PKB/Akt: A Critical Regulator of Lymphocyte Development and Function

Dissertation zur Erlangung des naturwissenschaftlichen Doktorgrades der Bayerischen Julius-Maximilians-Universität Würzburg

vorgelegt von
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aus
Seoul, Korea

Würzburg, 2005

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April 2005

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Acknowledgements

I am pleased to acknowledge and thank the many people who have helped and encouraged me during my PhD time. First of all I would like to thank Dr. Ursula Bommhardt for supervising my work and for giving me the opportunity to carry out my doctoral work in her lab, as well as for her support and personal care.

I am very thankful to Prof. Thomas Hünig, who gave me the chance to study in the Institute for Virology and Immunobiology, for many useful discussions and for his generous help.

I would like to thank Prof. Jörg Hacker for accepting to be my co-supervisor at the faculty of biology and his support with all the formalities concerning the doctoral thesis.

Thanks to Prof. Annelise Schimpl, for helpful discussions and advice and to Prof. Lars Nitschke, for help regarding B cell study.

I am delighted to thank Amiya for his help and fruitful co-operation and also to Yvonne Scheuring for helping me with kindness. I thank Sonja, Nicholas, Snighda, Anja and Caro for their help and the good times we had together in the lab. I also thank Soon-Hwan and Hee-Young who made my life enjoyable in Germany.

Special thanks to Thorsten for translating my summary into German and for giving me a delightful time in Germany. I also thank my colleagues in other labs in the Institute.

I'm very grateful to Dr. Jung-Hyun Park for his support and encouragement during this time.

I reserve the greatest thanks for my family, for their unlimited love and support.

Contents

1. Introduction

The immune response is divided into two branches: innate immunity, which comprises non-specific defence mechanisms that include physical barriers such as skin and phagocytic cells that attack foreign antigen in the body and adaptive immunity, which comprises antigen specific immune responses. Innate immunity involves the activation of granulocytes, macrophages, NK cells, and is initiated by the production of inflammatory cytokines. In the adaptive immune response, antigen specific lymphocytes T and B cells, proliferate in response to specific antigen and differentiate into T effector cells or antibody producing plasma cells that eliminate the invading pathogen.

1.1 T cell development

The thymus is divided into an outer cortex, where most of T cell differentiation takes place, and an inner medulla, where newly formed T cells undergo final maturation before exiting the thymus and seeding peripheral lymphoid organs. The thymus does not contain self-renewing hematopoietic stem cells. To maintain thymopoiesis in the adult, lymphoid progenitor cells reach the thymus from the blood stream and enter at the cortex-medullary boundary. These cells lack most of the surface molecules characteristic of mature T cells, and their T cell receptor (TCR) genes are still unrearranged. When these progenitor cells begin to express CD2, they are double negative (DN) for CD4 and CD8 expression. DN thymocytes can be further subdivided based on CD44 and CD25 cell surface expression: DN1, CD44+CD25-; DN2, CD44+CD25+; DN3, CD44-CD25+; and DN4, CD44-CD25-. DN thymocytes can give rise to either $\alpha\beta$ or $\gamma\delta$TCR expressing cells. Cells that proceed along the $\gamma\delta$TCR lineage express the so-called pre-TCR at the DN3 stage, which consists of a pre-TCRα and TCRβ chain, the latter of which is produced by DNA rearrangements that require expression of Recombination Activating Gene 1 (RAG1) and RAG2 proteins. The pre-TCR is expressed on the cell surface in association with CD3 molecules that provide the signalling components of T cell receptors (1). Signalling from the pre-TCR/CD3 complex leads to cell proliferation, arrest of further TCR β-chain gene rearrangements (β-chain allelic exclusion), and the expression of CD8 and CD4 molecules, a process known as β-selection. CD4/CD8 double positive (DP) T cells re-express RAG1 and RAG2 to rearrange TCRα chain genes, which replace the pre-Tα chain to form a functional $\alpha\beta$TCR. The $\alpha\beta$TCR associated CD3 complex consists of two CD3$\varepsilon\gamma$ and $\varepsilon\delta$-chains, and one $\zeta\zeta$ and $\eta\eta$ chain.

Maturation of thymocytes from the DP to the CD4+ or CD8+ single-positive (SP) stage requires signals generated by the concerted interaction of surface TCRs and CD4 or CD8 coreceptors with appropriate MHC/self-peptide molecules (2, 3). DP thymocytes undergo one of the three cell fates: death by neglect, positive selection or negative selection. Death by neglect is a passive form of cell death caused by the failure of a clonotypic □□TCR to engage peptide-MHC ligands. These cells fail to undergo positive selection and die because they do not receive a survival signal. Approximately 5-10% of all DP thymocytes survive these selection processes and mature to SP T cells that are exported from the thymus to form the peripheral T cell repertoire.

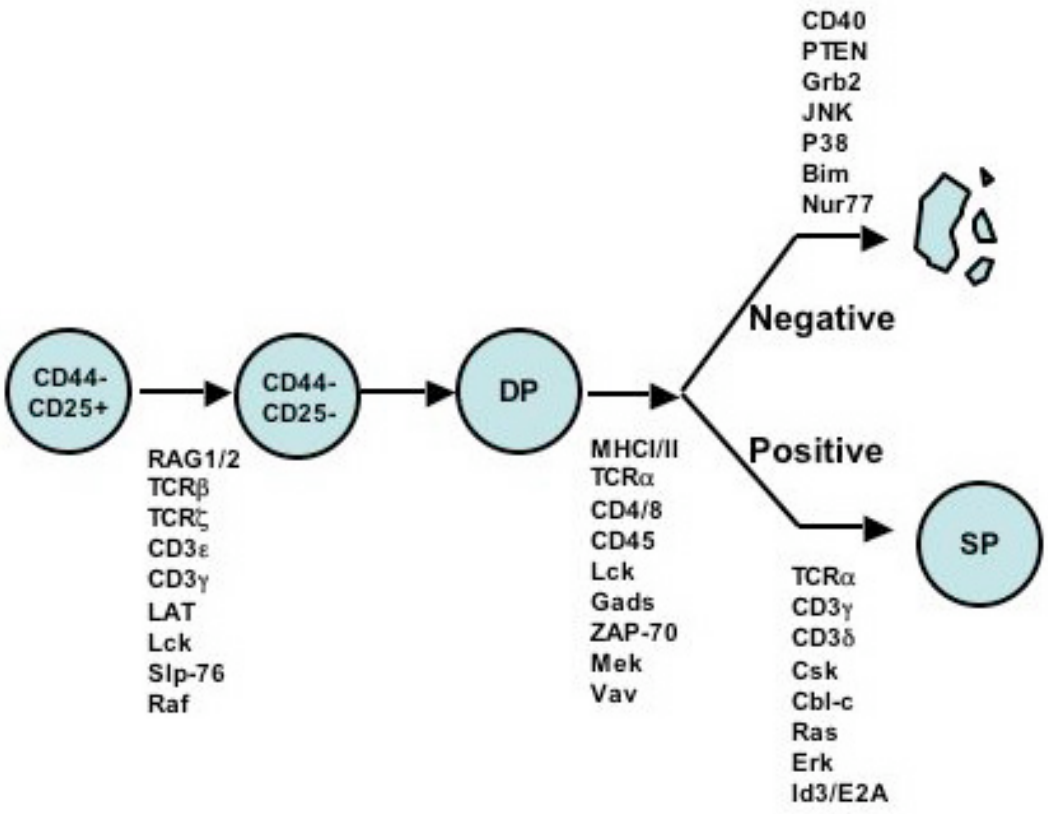

Fig 1.1 Selected molecules identified as critical regulators of selection checkpoints in T cell development (adapted from Annu.Rev.Immunol. 2003 vol.21).

Positive selection occurs when DP cells bind MHC class I or class II molecules plus self-peptides presented by cortical epithelial cells with an affinity high enough to get a survival signal. The transition from the DP to the SP stage is also accompanied by commitment to either the CD4 or CD8 T cell lineage, a decision influenced by the specificity of the TCR for MHC

and appropriate MHC-coreceptor match. For example, transgenic mice constitutively expressing MHC class I restricted TCRs or those expressing MHC class II restricted TCRs develop mainly CD8+ or CD4+ T cells, respectively.

Two main models, stochastic and instructional, have been proposed to explain how MHC specificity and coreceptor expression are linked during lineage commitment (4, 5). The stochastic model proposes that commitment to the CD4 or CD8 lineage occurs independently of TCR specificity for MHC. Expression of either CD4 or CD8 is terminated stochastically. Since MHC recognition by a specific TCR usually requires coengagement of class I molecules by CD8 and of class II molecules by CD4, only cells with an appropriate match of TCR and coreceptor for MHC will then mature in a second step.

The instructional model proposes that the recognition and coengagement of class I MHC by a specific TCR and CD8 activates a differentiation program that includes the turn-off of CD4, thus generating a CD8+ T cell, whereas engagement of TCR and CD4 co-receptor with MHC II shuts off CD8 expression.

The "strength of signal" model proposes that commitment to the CD4 versus CD8 lineage depends on the intensity of signalling from the TCR (6-8). CD4 coreceptor signals would contribute a "strong" signal and induce the generation of CD4+ T cells, whereas the "weaker" signals delivered by the CD8 coreceptor would result in the generation of CD8+ T cells. Furthermore, it has been shown that a short duration signal leads to the CD8 lineage, whereas more prolonged signals lead to the CD4 lineage ("kinetic model"). This results from the fact that CD4 molecules are more extensively associated with the lymphoid specific protein kinase Lck than CD8 molecules (9-11).

The Src family protein tyrosine kinase Lck is expressed mainly in T-lineage cells and throughout T cell development (12). It associates noncovalently with the cytoplasmic tails of the CD4 and CD8 coreceptor molecules and becomes catalytically activated when the coreceptors are cross-linked (11, 13, 14). Lck also enhances signals mediated through the TCR/CD3 complex (15), although a direct physical association between Lck and the TCR/CD3 complex has not been established. In mice, targeted disruption of the Lck gene, or transgenic expression of catalytically inactive Lck under the control of the *Lck* proximal promoter (which drives transgene expression in DN and DP thymocytes), interferes with both cellular expansion and allelic exclusion at the TCR chain gene locus during the transition from the DN to the DP stage (16, 17). Several Src and Syk family kinase proteins proximal to TCR signalling are critical in positive selection. Besides its critical role in -selection, the importance of Lck in positive selection has been established by temporally controlled expression of a dominant

negative and constitutively active form of Lck (18, 19). Several proteins negatively regulate TCR signalling at a proximal point in the signalling pathway. Some of these, including c-Cbl, SLAP, and Csk, oppose the process of positive selection, and their deficiency results in enhanced positive selection. c-Cbl and SLAP appear to regulate surface TCR levels and thereby influence TCR signalling (20-22). Csk is a kinase that negatively regulates Lck activity. Deficiency in Csk resulted in positive selection of mature CD4+ T cells in the absence of MHC or even a surface TCR, highlighting the importance of Lck in initiating TCR signals in the thymus (23). The ZAP-70 tyrosine kinase contains two N-terminal SH2-domains that bind phosphorylated ITAM motifs in the antigen receptor subunits and is activated by Lck. The phenotype of ZAP-70 deficient mice showed strongly impaired positive selection (24). One of the main targets of ZAP-70 kinase activity is the adaptor protein LAT (linker for activation of T cells), which is assumed to be critical for positive selection. However, since LAT deficiency results in profoundly impaired β-selection and arrest at the DN stage, LAT's role in positive selection is still obscure (25).

Positive selection is initiated by α/βTCR ligation of low affinity self-peptide/MHC complexes, thus structural components of the TCR are required. Indeed, TCRβ chain-deficient mice do not develop past the DP stage. The role of TCRζ, CD3γ, CD3δ, and TCRη in positive selection was more difficult to study because deficiency in these genes resulted in an early block in T cell differentiation before the DP stage (3, 26-29). CD3ζ-deficiency did not impair the generation of DP cells but profoundly blocked positive selection (27, 30). In CD3ζ-deficient mice activation of the MAP-kinase Erk was severely impaired, but activation of the MAP kinase p38 and JNK was unaffected. In mice restored with CD3ζ molecules that lacked the cytoplasmic tail, both positive selection and Erk activation were restored. This suggests that the extracellular and transmembrane regions of CD3ζ are critical for TCR signalling in positive selection.

Several recent studies have shown an important role for Notch signalling at various stages of T cell development (31). Notch1 signalling has been shown to promote the development of $\alpha\beta$ T lineage cells over that of the $\gamma\delta$ T cell lineage, to regulate rearrangement of the TCR-β gene (32), and to influence the CD4 versus CD8 T cell fate decision (33, 34).

Negative selection occurs when DP T cells bind to bone marrow derived APCs expressing MHC class I and class II plus self-peptides with high affinity to receive an apoptotic signal. Studies with superantigen-reactive thymocytes first showed that clonal deletion occurs at the

DP to SP transition (35, 36). For thymocytes that express MHC class II restricted TCRs, cells that are sensitive to negative selection can be found within SP thymocytes that express high levels of the heat stable antigen CD24 (37) and are considered to be semi-mature SP medullary thymocytes, which proliferate after antigen exposure. A recent study identified NUR77, an orphan steroid receptor, as a marker for clonal deletion (38). NUR77 expressing cells are found in the DP and SP stage and their numbers are increased among thymocytes undergoing negative selection.

Co-stimulatory molecule can influence apoptosis of DP thymocytes as monoclonal antibodies specific for CD28, CD5 and CD43 could enhance the apoptosis of semi-mature (CD4+CD8-$CD24^{hi}$) cells. Consistent with these finding is the observation that clonal deletion was reduced in CD28- or CD43-deficient neonatal mice that had been injected with the superantigen staphylococcal enterotoxin B (SEB).

After engagement of the TCR by the appropriate peptide-MHC complex, which triggers clonal expansion, CD4+ helper T (Th) cells rapidly undergo programmed differentiation. Naïve Th cells can differentiate into at least two functional subsets of cells during an immune response: Th1 cells, which mainly secrete IFN, and Th2 cells, which secrete IL-4, IL-5, IL-10 and IL-13 (39, 40). Th1 cells are responsible for cell-mediated immunity, whereas Th2 cells are responsible for humoral immune responses. Both Th cell subsets have been implicated in pathological responses. Th1 cells can mediate organ-specific autoimmunity and Th2 cells have been implicated in the pathogenesis of asthma and allergy.

In addition to the differentiation of CD4+ effector Th1 cells, adaptive Th1-type immune responses rely on the generation of a CD8+ effector T-cell pool. Following activation, naive CD8+ T cells undergo antigen-driven terminal differentiation in the periphery. Recent studies have shown that the transitions from naive to effector and effector to memory CD8+ T-cell populations are associated with marked changes in gene expression. The naive, antigen-inexperienced CD8+ T-cells undergo genetic remodelling that results in the expression of signature genes central to CD8+ effector T-cell function, including genes that encode cytokines and chemokines, and genes associated with cytolysis. IFN and tumour-necrosis factor (TNF) are the main cytokines produced by differentiated CD8+ effector T cells, and IFN has been shown to have a fundamental role in CD8+ T-cell-mediated immunity.

1.2. B cell development

B cell development is a highly regulated differentiation process whereby functional peripheral B cell subsets are produced from hematopoietic stem cells, in the fetal liver before birth and in the bone marrow (BM) afterwards. The BM contains B lineage cells of all stages of development, from earliest progenitors to mature recirculating B cells. The stages in primary B cell development are defined by the sequential rearrangement and expression of heavy- and light-chain immunoglobulin (Ig) genes. The earliest B lineage cells are known as pro-B cells, progenitor cells committed to the B-cell lineage, i.e., rearrangement of the Ig heavy chain locus takes place in pro-B cells. D_H to J_H joining at the early pro-B cell stage is followed by V_H to D_{JH} joining at the late pro B cell stage. Productive VDJ_H joining leads to the expression of an intact μ heavy chain, which is the hallmark of the next stage of development, the pre-B cell stage. The μ chain in large pre-B cells is expressed intracellular and possibly in small amounts at the cell surface, in combination with a surrogate light chain, forming the pre-B cell receptor (pre-BCR). Expression of the pre-BCR signals the cell to halt rearrangement of the heavy chain locus and production of the surrogate light chain, and to divide several times before giving rise to small pre-B cells, in which light chain rearrangement begins. Once a light chain gene is assembled and a complete IgM molecule is expressed on the cell surface, the cell is defined as an immature B cell. All development up to this point takes place in the bone marrow and is independent of antigen. Immature B cells undergo selection for self-tolerance and subsequently for the ability to survive in the peripheral lymphoid tissue. B cells that survive this selection undergo further differentiation to become mature B cells that express IgD in addition to IgM molecules.

As B cells develop from pro-B cells to mature B cells, they express surface proteins that are characteristic for each stage of differentiation. One of the first identifiable proteins expressed on the cell surface of B-lineage cells is CD45R (B220) (41, 42). Another protein that is expressed throughout B cell development is CD19, which acts as a costimulator in BCR signalling.

Circulating transitional B cells rapidly exit the blood stream and enter the peripheral lymphoid organs, most predominantly the spleen, which is the major site for final maturation of early peripheral B cells. There are at least two major subsets of transitional B cells, transitional type 1 (T1) and type 2 (T2) cells. Within the pool of mature B cells in the spleen, three main types of B cells can be defined: follicular (FO), marginal zone (MZ), and B-1 cells. Although the most important part of B cell development takes place in the BM, the spleen is also a critical site for B cell selection.

Crosslinking of the BCR induces Src family kinase-mediated tyrosine phosphorylation of BCR associated Ig and Ig molecules, followed by the recruitment and activation of the tyrosine kinase Syk. Syk in turn contributes to the tyrosine phosphorylation of specific integral membrane proteins, the most prominent being B-cell adapter for phosphoinositide 3-kinase (BCAP), membrane adaptor protein CD19, and linker for activation of B cells (LAB) (43-45). BCAP and, probably to a lesser extent, CD19 mediate phosphoinositide 3-kinase (PI3K) activation by recruiting the p85 subunit of PI3K via Src homology 2 (SH2) domain-phosphotyrosine interaction. Knockout mice for CD45, BCAP, Btk, BLNK, or PLC-2, all reveal the loss of B1 cells and of $IgM^{lo}IgD^{hi}$ mature FO B cells (46-49). MZ B cells are, however, spared.

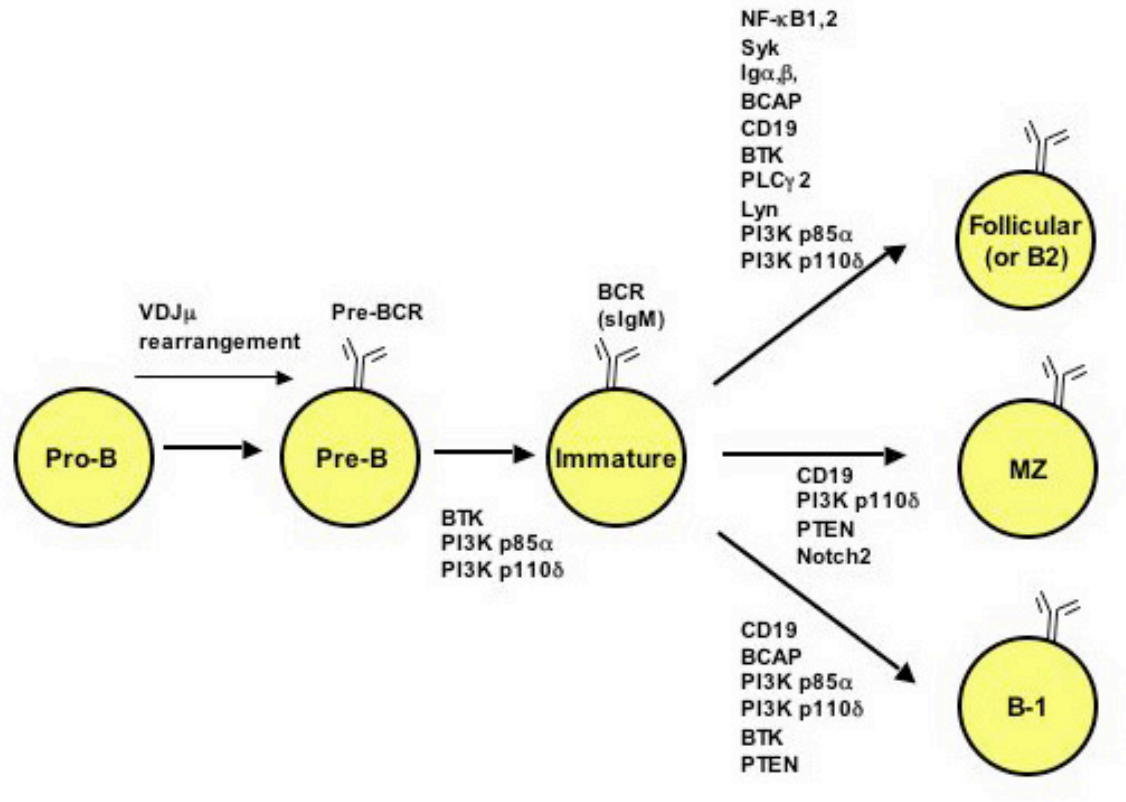

Fig 1.2 An overview of B cell development (adapted from Nature Reviews 2003 vol.3).

1.2.1 Follicular and marginal zone B cells

Mature FO B cells ($IgD^{hi}IgM^{lo}CD23^{+}CD21^{lo}$) are relatively short-lived cells with a half-life of around 2-3 months. In conditional B-cell specific Rag1 deficient mice, a fairly rapid decline in the FO B cell number was noted, whereas the number of MZ B cells remained virtually unchanged up to a year later (50). Thus it is presumed that MZ B cells ($IgM^{hi}IgD^{lo/-}CD23^{-}CD21^{hi}CD1d^{hi}$) are self-renewing. While FO B cells probably contribute to most T-dependent

germinal center (GC)-based responses that originate in spleen, lymph node and Peyer's patches, MZ B cells are presumed to be critical for T-independent responses to blood-borne pathogens (51). Because MZ B cells express high levels of B7-1 and B7-2, the ligands for CD28 on T cells, they might also present blood-borne antigens to naïve T cells and, thus possibly contribute to T-dependent responses (52). However, it has not been formally established whether MZ B cells can actually contribute to the generation of germinal center B cells.

Conditional deletion of Notch-2 (53) or of RBP-J (54), a downstream transcription factor in the Notch signalling pathway, both reveal a defect in MZ B-cell development with an increase in mature FO B cells. An increase in MZ B cells was observed in mice lacking Msx2-interacting nuclear target protein (MINT), a vertebrate antagonist of the Notch pathway (55). These data show, as found for immature T cells, Notch signalling can influence the lineage decision of immature B cells.

1.2.2 B-1 B cells

B-1 B cells can be distinguished from all other B cells by their surface phenotype. Originally, B-1 cells were identified by their expression of CD5. Subsequently, a population of peritoneal CD5 negative B cells was identified whose surface phenotype was in other respects identical to that of B-1 cells. In contrast to recirculating FO (or B-2) cells, they are CD45 ($B220^{low}$), IgM^{hi}, CD23-, CD43+, and IgD^{low}. They are also larger and exhibit more side scatter than do B-2 cells. In the peritoneal cavity, but not in the spleen, B-1 cells express C3 (CD11b, Mac-1). B-1 cells are absent from peripheral lymph nodes and variably make up about 5% of splenic B cells. However, they constitute a substantial fraction of B cells in the peritoneal and pleural cavities (56, 57). B-1 cells share many phenotypic characteristics of MZ cells and, like them, appear to develop in response to T independent antigens. It is clear that BCR signalling is involved in the enrichment of certain B-1 and MZ B cell clones, since this process is impaired in Xid-, CD19- and CD45-deficient mice (48). B-1 cells are essential for resistance to several pathogens and they play an important role in mucosal immunity.

1.2.3 Plasma cell differentiation

The mature effectors of the B-cell lineage are terminally differentiated non-dividing plasma cells (PCs), dedicated to secreting large amounts of immunoglobulins (58). Antigen, in combination with other signals, triggers naïve B cells in the splenic marginal zone (MZ) to proliferate and differentiate into mainly short-lived PCs secreting low affinity IgM as the first Ig response to pathogens. Subsequently, antigen and antigen specific Th cells cause naïve

follicular B cells to undergo proliferation, affinity maturation and isotype switch recombination in a GC reaction. This reaction ultimately produces PCs secreting high affinity antibody with predominantly switched isotypes (59). Transcription factors such as B-lymphocyte-induced maturation protein 1 (Blimp-1) and X-box binding protein 1 (XBP-1) are required for plasma cell development. They regulate sets of genes that induce immunoglobulin secretion, halt proliferation and block alternative B-cell fates.

Upon activation, mature B lymphocytes may undergo class-switch recombination (CSR) to produce a single, specific Ig isotype, which may include IgA, IgE, or one of the IgG subclasses. Although many extracellular signals play prominent roles in this process, cytokines such as IL-4, IFN□ and transforming growth factor □(TGF-□) appear to play particularly critical roles in B cell differentiation in part by directing the isotype specificity of CSR. For instance, IL-4 directs murine IgE and IgG1 isotype production by activating transcription factors such as STAT6, which bind to and transactivate the germ-line C□ and C□1 promoters (60). Isotype specificity of CSR presumably is determined at least in part through the regulation of these resulting sterile, germ-line RNA transcripts, which presumably make the target isotype locus accessible to the CSR machinery (61). Similarly, the cytokines IFN□ and TGF-□ are thought to regulate B cell CSR to the IL-4-independent IgG isotypes: TGF-□ appears to selectively stimulate CSR to IgG2b (62), whereas IFN□ appears to selectively stimulate CSR to IgG2a (63) but plays a controversial role in the regulation of IgG3 (64).

1.3 T cell and B cell receptor mediated signalling

To develop effector functions, lymphocytes need to be activated by their natural ligands. This process requires at least two stimuli, a primary stimulus, which is mediated by the immunoreceptor, and a costimulatory signal, which is mediated by accessory receptors (e.g. CD28 on T cells and CD19, FcRs on B cells).

T cell receptor signalling is implemented by the Src family kinase Lck, which is constitutively associated with the cytoplasmic domains of the co-receptor molecules CD4 and CD8 and Fyn, which associates with the cytoplasmic domains of CD3□ and CD3□ chains upon receptor clustering. Once Src family kinases are activated they in turn phosphorylate tandem tyrosine residues within specialized signalling motifs termed ITAMs (immunoreceptor tyrosine based activation motifs) (65). ITAMs are present in the cytoplasmic domains of CD3□, -□, and –□, and the TCR□-chains. Phosphorylated ITAMs provide the docking sites for the tandem SH2 domains of the Syk family protein tyrosine kinase (PTK) ZAP-70 (□-chain associated protein),

which is thereby targeted to the cell membrane and subsequently becomes activated through phosphorylation by Lck. ZAP-70 phosphorylates the adaptor proteins LAT and SLP-76, which then leads to the activation of phospholipase C-γ1 (PLC-γ1) by Tec kinases and the activation of Ras by GEFs (guanine-nucleotide exchange factors). The Ras-induced kinase cascade induces and activates Fos, a component of the AP-1 transcription factor. Activated PLCγ1 cleaves the membrane associated phosphatidylinositol 4, 5-bisphosphate (PIP_2), thus generating the second messengers inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). IP_3 increases intracellular Ca^{2+} levels, which leads to activation of the phosphatase calcineurin, which in turn activates the transcription factor NFAT (nuclear factor of activated T cells). DAG represents a classical activator of conventional and novel protein kinase C (PKC) isotypes, which activate the transcription factors NFκB and AP-1 (66, 67). The transcription factors NFκB, NFAT, AP-1 and others induce specific gene transcription, leading to cell proliferation and differentiation.

Signalling from the B cell receptor complex depends on the presence of ITAMs in the Igα and Igβ chains. After antigen ligation, tyrosines within these ITAMs become phosphorylated by the receptor associated Src family tyrosine kinase Lyn. The phosphorylated ITAMs bind with high affinity the protein tyrosine kinase Syk. Recruitment of Syk to phosphorylated ITAMs results in its activation, which facilitates the initiation of several different signalling pathways. Although Syk-deficient B cells have a profound defect in BCR-mediated activation of downstream signalling pathways, Src-family PTK activation and Igα/β phosphorylation are relatively intact (68). Thus, Syk is essential to couple the BCR to distal signal transduction elements. After binding to the phosphorylated ITAMs of the Igα chains Syk phosphorylates a number of other targets, CD19, BLNK (SLP65), PLCγ2, GEFs and Tec. The adaptor protein BLNK helps to recruit Tec kinases, which in turn phosphorylate and activate the enzyme PLCγ2. The BCR as well as TCR induced signalling cascade can be negatively regulated via the transmembrane adaptor protein PAG (phosphoprotein associated with glycosphingolipid enriched microdomains), which recruits the Src kinase Csk, a negative regulator for Src kinases.

1.4 TGF-β signalling

Transforming growth factor β (TGF-β) has a major role in cell proliferation, differentiation and apoptosis in many cell types. TGF-β modulates gene transcription through receptor-mediated activation of the Smad proteins, which are transcriptional activators that transmit the signal from the cell surface to the nucleus (69). TGF-β binding induces the formation of a

serine/threonine kinase complex that is composed of the TGF-β type I receptor (TβRI) and type II receptor (TβRII). Smad2 and Smad3 are specifically recognized and phosphorylated by TβRI and the closely related activin and nodal type I receptors, whereas Smad1, Smad5 and Smad8 are primarily recognized by morphogenetic protein type I receptors. Smad2 and Smad3 can interact with Smad4 and translocate to the nucleus, where they regulate the transcriptional response of TGF-β (70). Smad proteins remain in the nucleus only for the duration of the TGF-β stimulus. They seemingly undergo repeated cycles of dephosphorylation, shuttling back to the cytoplasm, rephosphorylation and re-entry into the nucleus (70, 71).

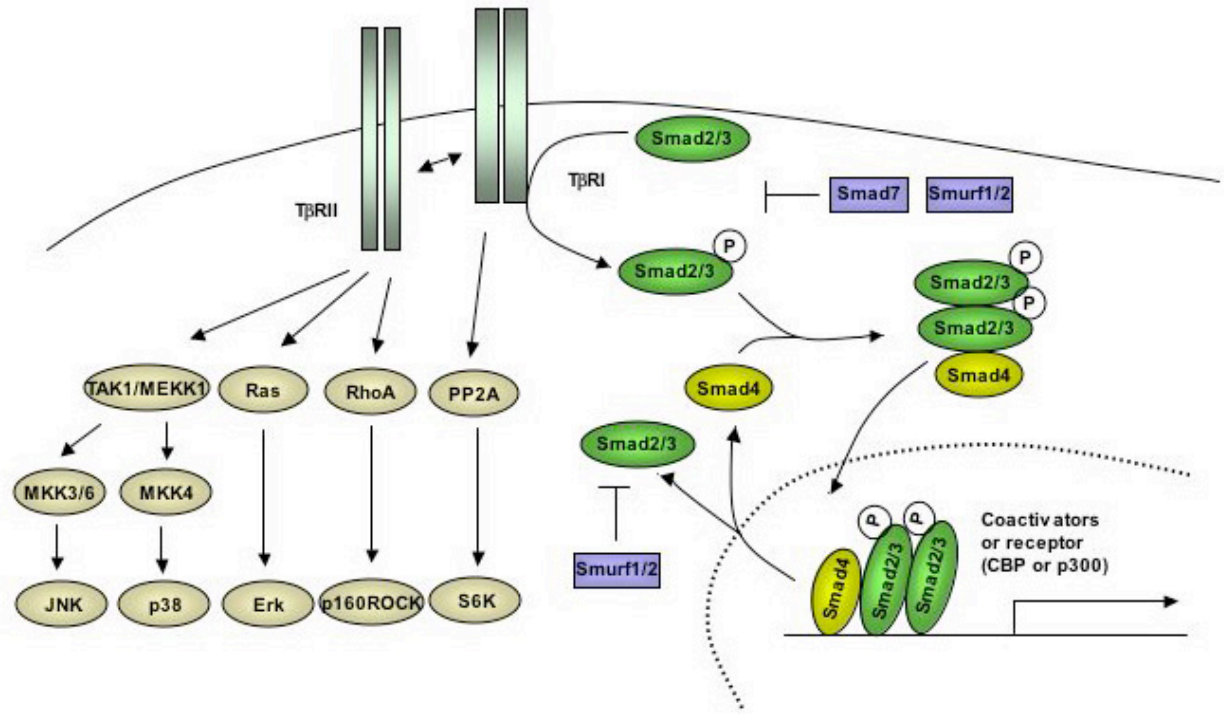

Fig 1.3 TGF-β receptor signalling through Smad-dependent or independent pathways.

Smad6 and Smad7 regulate activation of Smad2/3 (72, 73). Smad6 and Smad7 inhibit TGF-β family signalling through binding of their MH2 (MAD homology) domains to the type I receptor, thus preventing recruitment and phosphorylation of effector Smads (74, 75). Smad6 also interferes with Smad4, preventing the formation of an effector Smad complex. In addition, recruitment of a complex of Smad7 with Smurf1 or Smurf2 to the type I TGF-β receptor results in receptor ubiquitination by the Smurf proteins and targets the receptor for degradation (76, 77), possibly leading to inhibition of Smad2/3 activation.

Besides Smad–mediated transcription, TGF-β activates other signalling cascades, including the Erk, JNK and p38 MAPK pathways (78, 79). Studies using Smad4-deficient cells or dominant-

negative Smads support a notion that TGF-β-induced MAPK pathway activation is independent of Smads (78). In addition, mutated TGF-β type I receptors defective in Smad activation can activate p38 MAPK signalling in response to TGF-β (80). The molecular mechanisms of Erk, JNK or p38 MAPK activation by TGF-β and the biological consequences are however still poorly characterized.

1.5 PI3K-PKB signalling

Phosphatidylinositol-3 kinase (PI3K) signalling has been found in different cell systems to be fundamental in regulation of cell growth, differentiation, survival and adhesion/migration. In resting cells PI3K is a cytoplasmic enzyme, however its substrate are membrane localized phospholipids. Antigen receptor crosslinking initiates a cascade of tyrosine phosphorylation events of a number of kinases and adaptor protein, which also lead to recruitment of PI3K to the proximal signalling complex. These interactions lead to the increase of PI3K enzyme activity and position the lipid kinase near its substrates to allow local production of PI3K products, termed phosphatidylinositol 3,4,5-trisphosphate (PIP3). These lipids then promote further assembly of signalling complexes at the membrane by recruiting specific proteins harbouring domains that selectively bind 3-phosphoinositides. This membrane targeting signal is reversible and is opposed by specific lipid phosphatases, including PTEN (phosphatase and tensin homologue deleted on chromosome ten) and SHIP (SH2 domain-containing inositol 5-phosphatase).

PTEN hydrolyzes the 3-phosphate of PIP3 and plays a central role in limiting cellular levels of PIP3, thereby opposing proliferation and survival responses (81). SHIP1 and SHIP2 are phosphatases that selectively remove the 5-phosphate from PIP3 to generate PtdIns(3,4)P_2 (PIP2). Thus, SHIP1/2 activity may alter the spectrum of PI3K dependent signals rather than simply opposing all PI3K signalling. SHIP1 is selectively expressed in cells of the immune system and is important for setting activation thresholds and maintaining homeostasis of a variety of hematopoietic cell lineages (82). SHIP2 inhibits insulin-induced PKB activation and proliferation, suggesting that SHIP2 plays a negative role in insulin-induced mitogenesis and survival (83).

The PI3K family can be subdivided into three classes: class I, II, or III, depending on their subunit structure, regulation, and substrate selectivity (84). Class IA PI3Ks are the only enzymes capable of converting PIP2 to the critical second messenger PIP3 that are involved in signalling by antigen and co-stimulatory receptors. Class IA PI3Ks are heterodimeric enzymes

consisting of a regulatory subunit (p85α, p85β or p55γ) and a catalytic subunit (p110α, p110β or p110γ). Each of the catalytic subunits can associate with all of the regulatory subunits.

PI3K was shown to be important in B cell development. PI3K p85α-deficient mice have a partial maturation block at the pro-B cell stage and a reduced number of B cells in the spleen (85, 86). In addition, p85α-deficient mice lack CD5+ B-1 cells in the peritoneum (85, 86). p110δ-mutant mice have a similar phenotype, although variations occur between the mouse lines that have been generated (87, 88). Several studies showed that p110δ is required for the development of MZ B cells and B-1 cells. The lack of MZ and B-1 cells was of particular interest, as this phenotype is also observed in CD19-deficient mice (48, 89). CD19 is one of the main regulators of PI3K activity in B cells (90) and mice expressing a tyrosine to phenylalanine mutant of CD19 that cannot bind PI3K also lack B-1 and MZ B cells (91). These results indicate that a PI3K-transmitted signal from CD19 drives the differentiation of B-1 and MZ B cells. Sustained PI3K signalling is required for the development of MZ B cells, and this depends on both CD19 and p110δ signalling. This conclusion is supported by the observation that PTEN-deficiency can rescue the development of MZ and B-1 B cells in CD19-deficient mice (92).
T cell development in the thymus does not seem to be affected in p85α-deficient or p110δ-deficient mice, although positive and negative selection were not specifically examined (87, 93).

Protein kinase B (PKB), also known as Akt, is an ubiquitously expressed serine/threonine kinase that is recruited by its pleckstrin homology (PH) domain to membrane-localized PIP3 and PIP2, the second messenger products of PI3-kinase. Membrane localization of PKB leads to its activation via phosphorylation at Serine 473 in the carboxy-terminal regulatory domain and at threonine 308 in the catalytic domain mediated by phosphoinositide-dependent protein kinases (PDKs). Activated PKB dissociates from the plasma membrane and phosphorylates a variety of substrates in the cytoplasm and nucleus. PKB can be regulated either directly at the plasma membrane by interaction with several proteins such as the carboxy-terminal modulator protein (CTMP) (94) or indirectly as by the lipid phosphatase PTEN. Dysregulation of PTEN is associated with development of a variety of human cancers, autoimmune disorders and loss of tolerance (95-99) and increased PKB activity was implicated in most of these disease phenotypes.

In most cases examined, PKB activation promotes various cell responses that are associated with cell division, including increased cell size, suppression of apoptosis, inactivation of cell cycle inhibitors, and induction of cyclin and cytokine gene expression. In lymphocytes PKB is activated by cytokines (100), T cell receptor signalling (101), CD28 co-stimulation (102), integrin-linked kinase (103), CD38 (104) or ICAM-2 (105), among others.

Originally PKB was identified as the cellular counterpart of a viral oncogene (v-Akt), in which the PH domain is replaced by viral GAG sequences, leading to constitutive membrane targeting. Subsequently, transgenic models have confirmed that constitutive membrane targeting of PKB in T cells is sufficient to cause lymphoma and is associated with altered lymphocyte homeostasis and autoimmunity (106-108).

Recent experiments have illustrated that PKB has several substrates that mediate distinct aspects of the downstream signalling response. These substrates reside in different cellular compartments, including the cytoplasm, nucleus and mitochondrial membrane.

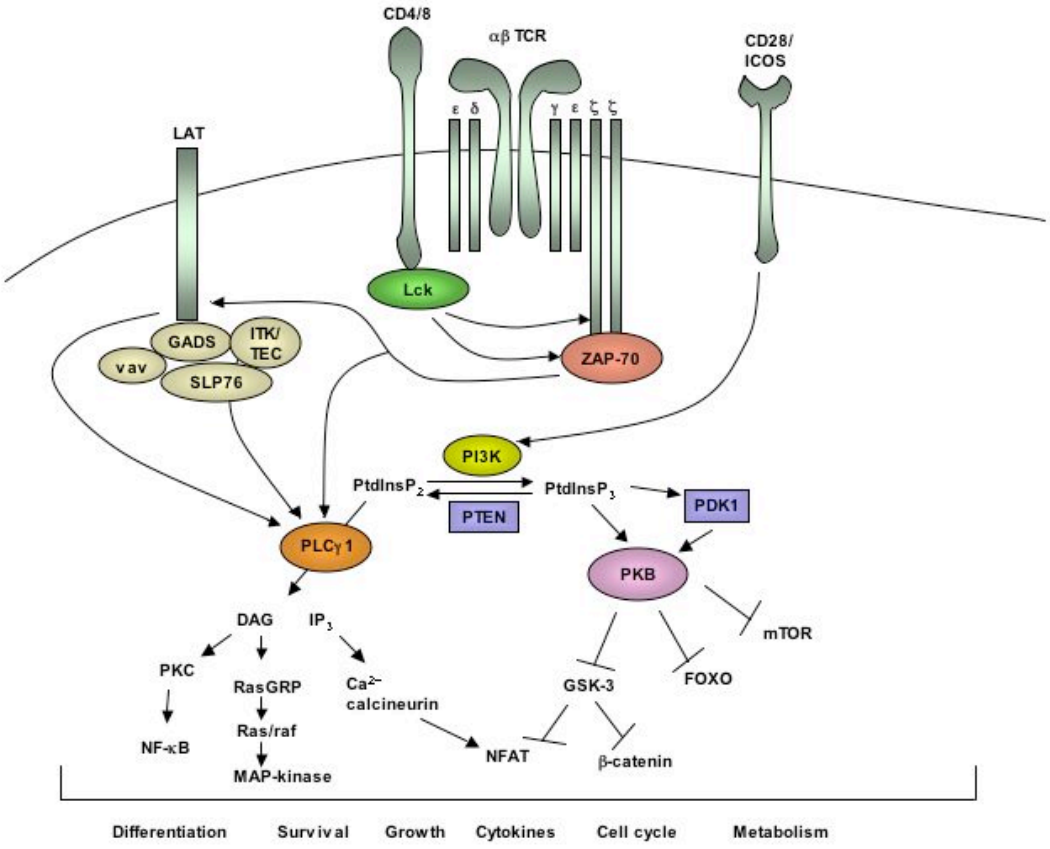

Fig 1.4 PI3K-PKB activation and signalling in T cells.

Glycogen synthase kinase-3 (GSK-3) is a substrate for PKB in many cell types and is rapidly phosphorylated upon lymphocyte activation in a PI3K-dependent manner (109).

Phosphorylation by PKB suppresses GSK-3 kinase activity in certain insulin-responsive cells, and this is important for augmenting glycogen synthesis. The transcription factor Nuclear Factor of Activated T cells (NFAT) is likely to be an important GSK-3 substrate in lymphocytes. GSK-3 phosphorylation of NFAT promotes its nuclear exit, thus preventing transcription of NFAT target genes. It has been speculated that PI3K-PKB activation by inhibiting GSK-3 activity contributes to NFAT nuclear accumulation (109, 110). In support of a possible negative role for GSK-3 in T cell activation, transgenic expression of constitutively active GSK-3β interferes with T cell proliferation (111). GSK-3 also regulates β-catenin, which is a key player in the Wnt signalling cascade. Activation of the Wnt cascade results in inhibition of the constitutive activity of GSK3-β (112). Consequently, β-catenin is no longer phosphorylated and can accumulate in the cytoplasm and nucleus, where it forms a complex with T-cell factor (TCF) family transcription factors to activate transcription of Wnt target genes (113, 114). Wnt signalling is required for normal thymocyte development, most dramatically at the pro-T cell stage (115, 116).

Transcription factors of the forkhead Box, Subgroup O (FOXO) family are an important group of PKB substrates (117). PKB phosphorylation inactivates FOXO factors by inducing their release from DNA, sequestration in the cytoplasm, and degradation. PKB-mediated FOXO inactivation has an evolutionarily conserved role in growth factor-driven increases in cellular metabolism, and is important for cell proliferation and survival in vertebrates. FOXO factors increase transcription of many target genes that are involved in cell cycle arrest, quiescence and apoptosis (i.e., $p27^{kip}$, Rb2/p130, Bim, TRAIL (tumor necrosis factor-related apoptosis inducing ligand), Fas ligand). FOXO1 is expressed in naïve T cells and B cells and is phosphorylated following mitogenic stimulation in a PI3K-dependent manner (118, 119). Forced expression of FOXO variants that cannot be phosphorylated by PKB promote cell-cycle arrest and apoptosis in activated lymphocytes, correlating with induction of $p27^{kip}$ and Bim (118, 119). These findings support the view that FOXO inactivation in lymphocytes is central to the ability of PI3K-PKB signalling to initiate the transition from quiescence into the cell cycle, and to sustain both cell cycle progression and survival in activated cells.

The target of rapamycin, TOR (mTOR in mammals, also known as FRAP, RAFT, or RAPT), is an evolutionarily conserved serine/threonine kinase that regulates both cell growth and cell cycle progression through its ability to integrate signals from nutrients (amino acids and energy) and growth factors (120). The best-characterized downstream targets of mTOR are two families of proteins that control protein translation, the ribosomal protein S6 kinases (S6Ks) and the eukaryotic initiation factor 4E (eIF4E)-binding proteins (4E-BPs).

Immunosuppressive effects of rapamycin highlight the importance of mTOR/S6K function for lymphocyte activation; moreover, in both T cells and B cells, stimulation via the antigen receptor and other mitogenic receptors causes phosphorylation and activation of S6K in a PI3K-dependent manner (121).

Under certain conditions, PKB can contribute to the activation of NF□□ (110). NF□□ activation is dependent on the phosphorylation and degradation of I□B, an inhibitor of NF□□, by the I□B kinase (IKK) complex. Transgenic expression of active PKB in T cells augments the ability of mitogens to stimulate the NF□□ pathway (107). It has been reported that PKB and PKC□ cooperate to activate the IKK complex, leading to I□B degradation (122). In this model, PKC□ is activated by TCR crosslinking, whereas PKB activation is primarily initiated by CD28 costimulation. A possible link between PKB and the IKK complex is Cot/Tpl2, a member of the MAP3K family of kinases. Cot was shown to be a direct PKB substrate; cotransfection studies in T cell lines and 293 cells further demonstrated that Cot promotes NF□□ pathway activation in a manner that is dependent on PKB phosphorylation (123).

Bad is a bcl-2 homology domain 3 (BH-3)-containing protein, the function of which is to activate the proapoptotic effect of bcl-2 family members such as Bax or Bak (124). Bad is inactivated by phosphorylation in response to growth factor stimulation (125), and PKB is one kinase responsible for this phosphorylation (126). Jones et al. demonstrated increased NF□□ activation and cell survival in a transgenic mouse model, but they also noted increased levels of Bcl-X_L, which is an antiapoptotic molecule (107). Thus, PKB plays an essential antiapoptotic role in survival signal transduction.

PKB is emerging as an important regulator of many different downstream functions, including inducible transcription, translation, glucose metabolism and survival. However, a number of lymphocyte-specific issues remain to be addressed.

Two of the three PKB genes have so far been disrupted in the mouse germ line. PKB□ knockout mice are viable but their growth is retarded, and PKB□-deficient thymocytes show increased apoptosis (127). PKB□-null mice are also viable and show defects in insulin signalling (128). Double knockout mice of PKB□/□ could develop to term, but die shortly after birth, showing a more obvious phenotype than the single knockout mice (129). The PKB□/□ double knockout mice were much smaller than wild type littermates and had impaired skin development, skeletal muscle atrophy and abnormal bone development.

Aim of the study:

When this work was started PKB's role in B and T lymphocytes was not known. The aim of this study therefore was to investigate how constitutively active PKB influences T and B cell development and lymphocyte functions, i.e. activation, proliferation, cytokine and immunoglobulin synthesis. As model system we used transgenic mice expressing a constitutively active form of PKB (myr PKB) in the T and B lymphocytes.

2. Materials

2.1 Chemicals, reagents and general materials

Acetone	AppliChem
Acrylamide (Gel 30)	AppliChem
Acrylamide (Gel 40)	Roth
Alu-Gel-S	Boehringer Ingelheim
7-amino-actinomycin D (7AAD)	Sigma
Agarose	AppliChem
Ammoniumpersulfate	Roth
AMP	Sigma
Anti-FITC microbead	Miltenyi Biotec
Anti-Rat IgG Fc dynalbeads	Qiagen
Baby rabbit complement	Cederlane
BCIP	Sigma
β-glycerophosphate	Sigma
β-mercaptoethanol	Gibco BRL
Boric acid	Roth
Brefeldin A	Sigma
Brij-98	Sigma
Bromophenol blue	Roth
BSA Fraction V	Roth
Calcium chloride	Roth
C2-ceramide	Sigma
Chloroform	Roth
Cyclosporin A	Calbiochem
Dexamethasone	Sigma
Diethanolamin	Merck
Diethylpyrocarbonate (DEPC)	Sigma
Dimethylsulfoxide (DMSO)	Sigma
1,4-Dithiothreitol (DTT)	MBI-Fermentas
dNTPs (dATP, dCTP, dGTP, dTTP)	Roth
EDTA	Roth
EGTA	Sigma

Enolase	Sigma
Ethanol	AppliChem
Ethidium Bromide (EtBr)	Roth
Etoposide	Calbiochem
F-127	Molecular Probes
Formamide	Sigma
Formaldehyde (37%)	Sigma
Glycerol	Roth
Glycine	Roth
HEPES	Sigma
Indo-1	Molecular Probes
Isopropanol	Roth
Low melting agarose	MBI-Fermentas
Magnesium chloride	Roth
Manganese chloride	Roth
Milk powder	Saliter
MOPS	Roth
NP-40	AppliChem
$OligodT_{(15)}$	Roche
Paraformaldehyde	Sigma
Percoll	Amersham
PMSF	Sigma
p-Nitrophenylphosphate	Sigma
Ponceau S	Sigma
Potassium chloride	Roth
Protein G Sepharose	Amersham
RPMI 1640 medium	Gibco BRL
Saponin	Sigma
Sodium acetate	Roth
Sodium azide	Roth
Sodium chloride	Roth
Sodium fluoride	Sigma
Sodium hydrogen phosphate	Roth
Sodium hydroxide	Roth

Sodium orthovanadate	Fluka
Sodium pyruvate	Gibco BRL
SDS	Roth
TEMED	Roth
Tris	AppliChem
Triton X-100	Roth
Trizol	Gibco BRL
Trypanblue	Sigma
Tween-20	Roth
Xylen cyanol FF	Roth

2.2 Enzymes and inhibitors

AEBSF (Pefabloc SC)	Roche
Leupeptin	Calbiochem
CsA	Calbiochem
FK506	Calbiochem
PD98059	Calbiochem
PP1	Calbiochem
Proteinase K	Sigma
Protease inhibitor tablet /complete mini	Roche
Reverse Transcriptase, SuperscriptII	Invitrogen
Taq DNA polymerase	Amersham

2.3 Antigens for immunization

TNP-OVA	Biosearch Technologies
TNP-Ficoll	Biosearch Technologies
SEB (Staphylococcal enterotoxin B)	Toxin Technology

2.4 Reagent kits

ECL-Western blot Detection kit	Pierce
RPA kit	BD Pharmingen
Probe labelling kit	BD Pharmingen

2.5 Radioactive materials

[□-^{32}P] ATP (3000Ci/nmol)	Amersham
[□-^{32}P] UTP (3000Ci/nmol)	Amersham
[^{3}□]-Thymidine	ICN Pharmaceuticals

2.6 Size markers

DNA size marker : GeneRuler 100 bp	MBI-Fermentas
Protein size marker : Full range Rainbow MW	Amersham

2.7 Antibodies

Antibodies for T cell isolation

Antigen	Antibody	Clone	Isotype	Source
CD4	Rat anti-mouse CD4	GK1.5	IgG2b	Hybridoma
CD8	Rat anti-mouse CD8	YTS169.4, TiB105	IgG2a	Hybridoma
CD19	Rat anti-mouse CD19	1D3	IgG2a	Hybridoma
MHC class II	Rat anti-mouse MHC II	2G9	IgG2a	Hybridoma
NK1.1	Rat anti-mouse NK1.1	4D11	IgG2a	Hybridoma

Antibodies for B cell isolation

Antigen	Antibody	Clone	Isotype	Source
CD4	Rat anti-mouse CD4	RL174.2	IgM	Hybridoma
CD8	Rat anti-mouse CD8	3.168.1	IgM	Hybridoma

Antibodies for FACS staining

Antigen	Antibody	Format	Clone	Source
CD3□	Hamster anti-mouse CD3□	FITC	145-2C11	BD Pharmingen
CD4	Rat anti-mouse CD4	FITC, PE, Biotin	GK1.5	BD Pharmingen
CD5	Rat anti-mouse CD5	Biotin	53-7.3	BD Pharmingen
CD8□	Rat anti-mouse CD8	FITC, Biotin	53-6.7	BD Pharmingen
CD21	Rat anti-mouse CD21	FITC	7G6	Hybridoma
CD23	Rat anti-mouse CD23	PE	B3B4	BD Pharmingen

CD24 (HSA)	Rat anti-mouse CD24	Biotin	M1/69	BD Pharmingen
CD25	Rat anti-mouse CD25	PE	7D4, PC61	BD Pharmingen
CD44	Rat anti-mouse CD44	FITC, Biotin	IM7	BD Pharmingen
CD45R (B220)	Rat anti-mouse CD45R	FITC, PE	RA3-6B2	BD Pharmingen
CD62L	Rat anti-mouse CD62L	Biotin	MEL-14	BD Pharmingen
CD69	Hamster anti-mouse CD69	Biotin	H1.2F3	BD Pharmingen
CD138 (Syndecan-1)	Rat anti-mouse CD138	PE	281-2	BD Pharmingen
TCR chain	Hamster anti-mouse TCR	Biotin	H57-597	Hybridoma
V 2 chain	Rat anti-mouse V 2	Biotin	B20.1	BD Pharmingen
V chain	Rat anti-mouse V HY-TCR specific	Biotin	T3.70	Hybridoma (Dr.T.Miyazaki)
V 5 chain	Rat anti-mouse V 5	Biotin	MR9-4	BD Pharmingen
V 8 chain	Mouse anti-mouse V 8	Biotin	F23.1	BD Pharmingen
IgM	Goat F(ab')$_2$ anti-mouse IgM	PE	polyclonal	SouthernBiotech
IgD	Rat anti-mouse IgD	FITC	11-26c.2a	BD Pharmingen
IL-4	Rat anti-mouse IL-4	PE	BVD4-1D11	BD Pharmingen
IL-10	Rat anti-mouse IL-10	FITC	JES5-16E3	BD Pharmingen
IFN	Rat anti-mouse IFN	PE	XMG1.2	BD Pharmingen
Isotype control	Rat IgG1,	FITC, PE, Biotin	R3-34	BD Pharmingen
Isotype control	Rat IgG2a,	FITC, PE	R35-95	BD Pharmingen

Antibodies for ELISA and ELISPOT

Antibody	Format	Manufacturer
Goat anti-mouse IgM	UNLB, AP	SouthernBiotech
Goat anti-mouse IgG	UNLB, AP	SouthernBiotech
Goat anti-mouse IgG1	UNLB, AP	SouthernBiotech
Goat anti-mouse IgG2a	UNLB, AP	SouthernBiotech

Goat anti-mouse IgG2b	UNLB, AP	SouthernBiotech
Goat anti-mouse IgG3	UNLB, AP	SouthernBiotech
Goat anti-mouse IgA	UNLB, AP	SouthernBiotech
Mouse IgM	UNLB	SouthernBiotech
Mouse IgG	UNLB	SouthernBiotech
Mouse IgG1	UNLB	SouthernBiotech
Mouse IgG2a	UNLB	SouthernBiotech
Mouse IgG2b	UNLB	SouthernBiotech
Mouse IgG3	UNLB	SouthernBiotech

Antibodies for Western blot

Antigen	Source	Dilution	Manufacturer
Phospho-PKB (Ser473)	Rabbit	1:1000	Cell Signaling
PKB	Rabbit	1:1000	Cell Signaling
Phospho-Erk	Rabbit	1:1000	Cell Signaling
Phospho-Raf	Rabbit	1:1000	Cell Signaling
Phospho-GSK-3□□	Rabbit	1:1000	Cell Signaling
Phospho-Tyrosine	Mouse	1:3000	Hybridoma (4G10)
Actin	Goat	1:1000	Santa Cruz
Cbl-c	Goat	1:500	Santa Cruz
Lck	Rabbit	1:5000	Brouns et al., 1993 (130)
LAT	Goat	1:500	Santa Cruz
CIS	Goat	1:500	Santa Cruz
NFATc1	mouse	1:2000	Alexis (7A6)
NF□B p50	Rabbit	1:1000	Santa Cruz
NF□B p65	Rabbit	1:1000	Santa Cruz
SLP-65	Rabbit	1:1000	From Dr. L.Nitschke Würzburg (131)
Phospho-PKC□ (Ser744,748)	Rabbit	1:1000	Cell Signaling
Phospho-PKC□□□(pan)	Rabbit	1:1000	Cell Signaling
Phospho-PKC□ (Ser643)	Rabbit	1:1000	Cell Signaling

Secondary antibodies for Western blot

Antigen	Source	Format	Dilution	Manufacturer
Goat IgG	Rabbit	HRP	1:3000	Dianova
Mouse IgG	Goat	HRP	1:3000	Dianova
Rabbit IgG	Goat	HRP	1:5000	Santa Cruz

2.8 Reagents for cell stimulation

CD3ε mAb (145-2C11)	BD Pharmingen
CD28 mAb (37.51)	BD Pharmingen
CD40 mAb (FGK)	BD Pharmingen
Fas mAb (Jo2)	BD Pharmingen
goat anti-mouse IgM F(ab')$_2$ Ab	Dianova
LPS	Sigma
PMA	Calbiochem
Ionomycin	Calbiochem
IL-4	from Prof. A.Schimpl, Würzburg
TGF-β (CHO expressed)	R&D Systems

2.9 Plastics

96, 48, 24, 12, 6 well plate	Greiner
96 well plate MaxiSorb plate (ELISA)	NUNC
25 well plate (ELISPOT)	Sterlin
6 cm cell culture dish	Greiner
15 ml, 50 ml tube	Greiner
FACS tube	Greiner
cell strainer	BD Falcon

2.10 Buffers and solutions

Balanced Salt Solution (BSS): BSS I (10X) :

Glucose	10 g
KH_2PO_4	0.6 g
$Na_2HPO_4 \cdot 2H_20$	2.3 g
Phenolred	0.1 g
add 1000 ml H_20	

BSS II (10X) :	$CaCl_2 \cdot 2H_20$	1.86 g	
	KCl	4 g	
	NaCl	80 g	
	$MgCl_2 \cdot 6H_20$	2 g	
	$MgSO_4 \cdot 7H_20$	2 g	
	add 1000 ml H_20		

Final solution : 1 vol. BSS I (10X) + 1 vol. BSS II (10X) + 8 vol. H_20

BSS/BSA BSS with 0.1% Bovine Serum Albumine (BSA)

Gey's solution:	Stock A :	NH_4Cl	35 g
		KCl	1.85 g
		$Na_2HPO_4 \cdot 12H_20$	1.5 g
		KH_2PO_4	0.12 g
		Glucose	5 g /1000 ml
	Stock B :	$MgCl_2 \cdot 6H_20$	0.42 g
		$MgSO_4 \cdot 7H_20$	0.14 g
		$CaCl_2$	0.34 g /100 ml
	Stock C :	$NaHCO_3$	2.25 g / 100 ml

Final sol : 70 vol. H_20 + 20 vol. stock A + 5 vol. stock B + 5 vol. stock C

FACS buffer 0.1% BSA

0.01% Azide in PBS

Phosphate Buffered Saline (PBS):	NaCl	8 g	
	KCl	0.2 g	
	Na_2HPO_4	1.15 g	
	KH_2PO_4	2 g	adjust to pH7.4 /1000 ml

RPMI 5% 500 ml RPMI1640 c with 50 ml SC

Supplement complex (SC)	5% heat inactivated FCS (30 min at 56°C)
	1:100 non-essential amino acids (Gibco)
	1:100 Na pyruvate (Gibco)
	0.07% L-Glutamin
	0.025% Penicillin

0.025% Streptomycin

$5x10^{-5}$M β-mercaptoethanol

TBE buffer (10x):

Tris	108g
Boric acid	55g
EDTA	3.7g

DNA gel loading buffer:

0.5% Xylene cyanol FF

0.5% Bromophenolblue

50% Glycerol

10 mM EDTA (pH 8.0)

RNA gel loading dye:

0.25% Xylene cyanol FF

0.25% Bromophenolblue

50% Glycerol

1 mM EDTA (pH 8.0)

2.11 Instruments

Agarose gel reader	Mitsubishi
CO_2 incubator	Heraeus Instruments
Centrifuge	Heraeus Instruments
β-counter	PerkinElmer
ELISA-Reader ″v-max″	Molecular Devices
ELISA washer	TECAN
FACScan	Beckton Dickinson
FACScalibur	Beckton Dickinson
FACS Vantage	Beckton Dickinson
Harvester ″MACH 3″	Tomtec
Heat block	Hartenstein
Microscope	Leica
pH meter	Hanna Instruments
Spectrophotometer	Pharmacia Biotech
Thermo-Cycler ″Biometra″	Biotron
Ultracentrifuge ″Combi plus″	Sorvall

2.12 Mice

Human CD2-myr PKB (myr PKB) transgenic (tg) mice were crossed to C57BL/6 mice for at least five generations.

OT1 (132), OT2 (133), HY (134) TCR tg mice and DBA/2 and CBA/J mice (Charles River Breeding Laboratories) were crossed with myr PKB tg mice. In this study usually 6-8 weeks old mice were used.

3. Methods

3.1 Methods in molecular biology

3.1.1 DNA work

3.1.1.1 Isolation of genomic DNA

For genotyping of mice genomic DNA was isolated from tail. About 1 cm of mouse tail was incubated with tail lysis buffer containing 10 µl proteinase K (20 mg/ml) at 56°C for O/N. The following day the lysate was boiled at 100°C for 5 min to inactivate proteinase K and then centrifuged for 10 min at 14000 rpm at 4°C. The supernatant was transfered to a fresh tube and 500 µl isopropanol was added. Using a blocked pasteur pipette, genomic DNA was collected and washed with 70% EtOH. Finally, the genomic DNA was dissolved in 200 µl H_2O.

Tail lysis buffer	:	50 mM Tris pH 8.0
		100 mM EDTA
		100 mM NaCl
		1% SDS

3.1.1.2 PCR (Polymerase chain reaction)

DNA fragments were amplified from isolated genomic DNA or cDNA by PCR using specific primer pairs. The PCR cocktail contained the following components :

10x buffer	2 µl	$MgCl_2$ (25 mM)	0.8 µl
dNTPs (10 mM)	0.4 µl	Taq polymerase (5 U/µl)	0.1 µl
Reverse primer (10 µM)	1 µl	DNA template	1 µl
Forward primer (10 µM)	1 µl		
		ddH2O	up to 20 µl

The PCR reaction was performed for 31 cycles using the following protocol :

Pre-denaturation at 95°C for 2 min
30 cycles of : denaturation at 95°C for 1 min
annealing at 55.5°C for 1 min
elongation at 72°C for 1 min
Post-elongation at 72°C for 5 min

The PCR conditions and number of cycles depended on the size of the PCR products and base pair composition of the primers. The sequences of the primers used and their optimal annealing temperatures are indicated below.

	Primer sequence (5'-3')	Temp. (°C)
PKB (transgene)	Fwd: AGATTTCCTGTCCCCTCTCAG Rev: TGTTGGACCCAGCTTTGCAG	55.5
Actin	Fwd: CCAGGTCATCACTATTGGCAAGGA Rev: GAGCAGTAATCTCCTTCTGCATCC	58
Notch1	Fwd: ACCAATGGCACCGGGGCTATGAAT Rev: GTGGCTGTGATGGTGGCTGTAAGT	62
Notch3	Fwd: AACCCCACCCACCCAGGATTTG Rev: GCAGGGCAGGCTTTGTATGTCG	64
Blimp-1	Fwd: GAAGAAACAGAATGGCAAGA Rev:AAGACACTTTCAGACTGGT	58
XBP-1	Fwd: CAGGAGTTAAGAACACGCTTGG Rev: TTAGACACTAATCAGCTGGGGG	60
AID	Fwd: GGCTGAGGTTAGGGTTCCATCTCAG Rev: GAGGGAGTCAAGAAAGTCACGCTGGA	62
T-bet	Fwd: GCCAGGGAACCGCTTATATG Rev: GACGATCATCTGGGTCACATTGT	56
TLR4	Fwd: CAAGAACATAGATCTGAGCTTCAACCC Rev: GCTGTCCAATAGGGAAGCTTTCTAGAG	62
TLR9	Fwd: CCGCAAGACTCTATTTGTGCTGG Rev: TGTCCCTAGTCAGGGCTGTACTCAG	64

c-Myc	Fwd: ACCAACAGGAACTATGACCTC Rev: AAGGACGTAGCGACCGCAAC	58

3.1.1.3 DNA gel electrophoresis

PCR products were identified in agarose gels; for PKB PCRs 1% agarose gels were routinely used.

Agarose	1.5 g			
1x TBE	150 ml	+	EtBr (10 mg/ml)	4 µl

3.1.2 RNA work

3.1.2.1 RNA isolation

$1x10^7$ primary or activated T or B cells were suspended in 1 ml TRIZOL and were stored at -20°C. The samples were thawed, 200 µl of chloroform were added and the samples were vortexed. After 5 min incubation at RT, the sample was centrifuged at 14000 rpm for 5 min and the upper phase was transferred to a fresh tube. RNA was precipitated by addition of 500 µl isopropanol. After centrifugation, RNA was washed with 75% EtOH and dissolved in 30 µl DEPC-treated water.

3.1.2.2 RNA gel electrophoresis

The integrity of the isolated RNA was analyzed in formaldehyde gels.

	Agarose	0.48 g	
	10x MOPS buffer	4 ml	
	H_2O	28.8 ml	⇒ dissolve in microwave
+	Formaldehyde	7.2 ml	

1 µg RNA was added to 5 µl formaldehyde buffer and incubated at 70°C for 20 min to denature the RNA. After adding 1 µl RNA-gel loading dye, samples were loaded on RNA gels.

10x MOPS buffer :	0.4 M MOPS
	0.1 M NaOAc
	0.01 M EDTA
Running buffer :	1x MOPS buffer
Formaldehyde buffer :	15% Formaldehyde
	50% Formamide
	10% 10x MOPS buffer

3.1.2.3 Determination of RNA concentration

The concentration of RNA was determined in an UV spectrophotometer. RNA was diluted 1:50 and measured at 260 nm wavelength. The absorption of 1 corresponds to a concentration of 40 µg/ml RNA.

3.1.2.4 RT-PCR (Reverse Transcription-PCR)

To synthesize cDNA, 1 µg RNA was mixed with 1 µl OligodT_{15} primer and the volume was adjusted to 12 µl with H_20. The mixture was incubated for 10 min at 70°C and then chilled on ice. Afterwards, 4 µl 5x transcription buffer, 2 µl 100 mM DTT and 1 µl 100 mM dNTPs were added and incubated for 2 min at 42°C. 1 µl reverse transcriptase (Superscript II) was added and incubated for 10 min at 25°C. Thereafter the reaction mix was incubated for 50 min at 42°C and the reaction was terminated by incubation at 70°C for 15 min.

3.1.2.5 RPA (RNase Protection Assay)

RPAs were used to detect and quantify the amount of specific mRNAs in samples from resting and activated T cells. All RPA reagents were taken from kits.

Probe synthesis : For synthesis of the RNA probe the following protocol was used:

1 µl RNasin
1 µl GACU nucleotide mix
2 µl 100 mM DTT
4 µl 5x transcription buffer
1 µl RPA template set
10 µl [α-^{32}P] UTP
1 µl T7 RNA polymerase (keep at -20°C until use; return to -20°C immediately)

All reagents were gently mixed and incubated for 1 hr at 37°C. The reaction was terminated by adding 2 µl DNase, mixed and incubated for 30 min at 37°C. To remove contaminating proteins, the following reagents were added, then mixed by vortexing and spinned in a microfuge for 10 min at RT.

26 µl 20 mM EDTA
50 µl chloroform:isoamyl alcohol (50:1)
2 µl yeast tRNA

The upper phase, containing the RNA, was transfered to a new tube. To precipitate the RNA, 50 µl 4M ammonium acetate and 250 µl ice-cold 96% EtOH were added, mixed, incubated for 2-3 min on dry ice and centrifuged for 15 min at 4°C. The supernatant was removed and washed with 100 µl ice cold 90% EtOH. The pellet was dried for 5 to 10 min at RT and the RNA template was suspended in 50 µl hybridization buffer. The RNA probe could be stored at -20°C for 2-3 days.

RNA hybridization : 5 µg RNA was mixed with 20 µl diluted probe and placed in a heat block prewarmed to 90°C. Samples were immediately transfered to a 56°C incubator and kept for 12-16 hrs for RNA hybridization.

RNAse treatment: For 20 samples, a RNase cocktail was prepared by mixing 2.5ml RNase buffer and 6 µl RNase A+ T1 mix. Each hybridized sample was mixed with 100 µl RNase cocktail, briefly centrifuged, and incubated for 45 min at 30°C. For 20 samples, a proteinase K cocktail was prepared by mixing 390 µl proteinase K buffer, 30 µl proteinase K and 30 µl yeast tRNA. After RNase treatment, 18 µl proteinase K cocktail was added, mixed and incubated for 15 min at 37°C. For eliminating proteins, 120 µl chloroform:isoamyl alcohol was added, the mixture was vortexed and microfuged for 5 min at RT. The upper phase was transfered to a new tube, and RNA was precipitated by adding 120 µl 4M ammoniumacetate and 650 µl ice-cold 96% ethanol. After centrifugation for 15 min at 4°C, the RNA pellet was washed with 90% ethanol and dried at RT. The pellet was dissoved in 4 µl loading buffer, denatured at 90°C for 3 min and kept on ice.

Gel resolution of RNAse protected probes: RNA was seperated in 0.4mm thick 5% polyacryamide gels.

Pre-mixture : 250 g Urea
75 ml 40% Acrylamide
50 ml 10x TBE
200 ml H_2O → filtrated with 0.4 µm filter

Gel : 40 ml pre-mixture
300 µl 10% APS
35 µl TEMED

The polymerized gel was pre-run at 30 mA for 45 min in 1x TBE buffer and samples were loaded on the gel along with the undigested RNA probe. The gel was run until the leading edge of the bromophenol blue stain reached three quarter of the gel, then vacuum dried for 2 hr at 80°C and exposed to X-ray film at -70°C.

3.2 Protein work

3.2.1 Preparation of protein extracts from primary lymphocytes

2-4x10^6 purified T or B cells were washed with PBS and lysed in 25 µl lysis buffer. The lysates were incubated for 30 min on ice and centrifuged at 14000 rpm for 10 min at 4°C. The supernatant was transfered to a fresh tube, 1x SDS protein sample buffer was added and after denaturing at 100°C for 5 min proteins were stored at -20°C.

Protein lysis buffer : 20 mM Hepes, 2 mM EGTA, 50 mM β-glycerophosphate
1% Triton X100, 10% glycerol, 0.04% azide
freshly add: 1 mM DTT, 1 mM orthovanadate, 2 µM leupeptin
0.4 mM PMSF, 50 mM NaF

4x Upper buffer: 0.5 M Tris-HCl (pH 6.8), 0.4% SDS

2x Protein loading buffer: 1 vol. 4 x upper buffer + 1 vol. 10% SDS
+ 1 vol. glycerol + 1 vol. H_20
+ 0.1 vol β-mercaptoethanol
+ 0.001% bromophenol blue

3.2.2 Immunoprecipitation (IP)

For IP, $1x10^8$ thymocytes or $2x10^7$ CD4+ T cells or B cells were used. Cells were lysed in 500 µl lysis buffer and incubated for 20 min on ice. The lysate was centrifuged at 14000 rpm for 10 min at 4°C and the supernatant was transferred to a fresh tube. 20 µl of packed Protein G sepharose beads were washed with lysis buffer and incubated with the lysate and the immunoprecipitating Ab for O/N at 4°C with rotation. The next day the lysate was washed 4x with lysis buffer and the sepharose beads were suspended in 50 µl of 2x SDS gel loading buffer, the mixture was boiled at 100°C for 5 min and frozen until analysis by SDS-PAGE.

Lysis buffer :	0.1% Brij-98 in TNE buffer
TNE buffer :	25 mM Tris pH 7.5
	150 mM NaCl
	5 mM EDTA
	(freshly add 1 mM DTT, 1 mM Na_3VO_4, protease inhibitor cocktail)

3.2.3 Isolation of lipid raft fractions by ultracentrifugation

$1x10^8$ freshly isolated thymocytes or purified CD4+ T cells from lymph nodes in 200 µl PBS were treated with 200 µl cold Brij-98 lysis buffer. The lysate was mixed with 400 µl 80% sucrose in TNE buffer. The solution was overlaid with 2.8 ml 30% sucrose in TNE buffer, followed by 400 µl TNE buffer and centrifuged at 50000 rpm for 22 hrs at 4°C. Proteins were harvested from the top in 500 µl fractions (total of 4 fractions) and precipitated with 750 µl acetone. The precipitates were resolved in 50 µl (lipid raft, insoluble fractions 1-3) or 250 µl (soluble, cytosolic fraction 4) SDS-PAGE sample buffer and boiled at 100°C for 5 min.

3.2.4 Nuclear and cytoplasmic extracts

$2x10^7$cells were suspended with 300 µl of buffer A and incubated for 3 min on ice. Cells were lysed by adding 0.5% NP-40. Samples were centrifuged at 14000 rpm for 3 min at 4°C and the supernatants were taken as cytoplasmic extracts. The pellets were washed 3x with buffer A and then 75 µl of buffer C was added. After shaking for 2 hrs at 4°C, nuclear extracts were collected by centrifugation at 14000 rpm for 20 min at 4°C and stored at -20°C.

Buffer A :	10 mM KCl
	10 mM HEPES (pH 7.9)
	0.1 mM EGTA (pH 7.9)
	0.1 mM EDTA (pH 7.9)
	freshly add 1 mM DTT, 1 mM Na_3VO_4, protease inhibitor cocktail
Buffer C :	420 mM NaCl
	20 mM HEPES (pH 7.9)
	1 mM EGTA (pH 7.9)
	1 mM EDTA (pH 7.9)
	freshly add 1 mM DTT, 1 mM Na_3VO_4, protease inhibitor cocktail

3.2.5 SDS-polyacrylamide gel electrophoresis

Protein extracts were separated on 8-12% denaturing SDS polyacrylamide gels. The separating gel mix was prepared, poured into the gel apparatus and overlaid with water. After the separating gel was polymerized, the overlay was decanted and and the stacking gel was poured. Protein samples were loaded and electrophoresed in 1x SDS PAGE running buffer.

	Separating gel			Stacking gel
	8%	10%	12%	4%
H_20	7.01 ml	6 ml	5 ml	3.6 ml
30% acrylamide	4 ml	5.01 ml	6 ml	0.65 ml
1.5 M Tris pH 8.8	3.75 ml	3.75 ml	3.75 ml	-
0.5 M Tris pH 6.8	-	-	-	0.623 ml
10% SDS	0.15 ml	0.15 ml	0.15 ml	0.05 ml
10% APS	0.075 ml	0.075 ml	0.075 ml	0.03 ml
TEMED	0.015 ml	0.015 ml	0.015 ml	0.005 ml
total	15 ml	15 ml	15 ml	5 ml

10x Running buffer :	100 ml 10% SDS	
	144.13 g Glycin	
	30.3 g Tris	add to 1000 ml H_2O

3.2.6 Western Blot

Proteins from the SDS-PAGE gel were electroblotted to a nitrocellulose membrane at 70 V for 2 hrs at 4°C. The membrane was stained with Ponceau S to control the transfer of the protein molecular weight marker and proteins. The membrane was then washed several times with H_20 and washing buffer to remove Ponceau S and incubated with blocking solution for 1 hr at RT. The nitrocellulose membrane was incubated with the primary Ab solution O/N at 4°C. The next day the membrane was washed with washing buffer 3x for 10 min each and incubated with horseradish-peroxidase-conjugated secondary Ab for 1 hr at RT. After incubation, the membrane was washed, treated with enhanced chemiluminescent substrate and exposed to X-ray film.

10x Transfer buffer : 29 g Tris, 145 g Glycin / 1000 ml
1x Transfer buffer : 1 vol. 10 x Transfer buffer + 2 vol. Methanol + 7 vol. H_2O
Blocking buffer : 5% milk powder in PBS-0.1% Tween
Washing buffer : 0.1% Tween in PBS
Primary Ab solution : Abs from Cell Signaling 5% BSA in washing buffer
other Abs 1% milk powder in washing buffer

3.2.7 Kinase assay

For Lck kinase assays, $5x10^7$ freshly isolated thymocytes were lysed immediately or starved for 2 hr in 1% FCS medium before stimulation with CD3 mAb (30 g/ml) for the indicated time points. Cells were lysed in 500 l Brij-98 lysis buffer and Lck was immunoprecipitated with Lck Ab and Protein-G-Sepharose. Lck precipitates were washed twice with lysis buffer and kinase assay buffer (0.1% Brij-98, 25 mM Hepes pH 7.4) before incubation with 30 l assay buffer containing 10 mM $MnCl_2$, 5 Ci [-^{32}P]-ATP (3000 Ci/mmol) and acidified enolase (5 g) for 20 min at 30°C. After addition of 30 ml 2x sample reducing buffer, Lck activity (autophosphorylation and enolase phosphorylation) was detected by 10% SDS-PAGE and exposure to X-ray film.

3.2.8 ELISA (Enzyme Linked Immunosorbent Assay)

Immunoglobulin titers were determined by ELISA. The day before analysis, 96 well MaxiSorb ELISA plates were coated with 1 g/ml of the indicated Ab (i.e., IgG, IgM, IgG2a)

for serum Ab titer of unimmunized mice or TNP-BSA (10 μg/ml) for TNP-specific Ab titer of TNP-Ova or TNP-Ficoll immunized mice in PBS (50 μl/well) for 2 hrs at 37°C. For blocking 200 μl 1% BSA in PBS was added to each well and incubated O/N at 4°C.

The next day ELISA plates were washed 3x with PBS and serum in 1:3 serial dilutions in 0.1% BSA PBS was added. For the standard, pooled sera from immunized mice or isotype standards (1 μg/ml) were serially diluted and added to the wells. After adding serum, plates were incubated for 90 min at 37°C, washed 3x with PBS and 1 μg/ml of alkaline phosphatase (AP)- conjugated isotype specific secondary Ab was added to each well. Plates were incubated for 1 hr at 37°C and washed with PBS. For revealing bound secondary Ab, 100 μl/well of substrate was added and after the development of yellow colour plates were measured at 405 nm absorbance.

Substrate : 1 mg/ml p-nitrophenylphosphate in diethanolamine buffer

diethanolamine buffer : 1 M diethanolamine

0.02% NaN_3

4 mM $MgCl_2 \cdot 6H_20$ / adjust pH to 9.8

3.3 Cellular methods

3.3.1 Isolation of cells from lymphoid organs

For single cell suspensions, total thymus, lymph nodes (LNs) or spleen were meshed through a cell strainer in 10 ml BSS/BSA buffer and centrifuged at 1300 rpm for 5 min at 4°C. The cell pellet was suspended in 10 ml BSS/BSA and the cell number was determined. For this purpose cells were diluted 1:50 in 0.04% trypan blue in PBS, counted and the cell number was calculated.

3.3.2 Isolation of CD4+ T cells from LN

For isolation of CD4+ T cells, LN cells were counted, centrifuged and resuspended in 0.5 ml PBS/BSA. 500 μl of each hybridoma supernatant against CD8, NK1.1, CD19, and MHC II were added and the cell mixture was incubated for 25 min on ice. Cells were washed 2x with PBS/BSA, resuspended in 500 μl PBS/BSA and incubated with goat anti-rat IgG coupled with magnetic beads (per $1x10^8$ cells 200 μl 10x concentrated beads) for 20 min at 4°C. During incubation cells were mixed intermittently. After incubation, 5 ml PBS/BSA were added to

the cells and the tube was attached to a magnet for 5 min. Cells that were not attached to beads,i.e. CD4+ T cells were transferred to a new tube, centrifuged and counted. Purity of isolated T cells was controlled by FACS analysis.

3.3.3 Isolation of naive ($CD62L^{high}$) CD4+ T cells

$1x10^7$ purified CD4+ T cells were stained with biotinylated CD62L Ab, followed by staining with streptavidin-FITC. Cells were washed with PBS/1% FCS, suspended in 90 µl PBS/1% FCS and incubated with 10 µl anti-FITC-microbeads for 15 min at 4°C. Cells were washed with PBS/1% FCS and resuspend in 1 ml PBS/1% FCS. To isolate labelled cells, a midiMACS LS separation column was attached to the MACS and loaded with the cell suspension. A tube under the column was used to collect the flow through. The column was washed with 3 ml PBS/1% FCS before labelled cells were loaded onto the column. CD62L negative cells passed through the column, which was washed 2x with 2 ml PBS/1% FCS. To collect CD62L positive cells, the column was removed from the magnet, additional 3 ml PBS/1% FCS were applied, and using the plunger supplied with the column positive cells were washed off from the column and were collected in a fresh tube.

3.3.4 Isolation of splenic B cells

The spleen was meshed through a cell strainer, cells were collected in 10 ml BSS/BSA buffer and centrifuged. 2 ml Gey's solution were added to the cell pellet, cells were resuspended and incubated for 5 min at 37°C (lysis of erythrocytes). Splenocytes were then washed with BSS/BSA and counted. For isolation of B cells, cells were suspended in 500 µl BSS and incubated with 500 µl anti-CD4 and anti-CD8 hybridoma supernatant for 25 min on ice. Thereafter cells were washed twice with BSS and suspended in 3 ml BSS. 330 µl baby rabbit complement was added and the cell suspension was incubated at 37°C for 45 min. Cells were mixed twice intermittently. After the incubation, cells were washed with BSS and suspended in a 80% percoll solution. Cells were overlaid with each 2.5 ml of 70%, 65%, 60%, and 50% percoll and BSS. Cells were centrifuged at 2000 rpm for 20 min without break and the interphase between the 60-65% and 65-70% layer of the gradient was collected. To control the purity of the isolated resting B cells, cells were stained with B220 and CD3µ Abs and analyzed by FACS.

90% percoll : 45 ml 100% percoll and 5 ml 9% NaCl

	90% percoll	0.9% NaCl	BSS
80%	8 ml	1 ml	
70%	7 ml		2 ml
65%	6.5 ml	2.5 ml	
60%	6 ml		3 ml
50%	5 ml	4 ml	

3.3.5 B-1 B cell isolation

5 ml of BSS were injected into the peritoneal cavity of a mouse and peritoneal cavity cells were collected with a syringe. Cells were washed with BSS/BSA and stained with CD5-biotin and IgM PE Abs followed by streptavidin-cychrome. B-1 and B-2 peritoneal B cells were electronically sorted using a FACSVantage.

3.3.6 FACS analysis

$8x10^5$ cells were transferred to a FACS tube and washed with FACS buffer (PBS/0.1% BSA/0.01% Azide) for staining. Cells were incubated with FITC-, PE- or biotin-conjugated Abs for 30 min on ice and then washed with FACS buffer. In case of biotinylated Abs cells were incubated with streptavidin-cychrome in a second staining step. Stained cells were analyzed on a FACSCalibur using CellQuest software (Becton Dickinson).

3.3.7 Intracellular cytokine staining

CD4+ T cells were stimulated for the indicated time points and restimulated with PMA (100 ng/ml) and ionomycin (100 ng/ml) in the presence of brefeldinA (10 µg/ml) for 4 hrs. Cells were washed with FACS buffer, fixed by adding 200 µl 4% paraformaldehyde in PBS and incubated for 30 min at RT, followed by washing with FACS buffer. Cells were then permeabilized by incubation in 1 ml saponin buffer (0.1% saponin, 1% FCS, 0.01% NaN_3 in PBS) for 10 min at RT. Afterwards cells were washed and centrifuged. Cytokine specific Abs (FITC- or PE-labelled) were diluted in saponin buffer and added to the fixed cells for 15 min on ice for intracellular staining of cytokines. Cells were washed with FACS buffer and analyzed by flow cytometry.

3.3.8 Measurement of Ca^{2+}-flux

Splenocytes were prepared as described before and $2.5x10^7$ cells were suspended in 5 ml RPMI/1% FCS medium. Indo-1 (50 µg) was suspended in 25 µl DMSO, 25 µl F-127 were added and the mixture was incubated for 5 min at RT in the dark. After addition of 113 µl FCS, the mixture was incubated for 5 min at RT in the dark. Splenic cells were incubated with 15 µl Indo-1 mix /ml cells for 45 min at 37°C and washed with RPMI/1% FCS medium. Indo-1 loaded splenic cells were then stained with CD5-PE and Mac-FITC Abs for 30 min on ice. After staining, cells were washed, resuspended in RPMI/1% FCS medium ($1x10^7$ cells/ml) and stimulated with the indicated concentration of IgM $F(ab')_2$ Abs. Increase in intracellular Ca^{2+} concentration in stimulated splenic B cells (gated on $CD5^-Mac1^-$ cells) was recorded for 5 min using a FACSVantage.

3.3.9 Proliferation assay

For proliferation assays, total thymocytes ($1x10^6$), purified CD4+ T cells ($2x10^5$) or purified B cells ($2x10^5$) were cultured in 96 well plates in triplicates in RPMI medium supplemented with 10% FCS. Thymocytes were stimulated with plate bound CD3µ mAb or with PMA and ionomycin for the indicated time points. CsA, FK506, PD98059 or PP1 were added at the beginning of culture at concentrations as stated. CD4+ T cells were stimulated on plates coated with CD3µ mAb or CD3µ plus CD28 mAbs with or without TGF-µ at concentrations as stated. B cells were stimulated with LPS, IgM $F(ab')_2$ Ab, CD40 mAb or CD40 mAb plus IL-4 with the indicated concentrations. On day 1, 2 or 3 cells were pulsed with 1 µCi [^{3}H]-thymidine/well and harvested after 10-16 hrs.

3.3.10 Measurement of apoptosis

Total thymocytes or purified LN CD4+ T cells (each $1x10^6$/ml) were cultured in RPMI medium with different concentrations of FCS or treated with various apoptotic stimuli as indicated. Cell viability was measured at the indicated time points by Annexin V-FITC and 7-amino-actinomycin D (7-AAD) staining and the percentage of live 7-AAD- and Annexin V-negative cells was determined. Apoptosis was induced with C2-ceramide, dexamethasone, fas mAb, etoposide or PMA. Apoptosis of thymocytes from OT1 and OT2 TCR tg mice and crosses with myr PKB tg mice was studied by adding ovalbumin peptide a.a.257-264 or a.a.323-339 to $5x10^5$ cells/well, respectively, in concentrations as indicated. In case of OT2 TCR bearing mice irradiated (30 Rad) splenic B cells ($1x10^6$ cells/well) were added as APCs.

3.3.11 TNP-OVA immunization

For analysis of T cell dependent B cell responses, mice were immunized with TNP(14)-conjugated ovalbumin. To precipitate TNP-OVA, 2 vol. of PBS and 2 vol. of ALUM (AL-Gel 2% AL-OH) were mixed and incubated for 1 hr at RT in the dark. Afterwards 1 vol. of PBS was added and the mixture was centrifuged at 1300 rpm for 5 min. Precipitated 100 µg TNP-OVA-ALUM were suspended in 200 µl PBS. Mice were injected with 200 µl TNP-Ova/ALUM i.p. at day 0 and boosted at day 14 with the same TNP-OVA/ALUM concentration. Mice were bled on day 0, 7, 14, 21 and 28 and serum was prepared. TNP-specific Ig was determined by ELISA with TNP-BSA coated ELISA plates as described under 3.2.8.

3.3.12 TNP-Ficoll immunization

To study a T cell independent B cell response mice were immunized i.p. with 10 µg TNP-Ficoll and sera were prepared on day 0, 5 and 7. TNP-specific Ig production was determined by ELISA with TNP-BSA coated ELISA plates as described under 3.2.8.

3.3.13 SEB immunization

Staphylococcal enterotoxin B (100 µg) was injected i.p. into mice on day 0. Blood taken from the tail vein was analyzed by flow cytometry on days 0, 1, 2, 3, 4 and 7. Blood samples were first treated with Gey's solution and cells were stained with CD4-PE, CD8-FITC and Vβ8-biotin or Vβ10-biotin Abs followed by streptavidin-cychrome.

3.3.14 ELISPOT (Enzyme-linked immunospot assay)

Immunoglobulin secreting B cells were analyzed by ELISPOT. 25 well plates were coated with 2 µg/ml isotype specific polyclonal Abs in 50 mM Tris-HCl (pH 9.5) for 2 hr at 37°C or O/N at 4°C, washed 3x with BSS and blocked with 1 ml BSS/BSA for 1 hr at 37°C. At the indicated time points, stimulated B cells were harvested, washed and suspended in RPMI/5% FCS medium in cell concentrations as indicated. Serially diluted B cells were added to the washed 25 well plate and incubated in a 5% CO_2 incubator O/N at 37°C. The following day cells were removed from the plates and plates were washed 3x with PBS/0.05% Tween and once with PBS. AP- conjugated goat anti-mouse Abs specific for the individual isotypes were diluted in PBS (1 µg/ml), and 1 ml was added to the wells. Plates were incubated for 3 hrs at 37°C with shaking. After the incubation period, plates were vigorously washed and 1 ml enzyme substrate was added to each well. Each spot represents one Ig producing B cell.

Substrate solution : 5 vol. BCIP/AMP solution + 1 vol. 3% low melting agarose

BCIP/AMP solution : 1 mg/ml BCIP in AMP solution

AMP solution : 47.9 ml AMP

75 mg $MgCl_2 \cdot 6H_2O$

50 µl Triton X-100 adjust to pH 10.25 / 500 ml H_2O

4. Results

4.1 Constitutively active protein kinase B enhances Lck and Erk activities and influences thymocyte selection and activation

4.1.1 Generation of transgenic mice expressing myr PKB in lymphocytes

To investigate the role of myr PKB in lymphocyte development and function, transgenic (tg) mouse lines expressing a human myr PKB cDNA (135) under the control of the human CD2 promoter and locus control region (LCR), (Fig. 4.1) were used in this study (established in Dr. Bommhardt's laboratory).

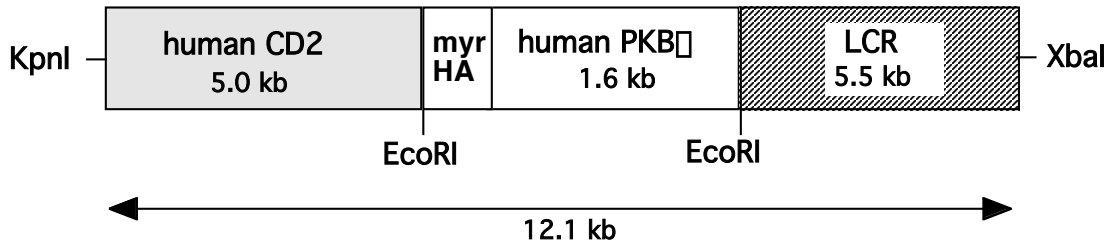

Fig 4.1 Generation of myr PKB tg mice.

Schematic representation of the cDNA construct used to generate myr PKB tg mice.

Myr PKB tg founder lines (PKB1, 2 and PKB6) were crossed to the C57/BL6 background. PKB6 and PKB2 mouse lines were used throughout this study. Myr PKB expression in thymocytes and peripheral CD4+ and CD8+ T cells was determined by Western blots using PKB and phospho-PKB (Ser 473) Abs (Fig. 4.2A and B).

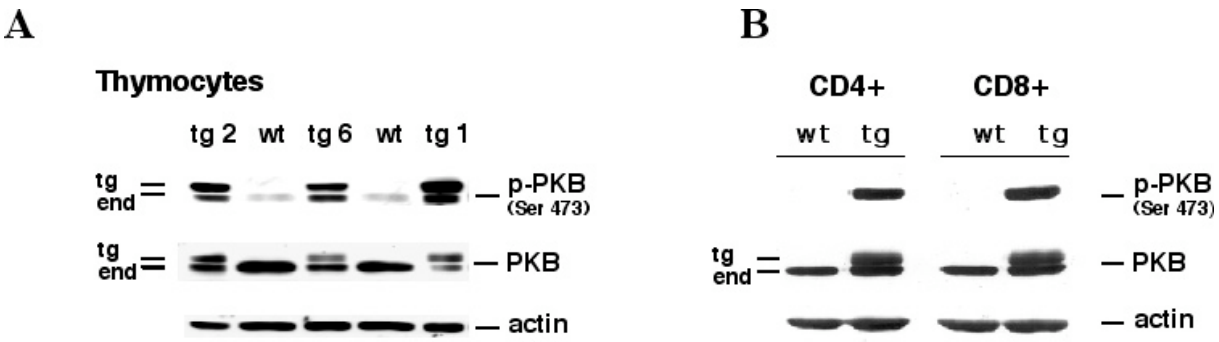

Fig 4.2 Expression of myr PKB in thymocytes and peripheral T cells.

A. Thymocytes from wild type (wt) and myr PKB tg lines (tg) were analyzed by Western blot using PKB and phospho-PKB (Ser 473) Abs. Results are shown for three different tg lines, PKB2, 6 and 1. Upper bands correspond to tg myr PKB, lower bands to endogenous (end) PKB. Actin Ab was used to control equal protein loading. B. Western blot analysis for phospho-PKB (Ser 473) expression in peripheral CD4+ and CD8+ T cells from wt and myr PKB2 tg mice.

4.1.2 Phenotype of myr PKB tg mice

First, we examined the cellular composition of thymus, spleen and lymph nodes from myr PKB tg and wt mice of different ages (Fig. 4.3 and Table 1).

Table 1. Myr PKB tg mice show a reduction in thymus size, CD8+ T cell numbers, and increased CD4:CD8 ratio with age.

Thymus

	wt				**PKB tg**			
(cell no x 10^7)	**total**	**CD4+**	**DP**	**CD8+**	**total**	**CD4+**	**DP**	**CD8+**
4-12 weeks	**16.19** (±6.51)	**1.26** (±0.79)	**13.86** (±5.85)	**0.45** (±0.30)	**17.13** (±6.70)	**1.47** (±0.71)	**14.36** (±6.39)	**0.67** (±0.35)
3-12 months	**6.28**** (±2.92)	**0.42** (±0.35)	**5.67**** (±2.91)	**0.11** (±0.10)	**4.38**** (±2.16)	**0.45** (±0.30)	**3.77**** (±2.11)	**0.13** (±0.08)

Spleen

	wt			**PKB tg**			**CD4:CD8 ratio**	
(cell no x 10^7)	**total**	**CD4+**	**CD8+**	**total**	**CD4+**	**CD8+**	**wt**	**PKB+/-**
4-12 weeks	**13.85** (±6.06)	**2.38***** (±1.39)	**1.10** (±0.79)	**14.62** (±4.18)	**4.67***** (±2.3)	**0.94** (±0.61)	**2.2**	**4.9**
3-12 months	**10.61** (±4.79)	**1.84***** (±1.23)	**0.77** (±0.59)	**11.38** (±5.09)	**4.51***** (±2.81)	**0.40**** (±0.18)	**2.4**	**11.3**

Cell numbers from thymi (n=28) and spleens (n=29) from wt or myr PKB tg mice aged either 4-12 weeks or 3-12 months were determined and cells were stained for CD4 and CD8 expression to detect T cells. Given are absolute cell numbers for the indicated subpopulations (x10^7) and the CD4:CD8 ratios from pooled young or aged mice. Student's t test **p<0.01 ***p<0.001.

Staining of thymocytes for CD4 and CD8 expression and analysis by flow cytometry showed that the composition of thymocyte subsets from young (4-12 weeks of age) myr PKB tg mice was comparable to that of littermate control mice or showed a moderate increase in CD4 and CD8 SP T cells (Fig. 4.3A and Table 1). A reduction in the number of DP thymocytes (to about 60% of wild type mice) was prominent in aged mice (3-12 months of age) and resulted in an overall reduction in thymocyte cellularity (Table 1). The development of DP thymocytes to mature SP thymocytes is associated with a series of phenotypic changes triggered by the TCR that can be defined using Abs reactive with the TCR, CD5 and CD69 antigens (136-138). Typically, a small percentage of DP thymocytes express higher levels of these markers, which are putative indicators for cells that have received either positive (139-141) or negative selection (142) signals in response to TCR engagements. Although thymi from myr PKB tg mice were grossly normal in size and morphology, a more detailed analysis revealed that myr PKB tg DP thymocytes have a higher percentage of cells that have upregulated TCR, CD5 and CD69 expression (Fig. 4.3A lower panel).

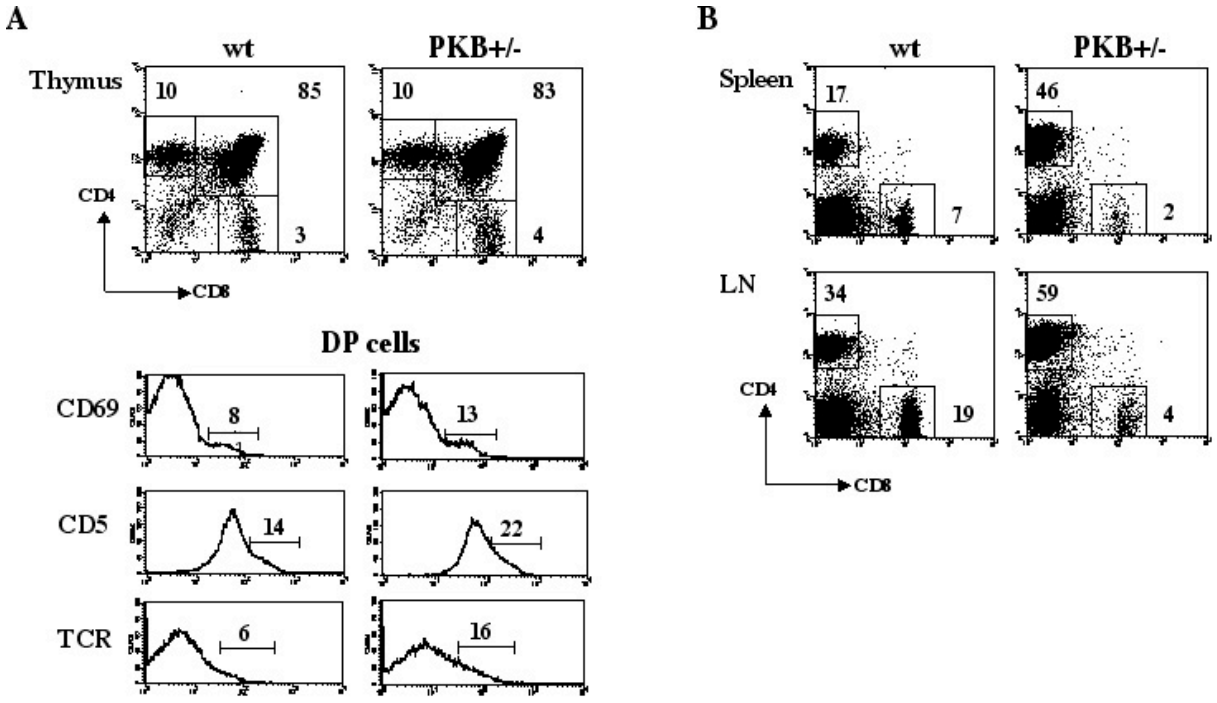

Fig 4.3 Analysis of cell subsets in thymus and peripheral lymphoid organs from myr PKB tg mice. Cell suspensions from A. thymus and B. spleen and lymph nodes of 8 weeks old mice were stained for CD4 and CD8 expression. Percentages of the individual subpopulations are indicated. Histograms in A. show expression of TCR, CD5 and CD69 after gating on DP thymocytes.

This suggests that in myr PKB tg mice more DP cells receive signals that qualify them for further differentiation or induction of apoptosis. Analysis of T cell subpopulations in the spleen and lymph nodes in myr PKB tg mice (Fig. 4.3B and Table 1) showed that the CD4 compartment was selectively enlarged, with 2-3-fold more splenic CD4+ T cells, whereas CD8+ T cells were reduced with age. A greater expansion or accumulation of CD4+ T cells led to an increase in the CD4:CD8 ratio which was about 5:1 in young mice (4-12 weeks) and 11:1 in older mice (3-12 months). Thus, myr PKB has differential effects on the homeostasis and/or generation of CD4+ and CD8+ T cells leading to a preferential increase in the peripheral CD4+ T cell compartment concomitant with a decline in the CD8+ T cell population.

4.1.3 Myr PKB tg thymocytes are hyperreactive to TCR stimulation and less sensitive to inhibition by CsA

To correlate the change in the level of PKB activity to functional responses we analyzed the proliferative potential of thymocytes in response to immobilized CD3 mAb and to the pharmacological agents PMA and ionomycin (IO), which are known to activate PKC and to induce calcium flux, respectively. As shown in Fig. 4.4A, stimulation of thymocytes with different concentrations of CD3 mAb resulted in higher ^{3}[H]-thymidine incorporation in myr PKB tg thymocytes. Astonishingly, thymocytes from myr PKB tg mice were capable to proliferate in response to PMA only, i.e. in the absence of the Ca^{2+}-fluxing agent ionomycin (Fig. 4.4B), whereas the two populations showed equivalent response to stimulation with PMA plus ionomycin. Moreover, in case of myr PKB tg mice the proliferative responses induced by CD3 mAb as well as those induced by PMA or PMA/IO were less sensitive to inhibition by the calcineurin inhibitors CsA or FK506 than those of wt thymocytes which were totally abolished (Fig. 4.4B). In addition, when TCR induced signaling was blocked by MEKinase inhibitor PD98059 (Fig. 4.4C) or Src-kinase inhibitor PP1 (Fig. 4.4D), myr PKB tg thymocytes again were more resistant to inhibition requiring 5-8-fold higher concentrations of inhibitor to achieve 50% inhibition of maximal proliferation. Thus, myr PKB synergizes with PMA to allow proliferation without a calcium signal and confers higher reactivity to limiting TCR signals coupled with CsA resistance.

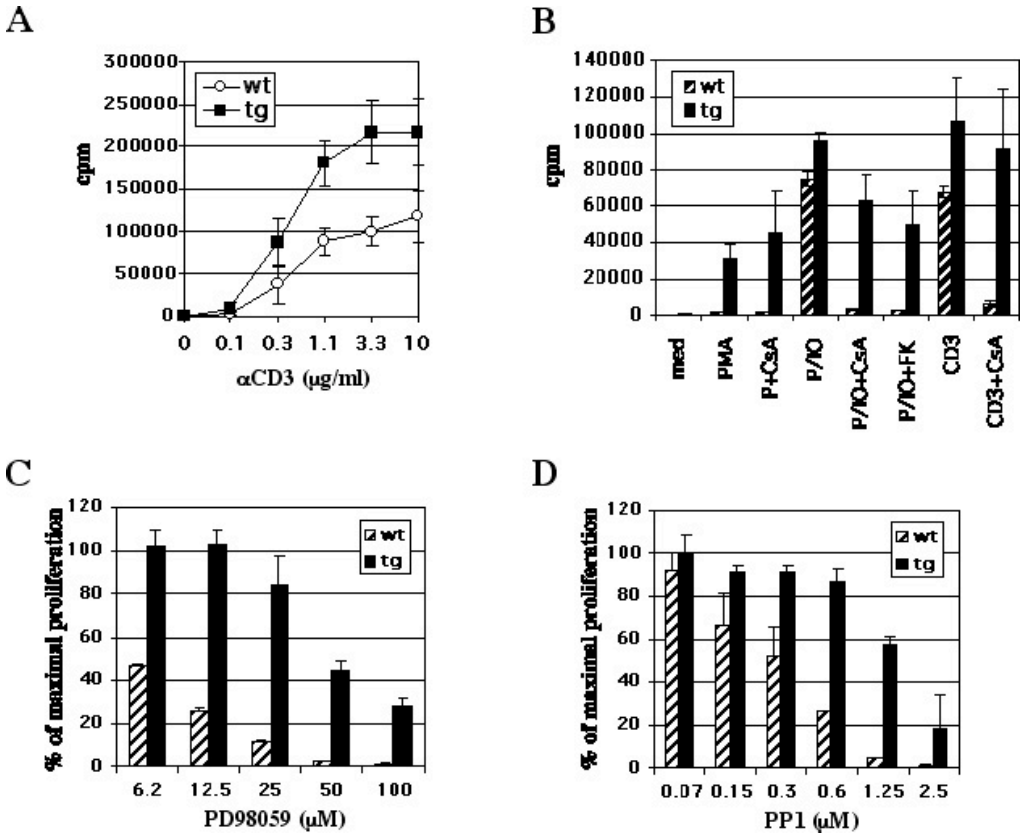

Fig 4.4 Myr PKB tg thymocytes are hyper-reactive against TCR stimulation and less sensitive to inhibition by CsA, FK506, MEK and Src-kinase inhibitors.

A. Equal numbers of total thymocytes from wild type or myr PKB heterozygous mice were activated with different concentrations of plate-bound CD3 mAb, and proliferation was measured by ^{3}[H]-thymidine incorporation. B. Thymocytes were cultured with PMA only (50 ng/ml), PMA plus ionomycin (P/IO, each 50 ng/ml) or CD3 mAb (CD3, 5 μg/ml) in the absence or presence of calcineurin inhibitors CsA (200 ng/ml) or FK506 (FK, 200 ng/ml). C. and D. Thymocytes were stimulated with CD3 mAb (5 μg/ml) in the absence or presence of MEK inhibitor PD98059 or Src-kinase inhibitor PP1 in concentrations as given. Data show the percent of maximal proliferation obtained by CD3 stimulation alone, which was set as 100%. In A-D proliferation was measured after 48 hrs and data represent averages from triplicate cultures from 2-3 individual wt or myr PKB tg mice.

4.1.4 Effects of myr PKB on survival of thymocytes and T cells

Next, we tested the response of thymocytes and peripheral T cells to various apoptotic stimuli. Apoptosis of thymocytes from young mice (6 weeks) was assayed after FCS withdrawal or treatment with PMA, ionomycin, CD3 or fas mAbs, dexamethasone, etoposide or UV-irradiation (Fig. 4.5A). For most treatments, the survival of myr PKB tg thymocytes was only slightly enhanced or identical with wild type cells. Survival was more significantly increased in

case of dexamethasone treatment. Titration of apoptosis inducing reagents and measurement of survival on day 2 (data not shown) reflected the differences observed as shown in Fig. 4.5A.

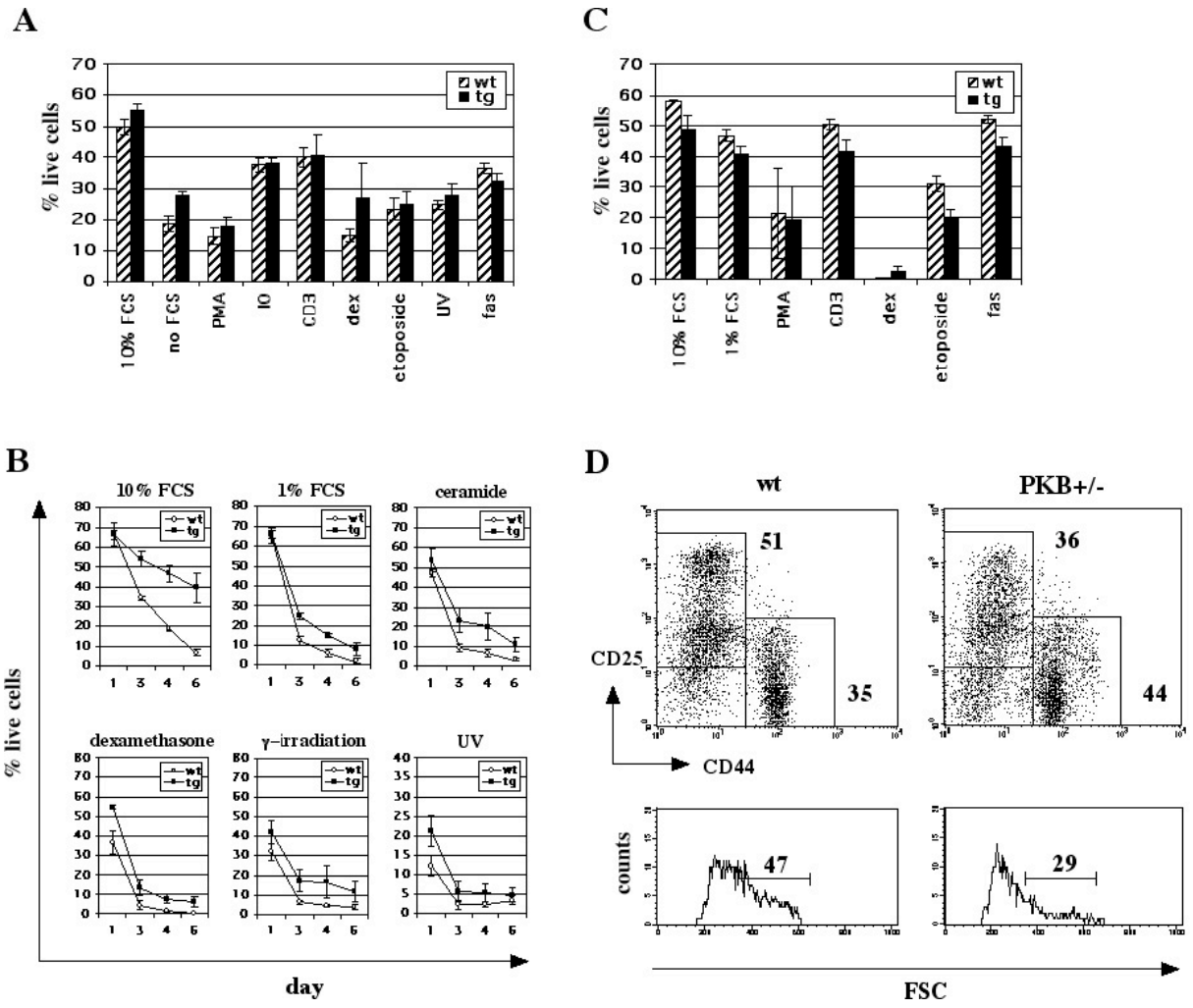

Fig 4.5 A-C. Effects of myr PKB on survival of thymocytes and peripheral CD4+ T cells.

A. Thymocytes from 6 weeks old mice were cultured in medium with 10% FCS or medium without FCS or were treated with PMA (50 ng/ml), ionomycin (IO, 50 ng/ml), CD3 mAb (10 □g/ml), dexamethasone (dex, 1 nM), etoposide (1 □M), UV-irradiation (0.01 J/cm^2) or fas mAb (3 □g/ml). The percentage of live (7-AAD and annexin V negative) cells was determined on day 1. B. Increased survival and reduced apoptosis in peripheral myr PKB tg CD4+ T cells. CD4+ T cells from wt and myr PKB tg mice were cultured in medium only containing 10% or 1% FCS or were treated with C2-ceramide (2 □M), dexamethasone (1 nM), □□irradiation (0.3 Gy) or UV-irradiation (0.01 J/cm^2) as indicated and live cells were determined on days indicated. Data were calculated from three individual mice and are representative of three independent experiments. C. Thymocytes from aged mice (7 months) were cultured in medium with 10% or 1% FCS or were treated with PMA (50 ng/ml), CD3 mAb (10 □g/ml), dexamethasone (dex, 3 nM), etoposide (0.1 □M), or fas mAb (9 □g/ml) and live cells were determined on day 1.

D. CD44/CD25 profiles of DN thymocytes from 10 months old wt and myr PKB tg mice. Isolated DN cells were stained with CD44 and CD25 mAbs and analysed by FACS. The percentage of cells present in each area is indicated. Histograms show cell size distribution within the CD25+CD44- population of wild type and myr PKB tg mice as measued by FACS. Numbers give the percent of large cells.

When peripheral CD4+ T cells were analysed (Fig. 4.5B), survival and resistance to apoptosis after treatment with ceramide, dexamethasone, UV- or γ-irradiation was distinctly higher in myr PKB tg CD4+ T cells. This indicates that myr PKB activity delivers stronger survival signals for mature peripheral T cells than for thymocytes.

Since aged myr PKB tg mice showed a reduction of DP cells, we analysed the apoptotic response of thymocytes from 7 months old mice (Fig. 4.5C). In contrast to young mice, DP thymocytes from aged myr PKB tg mice survived less well after serum withdrawal, CD3 and fas mAb or etoposide treatment. Sensitivity to dexamethasone treatment and PMA stimuli were comparable to thymocytes from young mice.

Besides decreased survival another possibility for the decrease in the DP population could be an inhibition in cellular expansion or differentiation of CD4-CD8-DN cells. Flow cytometric analysis of DN cells in aged myr PKB tg mice revealed that the percentage of CD25+CD44- (DN3) cells was reduced whereas the CD44+CD25- (DN) population was increased compared to wild type cells (Fig. 4.5D). The CD25+CD44- DN population of aged myr PKB tg mice contained a lower fraction of large cells as determined by forward side scatter analysis, indicating that they are less cycling. Altogether these observtions suggest that the decrease in the DP thymocyte population in aged myr PKB tg mice could result from a combinaton of diminished survival of DP cells and a partial block in expansion/differentiation of DN cells.

4.1.5 Myr PKB supports thymic selection of CD4+ T cells

To examine the role of myr PKB in positive selection of thymocytes with a single TCR specificity, we crossed myr PKB tg mice with OT2 or OT1 TCR tg mice. The majority of OT2 and OT1 TCR tg DP thymocytes bear TCRs specific for ovalbumin peptides presented by MHC class II or class I molecules, such that they are selected into the CD4 or CD8 lineage, respectively (132, 133). In myr PKB/OT2 double-tg mice (Fig. 4.6) we detected a marked increase in the percentage and number of CD4 SP cells but not CD8 SP thymocytes compared to OT2 mice not harbouring myr PKB. These CD4 SP thymocytes were fully mature as evidenced by the expression of the TCR transgenic Vβ2 chain, CD69, CD5 and HSA, i.e. surface antigens that are up- or downmodulated when positively selected DP thymocytes mature to SP cells. Since staining profiles of myr PKB/OT2 double-tg CD4 SP cells were comparable to those of OT2 CD4 SP cells, myr PKB enhances the efficiency of CD4 development.

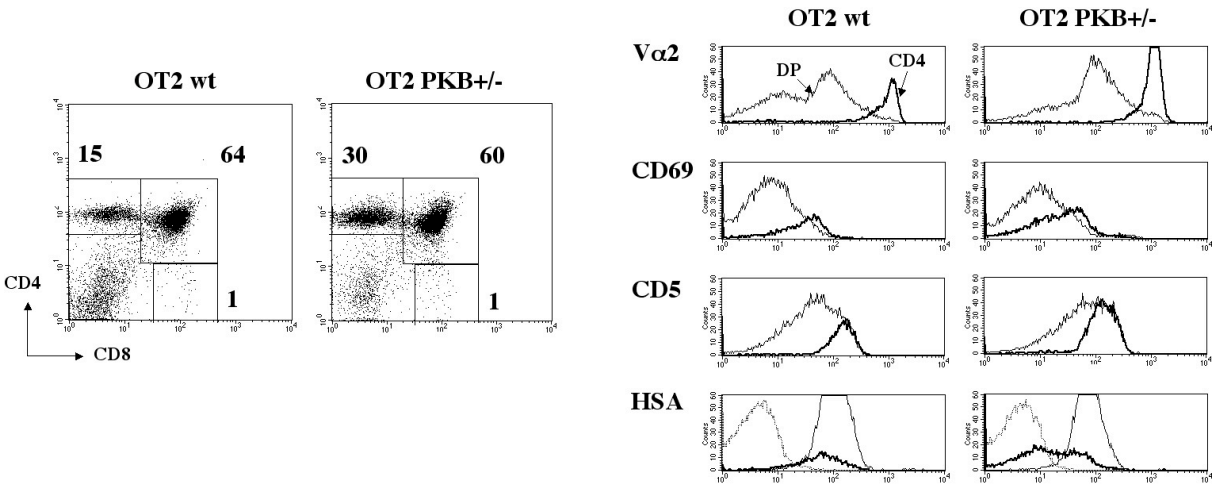

Fig 4.6 Enhanced positive selection of CD4+ T cells in myr PKB/OT2 TCR double-tg mice.
Thymi from 8 weeks old myr PKB tg mice crossed with OT2 TCR tg mice were stained for CD4 and CD8 expression. Percentages of cells in each quadrant are given. Histograms show expression of the tg TCR V□2 chain, CD69, CD5 and HSA on DP (thin line) and CD4 SP thymocytes (bold line). Dotted line indicates isotype control staining. Total cell numbers and quantitation of DP, CD8 and CD4 SP cells with comparable high expression of the OT2 TCR specific V□2 chain were for OT2 littermate mice (n=7, total $9.27x10^7$±1.38, DP $4.70x10^7$±1.2, CD4 SP $1.90x10^7$±0.78, CD8 SP $0.13x10^7$±0.10) and for OT2 PKB tg mice (n=9, total $12.07x10^7$±2.97, DP $6.35x10^7$±1.15, CD4 SP $4.06x10^7$±1.49, CD8 SP $0.17x10^7$±0.07).

The effect of myr PKB on positive selection of CD8 lineage cells was investigated in myr PKB/OT1 double-tg mice (Fig. 4.7A and 4.7B). Selection of OT1 CD8 SP T cells was not enhanced or even reduced in myr PKB/OT1 double-tg mice. Interestingly, in about 50% of myr PKB/OT1 double-tg mice a strong increase in the percentage and absolute cell number of OT1 CD4+ T cells was observed. Thymi from myr PKB/OT1 double-tg mice that did not show an overselection of CD4+ T cells phenotypically looked like OT1 wt mice but DP cells showed enhanced reactivity to deleting stimuli (see Fig. 4.10B). OT1 CD4 SP cells were phenotypically mature as assessed by expression of TCR tg chains, CD69 and CD5 which were comparable to mature CD8 SP T cells from OT1 mice (Fig. 4.7A). In parallel, in spleens from myr PKB/OT1 double-tg mice a 3-4-fold increase in OT1 V□2 TCR^{hi} CD4+ T cells and a decrease in OT1 CD8+ T cells was observed (Fig. 4.7B). Since the degree of CD4 overselection was variable, we analysed two other myr PKB tg lines crossed with OT1 mice, with similar expression levels of the PKB transgene, to exclude a dominant genetic effect from background genes. In these crosses maturation of CD4+ OT1 T cells was also observed in a high percentage but not in all mice (data not shown), indicating that CD4 overselection is linked to molecular mechanisms induced by myr PKB. Altogether, the data from the OT2 and OT1 experimental models suggest that myr PKB supports the selection and maturation towards the CD4 lineage.

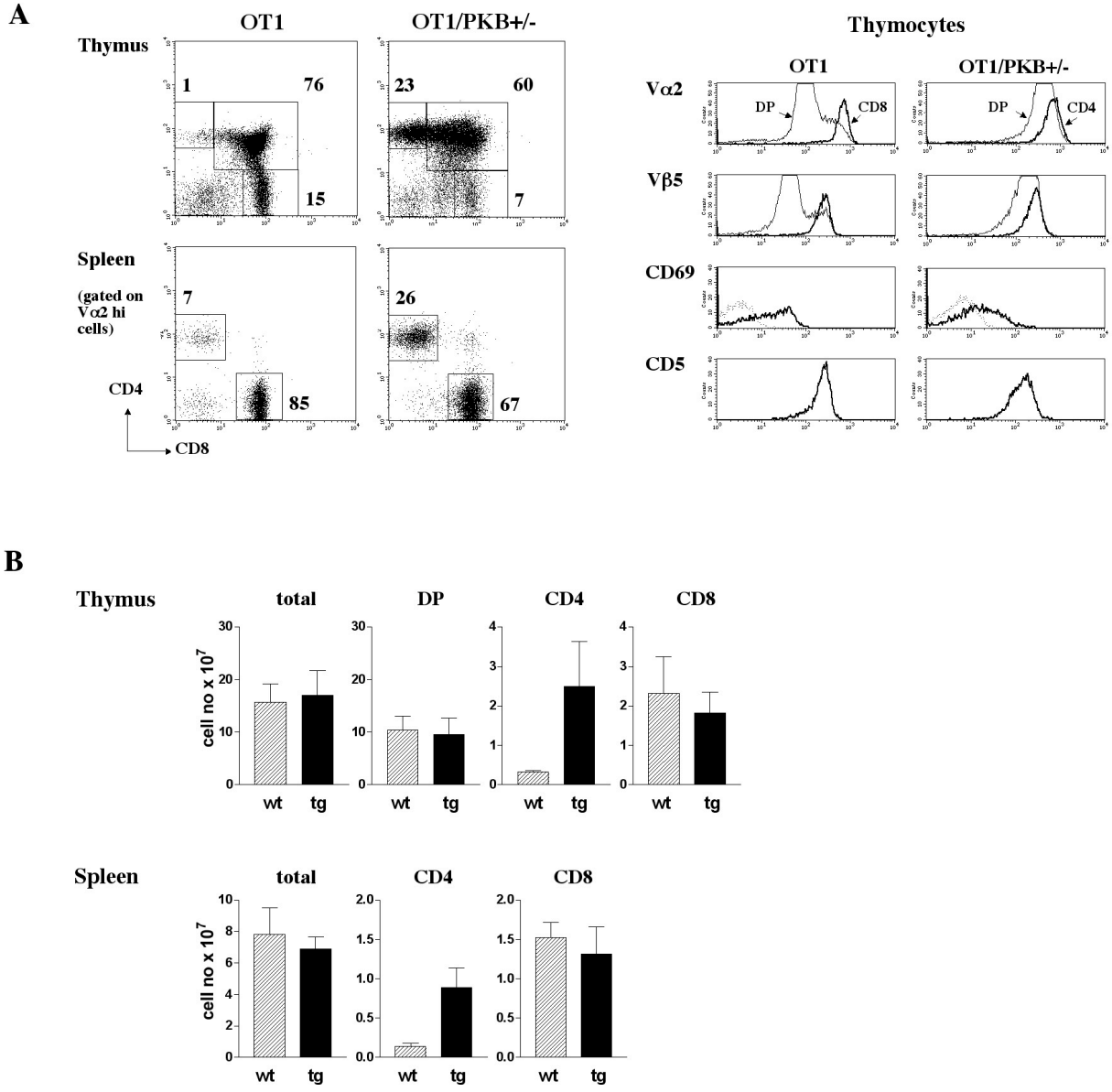

Fig 4.7 Myr PKB supports selection of OT1 CD4+ T cells.

A. Thymi and spleens from 6-8 weeks old OT1 mice or myr PKB tg mice crossed with OT1 TCR tg mice were stained for CD4 and CD8 expression. Percentages for thymocyte and splenic subpopulations are given. Plots for spleens are gated on OT1 TCR V□2^{hi} cells. Histograms for thymocytes were gated on CD8 SP (OT1 mice) or CD4 SP cells (OT1/myr PKB+/- mice) and show expression of OT1 TCR specific V□2 and V□5 chains and of CD69 and CD5 markers (bold lines). V□2 and V□5 expression is also shown for DP thymocytes (thin lines). Dotted line indicates isotype control staining. B. Total cell numbers for thymus and spleen from OT1 myr PKB double-tg mice (n=12, data include CD4 overselecting mice and myr PKB/OT1 double-tg mice looking phenotypically similar to wild type OT1 mice) and OT1 mice (n=12) were determined and the absolute cell numbers for V□2 positive (V□2^{hi} cells in case of mature cells) thymocyte and splenic subpopulations were calculated from percentages gained by FACS staining.

4.1.6 Effects of myr PKB on negative selection

To investigate the role of myr PKB in negative selection, we crossed myr PKB tg mice with HY TCR tg mice. In these mice thymocytes are positively selected in female but negatively selected in male mice (134). The latter results in small thymi mainly due to the marked deletion of DP thymocytes whereas positively selecting HY female mice show an increase in the CD8 SP population consistent with the recognition of MHC class I molecules by the HY TCR (Fig. 4.8). In myr PKB/HY double-tg female mice cell numbers for HY-specific CD8 SP thymocytes, as determined with the T3.70 mAb (recognising the tg Vβ chain), were comparable to HY female mice but numbers of CD4+ T3.70+ cells were increased about 3-4-fold. This enhanced selection of CD4+ T cells is similar to our observations in OT2 and OT1 TCR myr PKB double-tg mice. In negatively selecting male myr PKB/HY double-tg mice, on an average a 2-3-fold increase in the numbers of HY specific DP and CD8+ T cells was detected as well as a 2-4-fold increase in peripheral CD4+ and CD8+ T cells expressing autoreactive TCRs (data not shown). Although total cell numbers in male myr PKB/HY double-tg mice were still much lower than in female mice, myr PKB has a definite attenuating effect on negative selection.

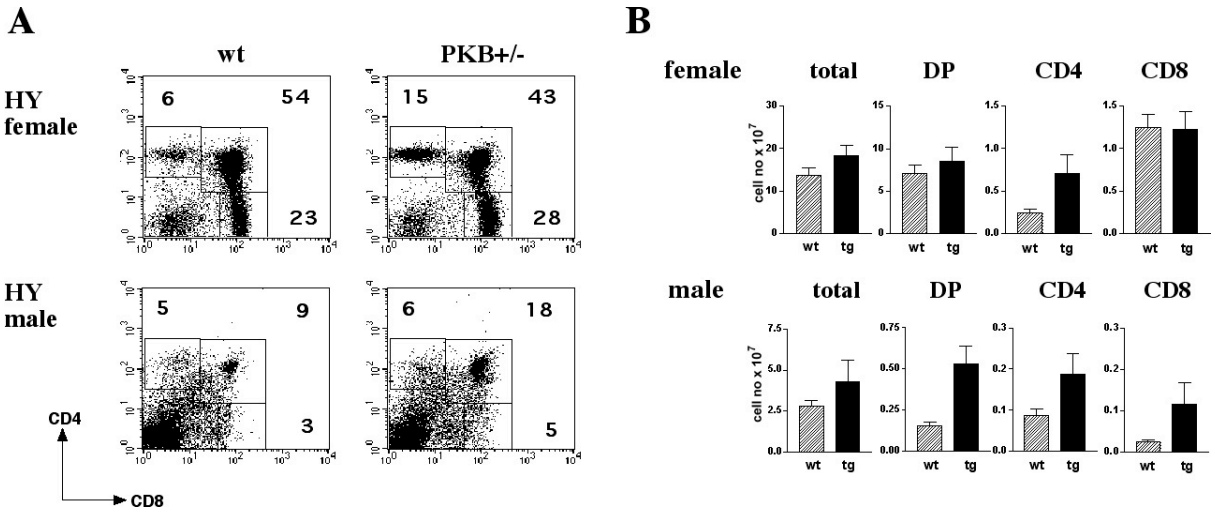

Fig 4.8 Effects of myr PKB on negative selection.

A. Thymi from female and male HY and HY myr PKB tg littermate mice were analysed by FACS for expression of CD4, CD8 and the HY TCR-specific Vβ chain using T3.70 mAb. B. The percentages for T3.70+ CD4+, T3.70+ DP and T3.70+ CD8+ cells were determined and absolute cell numbers for T3.70+ cells were calculated from total cell numbers; HY male mice n=12 and HY female mice n=7 for wt and myr PKB tg mice.

Since the HY system provides a very strong, early deletion stimulus, we investigated other model systems of negative selection. First, we analyzed deletion mediated by endogenous

superantigens (SAgs). Myr PKB tg mice (on C57/BL6 background) were bred with DBA/2 and CBA/J mice carrying the endogenous mouse mammary tumor viruses Mtv 6, 8 and 17, which in presence of I-E molecules induce deletion of SAg-reactive T cells bearing V□5 and V□11 TCRs (143). In analyses as shown in Fig. 4.9A, we did not detect a myr PKB dependent difference in the deletion of SAg reactive V□5+ or V□11+ CD4+ T cells in the thymus or periphery, suggesting that myr PKB does not alter *in vivo* negative selection of SAg reactive T cells.

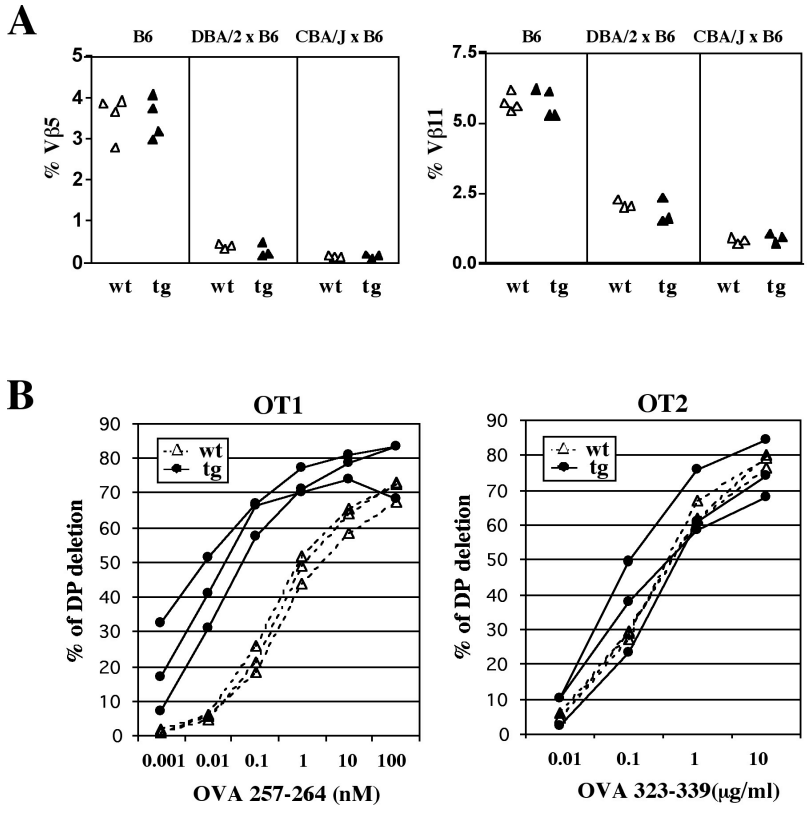

Fig 4.9 Effect of myr PKB on deletion of superantigen reactive T cells.

A. CD4+ T cells from wild type and myr PKB tg mice on C57/BL6 (B6) background and myr PKB tg mice crossed to DBA/2 and CBA/J mice (F1 generation) were analyzed for V□5 and V□11 expression. Mice were assayed at 5 weeks after birth. B. Influence of myr PKB on peptide antigen induced deletion of OT1 and OT2 DP thymocytes. Thymocytes from OT1 and OT2 mice (wt, broken lines) and myr PKB/OT1 or myr PKB/OT2 double-tg (tg, full lines) mice aged 6-7 weeks were cultured with the indicated concentrations of TCR-specific ovalbumin peptide antigen (OVA). On day one the percent of live DP cells was determined by CD4/CD8 labeling and FACS analysis. The graphs show the percent of peptide-induced deletion of DP cells compared to cultures without antigen.

The effect of myr PKB on negative selection in OT1 and OT2 mice was tested by culturing thymocytes from 6-7 weeks old mice with TCR specific agonistic peptides (144). As depicted in Fig. 4.9B, myr PKB distinctly enhanced negative selection induced by ovalbumin peptide 257-264 in OT1 mice. In contrast, deletion of thymocytes from OT2 mice, induced by ovalbumin peptide 323-339, on an average was not altered by myr PKB expression. Thus, in analysis of four experimental models myr PKB expression has differential effects, either partially blocking, enhancing or leaving negative selection unaltered.

4.1.7 Superantigen-induced deletion of peripheral T cells

Peripheral deletion of T cells was investigated by injection of mice with the superantigen staphylococcal enterotoxin B (SEB). SEB specifically recognizes TCRs containing the V□8 chain and leads to expansion and subsequent deletion of V□8+ peripheral T cells (145). As shown in Fig. 4.10A, a single dose of SEB (100 □g) injected into the peritoneal cavity of wt mice induced an expansion of V□8+CD4+ T cells that peaked on day 2 post injection, followed by deletion of this population by day 3. V□8+ CD4+ T cells from myr PKB tg mice expanded to the same degree as those from wt mice but were markedly more resistant to deletion. Control V□10+ CD4+ peripheral T cells, which do not respond to SEB, failed to undergo expansion and deletion in both mouse strains (Fig. 4.10B).

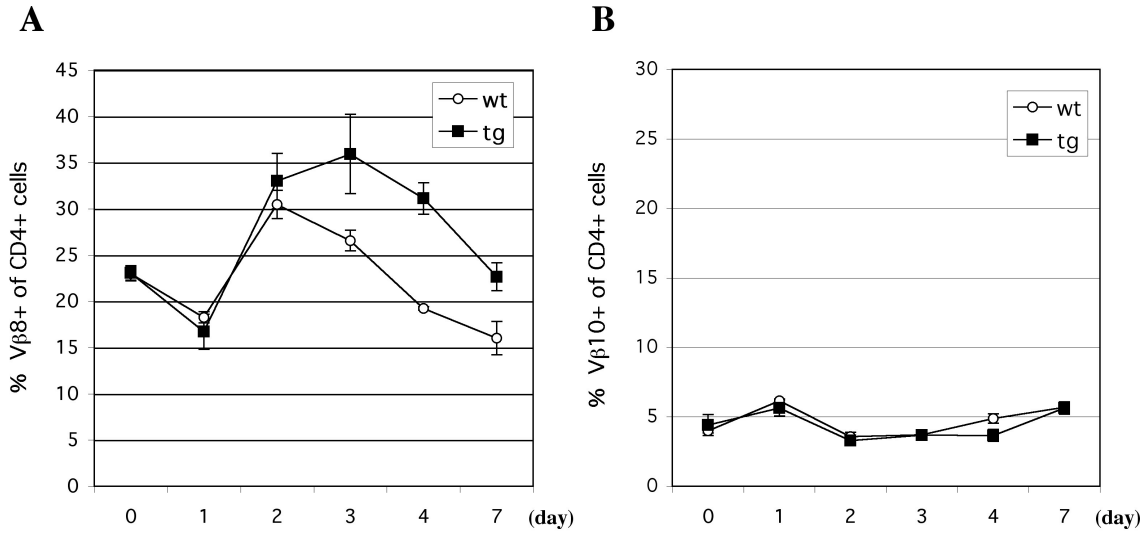

Fig 4.10 Impaired deletion of V□8+ T cells following SEB injection *in vivo*.

A. and B. Wild type and myr PKB tg mice were injected i.p. with SEB on day 0. Total blood cells were stained with V□8 and CD4 (A.) or with V□10 and CD4 (B.) Abs and analyzed by flow cytometry.

4.1.8 Signalling molecules affected by myr PKB expression

To define molecular events that could mediate the enhanced proliferation of thymocytes and the increase in maturation of CD4 lineage cells, we analysed the activity of the mitogenic activated protein kinase (MAPK) Erk and the Src-family kinase Lck, which have been shown to be central regulators of thymocyte selection and lineage choice. Strong and prolonged Lck and Erk signalling in particular favour development of the CD4 lineage but are needed less so for differentiation to the CD8 lineage (5). When freshly isolated thymocytes were analysed for Erk activation (Fig. 4.11A upper panel), myr PKB tg thymocytes showed higher endogenous levels of phospho-Erk than wt thymocytes. Furthermore, stimulation of thymocytes with CD3 mAbs (Fig. 4.11B) led to stronger and notably longer lasting Erk activation in myr PKB tg thymocytes. In addition, the levels of activated c-Raf, an upstream kinase of Erk, were also elevated showing that myr PKB acts as a positive regulator of the Raf-Mek-Erk signaling cascade.

For analysis of Lck activation we first used phosphotyrosine mAb in Western blots and observed that in rested non-stimulated myr PKB tg thymocytes tyrosine phosphorylation of Lck was as strong as in wt thymocytes after stimulation with CD3 mAb (Fig. 4.11B). Since phosphorylation can either activate or downmodulate Lck catalytic activity (146), the enhancement of Lck activity by myr PKB was confirmed by Lck kinase assay (Fig. 4.11B). As observed for Erk, increased Lck activity could also be detected in freshly isolated myr PKB tg thymocytes using Lck kinase assay and Abs that detect the activating Tyrosine 394 phosphorylation of Lck (Fig. 4.11A middle and lower panels). Thus, signals whose intensity and duration favour selection towards the CD4 lineage are clearly enhanced in myr PKB tg thymocytes and are likely to contribute to the observed increase in the selection of CD4 lineage cells found in myr PKB TCR double-tg mice.

In this context we also studied the activation of the c-cbl proto-oncogene which is highly expressed in thymocytes. Cbl-c has been shown to be an important regulator of TCR signaling with both a positive regulatory function as an adapter molecule and a negative function, inducing the degradation of proteins by its ubiquitin ligase activity (147). As shown in Fig. 4.11B, TCR triggering of thymocytes induced phosphorylation of Cbl-c. Interestingly, myr PKB enhanced the phosphorylation of Cbl-c suggesting that one way how myr PKB potentiates the Erk signaling cascade could be via activation of Cbl-c.

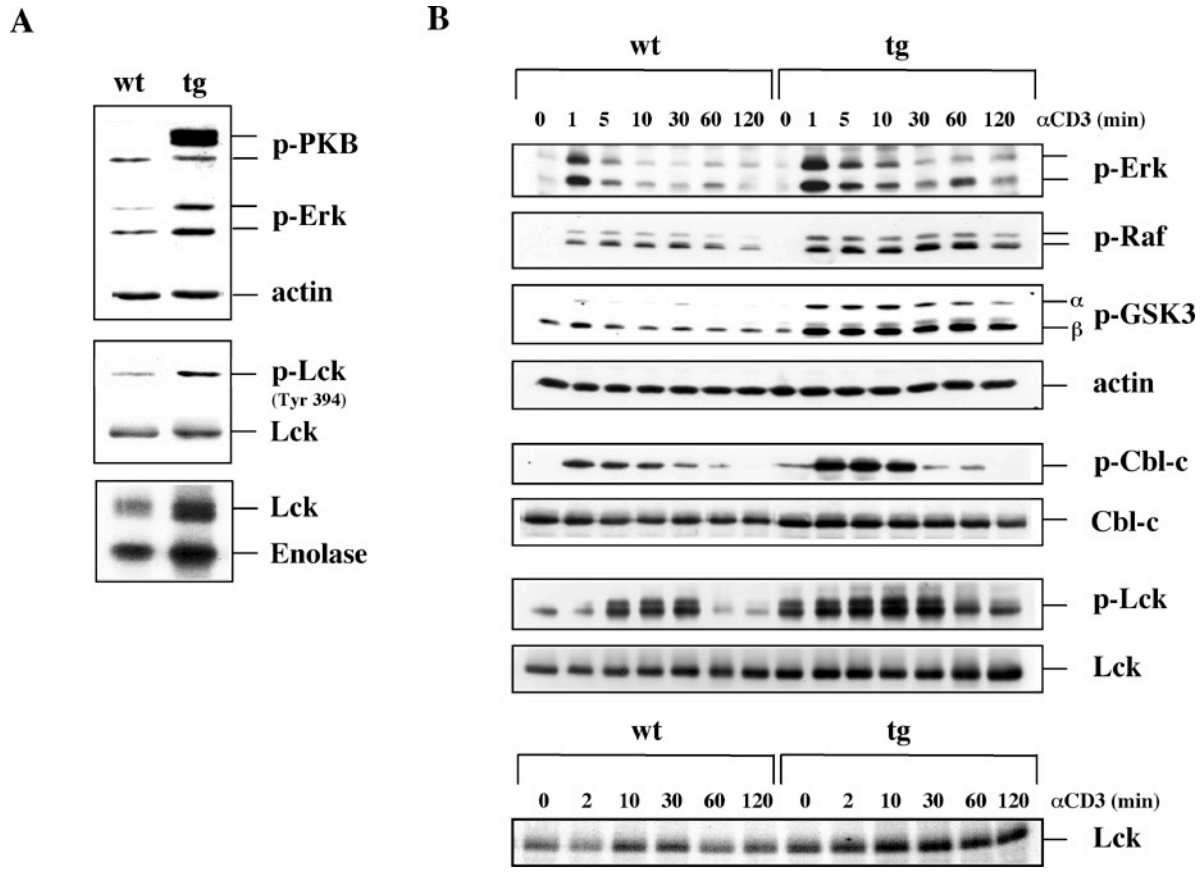

Fig 4.11 Active PKB enhances phosphorylation of Lck, Erk, Cbl-c and GSK3.

A. Freshly isolated thymocytes from control (wt) and myr PKB tg mice (tg) were lysed immediately and protein extracts were analysed by Western blot for activation of PKB and Erk (upper panel) and Lck (tyrosine 394, middle panel) using phosphospecific Abs. Blots were re-probed for actin and Lck to control protein loading. In the lower panel Lck tyrosine kinase activity from freshly isolated thymocytes was determined by immune complex kinase assay. In addition to autophosphorylation of Lck enolase was used as an indicator of transphosphorylation activity.

B. Thymocytes from myr PKB tg and wt mice were rested for 2 hrs in 1% FCS medium before activation with CD3 mAb for the time period indicated. Activation of Erk, Raf and GSK3 were determined in Western blot using phosphospecific Abs. Activation of Lck and Cbl-c was analysed with phosphotyrosine mAb. Protein content was controlled by re-probing blots with Cbl-c, Lck and actin Abs. In the lowest panel Lck was immunoprecipitated and Lck activity was determined by kinase assay.

Increased activity of Lck, Erk and possibly Cbl-c most likely contribute to the enhanced proliferation of myr PKB tg thymocytes. Since myr PKB tg T cells proliferated in the presence of the calcineurin inhibitors CsA or FK506 (Fig. 4.4), we analysed phosphorylation and thus inactivation of GSK3, which has been shown to oppose nuclear NFAT localization (148). We found that TCR triggering in normal thymocytes leads to fast and transient phosphorylation of GSK3. However, in myr PKB tg thymocytes phosphorylation of GSK3 was not only stronger

but also sustained over a much longer period (Fig. 4.11B). This suggests that a sustained block of GSK3 activity by myr PKB may render residual low calcineurin activity in the presence of CsA or FK506 sufficient to allow proliferation of myr PKB tg thymocytes in the presence of these immunosuppressants.

4.1.9 PKB is recruited to membrane lipid rafts after TCR/CD3 stimulation

Glycosphingolipid-enriched microdomains (GEMS), also known as membrane lipid rafts or low-density detergent insoluble glycolipid-rich membrane domains, are important structural membrane elements for the initiation of TCR mediated signalling (149).

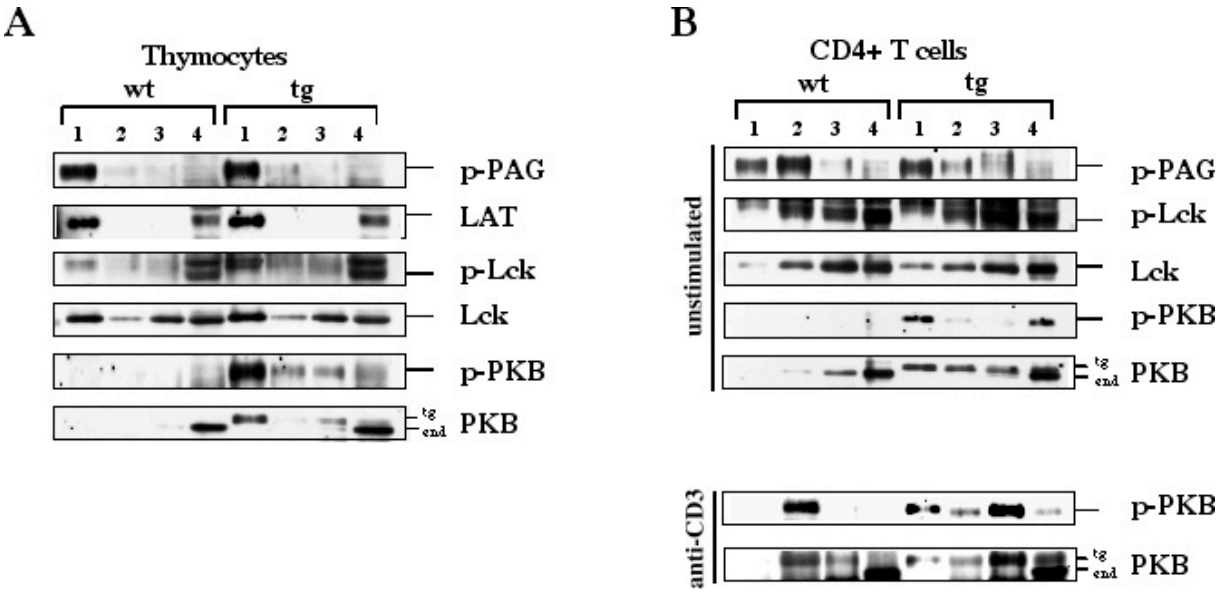

Fig 4.12 Active PKB localizes in membrane lipid rafts.

A. Localization of active PKB in lipid rafts. Indicated proteins in detergent insoluble lipid raft fractions (1 and 2) and detergent soluble fractions (3 and 4) isolated from thymocytes from wt and myr PKB tg mice were detected by Western blot. In anti-PKB blot the lower band corresponds to endogenous (end) PKB, the upper band to transgenic (tg) PKB. B. PKB translocates into lipid rafts after TCR/CD3 stimulation. Proteins in lipid raft and detergent soluble fractions from lymph node CD4+ T cells, either unstimulated (upper five panels) or stimulated for 30 min with CD3 mAb (lower two panels), were detected as described under A. Note that after activation endogenous PKB shows a mobility shift running at the same hight as tg PKB.

In order to define how myr PKB could regulate the activation of Lck and Erk, we studied the membrane localization of the tg protein. Low density detergent insoluble fractions and heavy soluble fractions from freshly isolated thymocytes were separated by ultracentrifugation and

anlaysed by western blotting (Fig. 4.12A). Whereas endogenous PKB in myr PKB tg and wt thymocytes was found only in the soluble fractions, transgenic phosphorylated PKB was locoalized predominantly in the lipid raft fraction. The latter was identified by the presence of the transmembrane adapter proteins PAG (phosphoprotein associated with GEMs) (150) and LAT (linker for activation of T cells) (151). Similar results were obtained for peripheral CD4+ T cells (Fig. 4.11B), however, in CD4+ T cells phosphorylated myr PKB was distributed in both, insoluble and soluble fractions. To analyze whether the presence of myr PKB in lipid rafts is a feature of TCR-mediated activation, we studied localization of PKB after TCR/CD3 stimulation (Fig. 4.12B, lower two panels). Intriguingly, we found that after TCR stimulation activated phosphorylated PKB is associated with lipid rafts in wild type CD4+ T cells similar to tg myr PKB. These results suggest a central role for PKB in TCR-induced formation of signaling complexes within lipid rafts.

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

To determine whether myr PKB influences cytokine production we performed RNase protection assays (RPA) and measured intracellular cytokine expression of T cells (Fig. 4.13A-C). For RPAs, purified CD4+ T cells were stimulated with CD3 mAb (5 μg/ml) for 4, 12, 24, 48, or 72 hrs and RNA was isolated. The RPAs showed that mRNA levels of Th1 cytokines, such as IFNγ, and notably also Th2 cytokines such as IL-4, IL-10 and IL-13 were increased after TCR/CD3 stimulation (Fig. 4.13A and B).

For detection of intracellular cytokine production isolated CD4+ T cells from wt and myr PKB tg mice were stimulated for 4 hrs with PMA plus ionomycin (IO) in the presence of brefeldin A or for 3 days with CD3 mAb, and then restimulated. After 4 hrs PMA/IO stimulation myr PKB tg CD4+ T cells showed about 2-fold increase in the percentage of cells expressing IL-4, IL-10 and IFNγ(Fig. 4.14C). The enhanced expression of cytokines in myr PKB tg CD4+ T cells was also apparent after 3 days TCR/CD3 stimulation. In both cases, more cells expressed the corresponding cytokine but individual cytokine production was also higher. Thus, intracellular cytokine staining supported the mRNA data from RPAs (Fig. 4.14C). Therefore, myr PKB has an overall positive regulatory effect on Th1 as well as Th2 cytokine production.

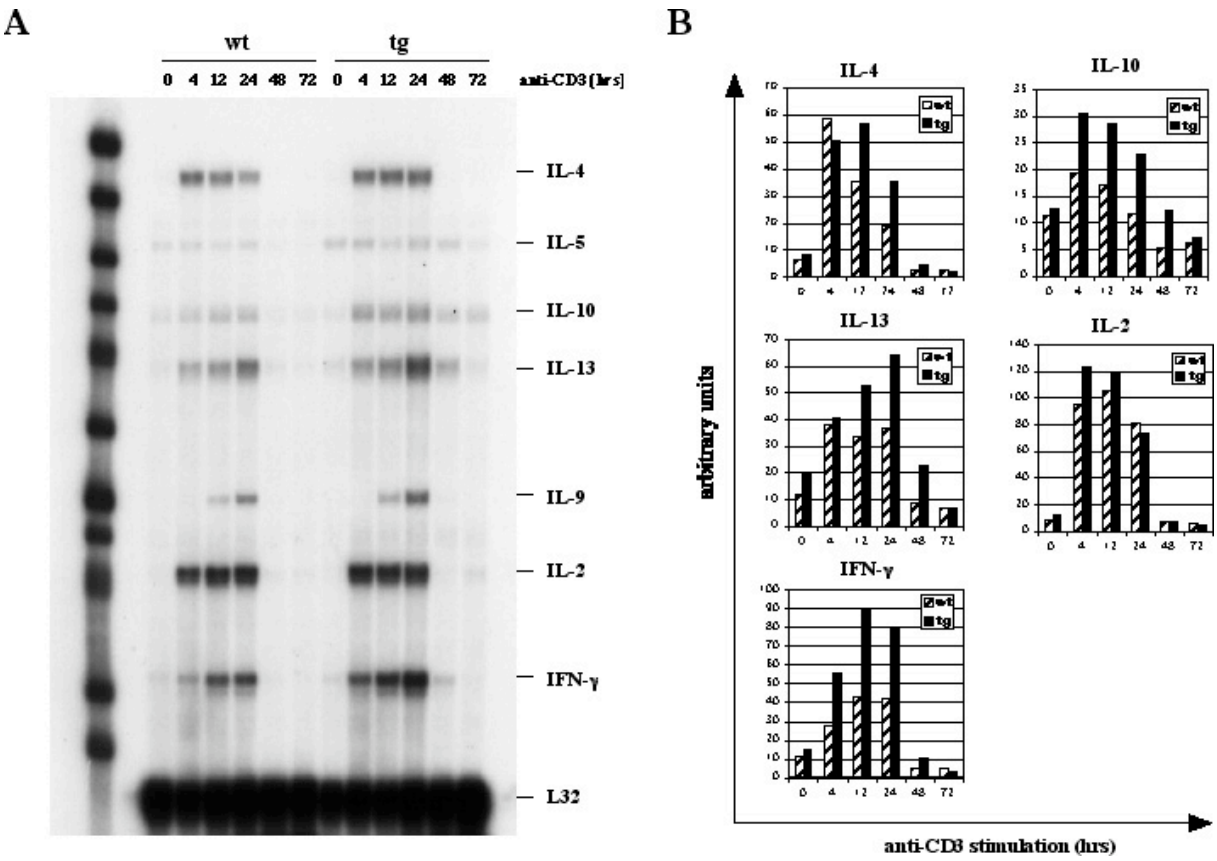

Fig 4.13 Myr PKB enhances the production of Th1 and Th2 cytokines.

A. CD4+ T cells from wt and myr PKB tg mice were left unstimulated or stimulated with plate bound CD3 mAb (5 □g/ml) for the indicated time points. RNA was isolated from each time point and cytokine mRNA expression level was determined by RPA. B. Evaluation of cytokine levels of RPA in A. Cytokine levels were normalized to the expression of the L32 house keeping gene and are given in arbitrary units.

Recently, a number of reports have emphasized that members of the suppressor of cytokine signalling family regulate cytokine signal transduction, thereby regulating immune responses and homeostasis (152-154). The expression of the family member cytokine-induced Src homology 2 protein (CIS) is induced in T cells by TCR stimulation, and overexpression of CIS in the CD4 T cell lineage was shown to enhance proliferative responses and survival of T cells (155). In view of the positive regulatory effects of myr PKB on proliferation and cytokine production, we studied the expression of CIS and detected accelerated and enhanced expression of CIS in myr PKB tg CD4+ T cells compared to wt cells (Fig. 4.14D). Thus, positive regulation of CIS may contribute to the enhanced functional responses of myr PKB tg T cells.

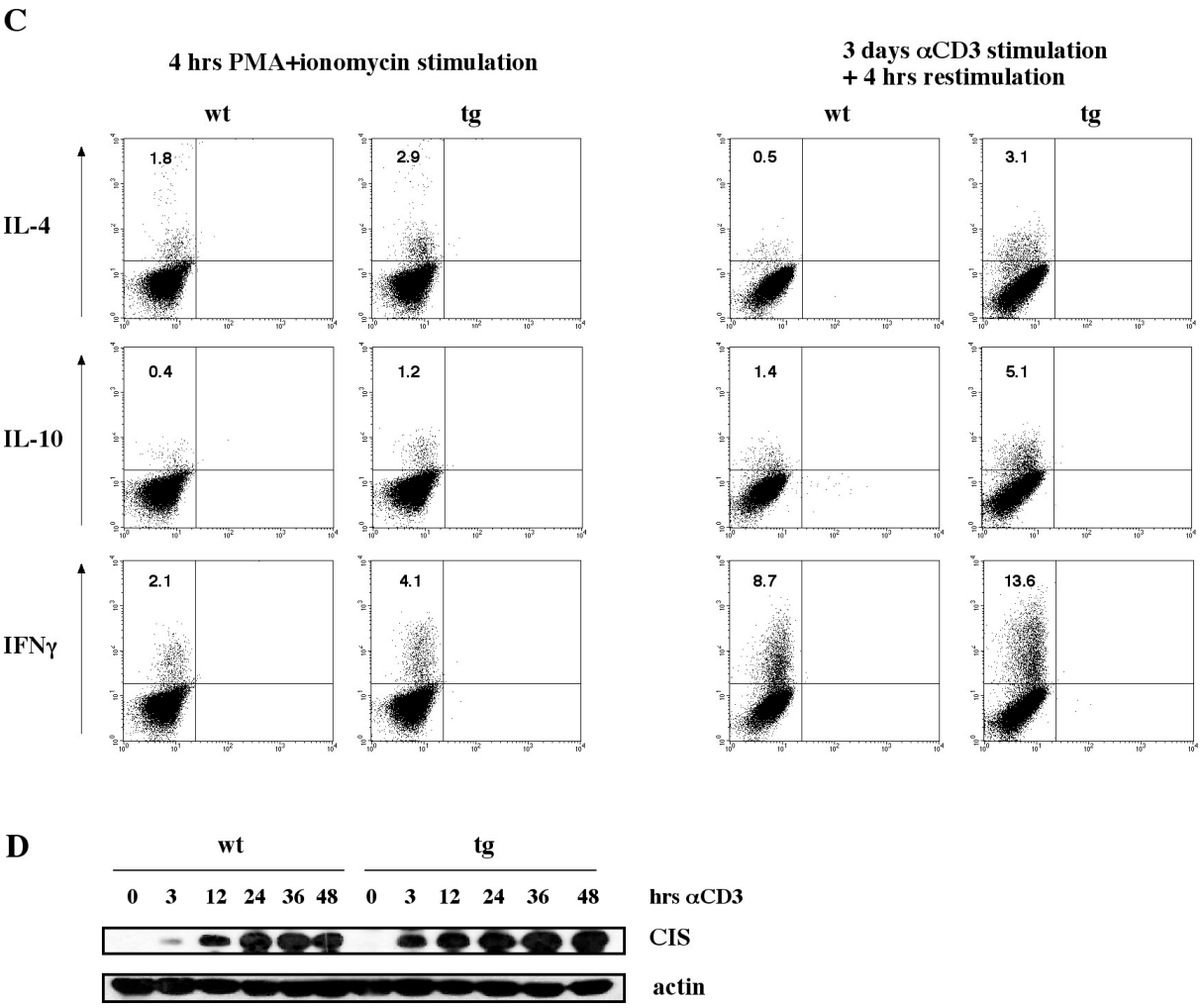

Fig 4.13 C. Purified CD4+ T cells from wt and myr PKB tg mice were stimulated for 4 hrs with PMA plus ionomycin (each 100 ng/ml) in the presence of brefeldin A (10 μg/ml) (left panel) or were stimulated for 3 days with immobilized CD3 mAb (5 μg/ml) and then restimulated with PMA plus ionomycin (each 100 ng/ml) for 4 hrs in the presence of brefeldin A (10 μg/ml) before cytokine analysis (right panel). Cytokine production was measured by intracellular staining for the indicated cytokine and flow cytometry. D. CD4+ T cells from wt and myr PKB tg mice were stimulated with CD3 mAb (5 μg/ml) for the indicated time points, and CIS expression was determined by Western blot. Actin expression is given as a control for protein loading.

4.1.11 Myr PKB ablates the inhibitory effects of TGF-β1 in T cell proliferation

Transforming growth factor-β1 (TGF-β1) is an immunosuppressive cytokine essential for the maintenance of immunological self-tolerance in the CD4+ T cell compartment. However, the molecular effects of TGF-β1 signalling on T-helper (Th) cell expansion are not well understood. In some studies TGF-β1 seems to inhibit T cell proliferation (156-158), while in others TGF-β1 was reported to augment Th cell proliferation (159-162). These data suggest the existence of physiological factors that regulate the responses of Th cells to TGF-β1, determining whether TGF-β1 inhibits or augments T cell expansion. To determine whether

myr PKB affects TGF-β1 signalling, myr PKB tg CD4+ T cells were stimulated with immobilized CD3 mAb or CD3 plus CD28 mAb in the presence of TGF-β1. As shown in Fig. 4.14A, proliferation of myr PKB tg CD4+ T cells was not affected or even augmented by TGF-β1 whereas proliferation of wt CD4+ T cell was drastically inhibited by TGF-β1. After costimulation with CD28 mAb TGF-β1 treatment did not block expansion of wt cells but even enhanced proliferation compared to activation with CD3 mAb only. This indicates that myr PKB can transduce signals similar to CD28 in TGF-β1 signalling and renders CD4+ T cells 'resistant' to inhibitory TGF-β1 signals.

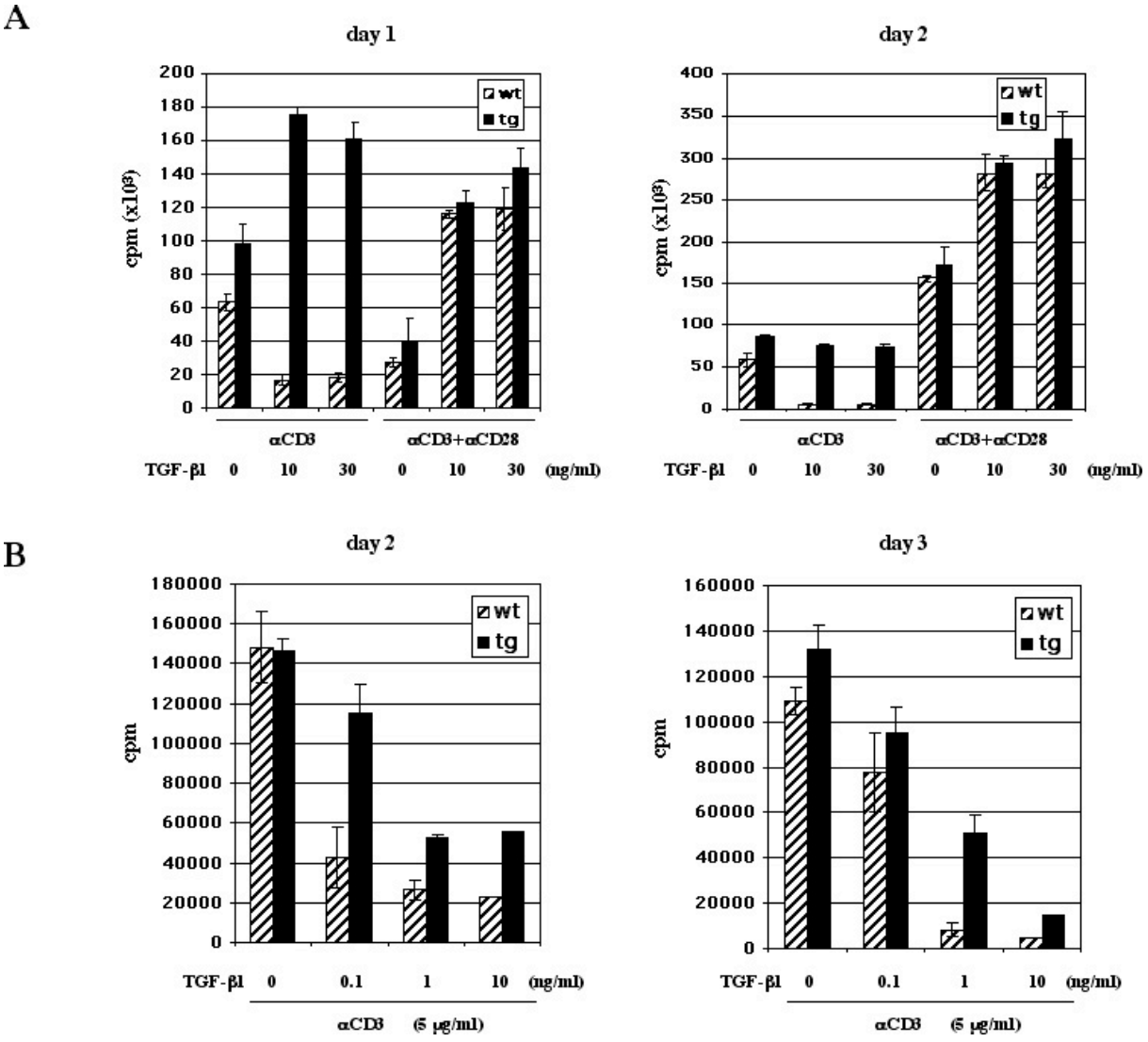

Fig 4.14 Myr PKB modulates the effect of TGF-β1 on CD4+ T cell proliferation.

A. CD4+ T cells from wt and myr PKB tg mice were stimulated with CD3 mAb (5 µg/ml) or CD3 (1 µg/ml) plus CD28 (5 µg/ml) mAb and TGFβ-1 as indicated. ^{3}[H]-thymidine incorporation was measured on day 1 or day 2 after initiation of cultures. B. Isolated CD4+ T cell were stained with CD62L mAb and CD62L^{hi} naive T cells were isolated by magnetic beads. Naive CD4+ T cells were stimulated with plate bound CD3 mAb (5 µg/ml) and TGF-β1 (0.1-10 ng/ml) as indicated and proliferation was measured on day 2 or 3.

It has been reported that TCR/CD3 induced proliferation of memory ($CD62L^{low}$) T cells is enhanced by TGF-β1 whereas TCR/CD3 induced proliferation of naive ($CD62L^{hi}$) T cells is inhibited by TGF-β1 (163). To examine whether the lack of inhibition of proliferation by TGF-β1 in myr PKB tg CD4+ T cells occurred because of a higher fraction of memory T cells, we isolated naive CD4+ T cells using magnetic cell sorting and measured their proliferation after stimulation with CD3 mAb and different doses of TGF-β1. As shown in Fig. 4.14B, myr PKB tg naive CD4+ T cells were less inhibited by TGF-β1 compared to wt naive CD4+ T cells.

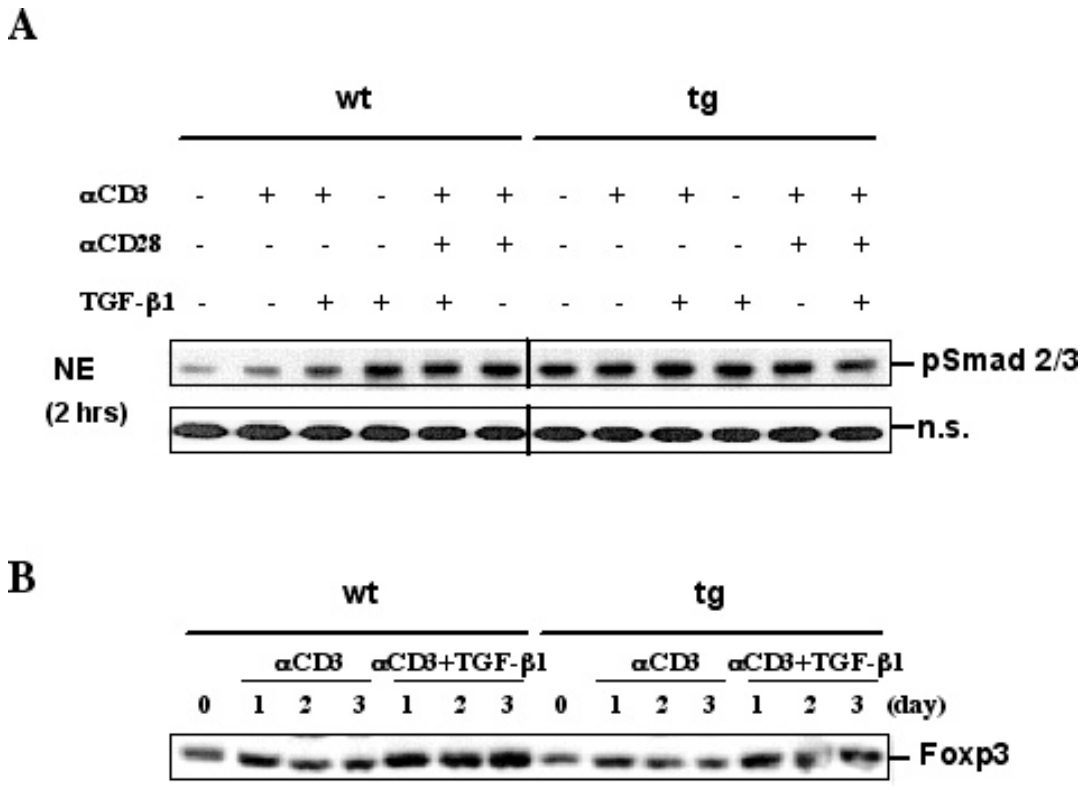

Fig 4.15 Expression of pSmad2/3 and Foxp3 in wt and myr PKB tg CD4+ T cells.

A. Purified CD4+ T cells from wt and myr PKB tg mice were cultured in medium only or stimulated with plate bound CD3 mAb (5 μg/ml) or with CD3 (1 μg/ml) plus CD28 (5 μg/ml) mAb in the presence or absence of TGF-β1 for 2 hrs. Nuclear protein extracts (NE) were analyzed for nuclear translocation of phospho-Smad2/3 by Western blot. B. CD4+ T cells were stimulated with CD3 mAb (5 μg/ml) in the presence or absence of TGF-β1 for the indicated time points. Total protein extracts were examined for Foxp3 expression by Western blot.

To determine the molecular mechanisms affected by myr PKB in the observed TGF-β1 resistance, we examined phospho-Smad2/3 protein expression. When purified CD4+ T cells from myr PKB tg and wt mice were stimulated with TCR/CD3 Ab alone or TCR plus CD28

Ab in the presence of TGF-β1 we found strong phosphorylation of Smad2/3 in myr PKB tg CD4+ T cells prior to CD3 stimulation and expression levels of pSmad2/3 were comparable to those found in wt cells after TGF-β1, or CD3 plus CD28 stimulation. This suggests myr PKB leads to constitutive activation of Smad2/3 proteins.

Foxp3, which encodes a transcription factor that is involved in the development of autoimmune and inflammatory syndromes in humans and mice, is specifically expressed in naturally arising CD4+CD25+ regulatory T cells. It has been shown that TGF-β induced Foxp3 down-regulates Smad7 expression in CD4+ T cells and thereby suppresses the expression of the key negative regulator of TGF-β1 signalling (164). Figure 4.15B shows that Foxp3 expression is enhanced in CD3-treated cells in the presence of TGF-β1. However, expression of Foxp3 in myr PKB tg CD4+ T cells was comparable to wt CD4+ T cell (Fig. 4.24B) indicating that myr PKB does not act on TGF-β1 signalling via enhanced expression of Foxp3.

4.2 Effect of myr PKB on B cell development and activation

4.2.1 Reduction of B cells in myr PKB tg mice

The CD2 minigene cassette was used to induce expression of myr PKB specifically in T cells, but additional expression has been reported to occur in pre-B and B cells since in the mouse system CD2 is also expressed on B cells (165). Spleens from myr PKB tg and wt mice were therefore stained for IgM and IgD expression to discriminate immature ($IgD^{low}IgM^{hi}$) and mature ($IgD^{hi}IgM^{low}$) B cell subsets (Fig 4.16A).

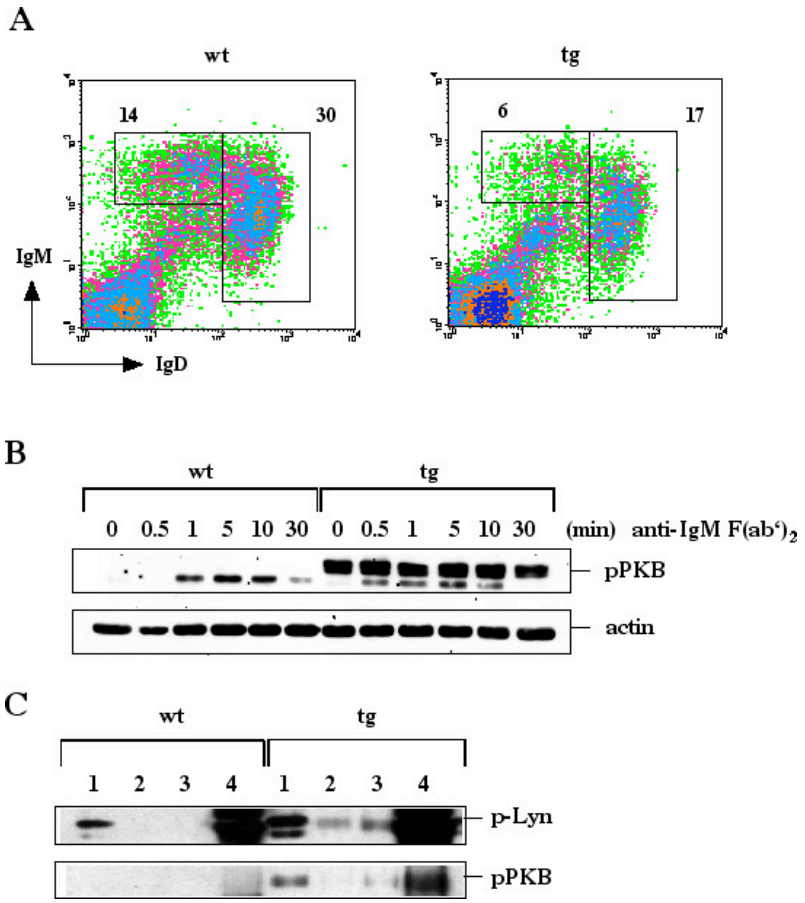

Fig 4.16 A. Analysis of B cells in spleens from myr PKB tg mice.

Cell suspensions from spleens of 6 weeks old mice were stained for IgM and IgD expression and analyzed by flow cytometry. Percentages of the individual subpopulations are indicated. B. Expression of myr PKB in B cells. Purified B cells from wt and myr PKB tg mice were stimulated with IgM $F(ab')_2$ Ab and expression of pPKB was determined by Western blot using phospho-PKB (Ser^{473}) Ab. Actin Ab was used to control equal protein loading. C. Myr PKB in B cells is found in lipid rafts. B cells were isolated and lipid raft and cytosolic fractions were prepared. Lanes 1 and 2 indicate lipid raft fractions and lanes 3 and 4 indicate soluble fractions. Proteins were detected by Western blot using phospho-tyrosine and phospho-PKB (Ser^{473}) Abs.

Myr PKB expression in splenic B cells was determined by Western blot using phospho-PKB (Ser^{473}) Ab (Fig. 4.16B). Analysis showed that similar to peripheral T cells, myr PKB in B cells is localized in insoluble lipid raft fractions as well as soluble cytosolic fractions. In B cells, the kinase Lyn has been shown to be localized in lipid rafts (166) and we therefore used Lyn as a marker to identify lipid raft fractions (Fig. 4.16C).

In myr PKB tg mice the subset of mature B cells was slightly decreased, not only percentage wise (Fig 4.16A), but also in absolute cell numbers, in both 6-8 weeks old and 6 months old myr PKB tg mice (Table 2). Splenic B cells can be subdivided into follicular ($CD21^{low}CD23^{hi}$) and marginal zone ($CD21^{hi}CD23^{-}$) B cells by phenotype, microanatomical localization and function. MZ B cells are a crucial component of the early immune response to blood-borne pathogens, even though they represent only about 5% of splenic B cells. Follicular B cells participate later in T-dependent antigen responses (52). MZ B cells exhibit rapid and robust proliferation and Ig secretory responses to stimulation with LPS, IgM and CD40 ligation. As shown in Table 2, myr PKB tg mice have a 25% reduction in follicular B cells but MZ B cell numbers were the same in 6-12 weeks old mice. However, in older mice (3-12 months) we noticed an increase in MZ B cells in myr PKB tg mice.

Table 2. Myr PKB tg mice have a reduction in mature B cells.

	6-12 weeks				
(cell no x 10^7)	**total**	**Immature ($IgM^{hi}IgD^{low}$)**	**Mature ($IgM^{low}IgD^{hi}$)**	**FO ($CD21^{low}CD23^{hi}$)**	**MZ ($CD21^{hi}CD23^{-}$)**
wt	**12.3** (±1.7)	**1.4** (±0.3)	**4.4** (±1.0)	**4.6** (±1.0)	**0.8** (±0.2)
tg	**14.4** (±3.6)	**1.2** (±0.4)	**2.7** (±0.5)	**3.5** (±0.8)	**0.7** (±0,1)
	3-12 months				
(cell no x 10^7)	**total**	**Immature**	**Mature**	**FO**	**MZ**
wt	**6.8** (±2.0)	**0.7** (±0.2)	**2.0** (±0.6)	**1.9** (±0.7)	**0.5** (±0.2)
tg	**11.9** (±6.2)	**0.7** (±0.3)	**1.4** (±0.5)	**1.4** (±0.4)	**0.8** (±0,3)

Cell numbers from spleens of wt or myr PKB tg mice aged either 6-12 weeks (n=6) or 3-12 months (n=10) were determined and cells were stained for IgM and IgD or CD21, CD23 and B220 expression to determine B cell subsets.

4.2.2 Myr PKB tg B cells are less reactive to BCR stimulation and show reduced Ca^{2+}-flux

To analyze the functional B cell response of myr PKB tg mice, we examined proliferation and Ca^{2+} mobilization after BCR stimulation. As shown in Fig 4.17A, stimulation of splenic B cells with different concentrations of IgM F(ab')$_2$ Ab resulted in reduced proliferation of myr PKB tg B cells, whereas proliferation after LPS or CD40 Ab stimulation was comparable to wt B cells. Interestingly, when B cells were stimulated with CD40 Ab in the presence of IL-4, myr PKB tg B cells showed enhanced proliferation (Fig. 4.17A). To measure Ca^{2+}-flux splenocytes were loaded with Indo-1 and B cells were activated with IgM F(ab')$_2$ Ab. Fig. 4.17B shows a representative Ca^{2+}-flux profile with myr PKB tg B cells clearly exhibiting reduced Ca^{2+}-mobilization after BCR triggering.

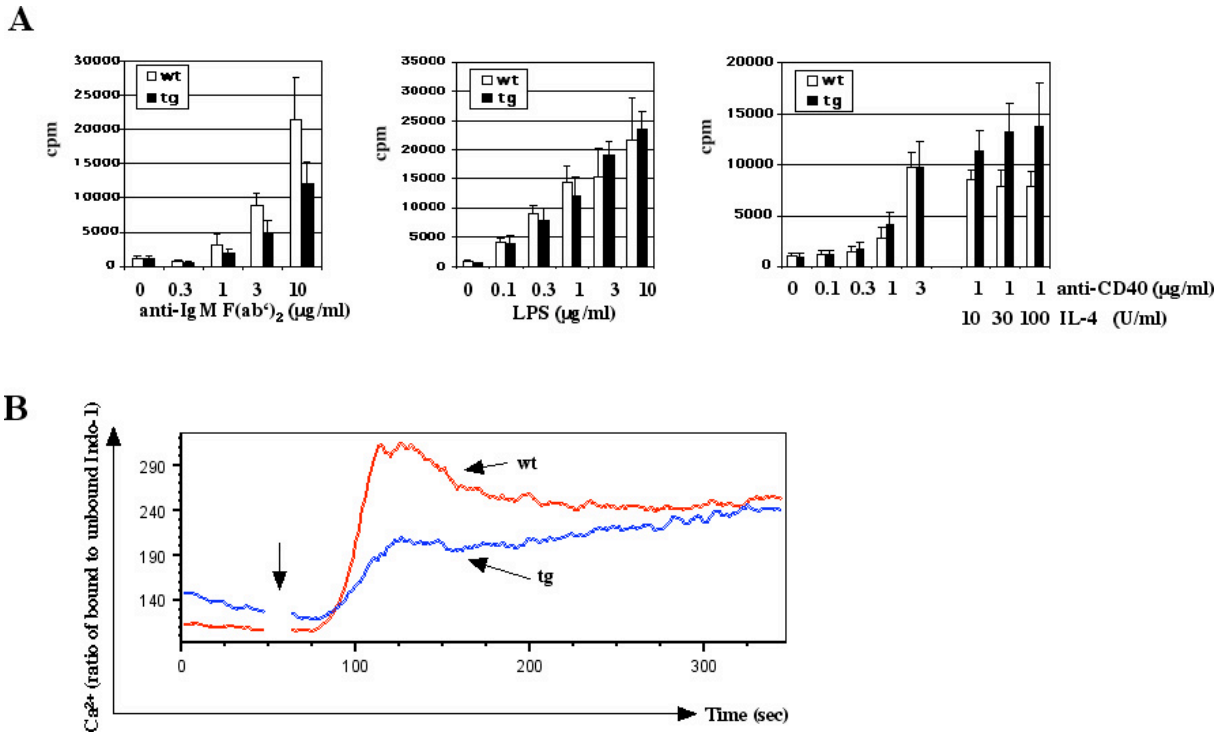

Fig 4.17 Reduced proliferation and Ca^{2+} mobilization of myr PKB tg B cells after BCR ligation.

A. Purified splenic B cells were stimulated with IgM F(ab')$_2$ Ab, LPS, CD40 Ab or CD-40 Ab plus IL-4 in concentrations as indicated. Proliferation was measured after 48 hrs and data represent averages from triplet cultures of three individual wt or myr PKB tg mice. B. Splenic B cells of wt and myr PKB tg mice loaded with Indo-1were stimulated with IgM F(ab')$_2$ Ab (3 µg/ml) at the time point indicated by the arrow. Ca^{2+}-mobilization was determined via the ratio of bound to unbound Indo-1.

4.2.3 BCR downstream signalling is enhanced in myr PKB tg B cells

As shown before in Fig. 4.16B, ligation of the BCR by IgM F(ab')$_2$ Ab leads to activation of PKB. BCR signaling involves the activation of multiple tyrosine kinases, including the Src family tyrosine kinases Lyn, Fyn, Blk and the tyrosine kinases Syk and Btk. BCR induced PKB phosphorylation seems to depend on tyrosine kinase activation, since PKB activation is completely ablated in DT40 cells (a chicken B cell line) lacking both Lyn and Syk tyrosine kinase (167). To examine the effect of myr PKB on BCR signalling, purified B cells from wt and myr PKB tg mice were stimulated with IgM F(ab')$_2$ Ab for short time periods and protein extracts were analyzed by Western blot. Compared to wt B cells, myr PKB tg B cells showed enhanced phosphorylation of the Src family tyrosine kinase Lyn while phosphorylation of Syk was slightly reduced after BCR stimulation (Fig. 4.18A). Activation of Lyn was already detected in unstimulated myr PKB tg B cells.

Protein kinase C (PKC), a family of lipid-activated serine kinases, has multiple functions in the regulation of growth control. PKCμ (also known as PKD) is ubiquitously expressed and involved in diverse cellular functions such as regulation of NF-κB activation, transport processes, G protein-mediated regulation of Golgi organization (168) as well as in anti-apoptotic functions (169). After BCR stimulation myr PKB tg B cells showed faster activation of PKCμ, which was apparent already after 0.5 min, but at later time point expression of p-PKCμ was similar (Fig. 4.18A).

Cbl-c has been shown to be associated with numerous signaling molecules (Src, Fyn, Lyn, Syk, ZAP-70, and PI3-kinase) as well as with several adaptor molecules (Shc, Crk, and Grb2). The association of Cbl-c with PI3-kinase suggests that Cbl-c could function as a scaffolding molecule that regulates activation of downstream signaling molecules. Myr PKB enhanced and prolonged the phosphorylation of Cbl-c in B cells after BCR triggering, similar to what was found for T cells (Fig. 4.18A). We also found that phosphorylation of GSK-3β, a direct target of PKB, was enhanced and sustained over longer periods in myr PKB tg B cells (Fig. 4.18A) similar to what we detected for T cells (Fig. 4.11B).

The adaptor protein SLP-65 (also known as BLNK, BASH or BCA) is a key participant in the formation of signaling complexes after BCR ligation and is required for both, the translocation of PLCγ2 from the cytosol to the plasma membrane and its subsequent phosphorylation and activation (131). Since SLP-65 deficient mice show an incomplete block in B cell development at the pre-B cell and immature B cell stages (170) , we analyzed SLP-65 activation in myr PKB tg B cells. In repeated experiments we found that SLP-65

phosphorylation was found to be enhanced after 1 min of stimulation in myr PKB tg B cells but at later time points (5 min) was similar to wt B cells (Fig. 4.18B).

These data altogether show that myr PKB enhances activation of important signalling molecules after BCR stimulation, although myr PKB tg B cells showed less proliferation and Ca^{2+}-mobilization than wt B cells.

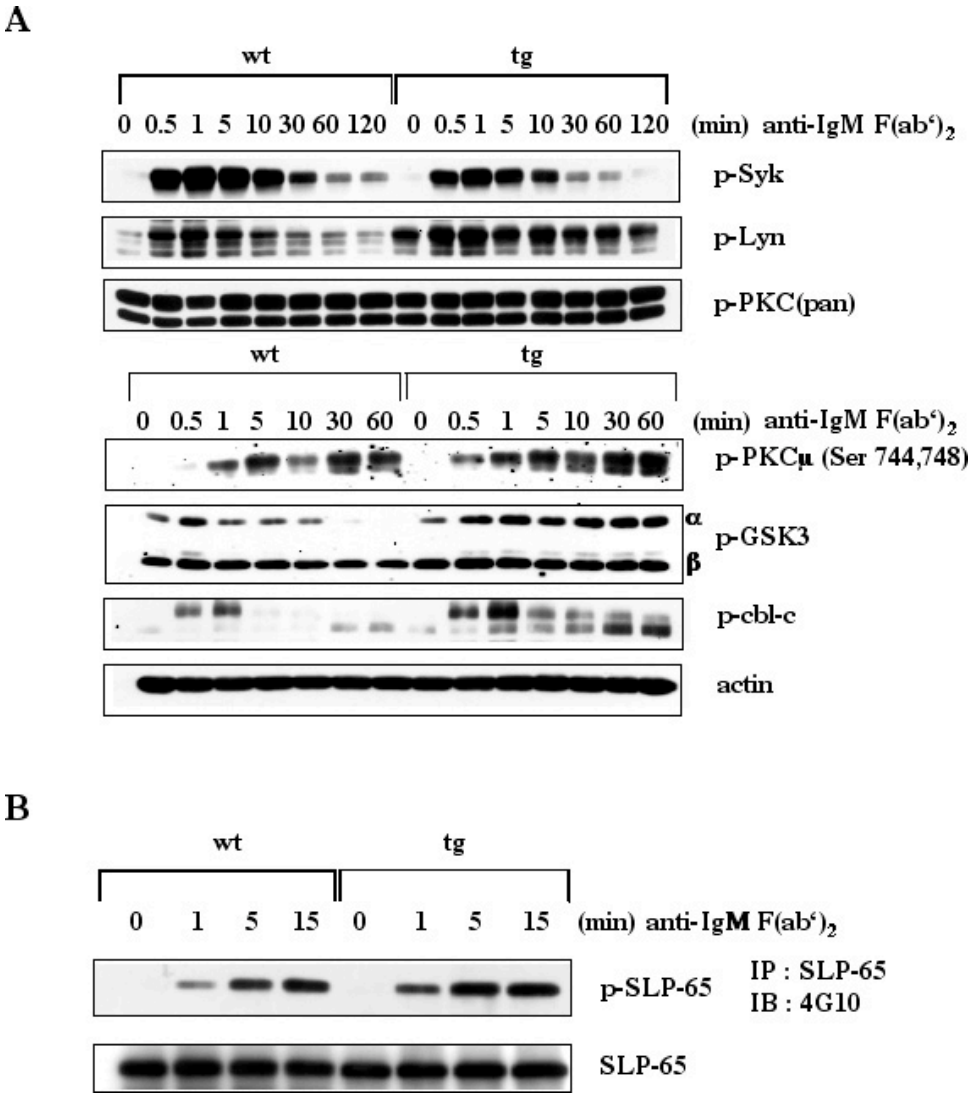

Fig 4.18 Myr PKB enhances phosphorylation of Lyn, PKC□, Cbl-c, GSK-3□ and SLP-65.

A. Purified B cells from wt and myr PKB tg mice were rested for 1h in 1% FCS medium before activation with IgM $F(ab')_2$ Ab for the time periods indicated. Phosphorylation of Syk, Lyn and Cbl-c was analyzed with phosphotyrosine (4G10) mAb and activation of PKC□ and GSK-3 was determined with phosphospecific Abs. Protein loading was controlled by reprobing blots with actin Ab. B. SLP-65 was immunoprecipitated from lysates of activated B cells and phosphorylation was determined by phosphotyrosine Ab.

4.2.4 Enhanced antigen specific immunoglobulin production in myr PKB tg mice

To investigate whether active PKB affects self-tolerance in the humoral response, serum Ig levels were assessed in 8 weeks old myr PKB tg mice. As shown in Fig. 4.19A, serum titers of IgM, IgG1, IgG2a and IgA were elevated in myr PKB tg mice compared to wt mice.

We then examined the humoral response of myr PKB tg and wt mice immunized with the thymus-dependent (TD) antigen TNP-OVA. TNP-specific Ig production was measured by ELISA. In myr PKB tg mice, we observed a strongly elevated IgM and IgG1 response, increase of IgG1 after secondary stimulation, and also a much stronger IgA response to TNP-OVA (Fig. 4.19B).

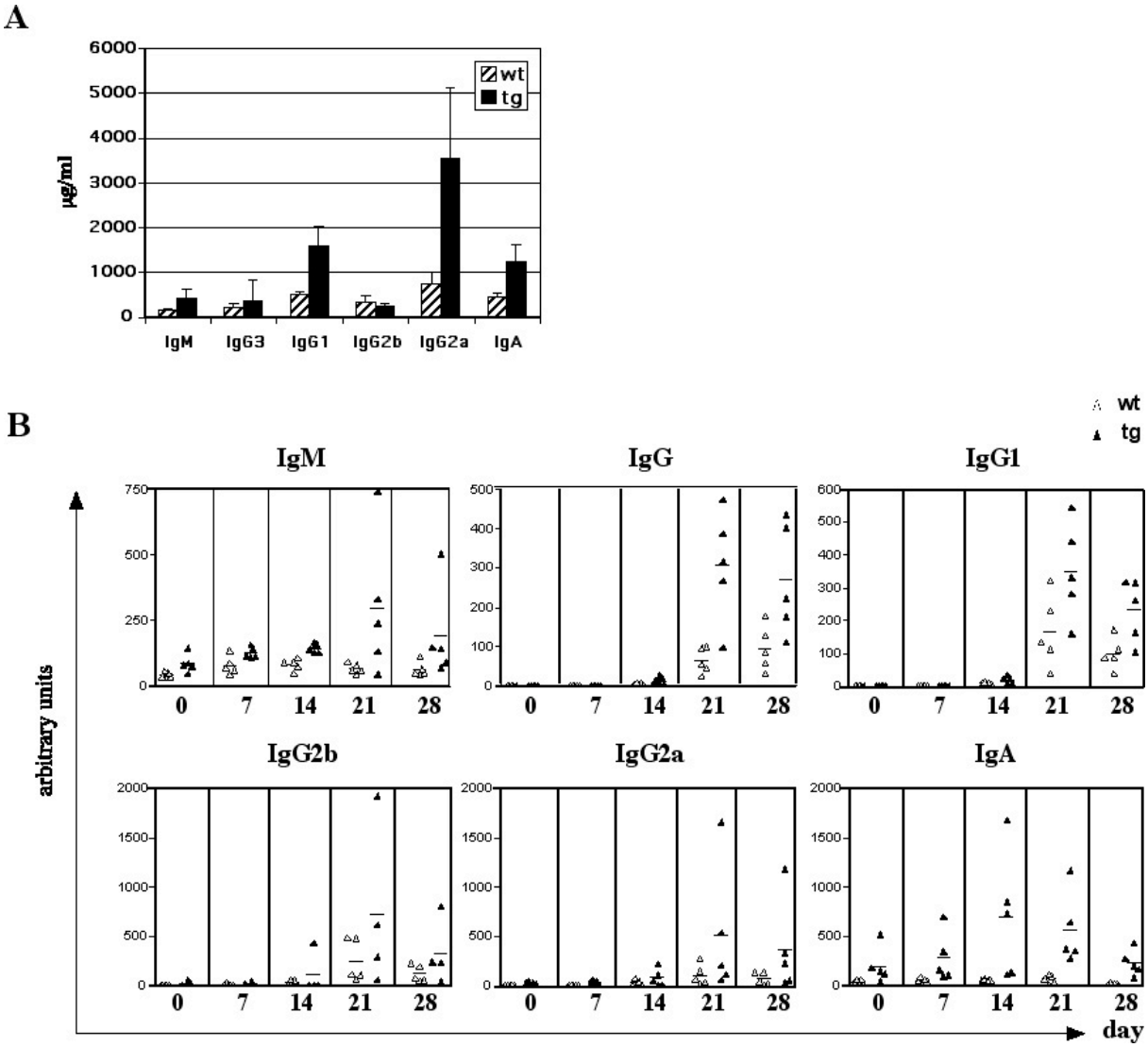

Fig 4.19 Enhanced antigen specific immunoglobulin production in myr PKB tg mice.

A. Serum immunoglobulin levels of unimmunized 8 weeks old mice. Sera from myr PKB tg (n=5) and wt (n=5) mice were analyzed for immunoglobulin isotypes by ELISA. B. Analysis of a T cell dependent immune response. Myr PKB tg and wt mice were immunized i.p. with 100 µg TNP-OVA at day 0, and boosted with the same antigen dose at day 14. Blood of the immunized mice was taken at days 0, 7, 14, 21 and 28 and production of TNP-OVA specific Ig isotype was determined by ELISA.

4.2.5 Immune response to TNP-Ficoll is enhanced in myr PKB tg mice

To study a T cell independent (TI) immune response, myr PKB tg and wt mice were immunized with TNP-Ficoll. Myr PKB tg mice showed a 2-4 fold higher production of TNP-specific IgG3 and IgG1 compared to wild type mice (Fig. 4.20). The IgM response was similar since unimmunized mice already showed higher IgM levels. Thus, in myr PKB tg mice B cell responses to TD and TI antigens are enhanced.

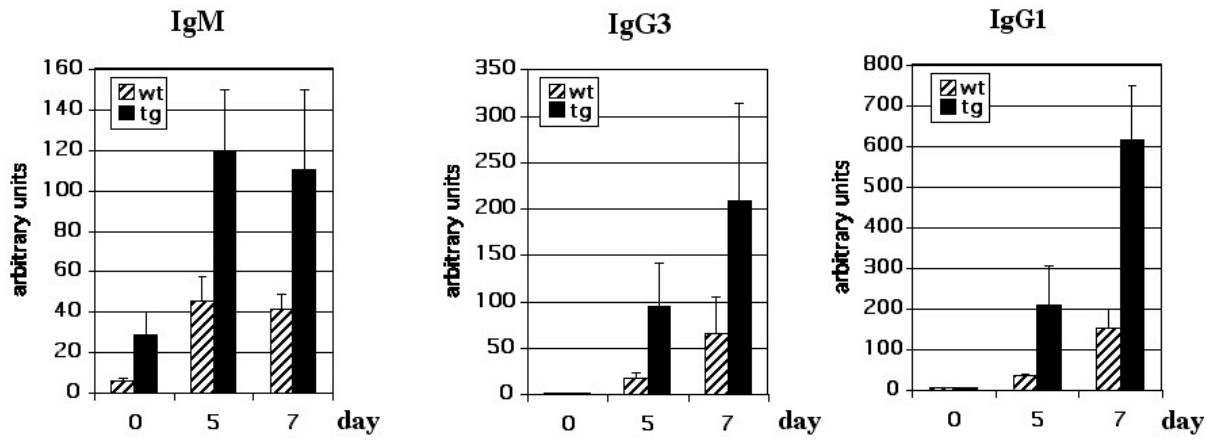

Fig 4.20 Enhanced immune response to TNP-Ficoll in myr PKB tg mice.

Myr PKB tg and wt mice were immunized i.p. with 10 □g TNP-Ficoll. Serum of immunized mice was prepared at days 0, 5 and 7 and TNP-specific Ab production was measured by ELISA.

4.2.6 Enhanced response of immunoglobulin secreting cells in myr PKB tg mice

To test if the elevated levels of serum Ig in myr PKB tg mice are due to an increase in the number of Ig-secreting cells or to an increased Ig production of individual cells, purified B cells from myr PKB tg and wt mice were analyzed by ELISPOT. As can be seen in Fig. 4.21A myr PKB tg B cells from unimmunized mice contained a higher fraction of B cells that secreted IgM and IgG than wt B cells.

Bacterial lipopolysaccharide (LPS) is among the few reagents known to activate both, proliferation and terminal differentiation of primary mouse B lymphocytes (171). Other B cell stimulatory agents which induce vigorous proliferation *in vitro*, such as Abs to the BCR or CD40, fail to induce Ig secretion and have been shown to prevent terminal differentiation of LPS-stimulated plasma cells (172). It has been reported that IL-4 also prevents efficient generation of IgM and IgG secreting cells and that addition of IL-4 leads to a drastic increase in surface IgG1 expression, although only less than 1% of IgG1 positive cells actually secrete IgG1 (173). It thus seems likely that IL-4 promotes the generation of IgG1 surface expression,

while at the same time inhibiting secretion of both IgM and IgG1 isotypes. To analyse the effect of IL-4 on Ig secretion of LPS stimulated B cells, myr PKB tg B cells and wt B cells were cultured for 3 days in the presence of LPS with or without IL-4 and then the numbers of Ig secreting cells were determined by ELISPOT. After LPS stimulation alone myr PKB tg B cells showed a slightly increased number of IgM and IgG3 secreting cells, whereas the number of IgG2b, IgG2a and IgA secreting cells was more clearly enhanced (2-4-fold). Addition of IL-4 suppressed Ig secretion in both cell types but for IgG2a which, interestingly, in myr PKB tg B cells was not shut off by IL-4 (Fig. 4.21B). We also measured IgG1 surface expression after LPS and LPS plus IL-4 stimulation but there was no difference between myr PKB tg and wt B cells (data not shown).

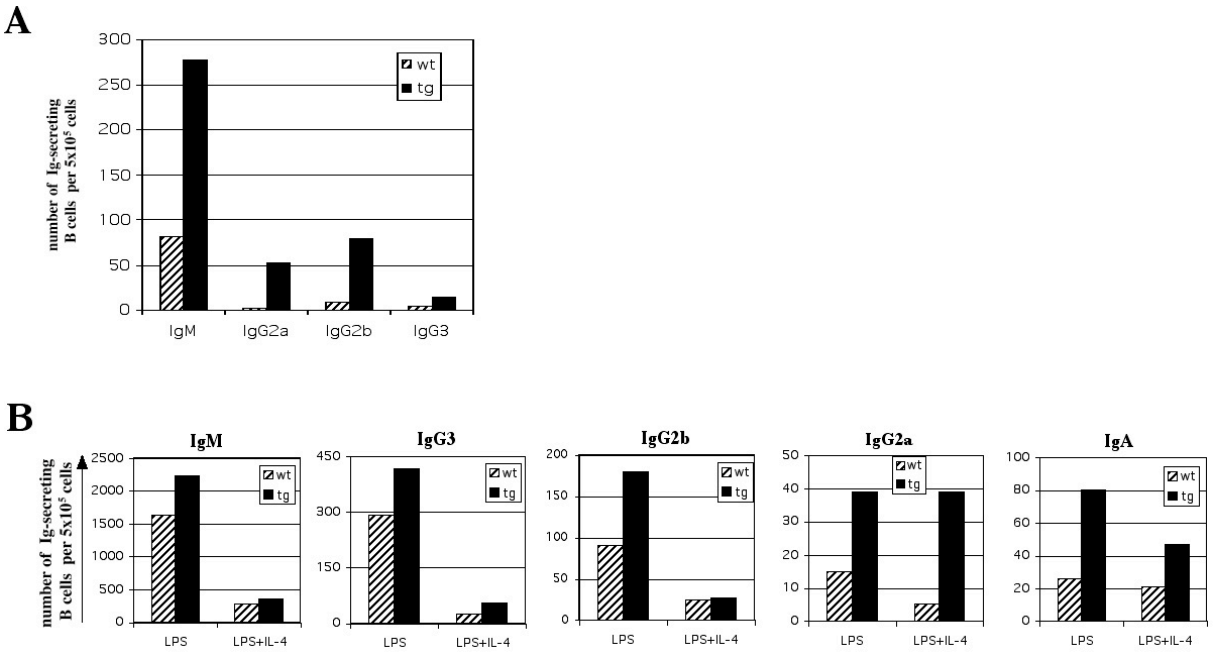

Fig 4.21 Determination of immunoglobulin secreting cells by ELISPOT.

A. Freshly isolated splenic B cells from myr PKB tg and wt mice were analyzed for Ig secretion by ELISPOT. B. Purified B cells from myr PKB tg and wt mice were stimulated with LPS (10 □g/ml) in the presence or absence of IL-4 (100 U/ml) for 3 days and Ig secreting cells were determined by ELISPOT. Results shown were derived from three pooled spleens per group and are representative of two independent experiments.

4.2.7 Enhanced expression of Blimp-1 mRNA in myr PKB tg B cells

BCR-, CD40- or IL-4-induced suppression of Ig secretion in LPS stimulated B cells, i.e. inhibition of plasma cell generation, has been shown to correlate with reduced expression of the transcription factor Blimp-1 (173, 174). To analyze Blimp-1 expression semiquantitative

RT-PCR was performed with freshly isolated or stimulated B cells from myr PKB tg and wt mice. Expression of Blimp-1, which is a master regulator of terminal B cell differentiation, was similar in LPS stimulated wt and myr PKB tg mice. However, after addition of IL-4 downregulation of Blimp-1 mRNA in myr PKB tg B cells was much less affected than in wt B cells (Fig. 4.22B lower panels). However, expression of XBP-1, which functions downstream of Blimp-1 activity and is involved in plasma cell differentiation and the unfolded protein response, was comparable in both cell types (Fig. 4.22B).

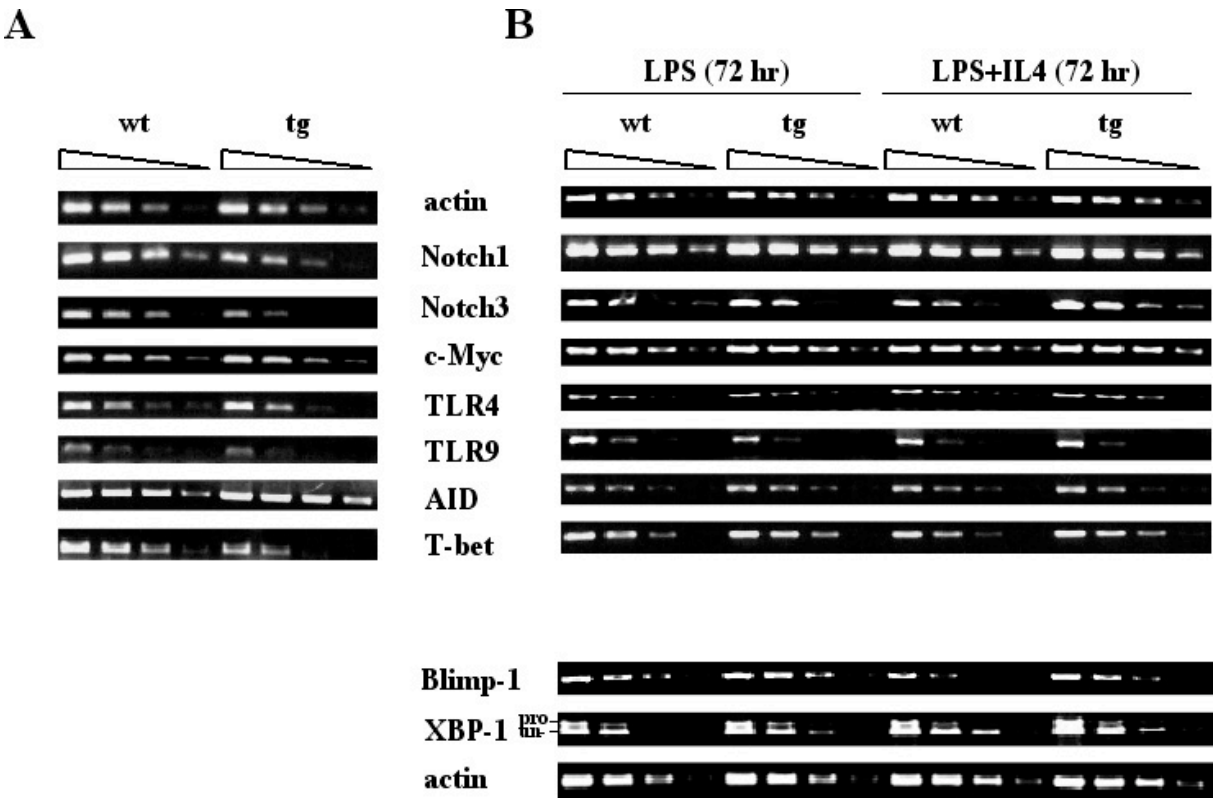

Fig 4.22 Comparison of gene expression in myr PKB tg and wt B cells by semiquantitative RT-PCR.

A. RNA was isolated from freshly purified B cells of myr PKB tg and wt mice. B. Purified B cells were stimulated with LPS (10 □g/ml) in the presence or absence of IL-4 (100 U/ml) for 3 days and RNA was isolated. In case of XBP-1, the PCR product was digested with *PstI* enzyme to separate the processed (pro) and unprocessed (un-) form of XBP-1. In A. and B. expression of the indicated gene was analyzed by semiquantitative RT-PCR. cDNA was serially diluted (1:3) and actin expression was used to control equal amounts of cDNA. All RT-PCRs were repeated in three independent experiments.

In addition to Blimp-1 and XBP-1, we analyzed the expression of other molecules that were anticipated to be differentially expressed in wt and myr PKB tg stimulated B cells. Notch proteins are known to direct developmental cell fate decisions in multiple organs. In

lymphocyte development, Notch signalling is critical for T/B lineage specification and for the generation of splenic MZ B cells. In unstimulated myr PKB tg B cells, mRNA expression of Notch1 and Notch3 was at least 3-fold less compared to wt B cells. In LPS stimulated B cells Notch1 and Notch3 expression was similar in wt and myr PKB tg B cells. However, after LPS plus IL-4 stimulation myr PKB tg B cells showed higher Notch3 expression (but not Notch1) than wt B cells (Fig. 4.22A and B).

c-Myc functions at a critical decision point of cell growth to favor proliferation and to block terminal differentiation (175). Toll-like receptor 4 (TLR4) on macrophages signals the presence of LPS by associating with CD14, the macrophage receptor for LPS, and TLR9 is involved in the recognition of CpG DNA (176, 177). It has been shown that T-bet mRNA is increased after CpG treatment but not after LPS stimulation. Furthermore, treatment of B cells with CpG inhibits Ig class switching induced by IL-4 and CD40 ligation, and this effect correlates with the induction of T-bet expression. T-bet is also required for switching to IgG2a since T-bet deficient B lymphocytes show impaired production of IgG2a, IgG2b, and IgG3 and are unable to generate germ line or postswitch IgG2a transcripts in response to IFN-γ (178). Activation-induced deaminase (AID) is a novel protein that is essential for class switch recombination (179). Analysis of the expression levels of these molecules in unstimulated and LPS treated B cells from wt or myr PKB tg mice did not show repeatable major differences (Fig. 4.22A and B).

4.2.8 Impaired B cell development in aged myr PKB tg mice

To investigate the influence of myr PKB on B cell development, we analyzed bone marrow from myr PKB tg and wt mice. Using staining against B220 and IgM molecules allowed us to distinguish pro/pre- ($B220^{low}IgM^{neg}$), immature ($B220^{low}IgM^{hi}$) and recirculating mature ($B220^{hi}IgM^{hi}$) B cells. 6-8 weeks old myr PKB tg mice showed overall normal B cell development, but total cell numbers were reduced and therefore all B cell subsets were reduced. However, aged myr PKB tg mice had a gross loss of pro/pre- and immature B cells (Fig. 4.23). This decrease in early B cell subsets was not only percentage wise, but also a reduction in absolute cell numbers although aged mice showed an increase in the total number of bone marrow cells (Table 3).

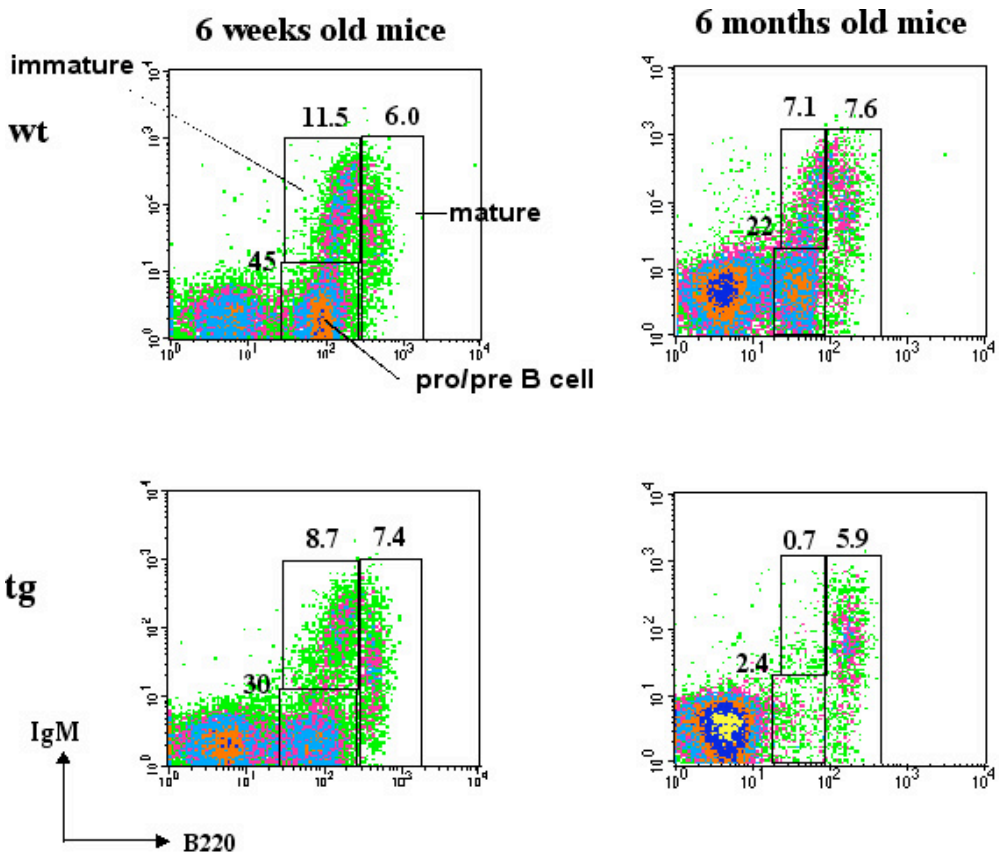

Fig 4.23 Aged myr PKB tg mice show impaired B cell development in the bone marrow.

Bone marrow cells were isolated from myr PKB tg and wt mice aged 6 weeks or 6 months, stained with B220-FITC and IgM-PE Abs and analyzed by flow cytometry.

Table 3. Aged myr PKB tg mice show a strong reduction in pro/pre- and immature B cell numbers in the bone marrow.

age	6-8 weeks				6 months			
(cell no x 10^6)	**total**	**Pro/pre**	**Immature**	**Mature**	**total**	**Pro/pre**	**Immature**	**Mature**
wt	19.0 (±3.5)	6.9 (±0.2)	1.6 (±0.1)	1.7 (±0.4)	12.9 (±4.6)	2.3 (±0.9)	0.9 (±0.4)	1.0 (±0.4)
tg	13.5 (±4.3)	4.3 (±1.2)	1.0 (±0.4)	1.1 (±0.3)	21.6 (±9.0)	0.6 (±0.3)	0.2 (±0.1)	0.6 (±0.5)

Total cell numbers from bone marrow of wt or myr PKB tg mice aged either 6-12 weeks (n=6) or 6 months (n=6) were determined and cell numbers for pro/pre-, immature and mature B cells were calculated from B220/IgM staining and FACS analysis.

4.2.9 Effect of myr PKB on B-1 cells

Myr PKB tg mice showed enhanced T cell independent immune responses (Fig. 4.18), which are dependent on the activity of MZ B cells and B-1 cells. Since the number of MZ B cells in

young adult myr PKB tg mice was normal, but myr PKB tg mice showed enhanced immune responses to TNP-Ficoll, we analyzed B-1 B cells in the peritoneal cavity.

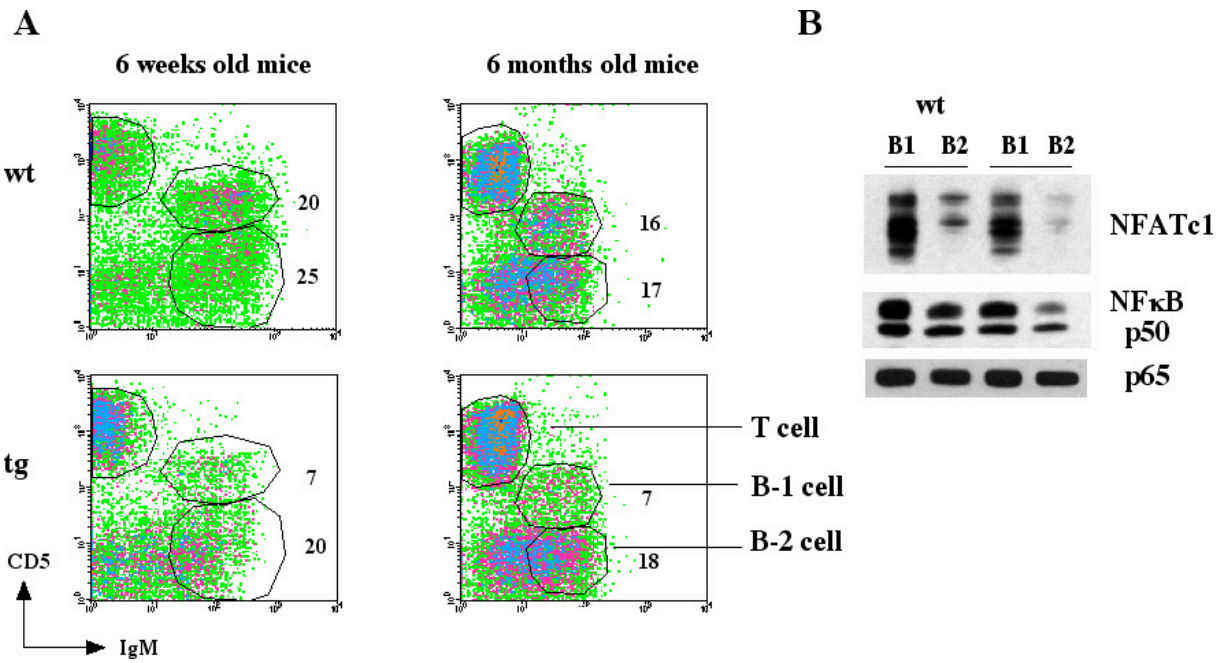

Fig 4.24 Reduction of B-1 cells in the peritoneum of myr PKB tg mice.

A. Lymphocytes from the peritoneal cavity of 6 weeks or 6 months old myr PKB tg and wt mice were analyzed for the expression of CD5 and IgM. The percentage of B-1 and B-2 cells is indicated. B. Peritoneal B-1 and B-2 cells were electronically sorted and whole protein extracts were analyzed for expression of NFATc1, NF□B p50 and p65 by Western blot.

B-1 cells are characterized by the expression of CD5 molecules and elicit T cell independent but Type II antigen dependent immune responses. Thus, IgM and IgG3 commonly have been considered to reflect the serum isotypes produced by B-1 cells (180).

Table 4. Reduction of peritoneal B-1 B cells in myr PKB tg mice.

age	6-8 weeks				6 months			
(cell no x 10^6)	**total**	**B-1**	**B-2**	**T**	**total**	**B-1**	**B-2**	**T**
wt	3.1 (±1.2)	0.8 (±0.4)	0.9 (±0.3)	0.6 (±0.1)	3.7 (±1.0)	0.7 (±0.3)	1.1 (±0.4)	0.8 (±0.3)
tg	3.3 (±1.5)	0.3 (±0.2)	0.9 (±0.5)	1.1 (±0.4)	6.1 (±2.1)	0.5 (±0.1)	1.9 (±0.1)	2.1 (±0.5)

Total cell numbers from the peritoneal cavity of wild type or myr PKB tg mice aged either 6-12 weeks (n=6) or 6 months (n=6) were determined and cells were stained for IgM and CD5 expression to detect B and T cell populations.

In 6-8 weeks old myr PKB tg mice we detected a reduction in the number of B-1cells ($CD5^+IgM^+$), whereas the number of B-2 cells ($CD5^-IgM^+$) was equal to wild type mice (Fig. 4.24 and Table 4). In aged myr PKB tg mice the total number of peritoneal cells was doubled compared to wt mice. Numbers of T cells and B-2 cells were also increased about 2-fold. The ratio of B-1 to B-2 B cells was about 1:3 in myr PKB tg mice while wt mice showed a 1:1 ratio of B-1 to B-2 B cells (Table 4). Therefore a strong reduction of B-1 S cell is also evident in aged myr PKB tg mice.

It has been reported that normal B-1 B cell development requires B cell intrinsic NFATc1 activity (181). We therefore electronically sorted B-1 and B-2 B cells and analyzed NFATc1 expression by Western blot. As reported, NFATc1 protein expression was increased in B-1 compared to B-2 B cells but we could not detect substantial difference of NFATc1 expression between wt and myr PKB tg B-1 B cells (Fig. 4.24B). However, we also noticed that as for NFATc1, expression of NF□Bp50 and NF□Bp65 was slightly reduced in B-2 B cells compared to B-1 B cells.

5. Discussion

5.1 Myr PKB in T cell development and activation

The purpose of this study was to determine how the serine threonine kinase PKB affects T and B cell development and activation.

Until now four PKB transgenic mouse lines have been described. The first reported PKB tg mouse line expressed a constitutively active form of PKB (gagPKB) in the T cell lineage (107). Following this study, two groups generated almost exactly the same mice to determine the mechanism of tumour induction (182) and metabolic consequences of activation of PKB in T cells (108).

In this study tg mice which overexpress myr PKB under the human CD2 promoter were analyzed. We found that membrane targeted PKB influences positive and negative selection of thymocytes, survival and activation/proliferation of T cells. Furthermore, we demonstrate a crosstalk between PKB and important T cell receptor downstream signalling molecules, which modulate the threshold of thymocyte selection and T cell activation.

We first observed that myr PKB tg thymocytes were hyperreactive to TCR/CD3 stimulation and showed resistance to the calcineurin inhibitors CsA and FK 506 in proliferation. Also, PKB tg thymocytes could proliferate in response to PMA only i.e. without an additional calcium signal provided by the ionophore ionomycin. RPA analysis and intracellular staining showed that myr PKB CD4+ T cells produce higher levels of Th1 cytokines like IFN and of Th2 cytokines like IL-4 and IL-5 compared to wt CD4+ T cells. These enhanced functional responses of myr PKB tg T cells could result from enhanced expression of CIS, which is known to positively regulate proliferative responses and survival of T cells (155).

Proteins that were found to be regulated by myr PKB in thymocytes include Lck, Raf, and Erk as well as c-Cbl and GSK-3, molecules that are known to set thresholds in thymocyte selection and T cell activation. According to the current models of selection, strength and duration of TCR mediated signalling determine lineage commitment or efficiency of selection of CD4+ or CD8+ T cells (5, 183), whereby strong or long lasting signals favor CD4 and weaker or short signals CD8 maturation. In particular, experiments using thymic organ culture (184, 185) and Lck transgenic mice (10, 186) have demonstrated that relatively small alterations in intrathymic Lck activity can significantly affect the CD4/CD8 lineage decision. Likewise, several studies using gentically modified mice and *in vitro* differentiation systems have shown that the strenght/duration of Raf-Mek-Erk signaling regulates positive selection, lineage commitment

and negative selection (5). Both, Erk1 knockout mice and animals deficient for the novel upsteam activators Ras guanyl-releasing protein (Ras GRP) show defects in positive selection (187). In addition, studies using CD3ζ-deficient mice indicate that CD3ζ functions in the coupling of TCR signals to the Erk pathway during positive selection (30). Erk is also known to influence CD4/CD8 lineage commitment. Development of CD4+ cells is enhanced when Erk activity is increased, while CD8 maturation is increased when Erk activity is decreased or low (188). Mice that lack the adapter molecule c-Cbl show enhanced positive selection and develop slpenomegaly, lymphadenopathy and hyperactivation of peripheral T cells, indicating that c-Cbl normally acts as a "brake" on TCR signalling (20, 21).

Here, we observed in three different TCR tg systems that myr PKB promotes positive selection of CD4+ T cells. In OT2 TCR tg mice, myr PKB's positive crosstalk on Lck-Raf-Erk signalling thus could increase the efficiency of CD4 selection or even allow some DP thymocytes to acquire the necessary threshold for selection, rescuing them from 'death by neglect'. We were unable to detect a reproducible significant effect of myr PKB on the maturation of OT2 or OT1 CD8+ T cells. Keeping in view that myr PKB strengthens Lck-Erk activation, TCR signals might lie above the threshold levels required for maturation of CD8+ T cells. On the other hand, stronger Lck-Erk signals could drive a high proportion of DP cells from OT1 myr PKB double-tg mice to develop into CD4+ T cells, as we indeed observed in some mice. The variability in overselection of CD4+ cells in OT1 mice, although the underlying mechanisms are unresolved, is most likely connected to myr PKB activity since we observed it in three myr PKB lines crossed to OT1 mice (data not shown). To date we could not discriminate whether overselection of OT1 CD4+ T cells is due to an initial switch of some DP cells to the CD4 lineage or rather a rescue of CD8 committed CD4+CD8^{lo} cells due to enhanced/prolonged Lck-Erk signalling. This also applies for the increased selection of CD4+ T cells seen in OT2 and HY female mice expressing the transgene. In case of OT1 mice, myr PKB clearly enhanced negative selection induced by peptide antigen, arguing for a higher sensitivity of OT1/myr PKB DP cells. Therefore, it is also imaginable that some DP cells that were destined for deletion might escape negative selection and mature instead to CD4+ T cells. Negative selection not only operates at the DP stage but also at the level of semi-mature CD4+CD8^{lo} cells, detecting a role for fas mediated deletion of these semimature cells depending on the antigen dose (189).

Fas signalling is reportedly involved in SEB-induced tolerance (190), and a defect in Fas signalling has been proposed as the basis of autoimmunity in PTEN mutant (98) and gag-PKB tg mice (191). Although deletion mediated by endogenous SAgs and the response of thymocytes to fas-ligation *in vitro* was not affected by myr PKB in mice with heterogeneous

TCRs, an effect of myr PKB on deletion of CD4+CD8^{lo} cells in OT1 mice cannot be totally excluded. However when we analyzed deletion of superantigen reactive peripheral T cells by injecting SEB we found that deletion of SEB reactive V□8+CD4+ T cells was markedly reduced in myr PKB tg mice compared to wt mice. Therefore, myr PKB provides signals, probably via fas, that impair peripheral deletion of CD4+ T cells and thus peripheral tolerance. This is in accordance with gagPKB tg mice which showed a reduction in fas-mediated deletion due to defective recruitment of caspase 8 to the death inducing signalling complex (107).

Maturation of DP cells is initiated by the ligaton of the TCR and a number of coinducer/costimulatory receptors including CD28 (192) and the net effect of these interactions will decide whether DP cells mature or undergo apoptosis. Interestingly, in a recent report it was shown that CD28 co-engagement of DP cells can either induce CD4 T cell maturation or negative selection, depending on the intensity of CD28 co-stimulation (193). Assuming that myr PKB reflects some aspects of CD28 signalling in thymocytes it is more conceivable that subtle differences in myr PKB expression and modification of downstream target proteins in individual cells could have differential effects on selection.

Considering that myr PKB inhibits the activtiy of GSK3, a kinase identified in the regulation of nuclear export of NFAT (148), and supports T cell proliferation in the presence of calcineurin inhibitors or the absence of significant calcium mobilization, it is also conceivable that differential regulation of NFAT proteins contributes to the altered phenotype in myr PKB tg mice. Various NFAT family members have been shown to be involved in thymocyte selection (194-196) and a role for calcineurin in thymic selection and activation has been reported in mice expressing a constitutively active form of calcineurin. In these mice T cells showed increased TCR sensitivity and calcium-independent proliferation as well as enhanced positive selection of CD4+ T cells (197), similar to what we observe in myr PKB tg mice. Since PKB has multiple targets, as observed in many different cell systems, future studies also have to address whether other proteins regulating thymic selection processes such as JNK (198), p38 (199) or Notch (200) are affected by myr PKB.

The influence of myr PKB on negative selection was studied in four model systems, whereby myr PKB either enhanced, reduced or had no effect on deletion. We conclude that the effect of myr PKB on negative selection is specific for each system analysed depending on the timing, the antigen and TCR affintity/avidity or whether antigen is presented by MHCI or MHCII molecules. Negative selection not only requires a high avidity TCR stimulus but also costimulatory signals from APCs which potentially can be provided by CD28 (201). For the

deletion of different autoantigens a complex array of variable costimulators seems to be necessary (202). Since some of these molecules, like CD28 or Fas, signal via PKB our results on negative selection in different model systems might reflect the complexity of molecules and their different downstream effector molecules as involved in negative selection.

In repeated *in vitro* experiments with thymocytes from 6-8 weeks old mice we did not detect very strong differences in apoptosis/survival between wild type and myr PKB tg thymocytes for most stimuli tested. Peripheral myr PKB tg T cells on the other hand showed better survival and were more resistant to induction of apoptosis to different reagents. This differential survival effect could result from small differences in thymocyte survival that cannot be detected in *in vitro* assays but are relevant *in vivo* or to so far unknown mechanisms that counteract survival functions in thymocytes from myr PKB tg mice. In a different approach, generating Lck-crePtenflox/- mice with T cell specific deletion of the tumor suppressor gene PTEN, PKB activity was greatly enhanced in T cells (99). With regard to survival and negative selection, Lck-crePtenflox/- mice had defects in negative selection *in vivo* using the HY TCR tg system but thymocyte apoptosis *in vitro* was also not affected when CD3 or fas mAbs were employed. Thus for certain stimuli in thymocyte apoptosis other mechanisms and molecules might be more critical.

In PKB tg mice generated using a gagPKB construct Jones et al. on the other hand detected a major enhancement of survival of thymocytes after treatment with various apoptosis inducing stimuli *in vitro*. In further contrast to our data, they did not observe a selection towards the CD4 lineage using the P14 TCR tg mouse model (107). This discrepancy most likely results from differences in expression levels or localization of tg PKB in addition to differences in the affinity/avidity of the tg TCR used (203, 204). As pointed out by these authors, the gagPKB tg protein detected in western blots was much smaller than the expected gagPKB fusion protein. This is probably due to cleavage of the gag sequence thus preventing targeting to the plasma membrane, although elevated levels of phosphorylated PKB were detected. Here, we show that in thymocytes myr PKB is predominantly localized in membrane lipid rafts, in close proximity to other raft resident proteins that are essential regulators of TCR signalling such as Lck or LAT. In relation to these data we favour the view that the different localisation of PKB and immediate availability of target proteins might be the critical factors that account for the observed differences in thymocyte survival and selection in the two PKB transgenic systems. The possibility that localization of PKB modulates its effector functions is further supported by our finding that survival was clearly enhanced in peripheral myr PKB tg T cells and that myr

PKB in CD4+ T cells was distributed in insoluble as well as soluble membrane fractions. Biochemical studies assessing the redistribution of signalling molecules into lipid rafts indicate that positive selection signals or TCR/CD3 stimulation alone can recruit TCR signalling components to the lipid raft fraction (149). Recently, Hill et al. detected a constitutively active PKB Serine 473 kinase activity enriched in plasma membrane rafts (205). Here, we show that TCR/CD3 stimulation of CD4+ T cells leads to redistribution of active PKB to the lipid raft fraction thus extending and highlighting the involvement of lipid rafts in TCR mediated PKB signalling.

The PKB signalling pathway also interacts with other signalling pathways known to be essential for normal development and activation, including the TGF-β/Smad pathway. TGF-β1 is a critical regulator of T cell responses *in vivo*. *In vitro*, TGF-β1 can either enhance or inhibit T cell proliferative responses, but the relevant factors that determine the T cell response to TGF-β1 remain obscure. It has been shown that engagement of the CD28 co-stimulatory receptor enhances proliferation when used with TGF-β1 at high doses (163). In our experiments CD3 plus CD28 stimulation of wt and myr PKB CD4+ tg T cells also enhanced proliferation in the presence of TGF-β1. However, TGF-β1 in combination with TCR/CD3 stimulation alone also enhanced proliferation of myr PKB tg CD4+ T cells whereas wt CD4+ T cells were completely inhibited by TGF-β1. These results support studies from our group that PKB can replace CD28 costimulatory signals. Naïve T cells respond differently to TGF-β1 compared to memory T cells. Therefore, to exclude possibility that myr PKB tg CD4+ T cells respond better to TGF-β1 because they contain more memory T cells, we analyzed the effect of TGF-β1 on naïve CD4+ T cells. We observed myr PKB tg naïve CD4+ T cells were less inhibited by TGF-β1 compared to wt naive CD4+ T cells.
Addition of high levels of IL-2 to cultures of naive human CD4+ T cells stimulated in the presence of TGF-β1 converts TGF-β1 from an inhibitor to an enhancer of proliferation (206). *In vitro*, early IFNγ production by differentiating Th1 cells blocks the inhibitory effect of TGF-β1 (207). Our RPA studies have shown that myr PKB tg CD4+ T cells produce higher levels of IL-2 and IFNγ after TCR/CD3 stimulation. Thus, it is conceivable that the enhanced cytokine production makes TGF-β1 act as an enhancer of proliferation in myr PKB tg T cells.

PTEN is rapidly downregulated by TGF-β in keratinocytes and pancreas (208, 209). PI3K/PKB suppresses phosphorylation of Smad3 and inhibits Smad3 dependent TGF-β signalling (210). Two recent reports demonstrate that in several human cell lines, PKB can inhibit TGF-β1

activities through direct interaction with Smad3. Because PKB is known to protect cells from TGF-β1 mediated apoptosis, Conery et al. (211) and Remy et al. (211, 212) first set up to identify members of the TGF-β1 signalling cascade that associate with PKB. Both, co-immunoprecipitation and protein complement fragmentation assays showed that Smad3 physically binds PKB. However, overexpression of Smad3 did not disrupt stimulus-induced phosphorylation of PKB on both of its regulatory sites (Thr 308 and Ser 473), nor its kinase activity (212). This mechanism therefore is unique in being independent of PKB kinase activity, unlike other pathways through which PKB protects against apoptosis (213).
To determine molecular mechanism involved in the resistance of myr PKB tg T cells to TGF-β1 in proliferation, we compared Smad3 expression in myr PKB tg and wt CD4+ T cells. Unstimulated CD4+ T cells from myr PKB tg mice showed much higher phosphorylation of Smad2/3 compared to that of wt CD4+ T cells. In contrast to other reports, we observed higher phosphorylation of Smad2/3 in the nucleus of myr PKB tg CD4+ T cells compared to wt CD4+ T cells after TCR/CD3 plus TGF-β1 stimulation. It has been shown that in the presence of CD28 engagement, TGF-β1 prevents entry into the apoptotic pathway, and only slightly inhibits the proportion of non-apoptotic naive CD4+ T cells in proliferation (163). In conformity with earlier reports we observed similar enhancement in the phosphorlyation of Smad2/3 after TCR/CD3 stimulation with CD28 costimulation in both wt and myr PKB CD4+ T cells. Further studies will define the connection between PKB and other Smad family members in cellular proliferation and apoptosis.

Collectively, our findings clearly show that PKB plays an important role in TCR initiated signal transduction by crosstalk with several important signalling molecules such as Lck, Erk, c-Cbl or GSK3, or CIS. By enhancing the strength/duration of Lck-Erk and other signals, PKB is vitally involved in the modulation of activation thresholds and selectional windows that govern T cell maturation and activation. Positive effects on these signalling molecules and resistance to TGF-β1 mediated negative signals may also contribute to the development of lymphomas and other cancer types, which are thought to result from overexpression of PKB (182).

5.2 Myr PKB in B cell development and activation

PI3K is a primary candidate for mediating B cell survival as treatment of B cells with inhibitors of PI3K lead to an increase in BCR-induced cell death (121). In mice deficient for the p110δ subunit of PI3K, the activation of PKB in B cells is impaired significantly (87, 88). BCR signalling activates PKB even in PLCγ2-deficient B cells, whereas inhibitors of PI3K block this activation completely (49), which indicates that PKB is downstream of PI3K, but not of PLCγ2. In our tg mice, we observed phosphorylation of endogenous PKB after BCR stimulation as well as strong expression of the myr PKB transgene. Like in peripheral T cells, myr PKB in B cells is partially localized in membrane lipid rafts, which in B cells can be identified by the presence of the kinase Lyn.

In B cell activation, PI3K/PKB seems to be activated by two pathways. The first pathway involves tyrosine phosphorylation of the transmembrane adaptor protein CD19 by Src kinases such as Lyn (214), followed by the recruitment of the p85α subunit of PI3K to CD19 (90). In the absence of CD19, PKB kinase activity is reduced and transient (215). In addition, coligation of CD19 with surface immunoglobulin leads to augmented PKB activity in a dose dependent manner ().Nonetheless, inducible PI3K activity is not completely ablated in the absence of CD19, suggesting a possible compensatory pathway. This pathway may rely on the recruitment and activation of the kinase Syk by the BCR associated signal transducers, Igα and Igβ, which activate PI3K via an intracellular adaptor such as BCAP (B cell adaptor for PI3K) (47). The role of BCAP in PI3K activation is not clear. Although the production of PIP_3 and thus PKB phosphorylation are impaired in BCAP-/- chicken DT40 B cells (43), IgM specific antibody-stimulated PKB phosphorylation is unaffected in BCAP-/- mouse cells (47). One possibility is that BCAP is only required for PI3K signalling in particular B cell subsets. Studies using the DT40 B cell line show that BCR-induced activation of PKB requires the tyrosine kinase Syk. In contrast, BCR crosslinking of Lyn-deficient B cells resulted in markedly enhanced activation of PKB compared to wild type B cells, indicating that Lyn acts as an endogenous antagonist of BCR-induced PKB activation (167). These results suggest that Lyn provides a mechanism for negative regulation and opposes the effect of Syk on BCR-mediated activation of PKB.

After BCR stimulation, myr PKB B cells showed reduced Ca^{2+} flux and decreased proliferation. In contrast to these observations, analysis of BCR downstream signalling molecules showed that myr PKB enhances Lyn activation whereas Syk activation was slightly reduced. Also, BCR mediated activation of the adaptor molecules c-Cbl and SLP-65 was also enhanced or accelerated compared to wt B cells. How these observations connect to the observed reduced B cell proliferation has to be determined by future studies.

When we analyzed the whole B cell compartment in myr PKB tg mice we observed a decrease in immature and mature B cell numbers but the MZ B cell subset was normal. Interestingly, myr PKB tg mice had a strong reduction of B-1 cells in peritoneum. Berland's group have shown that in B-1 cell development NFAT expression is essential (181), we analyzed NFAT expression in peritoneal B-1 and B-2 cells from wt and myr PKB tg mice. But NFAT expression was comparable indicating that myr PKB induced signals act on other molecules to disturb B-1 cell development. In aged myr PKB tg mice we observed a very strong reduction of pro/pre and immature B cell populations in the bone marrow, which indicates PKB is also critically involved in regulation of B cell development.

BCAP-deficient mice show a reduction of B-1 cells, but MZ B cell formation is normal and PI3K activation in follicular B cells is unimpaired (47). It has been reported that sustained PI3K signalling is required for the development of MZ B cells, and that this depends on both CD19 and p110δ (216). These reports were supported by the observation that PTEN-deficiency can rescue the development of MZ B cells and B-1 cells in CD19-deficient mice (92). Lyn-deficient mice have reduced BCR signalling thresholds and develop autoantibodies, splenomegaly due to myeloid hyperplasia, and increased B-1 cell numbers. Btk, a Tec family kinase, is activated by tyrosine phosphorylation and has a critical role in BCR signalling (217). Btk-deficient mice, as well as mice with the Xid mutation (a natural mutation in the PH domain of Btk, in which an arginine residue critical for the binding to PIP_3 is replaced by cystein), show that PI3K dependent regulation of Btk translocation to the plasma membrane is essential for Btk function (218, 219). The role of Btk in PKB activation is controversial. BCR-induced activation of PKB was normal in Btk-/- B cells, but was severely impaired in PI3K-/- B cells. Btk-deficient mice show impaired B-1 cell development (220). Initially suggested by studies of Xid mice, it is now generally noted that mutations impairing BCR signal strength often result in a reduced B-1 cell population, whereas enhanced BCR signalling may favor expansion of this subset (221). Future studies therefore have to address how myr PKB affects BCAP and Btk expression and how myr PKB B cells respond to CD19 signals.

MZ B cells and B-1 cells are specialized to respond to thymus independent (TI) antigens, but even though B-1 cells were reduced in myr PKB tg mice, the production of antigen specific IgG in response to TI and also to thymus dependent (TD) antigens was profoundly increased. Consistent with defects in B cell activation and development, PI3K p85α-deficient and p110δ-deficient mice have reduced antibody concentrations in the serum. The p85α single knockout

mice were shown to raise a normal immune response against TD antigens, but failed to response to TI antigens (86). By contrast, the p110δ-mutant mice had impaired responses to both TD and TI antigens (87, 88). TD humoral immune responses are also deficient in CD19-deficient mice. PTEN-deficient mice, where PKB activation is enhanced, show highly increased MZ and B-1 cell populations whereas the TI immune response is drastically decreased. Production of antigen specific IgG in immune responses to TD antigens is also severely impaired in the absence of PTEN (222). The results from myr PKB tg mice, however show a reduced B-1 cell population and enhanced TD and TI immune responses, which is opposite to the results from PTEN-deficient mice.

Following an initial exposure to TD protein antigens, two broad types of plasma cells develop. These plasma cells can be distinguished by longevity and evidence of affinity maturation. Short-lived plasma cells represent an early reaction to antigen exposure that is dependent on T cell help, but does not require a germinal center (GC) phase in development. The long-lived plasma cells are the product of GC reactions and can also isotype switch. The transcriptional repressor, Blimp-1 is the best characterized regulator of plasma cell development (174).Blimp-1 blocks the expression of many transcription factors that regulate BCR signalling, class switch recombination, cell proliferation and GC activities, while allowing the expression of only a few genes. XBP-1 is a transcription factor that appears to be required for the generation of plasma cells (223). Since plasma cells and serum Ig were markedly reduced in lymphoid chimeric mice in which B cells lacked XBP-1, even though normal numbers of B cells were present and TD immunization produced normal GCs.

Using ELISPOT, we found that the number of immunoglobulin secreting cells was highly elevated among stimulated myr PKB tg B cells compared to wt B cells. Ligation of LPS in combination with IL-4, either fails to induce or prevents Blimp-1 expression and thus terminal differentiation (173). Interestingly, we found that in myr PKB tg B cells IL-4 plus LPS treatment failed to downregulate Blimp-1 expression whereas XBP-1 expression was comparable. To detect differences in gene expression between wt and myr PKB tg B cells that could be involved in the higher production of Ig, we did RT-PCR for a number of genes. Notch 2 is known to be involved in the development of MZ B cells, but we did not observe any difference in Notch2 expression between wt and myr PKB tg B cells (data not shown) whereas higher expression of Notch3 was detected in myr PKB tg B cells after LPS plus IL-4 stimulation. Since nothing is known about the function of Notch3 in B cells these data may be of special interest for future investigation.

In conclusion, the analysis on B cells showed myr PKB expression leads to a reduction of immature and mature B cells subsets in the spleen and has a profound effect on development of peritoneal B cells and on B cell precursors in aged mice. Moreover, despite reduction of B-1 cells, TI and also TD immune responses were highly enhanced in myr PKB tg mice. Preliminary studies indicate that this may result from or to be connected to altered BCR downstream signalling events and potentially enhanced Blimp-1 and Notch 3 expression.

6. References

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Abbreviations

Ab	antibody
Ag	antigen
AID	activation-induced deaminase
APC	antigen presenting cell
AP-1	activator protein 1
7-AAD	7-amino actinomycin D
BCR	B-cell receptor
BCAP	B-cell adaptor for PI-3K
BCIP	5-bromo-4-chloro-3-indolyl phosphate dipotassium salt
Blimp-1	B-lymphocate-induced maturation protein 1
BLNK	B-cell linker protein
BSS	balanced salt solution
BSA	bovine serum albumin
Btk	Bruton's tyrosine kinase
CD	cluster of differentiation
CIS	cytokine-inducible SH2-containing protein
c-Myc	cellular homologue of avian myelocytosis virus oncogene
CsA	cyclosporin A
CSR	class-switch recombination
DAG	diacylglycerol
DC	dendritic cell
DN	double negative
DP	double positive
DTT	dithiothreitol
ERK	extracellular signal regulated kinase
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
FKHR	forkhead transcription factor
FO	follicular
FSC	forward scatter

GC	germinal center
GSK3	glycogen synthase kinase 3
HSA	heat stable antigen
IFN-□	interferon gamma
Ig	immunoglobulin
I□B	inhibitor of NF-□B
IKK	I□B kinase
IL	interleukin
ITAM	immunoreceptor tyrosine based activation motif
IP	immunoprecipitation
JNK	c-jun N-terminal kinase
ko	knock out
LAT	linker for activation of T cells
MAPK	mitogen-activated protein kinase
MHC	major histocompatibility complex
mTOR	mammalian target of rapamycin
Myr	myristoylated
MZ	marginal zone
NE	nuclear extract
NFAT	nuclear factor of activated T cell
NF-□B	nuclear factor □B
NK	natural killer
O/N	over-night
PAG	phosphoprotein associated with glycosphinogolipid enriched microdomains
PBS	phosphate buffered saline
PC	plasma cell
PDK	phosphoinositide dependent protein kinase
PI-3K	phosphatidylinositide 3 kinase
PIP2	phosphatidylinositol 3,4-bisphosphate
PKB	protein kinase B
PKC	protein kinase C
PLC□	phospholipase C gamma
PMA	phorbol 12-myristate 13-acetate

Ptd(3,4,5)P3	phosphatidylinositol 3,4,5-trisphosphate
PTEN	phosphatase and tensin homologue deleted on chromosome 10
PTK	protein tyrosine kinase
RAG	recombinase activating gene
RT	room temperature
SEB	staphylococcal enderotoxin B
SH2	Src homology 2
SHIP	SH2 domain-containing inositol 5-phosphatase
Smad	contraction of Sma and Mad (mothers against decapentaplegic)
SOCS	suppressor of cytokine signaling
SP	single positive
T1	transitional type 1
TCF-1	T cell factor-1
TCR	T-cell receptor
TD	thymus-dependent
tg	transgenic
TGF-□	transforming growth factor □
Th1	T helper type 1
Th2	T helper type 2
TI	thymus-independent
TLR	toll like receptor
TNF□	tumor necrosis factor □
wt	wild type
XBP-1	X-box binding protein 1
ZAP70	zeta associated protein 70

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Summary

Protein kinase B (PKB), a serine threonine kinase, is highly involved in the regulation of cellular proliferation and survival. To characterize PKB's function in lymphocyte development and activation, transgenic (tg) mice that express a membrane targeted constitutively active form of PKB (myr PKB) in T and B cells were analysed.

Thymocytes from myr PKB tg mice showed enhanced proliferation after T cell receptor (TCR) engagement compared to wild type (wt) mice. Astonishingly, myr PKB tg thymocytes were capable to proliferate in response to PMA only and were also less sensitive to inhibition by the calcineurin inhibitors CsA or FK506, which indicates the proliferative response of myr PKB tg T cells is relatively independent of calcium mobilisation and calcineurin activity. In addition, when TCR signalling was inhibited by the MEKinase inhibitor PD98059 or the Src-kinase inhibitor PP1 myr PKB tg thymocytes again were more resistant to inhibition. Western blot analysis revealed myr PKB enhances activation of the kinases Lck, Raf and Erk after TCR/CD3 stimulation. Thus, myr PKB renders proliferative responses of thymocytes more sensitive to TCR signals by positive regulation of the Lck-Raf-MEK-Erk signalling pathway. Studies on the cellular location of the tg protein showed myr PKB is located in membrane so-called "lipid rafts". Furthermore, we found that after TCR/CD3 ligation endogenous cytoplasmic PKB moves into "lipid rafts", which highlights PKB as a crucial mediator of TCR proximal signalling events.

Analysing three different TCR tg model systems for positive and negative selection of immature precursors in the thymus, we found myr PKB promotes positive selection of CD4+ but not CD8+ T cells. This most likely results from PKB's positive cross-talk on Lck-Raf-Erk signalling, which is known to influence thymocyte selection and CD4/CD8-lineage choice. Furthermore, myr PKB enhances phosphorylation of glycogen synthase kinase 3 (GSK3), a negative regulator of the transcription factor NFAT (nuclear factor of activated T cells) and T cell activation, and of the adapter protein c-Cbl.
Concerning negative selection, myr PKB enhanced (OT1 mice), reduced (HY mice) or had no influence (OT2 mice) on negative selection. Thus, myr PKB's effect on negative selection strongly depends on the model system analysed and this most likely results from differences in TCR affinity/avidity and TCR specificity for MHC.

Peripheral CD4+ T cells from myr PKB tg mice showed enhanced production of both Th1 and Th2 cytokines. Furthermore, after TCR/CD3 stimulation in the presence of TGF-β1, wt CD4+ T cells showed a drastic inhibition of proliferation, whereas myr PKB tg CD4+ T cells proliferated even better, i.e. they were resistant to the inhibitory TGF-β1 signals.

Expression of myr PKB in B cells leads to reduced Ca^{2+} flux and proliferation after BCR stimulation, but activation of Lyn, SLP-65, c-Cbl and GSK-3 were enhanced. When we analysed B cell subsets in myr PKB tg mice, a decrease in immature and mature B cells became obvious, whereas cell numbers for marginal zone (MZ) B cells were normal.
In aged myr PKB tg mice we detected a very strong reduction of pro/pre and immature B cell populations in the bone marrow, indicating PKB is very important for maintenance of B cell development. Furthermore, myr PKB also lead to a strong reduction of peritoneal B-1 cells. However, expression of NFATc1, which is required for B-1 cell development, was comparable between wt and myr PKB tg B-1 cells.
To analyse the effect of myr PKB on immunoglobulin production, mice were immunized with thymus dependent (TD) and independent (TI) antigens. In both cases, B cell responses were strongly elevated in myr PKB tg mice. Finally, RT-PCR analyses of *in vitro* expanded B cells revealed increased Blimp-1 and Notch3 expression in myr PKB tg B cells, which might be primary candidates involved in their enhanced effector function.

In summary, this study clearly shows an important cross-talk between PKB and various critical signalling molecules downstream of the TCR and BCR. Thereby active PKB modulates and regulates the thresholds for thymocyte selection and T cell activation as well as for B cell development and function.

Zusammenfassung

Proteinkinase B (PKB), eine Serin-Threonin Kinase, spielt bei der Regulation der Proliferation und des Überlebens vieler Zelltypen eine wichtige Rolle ein. Um die Funktion von PKB bei der Reifung und Aktivierung von Lymphozyten zu verstehen, wurden transgene (tg) Mäuse analysiert, die eine konstitutiv-aktive, myristoylierte Form der PKB (myr PKB) in der T- und B-Zelllinie exprimieren.

Thymozyten von myr PKB tg Mäusen zeigten im Vergleich zu wildtypischen (wt) Mäusen nach T-Zell-Rezeptor (TZR)-Stimulation eine deutlich erhöhte Proliferation. Myr PKB tg Thymozyten konnten zudem nur durch PMA zur Proliferation angeregt werden und waren auch weniger sensitiv gegenüber Inhibition durch die Calcineurin-Inhibitoren CsA und FK506. Dies weist darauf hin, dass die Aktivierung von T Zellen der myr PKB tg Mäuse relativ unabhängig von der Calcium-Mobilisierung und der Calcineurin-Aktivität ist. Wurde die TZR-Signalübertragung durch MEKinase-Inhibitor PD98059 oder den Src-Kinase-Inhibitor PP1 blockiert, so waren myr PKB tg Thymozyten wiederum sehr viel schlechter inhibierbar als wt Thymoyzten. Western-Blot-Analysen zeigten sodann, dass myr PKB nach TZR/CD3-Stimulation die Aktivierung der Kinasen Lck, Raf und Erk verstärkt. Somit führt aktive PKB über die positive Regulation des Lck-Raf-Mek-Erk Signalwegs zu einer erhöhten TZR-Sensitivität.
Weiterhin verstärkt myr PKB die Phosporylierung der Glykogen Synthase Kinase 3 (GSK3), ein negativer Regulator des Transkriptionsfaktors NFAT (Nucleärer Faktor Aktivierter T-Zellen) und der T-Zell-Aktivierung sowie des Adaptorproteins c-Cbl.
Unsere Untersuchungen zur zellulären Lokalisation von myr PKB ergaben, dass myr PKB in den sog. „lipid rafts“ der Membran lokalisiert ist. Weiterhin konnten wir zeigen, dass endogene cytoplasmatische PKB nach TZR/CD3-Stimulation in diese „lipid rafts“ wandert. Diese Daten weisen auf eine profunde Rolle von aktiver PKB bei der frühen und proximalen TZR-Signalübertragung hin.

Die Analysen drei verschiedener TZR-tg Modellsysteme zur Selektion von unreifen T-Zellvorläufern im Thymus zeigten, dass myr PKB die positive Selektion von CD4+ aber nicht von CD8+ T-Zellen fördert. Dies resultiert sehr wahrscheinlich aus der positiven Regulation des Lck-Raf-Erk Signalweges, welcher ein zentraler Regulator der Thymozytenselektion und CD4/CD8-Linienentscheidung ist. Was den Einfluss von myr PKB auf die negative Selektion

betrifft, so verstärkte (OT1-Mäuse), verminderte (HY-Mäuse) oder hatte diese keinen Effekt (OT2-Mäuse). Die Effekte von myr PKB auf die negative Selektion sind daher stark abhängig vom Modellsystem, d.h. von der TZR-Affinität/Avidität und der Spezifität der TZRs für MHC-Moleküle.

Periphere CD4+ T-Zellen von myr PKB tg Mäusen wiesen eine erhöhte Produktion von sowohl Th1- als auch Th2-Cytokinen auf. Erstaunlicherweise führte TZR/CD3-Stimulation in Anwesenheit von inhibitorischen TGF-β1-Signalen, ganz im Gegensatz zu wt T-Zellen, in myr PKB tg CD4+ T Zellen zu keiner Inhibition der Expansion, sondern sie proliferierten sogar stärker. Dies könnte mit der beobachteten erhöhten Zytokinproduktion von IL-2 und IFNγ und/oder der erhöhten Phosphorylierung der Smad2/3 Proteine in myr PKB tg CD4+ T Zellen in Verbindung stehen.

Die Expression von myr PKB in B-Zellen führte zu reduziertem Calcium-Flux und reduzierter Proliferation, wobei jedoch eine verstärkte Aktivierung von Lyn, SLP-65, c-Cbl und GSK-3 nachgewiesen werden konnte. Die Analyse der B-Zell-Populationen der myr PKB tg Mäuse zeigte eine Abnahme der unreifen und reifen B-Zellen in der Milz, jedoch war die Anzahl der Marginalzonen (MZ)-B-Zellen normal. Interessanterweise führte myr PKB zu einer sehr starken Reduktion peritonealer B-1-Zellen. Die Expression von NFATc1, welcher für die Entwicklung von B-1-Zellen benötig wird, war jedoch in den B-1-Zellpopulationen von wt und myr PKB tg Mäusen durchaus vergleichbar.
Ältere myr PKB tg Mäuse zeigten einen starken Verlust der pro-/prä- und unreifen B-Zellen des Knochenmarks. Dies weist stark darauf hin, dass PKB für die Aufrechterhaltung der B-Zellentwicklung entscheidend ist.
Darüber hinaus war, obgleich reduzierter B-Zellen, die Immunantwort auf Thymus-abhängige (TD) und –unabhängige (TI) Antigene in myr PKB tg Mäusen verstärkt. In RT-PCR Analysen von *in vitro* expandierten B-Zellen wurde sodann eine erhöhte Expression von Blimp-1 und Notch3 beobachtet, die zu der erhöhten Immunglobulin-Produktion der myrPKB tg B-Zellen beisteuern könnte.

Zusammenfassend zeigen diese Arbeiten, dass aktive PKB die Expression/Aktivität zahlreicher wichtiger Signalmoleküle der TZR- und BZR-induzierten Signalleitung reguliert und somit die Schwellenwerte für die Selektion und Aktivierung von T-Zellen sowie für die Entwicklung und Funktion von B-Zellen entscheidend moduliert.

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Oral and poster presentations

Na SY, Y. Scheuring, A. Marx, A. Patra, D. Kioussis, T. Hünig, U. Bommhardt: Constitutively active AKT/PKB induces lymphomas. 32nd annual meeting of the German Society of Immunology (DGFI), September, 2001, Dresden, Germany.

Amiya K. Patra, **SY 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.

Na SY, A .Patra, Y. Scheuring, U. Bommhardt: Effects of constitutively active PKB on B cell maturation and effector function. 34th annual meeting of the German Society of Immunology (DGFI), September, 2003, Berlin, Germany.

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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Place : Shin-Young Na

List of Publications

Amiya K. Patra, **Na SY**, 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).

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