13. Active PKB enhances phosphorylation of MAPKs, p38 and JNK, and of GSK3. (*A*) Wt and tg CD4+ T cells were cultured in medium only or were activated with anti-CD3 mAb (5 □g/ml) for 8 hrs. The level of phosphorylation of the indicated kinases in CE (*left panel*) and NE (*right panel*) was analyzed by Western blot using phospho-specific Abs. (*B*) Inhibition of MAPKs and PI3K does not lead to higher nuclear localization of NFATc1. Wt and tg CD4+ T cells were stimulated with anti-CD3 mAb for 4 hrs in the absence or the presence of the kinase inhibitors, PD98059 (PD), SB202190 (SB), LY294002 (LY), or staurosporine (stau), and NE was analyzed for NFATc1 nuclear translocation (233).

nuclear extracts of myr PKB T cells, but is detected only very weakly or not at all in case of cytoplasmic and nuclear extracts of wt cells. This correlates with myr PKB being located in the nucleus (Fig. 10E) and published data that active PKB can phosphorylate GSK3, thereby leading to its inactivation (237). However, although inactivation of GSK3 by phosphorylation

through PKB should foster nuclear retention of NFAT, the opposite, namely, grossly diminished nuclear NFATc1 or NFATp, was detected in myr PKB T cells. We therefore analyzed cytoplasmic and nuclear levels of active JNK and p38, which by rephosphorylating NFAT would induce its nuclear extrusion. As shown in Fig. 13A, myr PKB T cells elicit elevated levels of phosphorylated p38 and JNK proteins compared with wt cells upon stimulation. These data suggest that rephosphorylation of NFAT by these kinases could be involved in the altered cytoplasmic-nuclear shuttling of NFAT in myr PKB T cells.

4.7 Inhibition of MAPK/SAPK and phosphatidylinositol 3-kinase (PI-3K) pathways fails to repair the altered nuclear shuttling of NFATc1

In view of the reported involvement of JNK (197), p38 (198), and PI-3K (226) in opposing nuclear translocation of NFAT and our observation regarding higher pJNK and p-p38 levels in myr PKB T cells, we studied whether inhibition of these signaling pathways could repair the alteration. For this purpose we treated cells with the pharmacological reagents PD98059, SB202190, and LY294002, inhibitors of MAPKs and PI3K, respectively, as well as with staurosporine, a broad spectrum inhibitor of kinases. The concentrations of inhibitors used in these experiments clearly blocked induction of CD25 expression at 4 hrs of stimulation, the time point when protein extracts were prepared, and also inhibited proliferation when measured by [^{3}H]-thymidine incorporation at 24 hrs, indicating that they effectively blocked the respective kinases (Fig. 14A & B). As none of these inhibitors led to higher nuclear accumulation of NFATc1 (Fig. 13B), the deficiency in nuclear NFATc1 in myr PKB CD4+ T cells is regulated by mechanisms not involving PI3K or the MAPKs, extracellular signal-regulated kinase (ERK), p38, and JNK.

These observations indicated that the negative regulation of NFATs in myr PKB CD4+ T cells could have something to do with the constitutively active PKB. As PKB is a serine threonine kinase it could be possible that it directly regulates serine phosphorylations on NFAT by physically associating with it. We therefore hypothesized whether PKB itself could act as NFAT kinase and investigated its direct interaction with NFATc1. Immunoprecipitation experiments, as shown in Fig. 15A, clearly detected in vivo association of pPKB with NFATc1 in cytoplasmic extracts from tg CD4+ T cells. To confirm that this interaction in tg T cells is not due to overexpression of PKB but also occurs in wt T cells we analyzed PKB and NFAT interaction by immunoprecipitation studies with anti-pPKB and

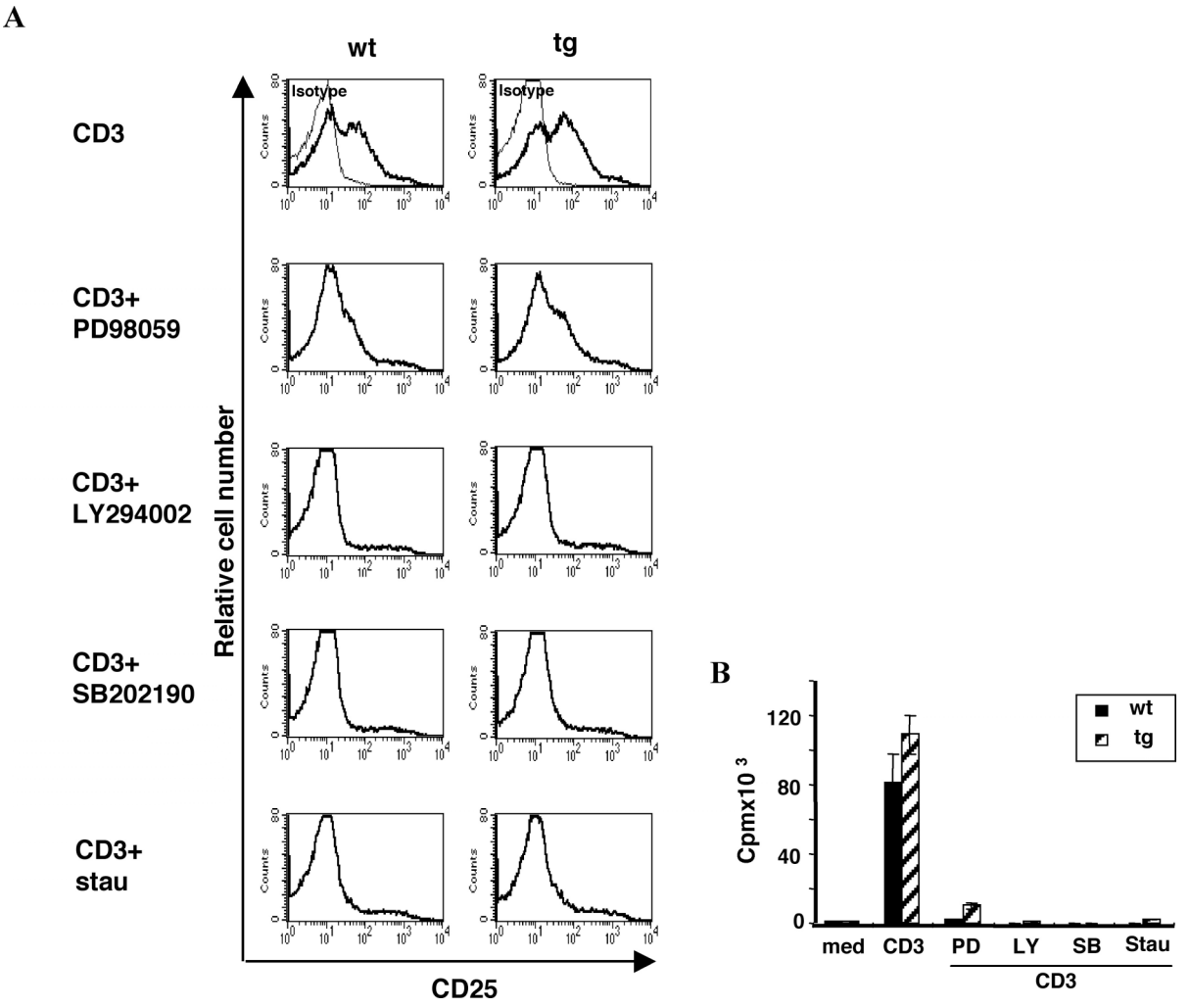


Figure 14. Specificity of the kinase inhibitors in downregulation of CD25 and inhibition of proliferation. (*A*) Inhibition of the early activation marker CD25. Wt and tg CD4+ T cells were stimulated with anti-CD3 mAb for 4 hrs in the absence or the presence of the kinase inhibitors PD98059 (PD), LY294002 (LY), SB202190 (SB), or staurosporine (stau), and expression of CD25 was analyzed by FACS. (*B*) Inhibition of proliferation in the presence of kinase inhibitors. Purified CD4+ T cells from wt and tg mice were cultured in medium only or stimulated with plate-bound anti-CD3 mAb (CD3) at a concentration of 10 □g/ml in the presence or the absence of the indicated kinase inhibitors. [^{3}H]thymidine incorporation was measured 24 hrs after initiation of cultures. Data show averages from triplicate cultures of two wt and tg mice each.

anti-PKB Abs in unstimulated and stimulated wt T cells (Fig. 15B). In unstimulated wt T cells, significant coprecipitation of NFATc1 with PKB could only be detected with anti-PKB, but not with anti-pPKB, Abs. This probably reflects the minute amounts of active PKB present in unstimulated T cells, as in stimulated wt cells immunoprecipitation with anti-pPKB Abs also resulted in pull-down of NFATc1. Together these data show that transgenic PKB as well as endogenous PKB interact with NFATc1.

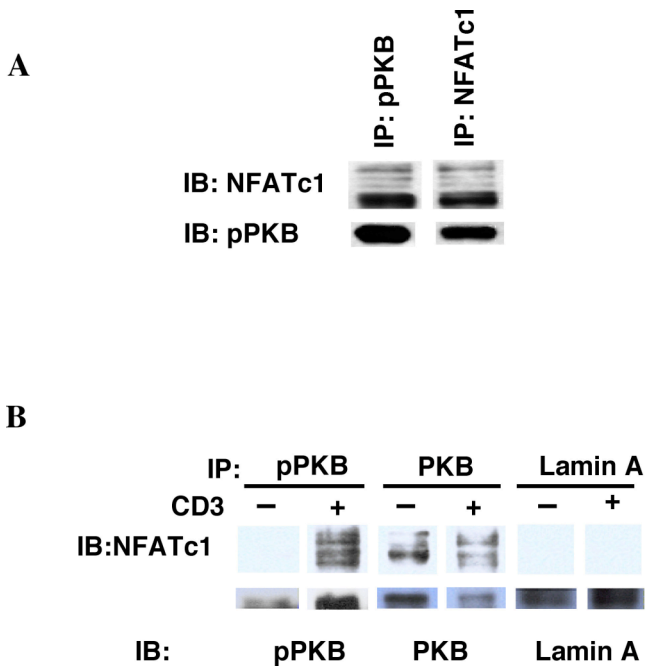


Figure 15. PKB coprecipitates with NFATc1. (*A*) Cytoplasmic protein extracts from unstimulated myr PKB CD4+ T cells were immunoprecipitated with either anti-phospho-PKB or anti-NFATc1 Abs. Immune complexes were resolved by Western blot and probed for interaction with NFATc1 and pPKB, respectively. (*B*) Interaction of NFATc1 with endogenous PKB in unstimulated and stimulated (5 μg/ml anti-CD3 mAb for 4 hrs) wt CD4+ T cells was analyzed in immunoprecipitation assays. Anti-Lamin A Ab was used to control the specificity of the interaction (233).

4.8 Myr PKB impairs TCR-induced nuclear translocation of RelB and NF-κBp65, but not NF-κBp50, proteins

Next we investigated whether the striking effect on nuclear translocation is confined to NFAT proteins or also occurs for members of the NF-κB family, which in various cell systems and with different stimuli have been shown to become activated via PKB (238, 239). Similar to NFAT analysis, CE and NE from wt and myr PKB CD4+ T cells stimulated in the presence or the absence of CsA were tested for nuclear translocation of NF-κBp50 and p65 proteins. Induction of cytoplasmic NF-κBp50, nuclear translocation, and inhibitory effects of CsA on it were similar for both wt and myr PKB CD4+ T cells (Fig. 16A). However, in strong contrast to NF-κBp50, nuclear translocation of NF-κBp65 in the case of myr PKB CD4+ T cells at 16 hrs of activation was markedly diminished, with nuclear NF-κBp65 levels similar to those in unstimulated or CsA-treated wt cells. Because of this differential effect of myr PKB on NF-κB subunits, we investigated translocation of RelB, another member of the NF-κB family. As shown in Fig. 16B, in resting cells and in response to both stimulation with CD3 alone and

with CD3 plus CD28, no nuclear RelB could be detected in myr PKB CD4+ T cells. Again, this is in stark contrast to wt cells, which showed the expected induction of RelB protein after TCR engagement. In repeated experiments nuclear levels of NF-κBp65, RelB, and NFAT proteins were always drastically diminished, but not completely absent, indicating that nuclear shuttling is not completely shut off, but is down-regulated to a major extent.

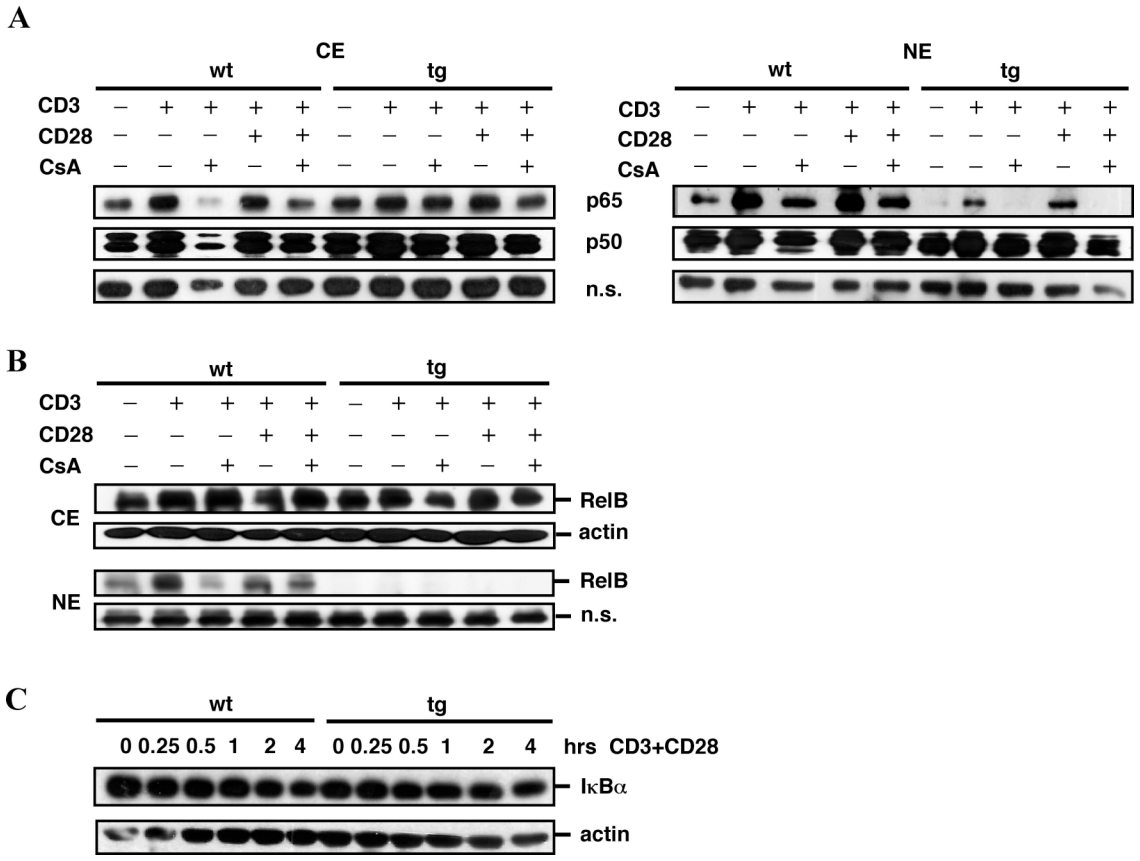


Figure 16. Myr PKB diminishes nuclear translocation of NF-κB subunits p65 and RelB. *A* and *B*, Western blot analysis of NF-κBp50, p65, and RelB in CE and NE from wt and tg CD4+ T cells stimulated as described in Fig. 12. NF-κBp50 and p65 expression is shown for cells stimulated for 8 hrs (*A*), and RelB expression is shown for cells 4 hrs after activation (*B*). n.s., Nonspecific loading control. (*C*) Similar degradation of IκBα after TCR/CD28 stimulation. Wt and tg CD4+ T cells, pretreated with cycloheximide for 15 min, were stimulated with anti-CD3 plus anti-CD28 mAbs for the time periods indicated. Whole cell extracts were analyzed for IκBα degradation. Protein loading was controlled by actin expression (Fig. 16C was provided by Na Shin-Young, AG: Bommhardt) (233).

As activation of NF-κBp65 and RelB, but not of NF-κBp50, proteins is regulated via IκBα proteins, we compared the degradation of IκBα in wt and tg T cells. As shown in Fig. 16C, degradation of IκBα after CD3/CD28 stimulation in myr PKB CD4+ T cells was only slightly reduced. This suggests that myr PKB-mediated mechanisms other than targeting IκBα degradation are the major factors in the substantial loss of nuclear NF-κBp65 and RelB proteins.

Part II. Effect of PKB on early thymocyte development: *crosstalk between PKB and calcineurin signaling*

4.9 Generation of transgenic mice

To study the putative role of PKB in regulating CN signalling during early thymic development, we analysed mice double-transgenic for active PKB (137) and a constitutively active version of the large CN subunit A, designated as ΔCam (230). ΔCam lacking CN's calmodulin binding and autoinhibitory domains was expressed in early thymocytes under control of the proximal $p56^{lck}$ promoter (Fig. 17A). Compared to the expression of endogenous CN in wt mice, expression of ΔCam was elevated about twentyfive to thirty fold in the ΔCam tg mouse line used for all further analyses (Fig. 17B). ΔCam mice were crossed with mice expressing a myristoylated constitutively active version of human PKB (myr PKB) under control of the human CD2 promoter, leading to high level expression of active PKB in ΔCam/PKB DN thymocytes (Fig. 17C).

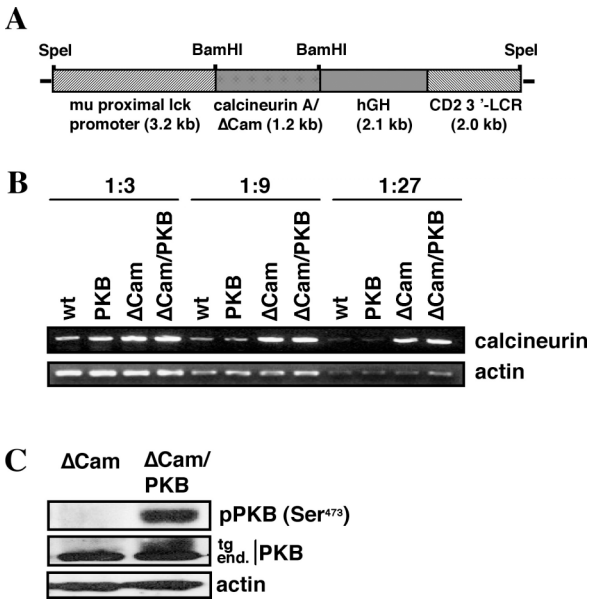


Figure 17. Generation of calcineurin A/myr PKB double transgenic mice. (*A*) Schematic representation of the injection fragment used to generate calcineurin A transgenic mice. The human calcineurin A catalytic subunit containing a deletion of the calmodulin binding and autoinhibitory domain (ΔCam) was cloned under the control of the proximal $p56^{Lck}$ promoter and CD2 locus control region (LCR). The polyadenylation site was provided by a 2.1 kb fragment of the human growth hormone gene. (*B*) RT-PCR analysis of calcineurin expression in CD4-CD8- double negative (DN) cells of wild type, PKB tg, ΔCam tg and ΔCam/PKB double tg mice. PKB tg mice express a constitutively active form of human PKB (myr PKB) under the human CD2 promoter. cDNA was serially diluted and actin expression was used to control amounts of cDNA applied. (*C*) Expression of phosphorylated active PKB, total PKB and actin, serving as loading control, was analyzed in isolated DN cells of ΔCam and ΔCam/PKB double tg mice by western blot. The lower PKB band represents endogenous PKB (end), the upper band transgenic (tg) PKB.

Phenotypically, ΔCam mice did not show any outward abnormality but the total cellularity in all the lymphoid organs was severely reduced (Fig. 18). Analysis of thymic cellularity shows that compared to wt littermate control mice, ΔCam mice exhibited on average a twenty five fold reduction in the number of thymocytes. Reduction in cellularity was also prominent in the lymph node (6 to 7 fold) and spleen (nearly 3 fold) compared to control mice. Although the CD4 to CD8 ratio remained similar, there was a sharp decrease in the percentage and absolute numbers of both cell types in case of ΔCam mice. The ratio of T verses B cells in LN and spleen and the number and ratio of immature to mature B cells in spleen were not significantly altered (data not shown).

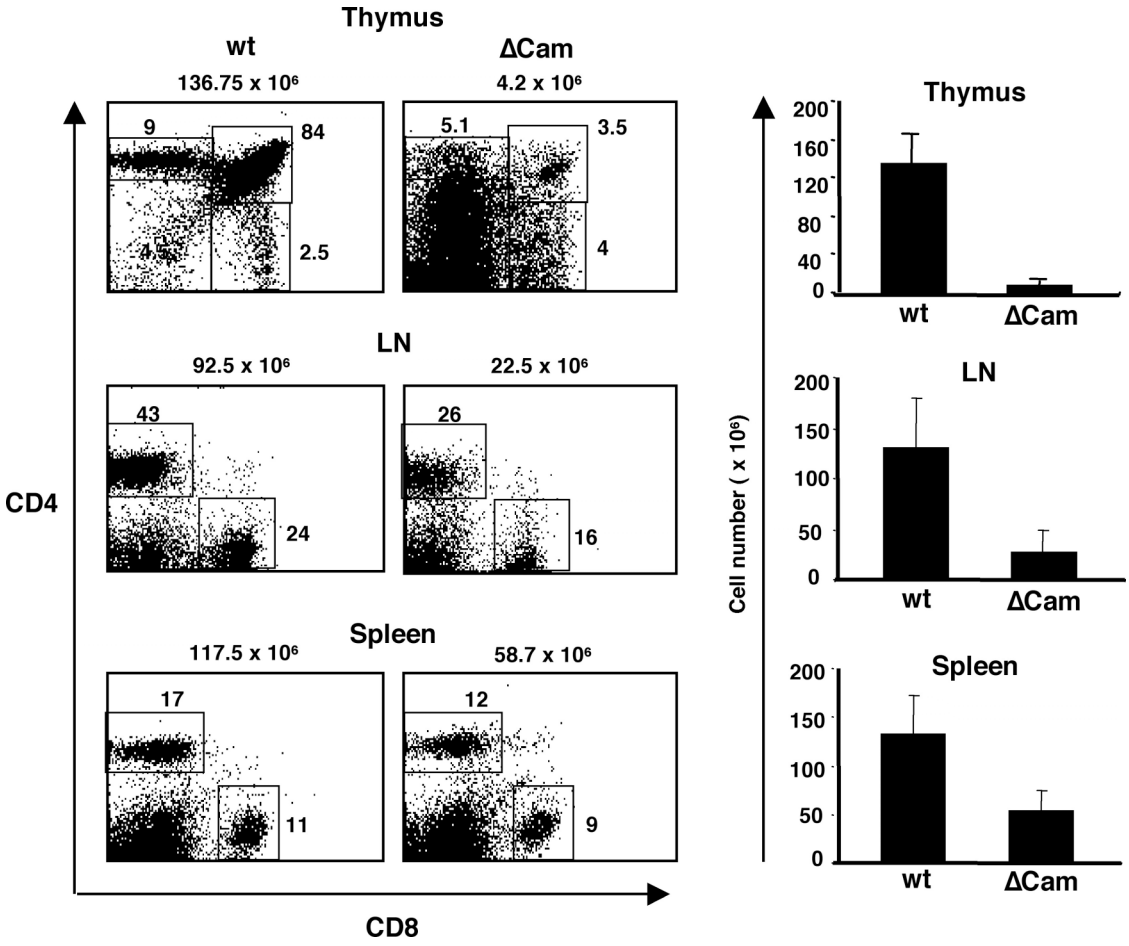


Figure 18. Reduced cellularity in the lymphoid compartment of ΔCam mice. Thymocytes (upper panel), lymph node cells (middle panel) and splenocytes (lower panel) from wt and ΔCam mice were stained for CD4 and CD8 surface expression. Numbers atop each plot represent total cellularity and numbers within plots give the percentage of the individual subsets. Bar diagrams to the right of each panel show absolute cell numbers for indicated organs from wt and ΔCam tg mice. Data shown represent 7-14 individual mice analyzed per organ.

4.10 PKB rescues the block in early thymocyte development induced by constitutively active calcineurin

Analysis of thymocyte sub-populations revealed a drastic loss in DP cells (5% versus 85%) and a marked increase in the percentage of DN cells. The impact of ΔCam on thymic cellularity was already obvious in newborn mice indicating that the loss in DP thymocytes is due to a block in early thymocyte differentiation (Fig. 19A & B).

Further characterisation of the DN population into DN1-4 subsets revealed that almost 90% of ΔCam DN thymocytes are CD25+CD44- DN3 cells. Therefore, the loss of DP cells in ΔCam mice is due to a developmental arrest at the transition from DN3 to DN4 cells (Fig. 19 C). Strikingly, simultaneous expression of myr PKB largely restored thymic cellularity, allowed efficient T cell maturation and significantly alleviated the DN3 arrest in ΔCam mice, which resulted in a phenotype closely resembling to that of littermate controls (Fig. 19A-C). Thus, active PKB releases the DN3 to DN4 transitional block, presumably by counteracting CN-induced downstream signalling events including activation of NFAT proteins.

To prove the observed dramatic effect in term of rescue of the thymic phenotype is specific to PKB activity or just a mere transgenic artefact, we generated ΔCam/PKB double tg mice with a second PKB tg line (PKB5). Results from the thymic analysis of ΔCam/PKB5 double tg mice were similar to that of ΔCam/PKB2 double tg mice (Fig. 20A & B). These observations argue that the effective rescue of DN3 cells and ultimately the thymic phenotype in ΔCam mice is mediated via active PKB.

4.11 Phenotype of ΔCam and ΔCam/PKB DN3 cells

To understand the molecular mechanisms whereby myr PKB can antagonize the differentiation block imposed by active CN, we first investigated several surface antigens that are modulated during progression from DN3 to DP cells. Analysis of CD4-CD8-CD25+ DN3 cells showed that a higher percentage of cells exhibited enhanced expression of CD2 and CD5 in ΔCam tg mice compared to DN3 cells from PKB tg mice or wild type littermates (Fig. 21 A). The elevated expression of these two markers indicates that the ΔCam DN3 cells have received at least part of the activation signal essential during DN3 to DN4 transition. The majority of ΔCam DN3 cells also showed lower levels of HSA (CD24) whereas IL7R expression was similar to control cells. Cell size is an indicator of the cycling status of cells. Larger size cells are either cycling or are ready to cycle and smaller size cells are non-cycling cells. But there was no difference in the cell size from ΔCam or ΔCam/PKB DN3 cells compared to the control DN3 cells (Fig. 21A lower panel). Notably, myr PKB reverted the

expression of the surface antigens in ΔCam DN3 cells towards the "normal" phenotype indicating that in the presence of myr PKB ΔCam DN3 cells underwent normal differentiation.

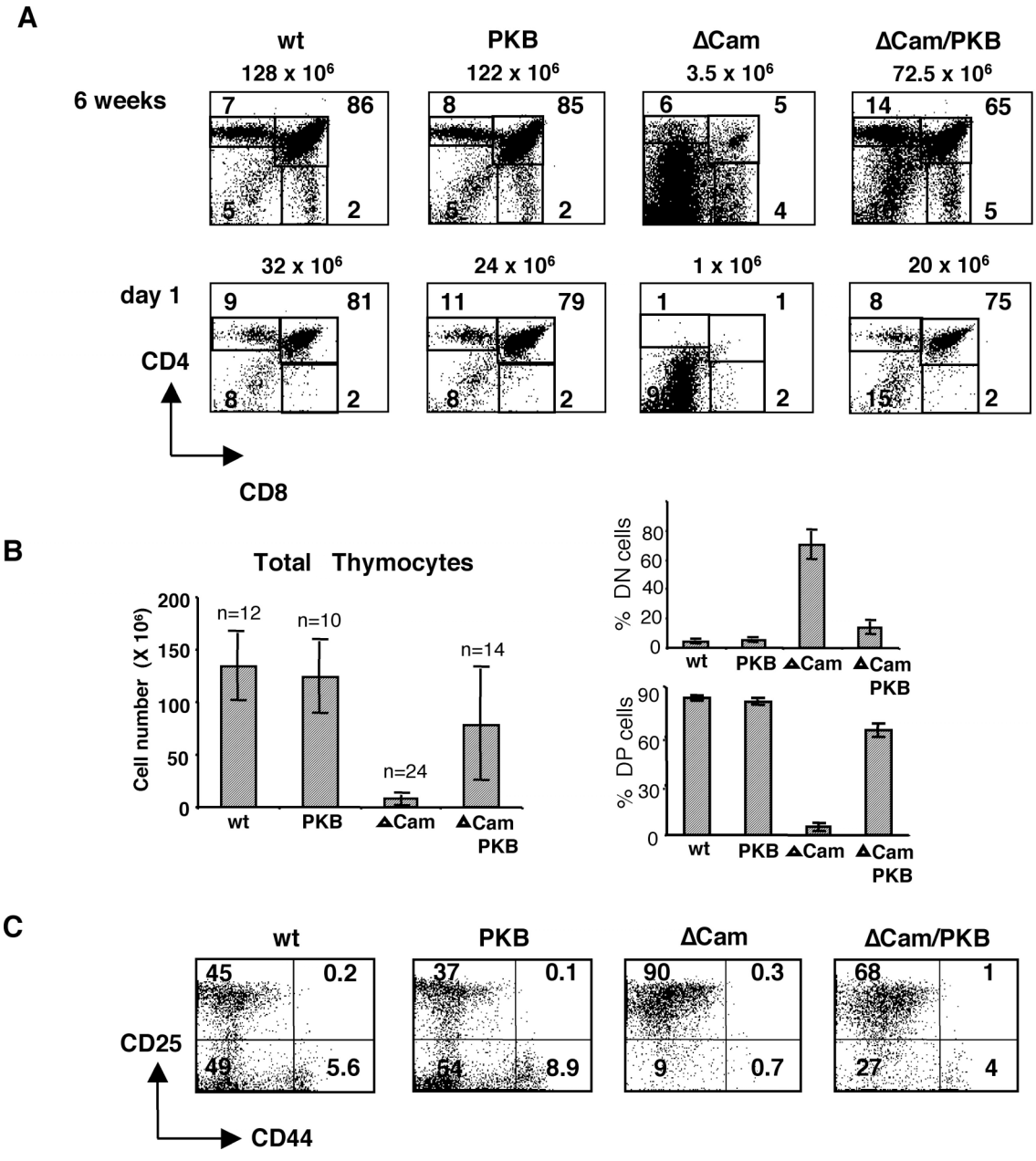


Figure 19. Active PKB rescues thymocyte development in ΔCam tg mice. (*A*) Distribution of thymocyte subsets in wt, PKB tg, ΔCam tg and ΔCam/PKB double tg mice, either aged 6 weeks (upper panel) or 1 day (lower panel). Single cell suspensions of thymocytes were stained for CD4 and CD8 surface expression and analyzed by flow cytometry. Numbers give the percentage of cells in the corresponding thymocyte subset. Thymocyte cellularity for individual mice is provided at the top of each plot. (*B*) Absolute cell numbers and percentages of DN and DP cells in wt, PKB tg, ΔCam tg and ΔCam/PKB double transgenic mice. Data shown represent 10-24 individual mice per group. (*C*) PKB rescues the developmental block at the DN3 stage induced by constitutively active calcineurin. DN thymocytes from the four indicated mouse strains were analyzed for CD25 and CD44 expression after gating on cells negative for CD4 and CD8 expression. The percentages of the individual DN1-DN4 subsets are indicated in the quadrants.

T cell development involves several processes that occur in different anatomic sites within the thymus. Therefore, thymocytes must have the ability to respond to signals received from stromal cells and adopt either adhesive or motile behavior (240). Cell adhesion molecules play an important role in these processes and thymocytes employ various integrins at different developmental stage for adhesion or motility. Analysis of expression of integrins exhibited much higher expression of LFA-1 (CD11) and CD18 (integrin β2 chain) on ΔCam DN and DP thymocytes compared to wt or PKB tg controls (Fig. 21B, left and middle panel). Expression of CD49d (integrin α4 chain) was also higher in ΔCam mice than in control mice. Active PKB only partially reduced the expression level of all these integrins in the double tg mice (Fig. 21B, left, middle and right panel), indicating that PKB has only little influence in the regulation of these integrins.

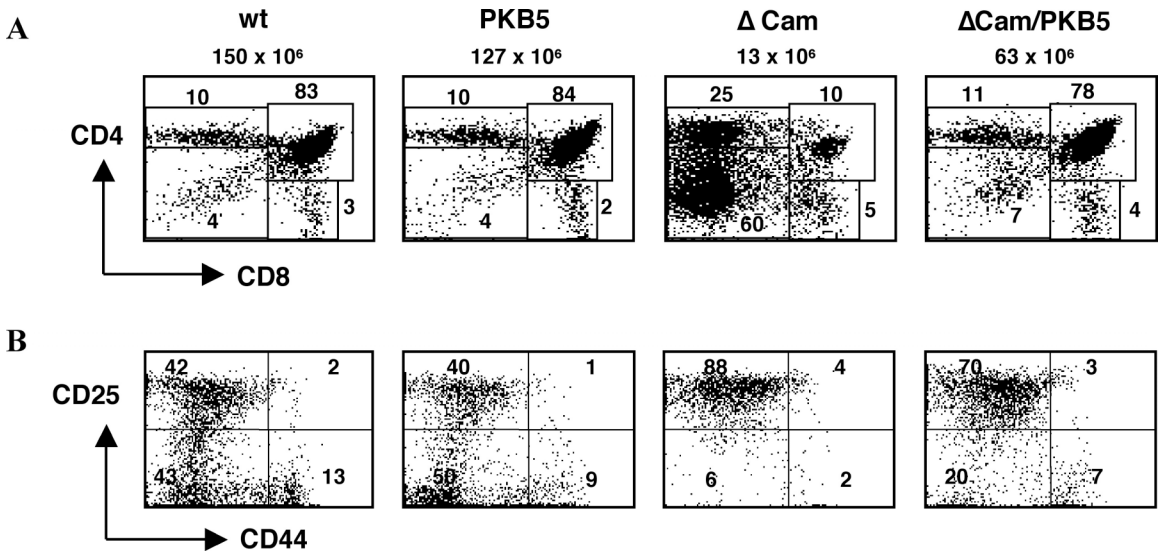


Figure 20. Rescue of thymic phenotype in ΔCam/PKB5 double tg mice. (*A*) Distribution of thymocyte subsets in wt, PKB5 tg, ΔCam tg and ΔCam/PKB5 double tg mice, aged 6 weeks. Single cell suspensions of thymocytes were stained for CD4 and CD8 surface expression and analyzed by flow cytometry. Numbers give the percentage of cells in the corresponding thymocyte subset. Thymocyte cellularity for individual mice is provided at the top of each plot. (*B*) PKB5 rescues the developmental block at the DN3 stage induced by constitutively active calcineurin. DN thymocytes from the four indicated mouse strains were analyzed for CD25 and CD44 after gating on cells negative for CD4 and CD8 expression. The percentages of the individual DN1-DN4 subsets are indicated in the quadrants.

4.12 Normal Apoptosis and cell cycle in ΔCam DN thymocytes

The reduced thymocyte cellularity in ΔCam mice could be due to; either increased apoptosis, reduced cell cycle or a combination of both. To clarify the effect of ΔCam on the survival of DN cells, we examined levels of the anti-apoptotic protein Bcl_2 and of apoptosis by annexin V staining. Bcl_2 expression has been described to be high in DN cells and low in most DP cells

(241). Accordingly, DN3 cells of wt and myr PKB tg mice showed two populations with higher and lower levels of Bcl_2 (Fig. 22A). ΔCam expressing DN cells only showed one population with very high Bcl_2 levels that were even above those detected in control mice. In

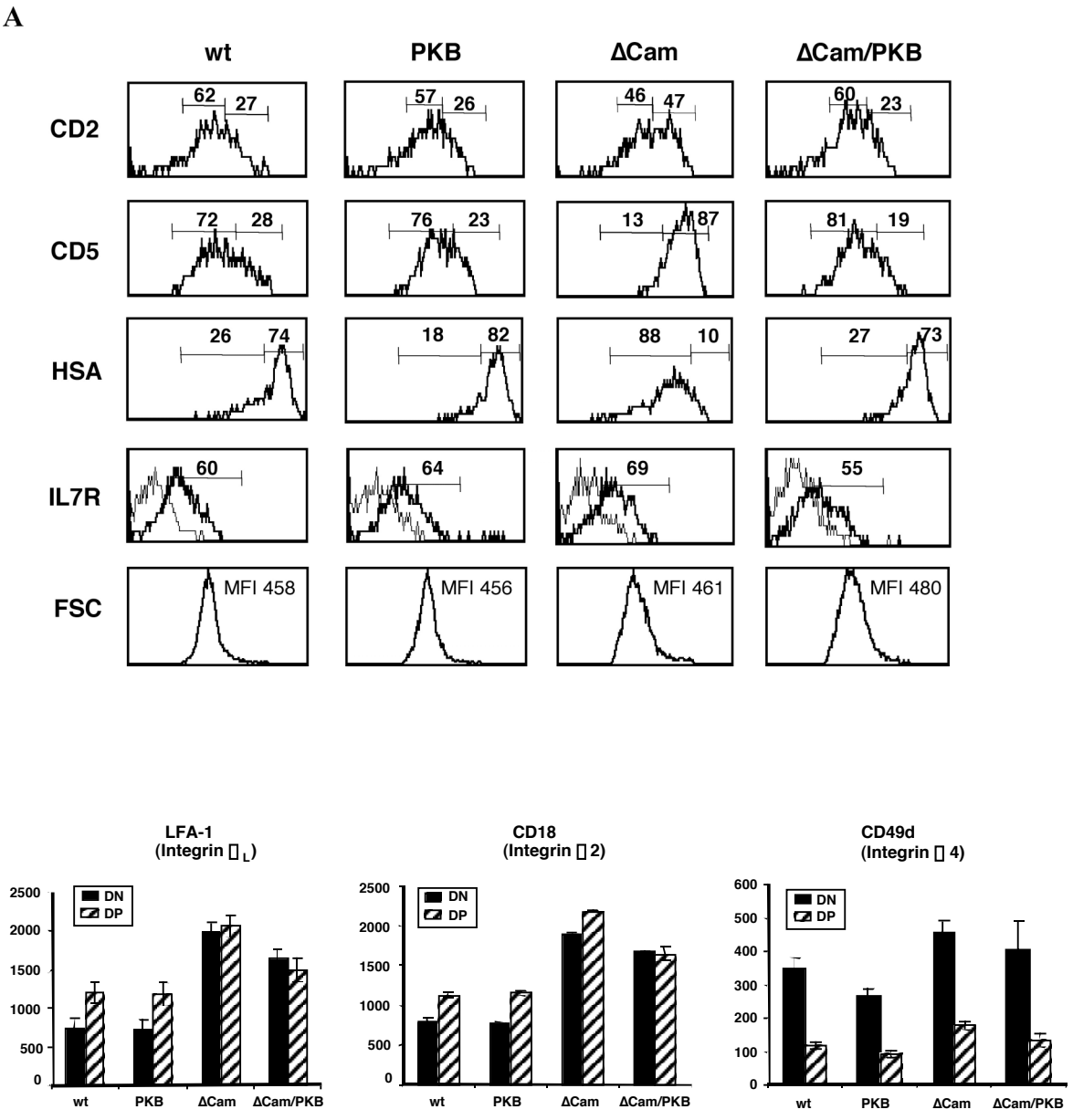


Figure 21. Active PKB reverses the effects induced by ΔCam in DN cells. (*A*) Histograms show expression of CD2, CD5, HSA, IL7R and cell size of CD25+CD44- DN3 cells of wt, PKB, ΔCam and ΔCam/PKB tg mice. Numbers in each histogram represent the percentage of cells showing medium and high expression of each marker or in case of FSC the mean fluorescence intensity (MFI). (*B*) Analysis of integrin expression on DN and DP thymocytes. Thymocytes from 6 weeks old mice in each group were surface stained for CD4, CD8 and various integrins. Plots show analysis of LFA-1 (left), CD18 (middle) and CD49d (right) expression on DN and DP thymocytes.

presence of myr PKB, Bcl_2 expression was intermediate between ΔCam and wt DN3 cells. In addition, a population with lower Bcl_2 expression was now detectable as found in wt cells,

presumably representing those cells that have progressed in differentiation towards the DN4 stage. Annexin V staining did not show any drastic difference in apoptosis between ΔCam mice to that of the ΔCam/PKB double transgenic or the control mice. Only an about 2-fold increase in apoptosis of DN3 cells from ΔCam mice was detected compared to the control or double tg mice (Fig. 22B). Thus, inhibition of DN3 progression by ΔCam is accompanied by strong induction of Bcl_2 and only slightly enhanced apoptosis of DN3 cells.

The cell cycle profile of ΔCam DN cells also did not show any drastic abnormality. Whereas in the control and in the double tg mice around 15% of DN cells were in the cycling ($S+G_2+M$) phase, it was slightly reduced (11%) in case of ΔCam DN cells (Fig. 22C). Combinedly these data show the defect in ΔCam thymocyte development is not because of dysregulated apoptosis or cell cycle but due to some other anomalies.

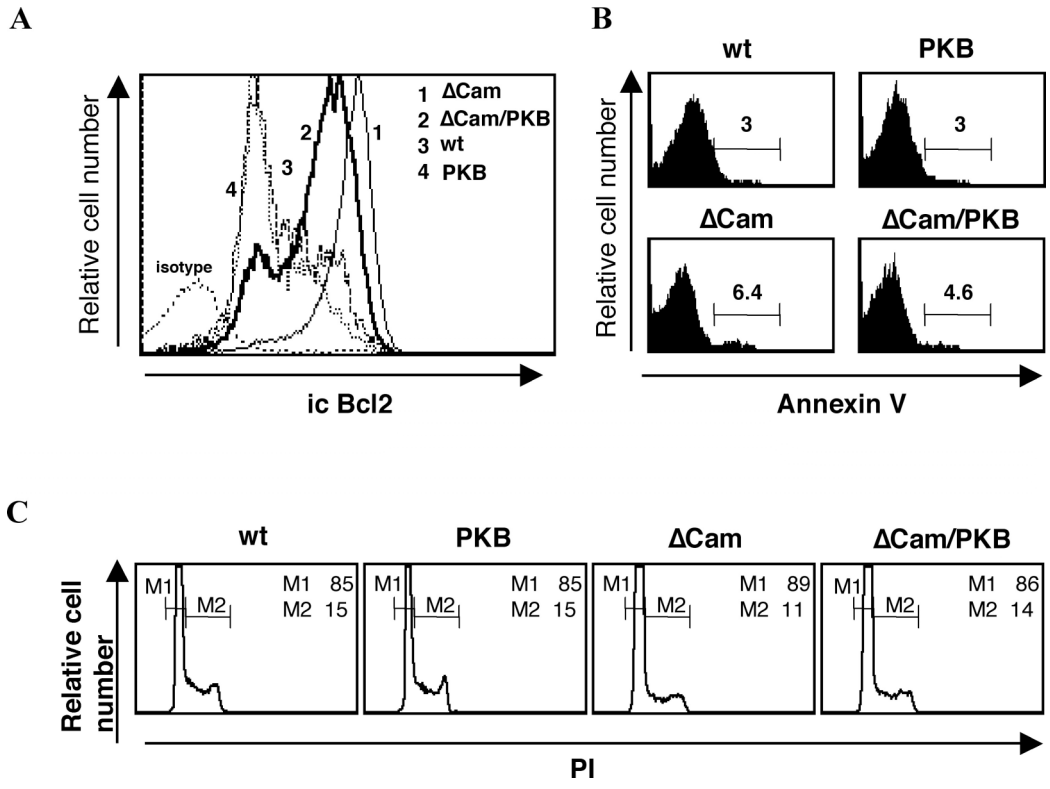


Figure 22. Apoptosis and cell cycle in ΔCam DN thymocytes. (*A*) Higher intracellular Bcl_2 expression in DN thymocytes from ΔCam compared to ΔCam/PKB, wt and PKB tg mice. (*B*) Profiles of Annexin V stained DN3 thymocytes in each group. Numbers in each histogram indicate percentage of Annexin V positive cells. (*C*) Cell cycle profile of ΔCam tg mice. Isolated DN thymocytes from indicated mice were stained with PI and were analyzed for cell cycle status. M1 and M2 gates represent cells at G_0/G_1 (non cycling) and $S/G_2/$ and M phases (cycling) of cell cycle, respectively. Numbers in each plot represent percentage of cells in noncycling and cycling stages.

4.13 ΔCam DN3 cells are normal in CD3ε chain expression

DN3 to DN4 transition during thymocyte development is dependent on signaling from the pTCR/CD3 complex. Mutation or deletion of various CD3 complex components has been shown to block thymocyte development at the DN3 stage (242-244). We therefore reasoned to explore whether thymocytes from ΔCam mice have any abnormality in the signaling from the pTCR/CD3 complex. Intracellular staining for of CD3ε in DN3 cells from ΔCam mice showed a similar level of expression as found for control and double tg DN3 cells (Fig. 23A, upper panel). Also, there was no apparent difference in CD3ε expression in the DN4 cells from each group (Fig. 23A, lower panel). Simultaneously when we looked at downstream signaling molecules we found phospho ERK levels were clearly increased indicating enhanced pTCR/CD3 signaling (Fig. 23B).

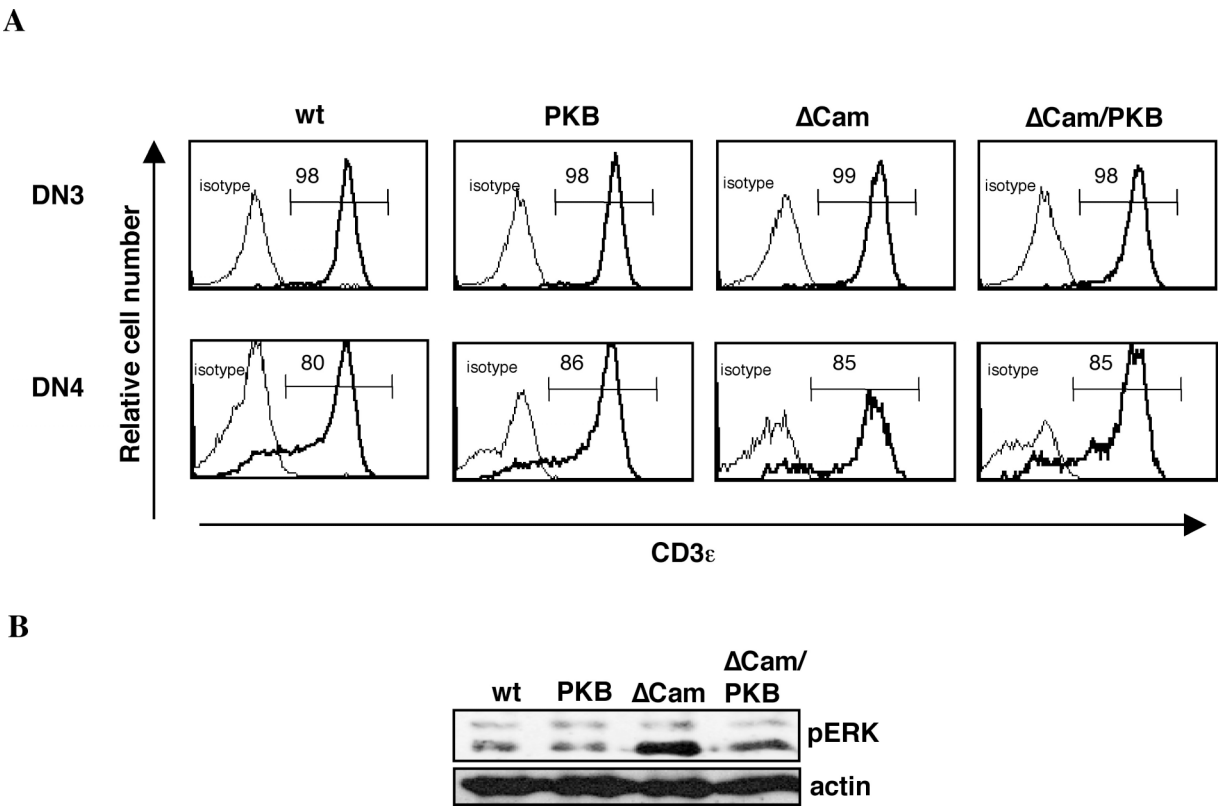


Figure 23. ΔCam DN3 cells show normal CD3ε chain expression but enhanced ERK activation. (*A*) Expression of CD3ε chain in DN3 and DN4 cells from wt, PKB, ΔCam and ΔCam /PKB double tg mice. Numbers in each histogram indicate percent of thymocytes expressing high level of CD3ε. (*B*) Higher phosphoERK level in ΔCam DN thymocytes. Western blot analysis of WCE from isolated DN thymocytes from indicated mice for pERK expression. Actin was used to show protein-loading control.

4.14 Myr PKB rescues Rag1 and icTCRβ chain expression in ΔCam DN3 cells

Based on the finding that despite up-regulation of certain surface molecules and enhanced ERK activation, ΔCam DN3 cells failed to develop into DN4 cells, we suspected defects in expression of the TCRβ chain. FACS analysis showed that only 0.17% of ΔCam DN3 cells expressed high levels of icTCRβ compared to 13-15 % in wt or PKB tg DN3 cells (Fig. 24A). Likewise, whereas almost all DP cells of wt and PKB tg mice were positive for icTCRβ, only 43% of the few DP cells arising in ΔCam mice showed high levels of icTCRβ. Strikingly, simultaneous expression of myr PKB increased the percentage of ΔCam DN3 cells expressing icTCRβ chain by 20-fold and restored icTCRβ expression in DP cells to normal levels. Thus, active PKB reverts the defects in icTCRβ chain expression imposed by active CN.

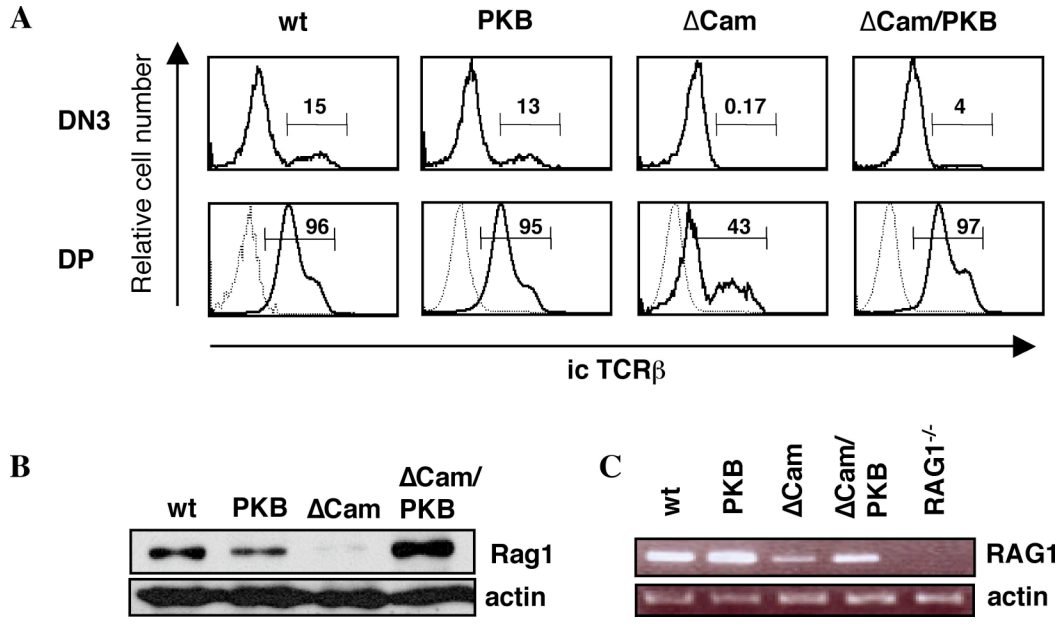


Figure 24. Expression of active PKB significantly enhances icTCRβ and Rag1 expression in ΔCam DN cells. (*A*) Expression of intracellular TCRβ (icTCRβ) in DN3 and DP cells from wt, PKB, ΔCam and ΔCam/PKB double tg mice. Numbers in each histogram indicate the percentage of thymocytes expressing icTCRβ. (*B*) Myr PKB rescues the defect in Rag1 expression imposed by ΔCam. Whole cell extracts of unstimulated DN thymocytes were fractionated by SDS-PAGE and immunoblotted with Rag1 antibody. Protein loading was controlled by actin antibody. (*C*) The ΔCam induced block of Rag1 expression is transcriptional. Total RNA from unstimulated DN thymocytes was used for analysis of RAG1 gene expression by RT-PCR. β-actin RT-PCR shows equal quantity of cDNA used.

To dissect the defect in TCRβ chain expression, we analysed the expression of Rag1 protein, which is essential for the recombinatorial rearrangements at the TCRβ chain locus. In ΔCam DN cells we detected extremely low Rag1 protein levels. However, Rag1 expression became normal when PKB was expressed simultaneously (Fig. 24B). Further RT-PCR studies

revealed the absence of Rag1 protein expression in ΔCam DN cells to be linked with reduced Rag1 mRNA levels (Fig. 24C), suggesting that active CN regulates RAG1 expression via transcriptional and/or post-transcriptional mechanisms. Again, in PKB/ΔCam DN cells Rag1 mRNA levels approached those found in wt or PKB tg cells (Fig. 24C). In conclusion, ΔCam induces a block in early thymocyte differentiation by drastically reducing RAG1 expression and preventing efficient TCR gene rearrangement. Active PKB either by directly acting on CN, its targets or some unknown proteins, overcomes the negative regulatory effects induced by ΔCam, allowing DN3 cells to efficiently express Rag1, rearrange TCR chains and to progress in differentiation to DP cells.

4.15 TCR transgenes can partially overcome the ΔCam-mediated DN3 arrest

Since, inefficient or lack of pre-TCR assembly due to defective Rag1 expression seems to be a major defect in ΔCam DN3 cells, we analysed whether a functionally rearranged tg TCR will be able to overcome the differentiation block. Therefore, we crossed ΔCam mice with OT1 TCR tg mice and analysed cellularity and thymocyte subsets in these ΔCam/OT1 double tg mice. As shown in Fig. 25 and Table 3 below, expression of the OT1 tg TCR increased the

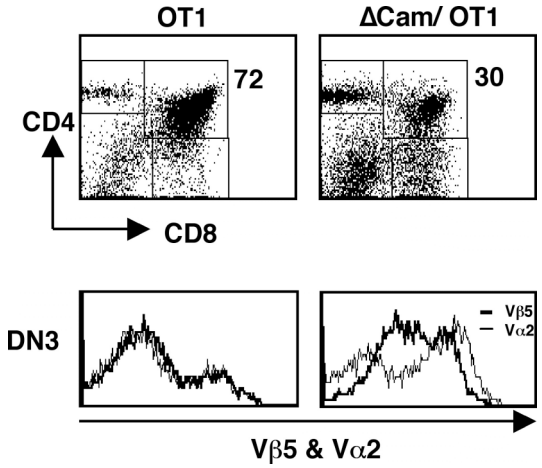


Figure 25. Expression of the transgenic OT1 TCR is not sufficient to rescue the thymic phenotype in ΔCam tg mice. FACS profile of CD4 and CD8 stained thymocytes from 6 weeks old OT1 and ΔCam/OT1 mice. Numbers indicate the percentage of DP cells (upper panel). Histograms (lower panel) show expression levels of the OT1 TCR specific V5 and V2 chains in DN3 thymocytes from OT1 and ΔCam/OT1 mice.

Table 3. Effect of OT1 TCR transgene expression on thymic phenotype of ΔCam mice

n = 4	**OT1**		**ΔCam /OT1**		**ΔCam**	
Total (x 10^6)	157.5	(± 14.6)	14.0	(± 20.0)	3.3	(± 2.5)
% DP	79.2	(± 0.9)	30.0	(± 19.8)	5.4	(± 1.5)
% DN3 of DN	19.4	(± 1.8)	86.2	(± 4.2)	88.4	(± 1.1)

percentage of DP cells in ΔCam mice maximally about 6-fold and the total cell number about 3 fold. Concerning expression of the OT1 TCR specific V□5 and V□2 chains, we observed that in the double tg mice a higher fraction of DN3 cells expressed the tg TCR chains compared to OT1 DN3 cells but in overall the percentage of DN3 cells within the total DN population was not significantly altered. In comparison to the dramatic rescue of maturation of DP cells and thymic celluarity by active PKB, we concluded that expression of a pre-rearranged TCR alone supports but is insufficient to completely overcome the DN3 arrest induced by ΔCam. This implies that active PKB evokes mechanisms in addition to controlling RAG1 and TCR□ chain expression.

4.16 CsA treatment releases the CN-induced DN3 block in differentiation

Since our previous studies suggested a direct interaction between PKB and NFATs (233), we decided to focus on NFAT regulation in the double tg ΔCam/PKB mice. First, we analyzed

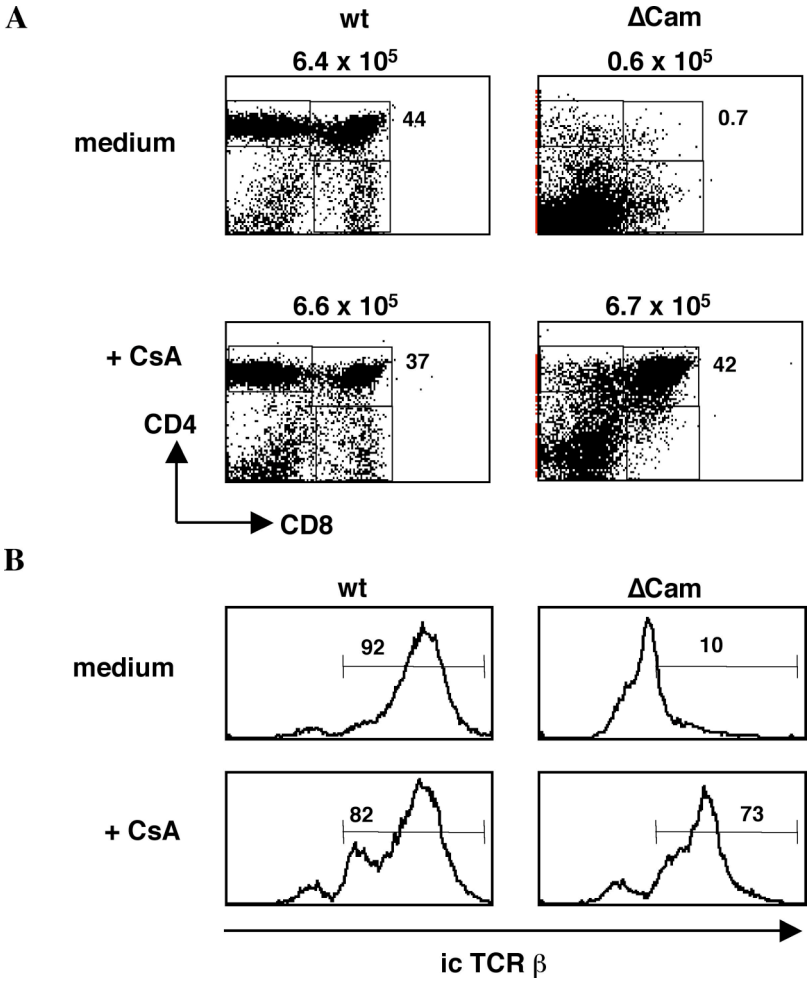


Figure 26. Inhibition of calcineurin by CsA allows normal differentiation of ΔCam DN3 cells. (*A*) Thymic lobes from newborn ΔCam and wt mice were cultured without or with CsA (100 ng/ml) for 4 days. Thymocytes were stained for CD4 and CD8 and thymocyte subsets were determined by FACS analysis. Numbers at the top of each plot represent total cellularity. (*B*) Thymocytes from lobe cultures described in (*A*) were analyzed for icTCR□ chain expression. Histograms are gated on total DN cells and the numbers in each histogram indicate percentage of icTCR□ positive cells.

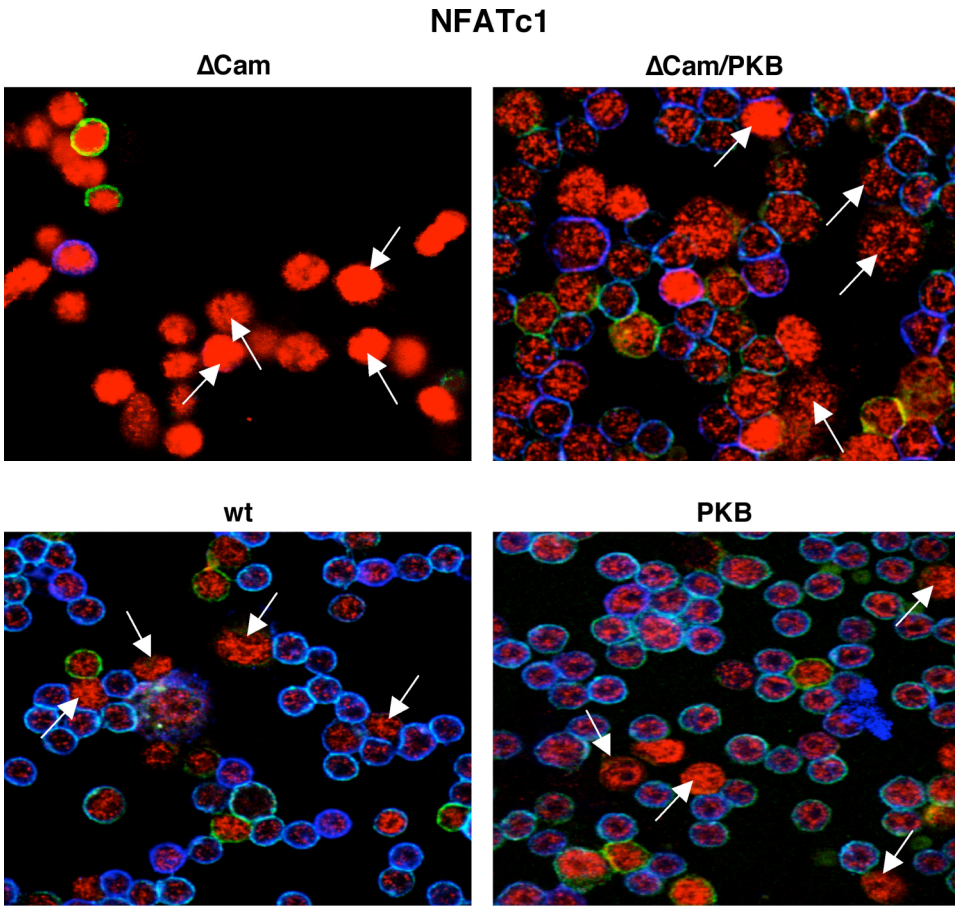
whether inhibition of CN and therefore inhibition of NFAT activation, would restore T cell differentiation in ΔCam mice. For this purpose we cultured newborn thymic lobes from ΔCam and wild type mice in the presence or absence of CsA, an efficient inhibitor of CN-driven NFAT activation. As shown in Fig. 26A, CsA treatment, in contrast to untreated ΔCam lobes, led within 4 days to a 10-fold increase in cellularity and enabled differentiation of DN cells to the DP stage (42% versus 0.7% DP in untreated lobes). Likewise, CsA treatment efficiently restored high level expression of icTCR chain in ΔCam DN cells (Fig. 26B). Thus, inhibition of calcineurin/NFAT activity allows normal expansion and differentiation of otherwise arrested ΔCam DN3 cells.

4.17 Myr PKB rescues the thymic phenotype in ΔCam tg mice by regulating NFAT proteins

In accordance with CN's role in regulating NFAT proteins and PKB's pivotal effects in ΔCam tg mice, we next asked whether myr PKB counteracts the effects induced by ΔCam by inhibition of NFAT activation. Using confocal microscopy, we studied the expression of NFATc1 and NFATc3 in DN cells of ΔCam and ΔCam/PKB tg mice. Thymocytes were stained for CD4 and CD8, and NFAT expression was evaluated in CD4-CD8- cells. As evident from Fig. 27A and B, in ΔCam tg mice NFATc1 expression was very strong in the majority (about 80%) of DN cells and only about 20% of cells showed lower levels of NFATc1. In NFATc1 high expressing cells NFATc1 was totally nuclear as judged by staining of nuclei with DAPI (data not shown). Intriguingly, in ΔCam/PKB tg mice about 70% of DN cells showed lower levels of NFATc1 and only 30% of cells showed nuclear levels as high as found in ΔCam DN cells. Importantly, this inverted ratio of $NFATc1^{hi}$ versus $NFATc1^{lo}$ cells corresponded to the expression levels of NFATc1 in DN cells from wt or myr PKB tg mice.

Since various NFAT family members seem to play different roles during the progressive stages of thymocyte development, we also studied the expression of NFATc3 in the respective mouse lines. NFATc3 has been shown to be mainly thymocyte specific. As observed for NFATc1, the nuclear load of NFATc3 was very high in almost all DN cells of ΔCam tg mice. In stark contrast, NFATc3 expression was low in the majority of DN cells of ΔCam mice when myr PKB was present (Fig. 28A). In addition, NFATc3 levels in DN cells of ΔCam/PKB tg mice corresponded to those in DN cells of wild type or PKB tg mice (data not shown). From these experiments it is obvious that one pivotal action of myr PKB is the reduction of NFATc1 and NFATc3 activation in ΔCam DN cells.

A



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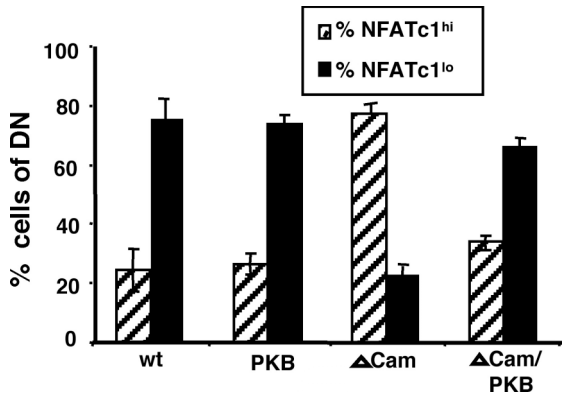

Figure 27. Myr PKB regulates nuclear NFATc1 levels in DN thymocytes. (*A*) Distribution of NFATc1 in DN thymocytes. Confocal images of thymocytes surface stained for CD4 (blue) and CD8 (green) and intracellular NFATc1 (red) from wt, PKB tg, ΔCam and ΔCam/PKB double tg mice. Arrows indicate representative DN cells in each image showing nuclear and cytoplasmic distribution of NFATc1. (*B*) Proportion of DN cells showing varying NFATc1 level. DN cells from each individual mouse were evaluated on the basis of high or low NFATc1 levels. Data represent percent of NFATc1^{hi} and NFATc1^{lo} cells from 150-350 DN cells counted for each group.

4.18 Higher nuclear level of NFATc1 and NFATc3 in Rag$^{-/-}$ DN3 thymocytes

In Rag k.o. mice thymocyte development is blocked at CD44-CD25+DN3 stage due to absence of TCR rearrangement and the pre TCR signaling. ΔCam DN3 thymocytes are severely deficient in Rag expression and activity. Thus functionally ΔCam DN3 cells are similar to Rag1 ko DN3 cells. Since ΔCam DN thymocytes (which are mainly DN3) show high nuclear NFATc1 and NFATc3 levels, we explored NFAT levels in Rag1 k.o. thymocytes. Surprisingly, confocal microscopy showed high levels of nuclear NFATc1 whereas NFATc3 levels were low in Rag1 k.o. DN3 cells, although these cells definitely

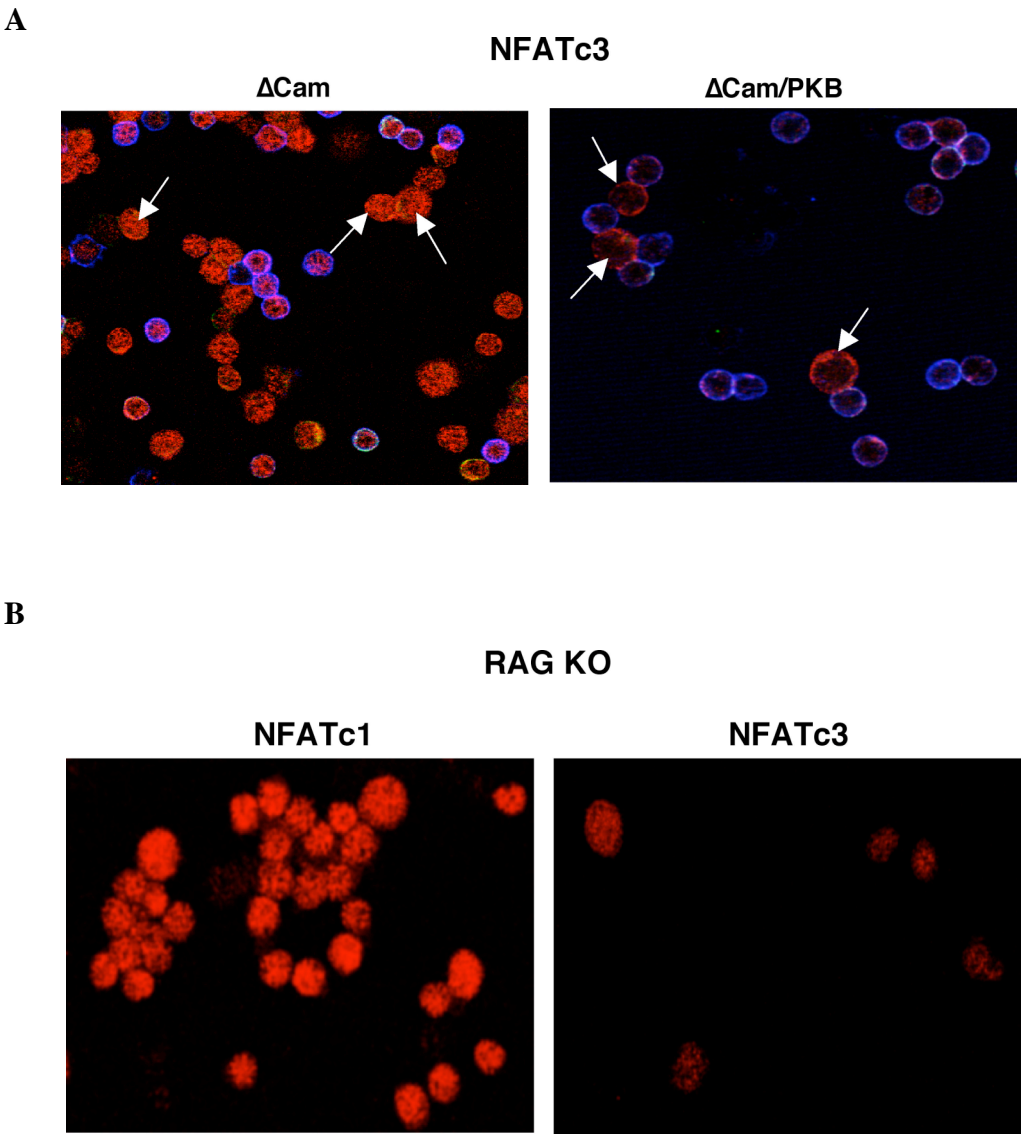

Figure 28. NFATc3 nuclear translocation is also regulated by myr PKB. (*A*) Distribution of NFATc3 in representative DN thymocytes from ΔCam tg and ΔCam/PKB double tg mice. (*B*) Confocal images show distribution of NFATc1 and c3 in Rag1 ko thymocytes.

lack a functional pre TCR (Fig. 28B). The functional significance and the signals that induce NFAT has to be analyzed in future studies.

4.19 Active PKB induced lymphoma show dysregulation in NFAT nuclear translocation

PKB tg CD4+ T cells show a severe reduction in nuclear translocation of NFATc1 and NFATp (233). TCR ligation alone or in combination with CD28 costimulation could not reverse this effect (Fig. 12A & B). Also in ΔCam/PKB DN thymocytes the nuclear accumulation of NFATc1 and NFATc3 is significantly regulated and this reduction in nuclear NFATs is responsible for the rescue of thymic phenotype in the double tg mice. Hence, we hypothesized NFATs could be directly phosphorylated by PKB. In vitro kinase assay show PKB could infact phosphorylate multiple serine residues in NFATc1 (S.Y. Na, personal communication).

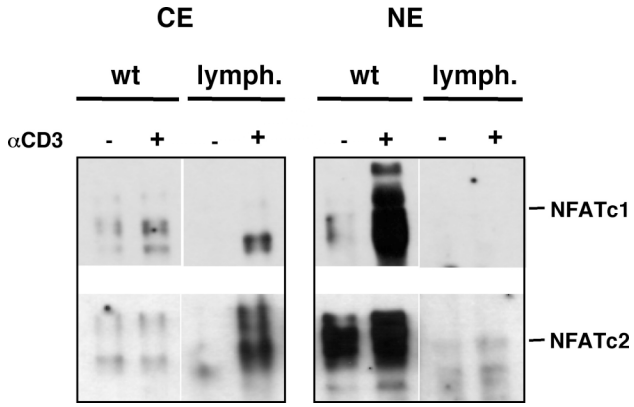

Figure 29. PKB induced T cell lymphoma exhibits impaired NFAT regulation. Nuclear (NE) and cytoplasmic (CE) distribution of NFATc1 and NFATc2 in CD4+T cells from wt mice and electronically sorted CD4+ Vβ6+ lymphoma cells stimulated with plate-coated CD3ε Ab for 4 hrs.

Both NFATs and PKB have been shown to regulate growth and proliferation. From our observations in thymocytes and CD4+T cells it is becoming increasingly clear these two molecules could play a role in tumorigenesis. In PKB induced lymphomas, which develop in myr PKB homozygous mice we suspected that NFAT activation should be altered. Sorted CD4+ cells from mono/oligoclonal T cell lymphoma (all positive for Vβ6 chain) and polyclonal wt type CD4+ T cells were activated with CD3ε Ab and analysed for NFATc1 and NFATc2 activation. The outcome was exactly similar to that in PKB tg CD4+ T cells. In contrast to strong nuclear NFAT localization in wild type CD4+ T cells, only marginal levels of nuclear NFATc1 and NFATc2 could be detected in the lymphoma cells (Fig. 29). Thus, myr PKB induced CD4+ T cell lymphoma is characterised by nuclear deficiency of NFATc1 and NFATc2 proteins.

Chapter 5.
Discussion

5. Discussion

5.1 Myr PKB in T cell activation, cell cycle progression and cytokine production

In this study on the effects of a constitutively active PKB on T cell development and function, we demonstrate that myr PKB tg T cells show a marked difference in a number of physiological parameters compared to wt T cells. First, they exhibit a distinct advantage in proliferation towards weak or suboptimal TCR signals (Fig. 9A & B). Second, proliferation of myr PKB tg T cells is significantly resistant to the immunosuppressive agent cyclosporin A (CsA) (Fig. 9A & B), an observation which could contribute towards better understanding of the phenomena of inflammation and hypersensitivity. Third, myr PKB tg T cells undergo cell cycle at a distinctly higher rate than wt T cells (Fig. 10A & B). Fourth, myr PKB tg T cells exhibit higher efficiency in production of cytokines of both Th1 and Th2 type and cytokine production is also less affected to CsA treatment (Fig. 11). Fifth and most importantly, myr PKB tg T cells exhibit all the above mentioned properties independent of CD28 costimulatory signals (Figs. 9, 10 & 11).

CD28 is the primary T-cell costimulatory receptor, the engagement of which is necessary for TCR induced proliferation of naive T cells. A number of studies have demonstrated that CD28, upon interaction with its ligands B7.1 (CD80) or B7.2 (CD86), is able to enhance interleukin-2 (IL-2) synthesis (245,246). One of the key events in T cell proliferation is the activation of the IL-2 gene promoter, which includes consensus binding sites for multiple transcription factors, like AP-1, NF-□B, NFAT and cAMP response element binding protein (CREB). Cooperative interaction between these factors is required for efficient IL-2 production. However, PKB tg CD4+ T cells show optimal proliferation to a ten fold lower TCR signal to that of wt indicating lower threshold of TCR signaling for activation and proliferation.

Expression of an active gagPKB□ construct in T cells was shown to alter T cell homeostasis, indirectly influence B cell homeostasis and to promote inflammation in vivo (175). T cells from transgenic mice expressing a CA-PKB mutant displayed resistance to a variety of apoptotic stimuli (174). PTEN, which inhibits PKB activation, is an essential mediator of the CD95 response and a repressor of autoimmunity, as CD95-mediated apoptosis was impaired in PTEN heterozygous mice, and T lymphocytes from these mice showed reduced activation-induced cell death and increased proliferation upon activation (176, 247). $PTEN^{-/-}$ mice were shown to die during embryogenesis as a result of a failure in

developmental apoptosis (248,249). Surprisingly, ablation of PKBα by itself does not have the severe phenotype, as expected from the phenotypes of PTEN$^{-/-}$ mice or transgenic mice, expressing CA-PKB (126). PKBβ and PKBγ may substitute to some extent for the loss-of-function of PKBα. However, taken together the role of PKB in cell proliferation and survival is rather clear. In this regard, signaling models based on results from Jurkat T-cells may underestimate the role of the PI-3K/PKB pathway as these cells are deficient of PTEN (250,251) and therefore PKB kinase activity is constitutively active.

Our observation concerning cytokine production shows, PKB tg CD4+ T cells not only produce higher IL-2 in the absence of CD28 costimulation, but also produce higher levels of Th1 cytokines like TNF-α and IFN-γ and Th2 cytokines like IL-4 and IL-5 compared to wt CD4+ T cells. Among the Th1 and Th2 cytokines PKB tg CD4+ T cells seem to have a bias towards Th1 cytokines as evident from TNF-α and IFN-γ production (Fig. 11). Implications of this huge production of Th1 cytokines are not yet known.

CD28 co-stimulation was also shown to directly regulate cell cyle entry and progression through the G1 phase in an IL-2-independent manner by downregulation of the cyclin-dependent kinase (cdk) inhibitor p27^{KIP1}. Subsequent progression to the S phase is mediated via both IL-2 dependent and IL-2 independent mechanisms (252). Cell cycle progression is a complex process that is activated by cyclins that associate with catalytically active cdks and is inhibited by cdk inhibitors (253). Induction of D-type cyclins occurs during G1 phase, induction of cyclin E at the late G1 restriction point, induction of cyclin A at the S phase entry and cyclin B1 at the M phase entry. The orderly progression of the cells through the cell cycle is controlled by the timely expression of cyclins, the activation of cdk enzymatic activity and the subsequent phosphorylation of the relevant substrates.

Cell division profile from PKB tg CD4+ T cells in response to TCR stimulation in absence of CD28 costimulation are very similar to the profiles from wt CD4+ T cells stimulated with TCR plus CD28 (Fig. 10A & C). The independence of CD28 costimulation is manifested in the higher levels of phosphorylated cyclins D3 and B1 in myr PKB T cells, which would contribute to faster entry into the G1 and M phase of cell cycle compared to wt cells. PKB has been shown to regulate cell division and cell cycle progression by acting on a number of different molecules involved in these processes. Prominent among them are the cell cycle inhibitors p21^{CIP1} and p27^{KIP1} (162-166). Besides it also regulates the checkpoint kinase 1 and 2 (chk1 & 2) (254,255) and various forkhead transcription factors (234). Forkhead proteins are generally nuclear in resting cells and inhibit cell cycle progression at the M phase. One of the target genes of forkhead transcription factors is the cdk inhibitor

$p27^{KIP1}$ (256). PKB inactivates forkhead proteins by phosphorylation, facilitates their nuclear exclusion and thereby augments entry into M phase and subsequent cell cycle progression. Both $p27^{KIP1}$ and forkhead have been shown to be direct targets of PKB in many different cell systems. Accordingly, we observe phosphorylated forkhead protein (FKHR) in the cytoplasm of resting myr PKB tg but not wt CD4+ T cells.

This observation along with the higher TCR sensitivity, increased cytokine production and increased levels of phosphorylated cyclins indicate that expression of constitutively active PKB replaces CD28 costimulatory signals to a great extent in various T cell functions. Myr PKB induced hyperproliferation when complimented with other cell cycle deregulatory mechanisms could contribute to the development of various forms of cancer.

5.2 Negative regulation of NFAT and NF-κB by myr PKB

The most important finding of this study is that the positive effects of myr PKB on T cell responses are coupled with a negative regulatory role of myr PKB on the nuclear localization of NFAT and NF-κB proteins. As coprecipitation experiments reveal an interaction betweem PKB and NFATc1 in vivo (Fig.15), active PKB could modify this transcription factor either directly or via some substrate associated within a multiprotein complex. To date we do not know whether the functional loss of nuclear accumulation of NFAT proteins is the result of inhibition of nuclear import or enhancement of nuclear export. Mutations in the NLS sequences of NFATs have been reported to reduce their nuclear localization (185,186). Thus, in the simplest scenario, sustained activation of PKB could interfere with unmasking of the NLSs and thereby retaining NFAT in the cytoplasm.

Recently, analysis of the regulation of nuclear shuttling of NFAT proteins has identified a number of NFAT kinases that oppose NFAT nuclear translocation, including GSK3 and the MAPKs, JNK and p38 (197, 198). However, the exact mechanisms of the regulation of NFAT activation and its subcellular localization by these kinases are not completely understood. GSK3 itself is a known target of PKB signaling, and phosphorylation leads to its inactivation (237). Although we detected higher levels of phosphorylated GSK3 in the nucleus of myr PKB T cells compared with wt cells, accumulation of nuclear NFAT was reduced, rather than increased. Considering the data on stronger activation of p38 and JNK in myr PKB T cells, one possibility could be that phosphorylation of NFAT by these kinases, which would foster nuclear extrusion, dominates over nuclear retention signals expected to be provided by inactive GSK3. However, in experiments in which activation of MAPKs and PI-3K was inhibited by pharmacological inhibitors, we did not observe any reversal of the block

in nuclear translocation of NFATc1 in tg T cells (Fig 13B). Thus, the enhanced JNK and p38 activities in myr PKB CD4+ T cells could contribute to the T cell hyper-responsiveness without being involved in the regulation of NFATs.

NFAT proteins are normally thought to be positive regulators of transcription, thereby regulating lymphoid homeostasis (257). However, studies from NFAT knockout mice also document a negative role of NFAT proteins in T cell function (258-261). In this context, in mice doubly deficient in NFATp and NFATc3 Th cells show hyper-responsiveness independent of CD28 coengagement (262), similar to our PKB transgenic system in which hyper-responsiveness of T cells and enhanced production of Th1 and Th2 cytokines are coupled with reduced nuclear NFAT activity. Recently, a direct association of NFATp with the histone deacetylase 1 in regulation of the cdk4 promoter has implicated NFAT in the down-modulation of immune responses when cells return to a quiescent state (263). Furthermore, NFAT has been indicated to be involved in the expression of stage-specific cyclins, unveiling a role for NFAT in the regulation of the cell cycle (264). We therefore propose that by direct interaction, active PKB regulates NFAT localization and thereby partially exerts its positive regulatory role on T cell activation and effector function. The modified regulation of subcellular localization of NFAT and other to date unknown proteins in cells with constitutively active PKB may contribute to the development of T cell lymphomas, as observed in our myr PKB homozygous mice (data not shown) and other reports where overexpression of active PKB is associated with tumor development (247, 265, 266).

Our observations concerning myr PKB's negative regulatory role on NF-κBp65 and RelB (Fig.16A & B) are in contrast with studies in which PI-3K/PKB signaling in transient transfection systems and using PI3K inhibitors was found to enhance NF-κB activation (155, 238, 239, 267). Some of these studies point to a possible role for PI-3K/PKB signaling in activating the IκB kinase complex and NF-κB *trans*-activational activities. Phosphorylation and ubiquitination-dependent degradation of IκBα, the cytoplasmic inhibitor of NF-κB, via the inducible IκB kinase complex (IKK), liberates NF-κB to translocate into the nucleus and to transcriptionally activate its target genes, including the IL-2 gene. In Jurkat T cells, PKB was also shown to regulate signaling pathways that lead to induction of NF-κB. However, PKB was not sufficient by itself to induce NF-κB reporters, since signals from other pathways, in particular phorbol ester, the pleiotropic PKC activator, was found to be required, implicating a functional PKB-PKC connection (238). In T cells the isotype PKC theta is known to selectively activate NF-κB (268, 269). Recently, it has become clear that PKC theta

cooperates physically and functionally with PKB/AKT1 in NF-B signaling in TCR/CD28 stimulated T cells (270). Moreover plasma membrane targeting of PKC theta and PKB exerted synergistic transactivation of the IKK/IB/NF-B signaling cascade independent of T cell activation (270). Along this line, it was shown that PKB can provide the CD28 costimulatory signal for RE/AP activation, a region of the IL-2 promoter, known to be activated by signals that originate from the TCR and CD28, and known to include NF-B binding sites (239).

However, a number of other research groups found no evidence for PI-3K signaling in NF-B activation (271,272). The opposing results probably reflect the use of different experimental systems, constructs, and stimuli. In T cells expressing a transgenic gagPKB construct, IB degradation and DNA binding of NF-B were enhanced compared with those in wt cells (174). We assume that the differences between our myr PKB and the gagPKB transgenic systems result from differences in temporal and spatial expression levels of active PKB. In our transgenic system the role of active PKB seems to entail the down-modulation of certain NF-B and also NFAT responses, rather than enhancing TCR-mediated activation of these proteins. Hence, our findings may reflect differences in the overall activation/differentiation status of myr PKB T cells compared with wt cells or cell lines transiently transfected with active PKB. Our observations thus would reveal the function of PKB in down-modulation of transcriptional activities such as might occur when activated T cells return to a resting state or during specific differentiation processes.

To date we do not know how myr PKB affects NF-Bp65/RelB nuclear translocation, and a variety of different mechanisms can be envisaged. In addition to the regulation via NF-B:IB complexes, it is now evident that NF-B/Rel proteins are regulated post-translationally via phosphorylation (272) or acetylation events (273). Thus, as the overall IB degradation in myr PKB T cells was normal, it is likely that myr PKB or one of its substrates acts downstream of IB degradation to inhibit nuclear translocation of NF-B. In view of altered regulation of NFAT as well as NF-B family members, it may also be possible that myr PKB acts by a similar mechanism on the Rel homology domain, which is common in both classes of transcription factors. NF-Bp50 may not be affected, because its activation depends on processing of a larger precursor protein and also lacks the C-terminal *trans*-activation domain(s) that might be critical.

From a host of studies we know that a delicate balance between positive and negative regulatory mechanisms determines the specificity and magnitude of immune responses. Despite all the above considerations, the novel finding that active PKB is involved in the

negative regulation of nuclear transcription factors highlights the role of PKB in processes related to the inactivation of gene expression programs through the subcellular localization of transcription factors.

5.3 Crosstalk between PKB and calcineurin in modulating early thymocyte development

The precise molecular mechanisms involved in DN3 to DN4 transition during early thymocyte development are poorly understood. Pre-TCR signaling and transition of DN to DP cells has been shown to involve calcium flux and NFAT signaling (95). Based on our previous data that PKB can target NFAT activation (233) in peripheral T cells, here we show that PKB is an essential regulator of calcineurin-NFAT signaling in early T cell differentiation. We concur with the earlier observation of Hayden-Martinez et al. (212) that over-expression of active calcineurin (ΔCam) induces a block at the DN3-DN4 transition with a resultant absence of DP cells (Fig.19A & B). Studies from gene deficient mice show a DN3 to DN4 block could be due to an impairment in pTCR assembly and/or due to a defect in any of the molecules in the signaling pathway immediately downstream to pTCR (242-244, 63, 64, 11, 13, 71, 72). We did not detect any perturbation in CD3ζ chain expression in DN3, DN4 and DP cells arguing against early defects in signaling downstream of the pTCR (Fig. 23A and data not shown). In fact, ΔCam DN cells show higher pERK levels compared to control or double tg cells (Fig. 23B). We show on molecular terms that the severe block at the pre TCR checkpoint is due to defective Rag expression and icTCRβ chain formation (Fig. 24A-C). Since we observed a drastic increase of NFATc1 and NFATc3 expression in DN cells of ΔCam mice, it is obvious that spatial/temporal expression of the individual NFAT members must be tightly regulated during β-selection in order to proceed with normal T cell development.

Notably, we were able to directly link PKB signaling with calcineurin/NFAT activation in early T cell differentiation since simultaneous expression of myr PKB reverted the severe developmental arrest of ΔCam DN3 cells. The rescue of cellularity by PKB is not only restricted to the thymus but also prominent in the peripheral lymphoid organs (Fig.18). Although active PKB likely plays multiple roles in this interplay, one essential outcome of PKB's action is downregulation of NFATc1 and NFATc3 activity in DN cells of ΔCam mice (Figs. 27 & 28). Since in ΔCam DN cells NFATc1 and NFATc2 levels were drastically increased, it seems that either their individual or combined continual activation essentially forbids TCRβ chain rearrangement and hence pre-TCR formation. In this scenario, down-

modulation of calcineurin-induced NFAT activity to appropriate levels by active PKB seems to be a prerequisite to proceed with RAG expression and recombinatorial processes similar to what was observed when ΔCam DN cells were treated with the calcineurin inhibitor CsA.

Calcineurin/NFAT signaling is known to have positive but also negative effects on gene transcription. For instance, negative regulation is found for the CDK4 gene, which is repressed under basal conditions by NFAT (263). Although ΔCam shuts off Rag and TCR chain formation, it simultaneously at least partially signals in differentiation. This is obvious from our observations that despite presumably defective or incomplete pre-TCR signaling, ΔCam DN3 cells show up-regulation of the surface antigens CD2 and CD5 to levels characteristic for DN4 cells. Also ΔCam DN cells express much higher levels of integrins LFA-1 and CD18 compared to control cells (Fig. 21B). Integrins are heterodimeric membrane glycoproteins, which are crucial in the regulation of haemopoiesis and thymic maturation, the direction and control of leukocyte adhesion and migration through tissues and in the development of inflammatory responses (274). Also, integrins, especially LFA-1 has been shown to be an important component of the immunological synapse that is formed between an APC and a T cell. The significance of the expression of such high levels of integrins in ΔCam cells on the overall thymic phenotype needs further experimentation.

The severe block in DN3 to DN4 transition in ΔCam mice does not seem to be because of increased cell death or reduced cell cycle. ΔCam DN3 cells express very high levels of Bcl_2 (Fig. 22A), an active anti-apoptotic molecule which should counteract enhanced cell death. Apoptosis analysis by annexinV staining further clarifies that fact (Fig. 22B). Also there was no significant alteration in the cell cycle status of ΔCam DN cells compared to control mice or the double tg mice (Fig. 22C).

Besides regulation of Rag and TCR chain expression, continuous calcineurin/NFAT activation seems to have additional inhibitory effects since simultaneous expression of the fully rearranged OT1 TCR in ΔCam mice only weakly restored differentiation of ΔCam DN3 cells to the DP stage (Fig. 25). Thus, we propose that independent of its role in regulating expression of pre TCR components, consistently high NFAT activity in DN cells exerts additional inhibitory effects thereby preventing DN3 to DN4 transition. In contrast, active PKB reverted thymocyte differentiation in ΔCam mice almost to normality. This is achieved by controlling the inhibitory NFAT activities and presumably other critical regulators of DN3-DN4 transition, which may involve Notch (275, 276) or Wnt signalling (277, 278) or some other transcription factors.

It has become evident that signals from the pre-TCR bifurcate into those that regulate pre-T cell survival and proliferation and those that induce allelic exclusion, a process in which pre-TCR induced signals terminate further Vβ-Dβ-Jβ rearrangements (50, 55). To date only few molecules are known that are essential or mimic the ability of the pre TCR to terminate TCRβ chain rearrangement, among them the adaptor molecule SLP76 (279) Notch 1 (275, 276) and cytosolic PKD (280). Seen from this perspective, sustained levels of active NFAT in ΔCam mice seem to mimic those part(s) of pre-TCR signaling, which can initiate allelic exclusion and in ΔCam mice therefore lead to premature shut-down of RAG expression and thus inhibition of TCRβ chain formation. Rag expression is regulated at the transcriptional and post-transcriptional level. In T cells c-myb (281) and GATA3 (282) are among the few known transcription factors regulating Rag expression.

PKB's action on NFATs could be direct or indirect via some unknown target proteins. Database analysis for putative PKB phosphorylation sites on NFATs shows multiple phosphorylation sites spanning the calcineurin binding domain and the rel homology domain. We have previously shown that PKB co-precipitates with NFAT, indicating a physical association between the two molecules (233). Thus, PKB by directly interacting with NFAT might phosphorylate single or multiple serine residues critical for NFAT regulation. This seems to be true as invitro kinase assay shows multiple serine phosphorylation on NFATc1 by PKB (S.Y. Na, personal communication). Our data suggest that multiple serine phosphorylation by PKB interferes with proper NFAT activation either by interfering with calcineurin binding, inhibition of dephosphorylation of critical serine residues within the serine/proline rich region of NFAT or interference with proper exposure of the nuclear localization signal. These issues have to be clarified in future experiments.

5.4 NFAT and PKB in tumor development and progression

NFATs can act as positive and negative regulators of growth and proliferation (263, 264, 283, 284). Upon activation and nuclear translocation they can upregulate or downregulate certain sets of gene expression depending on the situation and the context. After antigenic challenge T cells upregulate the expression of a number of genes, among these the well-characterized cytokine genes have been shown to be dependent on NFAT activation. NFATs also regulate the expression of Fas ligand (Fas L) gene once T cells are activated. After the withdrawl of the stimulus most of the activated T cells undergo apoptosis, a process known as activation induced cell death (AICD), in order to maintain T cell homeostasis. Thus, NFAT via upregulation of Fas L expression regulates uncontrolled T cell proliferation. This became

obvious in various NFAT single or double knock out mice, where T cells are hyperreactive and these mice develop various lymphoproliferative disorders (258, 259, 262).

Further, it has been shown that NFAT can control cell cycle by repressing cyclin dependent kinase-4 (CDK4) gene expression (263). In this situation NFAT does not require AP-1 elements but only requires calcium-dependent signals to stabilize its association with the CDK4 promoter. A reduction in acetylation of histone H3 within the CDK4 promoter and a direct association of NFATc2 with histone deacetylase1 (HDAC1) has been observed. This suggests that NFATc2-dependent repression of the CDK4 promoter involves recruitment of an HDAC family member by NFATc2, which displaces E2F family members bound to the CDK4 promoter and modulates the acetylation status of the core histone H3 within the CDK4 promoter (263). HDAC recruitment by NFAT may also contribute to silencing of many other genes.

NFAT is also involved in regulation of various cyclins, which are the critical components of an ordered cell cycle progression. NFATc2$^{-/-}$ T cells are hyperproliferative and this is associated with an altered cell cycle control. These T cells overexpress cyclins A2, B1, E and F upon stimulation. Cyclins A2 and E are expressed at early and late stages of G1 phase of the cell cycle respectively. These two cyclins are implicated in propelling cells to enter into S phase, and cyclin A2 also promotes passage through S into G2 and M phases (285-287). It was reported that overexpression of cyclin E accelerates the G1/S phase transition, which results in the shortening of the cell cycle time (288, 289). This is further supported by the evidence from NFATc2$^{-/-}$ x NFATc3$^{-/-}$ double-knockout mice where it has been shown that the hyperproliferative response of CD4+ T cells is related to a shortened time of cell division (262). Further, cyclin A and cyclin E are overexpressed in some types of lymphoproliferative diseases, such as leukemias (289-291). Thus, dysregulated NFAT activity could have wide ranging effect on normal cellular functioning. Absence of NFAT nuclear activity could lead to uninhibited cellular proliferation and tumorigenesis.

Evidence from a number of studies suggests that PKB perturbations play an important role in human malignancy. The first evidence concerning PKB's involvement in cancers emerged when amplification and overexpression of PKB was detected in human ovarian tumors and cell lines (120). Subsequent studies showed PKB amplification and/or mRNA overexpression in 10-20% of human ovarian and pancreatic cancers (292, 293) and activation of the PKB kinase in nearly 40% of ovarian cancers (294). Amplification of PKB was observed in human gastric cancer (116) and PKB kinase activity is often increased in prostate and breast cancers. Enhanced activity of PKB was found in estrogen receptor-

deficient breast cancer and androgen-insensitive prostate cancer cell lines (295) suggesting that PKB may contribute to the aggressiveness of steroid hormone-insensitive cancers.

The involvement of PKB in diverse tumorigenic activities suggests that PKB activation alone might be sufficient to induce cancer. However, whereas overexpression of myristoylated forms of PKB, PKB, and PKB are strongly oncogenic, wild-type forms of PKB are poorly transforming (124, 296).

Overexpression and overactivity of PKB has also been found in malignancies of the lymphoid system. PKB tg mice (266) or mice lacking PTEN (247) where PKB activity is enhanced, develop lymphoma. Transgenic mice expressing myrPKB from a proximal Lck promoter develop thymomas at an early age, whereas transgenic mice expressing constitutively active Lck-PKBE40K develop primarily tumors of the peripheral lymphoid organs later in life (265). Thymomas arising from myrPKB transgenic mice express high levels of cyclin D3. The delayed appearance of myrPKB and PKBE40K-induced lymphomas suggests that tumor induction by constitutively active PKB transgenes is a multiple-step process.

The ideal case to study whether PKB mediated lymphomas do show a dysregulation in NFAT activity and thereby probably exacerbates lymphoma progression is to analyze the lymphomas that develope in our myr PKB homozygous mice. Both NFAT deficient as well as PKB tg T cells show higher expression of various cyclins indicating alteration of cell cycle in both the situations. Thus, a common key to cell cycle dysregulation in the form of overexpression and overactive cyclins can be envisaged in absence of NFAT and in enhanced activity of PKB, which is exactly the situation in myr PKB transgenic mice. The fact became clear and consistent with our hypothesis and observation in PKB transgenic CD4+ T cells (Fig. 12A & B) so far, as CD4+ T cells from lymphoma show near absence of NFATc1 and NFATc2 in the nucleus (Fig. 29). Thus at least one mechanism responsible for PKB induced lymphomagenesis and progression seems to be a severe dysregulation of NFAT activation and nuclear translocation.

Altogether, our study clearly defined the importance of a serine/threonine phosphatase, calcineurin, and a serine/threonine kinase, PKB, counteracting each other with NFAT as the common substrate and setting the signaling threshold to modulate early thymocyte development. Also we could prove PKB acting as an effective NFAT kinase could spectacularly rescue thymocyte development in ΔCam mice by restoring Rag gene expression and subsequent TCR gene rearrangement and functional TCR chain expression. However, PKB is not enough to reverse it completely as still significantly higher percent of cells are

blocked at the DN3 stage. This could be due to additional problems in extra to lack of rag gene expression and β-selection or impairment of some other signaling molecules or pathways involved in early thymocyte development, which we are currently studying.

PKB mediated negative regulation of NFAT activation, together with T cell hyperreactivity, enhanced cell cycle progression and the concomitant negative regulation of NFAT activation strengthen the point of PKB's involvement in lymphoma or thymoma genesis and progression. This study will shed new lights on our understanding of PKB's role in oncogenesis in the immune system in particular and in other systems in general.

Chapter 6.
References

6. References

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Abbreviations

Ab	antibody
Ag	antigen
APC	antigen presenting cell
AP-1	activator protein 1
7-AAD	7-Amino actinomycin D
bHLH	basic helix-loop-helix
BRCA-1	breast cancer susceptibility gene product 1
BSS	balanced salt solution
BSA	bovine serum albumin
Ca^{2+}	calcium ion
cAMP	cyclic AMP
CBP	cAMP binding protein
CBA	cytokine bead array
CD	cluster of differentiation
CDK	cyclin dependent kinase
CE	cytoplasmic extract
CFSE	5-(and-6)-carboxyfluoresceinediacetate succinimidyl ester
CIS	cytokine-induced Src homology 2 protein
CK	casein kinase
CN	calcineurin
CsA	cyclosporin A
cRPMI-1640	complete RPMI-1640 medium
CREB	cAMP response element binding protein
CTL	cytotoxic T-lymphocyte
cTEC	cortical thymic epithelial cell
DAPI	4'-6-Diamidino-2-phenylindole
DBD	DNA binding domain
ΔCam	calcineurin mutant, deleted autoinhibitory domain
DC	dendritic cell
DN	double negative
DP	double positive

DTT	dithiothreitol
ELISA	enzyme linked immunosorbent assay
ERK	extracellular signal regulated kinase
eNOS	endothelial nitric oxide synthase
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
FKHR	forkhead transcription factor
FSC	forward scatter
GATA3	GATA associated protein 3
GNEF	guanine nucleotide exchange factor
GSK3	glycogen synthase kinase 3
HDAC	histonedeacetylase
HEB	human E-box binding factor
HSA	heat stable antigen
hTERT	human telomerase catalytic subunit gene
ic	intracellular
Id	inhibitor of DNA binding
IFN-□	interferon gamma
I□B	inhibitor of NF-□B
IKK	I□B kinase
IL	interleukin
ILK	integrin-linked kinase
IP	immunoprecipitation
JNK	c-jun N-terminal kinase
ko	knock out
LFA-1	leukocyte function antigen-1
LEF-1	lymphocyte enhancer factor-1
MHC	major histocompatibility complex
mTOR	mammalian target of rapamycin
NE	nuclear extract
NES	nuclear export signal
NFAT	nuclear factor of activated T cell
NF-□B	nuclear factor □B
NHR	NFAT homology region

NK cell	natural killer cell
NLS	nuclear localization signal
NTOC	neonatal thymic organ culture
o.n.	over-night
PBS	phosphate buffered saline
PDE-3B	phosphodiesterase-3B
PDK1	phosphoinositide dependent kinase1
PFK	phosphofructokinase
PI-3K	phosphatidylinositol 3-kinase
PI	propidium iodide
PKB	protein kinase B
PKA	protein kinase A
PMA	phorbol 12-myristate 13-acetate
Ptd(3,4,5)P3	phosphatidylinositol 3,4,5-trisphosphate
RAG1	recombinase activating gene-1
RHR	rel homology region
RT	room temperature
SAPK	stress mediated MAP-kinase
SCL	stem cell leukemia
SOCS	suppressor of cytokine signaling
SP	single positive
TCF-1	T cell factor-1
TCR	T-cell receptor
tg	transgenic
TGF	transforming growth factor
Th1	T helper type 1
Th2	Thelper type 2
TNF-	tumor necrosis factor
WCE	whole cell extract
wt	wild type
ZAP70	zeta associated protein 70

List of Figures and Tables

List of Tables

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Oral and Poster presentations

1. **Amiya K. Patra,** S.Y. Na, Y. Scheuring, D.Kiousis, T. Hunig and U. Bommhardt: Protein kinase B provides co-stimulation independent of CD28 in T lymphocyte activation/proliferation. Oral and poster presentation in the workshop on "Lymphocyte activation" at 33rd annual meeting of the German society of Immunology (DGFI), September 25-28, 2002, Marburg, Germany.
2. **Amiya K. Patra,** Shin-Young Na and Ursula Bommhardt: Active Protein Kinase B (PKB) Regulates nuclear localization of NFAT proteins in T lymphocytes. Poster presentation at 2nd international conference on "Strategies for Immune therapy", February 29-March 3, 2004, Wurzburg, Germany.

Declaration

I hereby declare all the above informations are true to the best of my knowledge.

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Vorträge und Poster-Präsentationen:

1. **Amiya K. Patra,** S.Y. Na, Y. Scheuring, D.Kiousis, T. Hünig and U. Bommhardt: „Protein kinase B provides co-stimulation independent of CD28 in T lymphocyte activation/proliferation“. Vortrag und Poster-Präsentation im Workshop „Lymphocyte activation” bei der 33. Jahrestagung der Deutschen Gesellschaft für Immunologie (DGFI) vom 25.-28. September 2002 in Marburg.
2. **Amiya K. Patra,** Shin-Young Na and Ursula Bommhardt: „Active Protein Kinase B (PKB) Regulates nuclear localization of NFAT proteins in T lymphocytes“. Poster-Präsentation im Rahmen der 2. Internationalen Konferenz „Strategies for Immune therapy”, 29. Februar bis 3. März 2004 in Würzburg.

Ehrenwörtliche Erklärung

Ich erkläre hiermit die Richtigkeit der obigen Angaben nach bestem Wissen und Gewissen.

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List of Publications

1. **Amiya K. Patra**, S.Y. Na, Edgar Serfling, Thomas Hunig and Ursula Bommhardt: Protein Kinase B rescues thymic phenotype by regulating calcineurin-NFAT signalling during early thymocyte development (manuscript in preparation).

2. **Patra, A. K.**, S. Y. Na, U. Bommhardt. 2004. Active protein kinase B regulates TCR responsiveness by modulating cytoplasmic-nuclear localization of NFAT and NF-□B proteins. *J. Immunol. 172:4812.*

3. Na, S. Y., **A. Patra**, Y. Scheuring, A. Marx, M. Tolaini, D. Kioussis, B. Hemmings, T. Hunig, U. Bommhardt. 2003. Constitutively active protein kinase B enhances Lck and Erk activities and influences thymocyte selection and activation. *J. Immunol.171:1285.*