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Patra, AK

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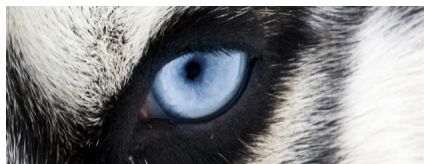
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## **Constitutively Active Protein Kinase B Enhances Lck and Erk Activities and Influences Thymocyte Selection and Activation**

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# Constitutively Active Protein Kinase B Enhances Lck and Erk Activities and Influences Thymocyte Selection and Activation<sup>1</sup>

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Protein kinase B (PKB), a serine threonine kinase is critically involved in cellular proliferation and survival. To characterize its role in T cell development *in vivo*, we have analyzed transgenic mice that express a membrane-targeted constitutively active version of PKB (myr PKB) in thymocytes and peripheral T cells. We report that myr PKB renders proliferative responses of thymocytes more sensitive to TCR signals by increased and sustained activation of Src kinase Lck and the extracellular signal-regulated kinase/mitogen-activated protein kinase pathway. In addition, the proliferative response of myr PKB T cells is relatively independent of calcium mobilization and calcineurin activity. We also find that myr PKB enhances phosphorylation of glycogen synthase kinase 3, a negative regulator of NFAT and T cell activation, and the recruitment of the adapter protein Cbl-c. Interestingly, we demonstrate that upon TCR/CD3 stimulation of wild-type T cells PKB is translocated into lipid rafts, adding a new role for PKB in TCR-initiated signalosome formation in T cell activation. Localization of transgenic PKB in lipid rafts could contribute to the higher TCR sensitivity of myr PKB thymocytes which is reflected in an increase in positive selection toward the CD4 lineage and variable effects on negative selection depending on the model system analyzed. Thus, our observations clearly indicate a cross-talk between PKB and important signaling molecules downstream of TCR that modulate the thresholds of thymocyte selection and T cell activation. *The Journal of Immunology*, 2003, 171: 1285–1296.

Clonal selection of immature T cells occurs at the CD4<sup>+</sup>CD8<sup>+</sup> (double-positive (DP))<sup>3</sup> stage. Signaling molecules central in determining the cell fate of DP thymocytes include the Src family kinase Lck and the mitogen-activated protein kinases (MAPKs). The Ras-Raf-MEK-extracellular signal-regulated kinase (Erk) signaling cascade in particular has been shown to regulate positive and negative selection as well as CD4 vs CD8 lineage choice (1). It is largely unknown how the MAPK pathways cross-talk with other signaling cascades. Phosphatidylinositol-3 kinase (PI3K) signaling has been found in different cell systems to be fundamental in regulation of cell growth, differentiation, survival, and adhesion/migration (2). A prominent target of PI3K is protein kinase B (PKB), which mediates many cellular responses of PI3K including protection against apoptosis (3–5). A cross-talk between the PI3K-PKB and Raf-MEK-Erk pathways has been reported on multiple levels. Whereas some

studies in cell lines show that PI3K/PKB enhances or synergizes with Raf-MEK-Erk signaling (6, 7), other studies, mainly in muscle cells, suggest that PKB abrogates Raf activity on downstream targets (8, 9); these discrepancies probably reflect differences in cell type and the differentiation state of the cell.

PKB, a serine threonine kinase, is recruited by its pleckstrin homology domain to membrane-localized phosphatidylinositol 3,4,5-triphosphate and PtdIns(3,4)P<sub>2</sub>, the second messenger products of PI3-kinase. Membrane localization of PKB leads to its activation via phosphorylation at serine 473 in the C-terminal regulatory domain and at threonine 308 in the catalytic domain mediated by phosphoinositide-dependent protein kinases. 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 C-terminal modulator protein (10) or indirectly as by the lipid phosphatase and tensin homologue (PTEN), which reduces the amounts of PtdInsP<sub>3</sub> at the membrane. Dysregulation of PTEN is associated with development of a variety of human cancers, autoimmune disorders, and loss of tolerance (11–15) and increased PKB activity was implicated in most of these disease phenotypes. Two of the three PKB genes have so far been disrupted in the mouse germline. *PKBα* knockout mice are viable but their growth is retarded, and PKB-deficient thymocytes show increased apoptosis (16). *PKBβ*-null mice are also viable and show defects in insulin signaling (17). In lymphocytes PKB is activated by cytokines (18), TCR signaling (19), CD28 costimulation (20), integrin-linked kinase (21), CD38 (22), or ICAM-2 (23), among others. The antiapoptotic proteins Bcl-x<sub>L</sub> and Bcl-2 and the transcription factor NF-κB are key targets of PKB in T cells, resulting in enhanced T cell survival (24, 25). Besides the prominent role of PKB in regulating cell survival or cell size (26, 27), recent work has highlighted PKB as a potentiator of proliferation. PKB effectors involved in cell cycle regulation include proteins like E2F and cyclins (28, 29) and the transcription factors of

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<sup>3</sup> Abbreviations used in this paper: DP, double-positive; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol-3 kinase; PKB, protein kinase B; PTEN, phosphatase and tensin homologue; CsA, cyclosporin A; HA, hemagglutinin; DN, double-negative; SP, single-positive; GSK3, glycogen synthase kinase 3; LAT, linker for activation of T cells; LN, lymph node; 7-AAD, 7-amino-actinomycin D; Erk, extracellular signal-regulated kinase; MEK, mitogen-activated protein/Erk kinase; HSA, heat-stable Ag; SAg, superantigen; PAG, phosphoprotein associated with glycosphingolipid-enriched microdomains.

the forkhead family (30). Both, inhibition of apoptosis and increased cellular proliferation are two distinct, although interconnected, mechanisms through which PKB promotes cellular transformation and cancer progression.

To address the effects of PKB on developmental events *in vivo*, we generated transgenic mice harboring a constitutively active PKB by means of a myristoylation tag (myr PKB) under the human CD2 promoter leading to expression of myr PKB in thymocytes and mature T cells. We found that overexpression of myr PKB promotes the efficiency of positive selection fostering selection of CD4<sup>+</sup> T cells in TCR transgenic mice, most notably in mice bearing TCRs with MHC class I-restricted specificity. In negative selection myr PKB either reduced, enhanced, or had no effect, depending on the experimental model investigated. Furthermore, myr PKB transgenic thymocytes showed a reduced dependence on calcium mobilization and a higher resistance to inhibition by the immunosuppressants cyclosporin A (CsA) and FK506 in T cell activation. Further studies revealed that TCR/CD3 stimulation leads to recruitment of endogenous active PKB into detergent insoluble glycolipid-rich membrane domains, so-called lipid rafts. Localization of myr PKB in lipid rafts could contribute to the enhanced activation of TCR downstream signaling molecules, whose differential activities contribute to the altered phenotype in T cell selection and activation in myr PKB transgenic mice.

## Materials and Methods

### Generation of transgenic mice

Construction of human *PKB $\alpha$*  cDNA harboring the hemagglutinin (HA) epitope tag and the *lck* myristoylation/palmitoylation signal (MGCWSS NPEDD) for membrane targeting has been described previously (31). Myr-HA-*PKB $\alpha$*  cDNA (myr PKB) was inserted into the *EcoRI* site of the human CD2 minigene cassette (32). The linearized hCD2-myr-HA-PKB fragment was microinjected into fertilized eggs from (CBA/J  $\times$  C57BL/10) F<sub>1</sub> animals. Transgenic founder mice were identified by DNA slot blots using a [<sup>32</sup>P]DNA probe specific for the human CD2 cassette. Stable PKB transgenic lines were established by crossing transgenic mice to C57BL/6 mice. Transgenic mice were identified by PCR of genomic tail DNA. Mice used throughout the study were offspring from two founder lines (PKB2 and PKB6) which were crossed to C57BL/6 mice for at least three generations. Mice used were 6–8 wk old if not specified otherwise. No transformation was observed in myr PKB heterozygous mice aged up to 8 mo.

### Mice

OT1 (33), OT2 (34), HY (35) TCR transgenic mice and DBA/2 and CBA/J mice (Charles River Breeding Laboratories, Wiga, Sulzfeld, Germany) were crossed with myr PKB transgenic mice.

### Isolation of cells and proliferation

Thymi were forced through a fine mesh filter to obtain single cell suspensions. For proliferation assays, total thymocytes ( $1 \times 10^6$ ) were cultured in 96-well plates in triplicates in complete RPMI 1640 supplemented with 10% FCS. Cells were stimulated with plate bound anti-CD3 mAb (145.2C11; BD PharMingen, San Diego, CA) or with PMA and ionomycin (both Calbiochem, San Diego, CA) for the indicated time points. CsA, FK506, PD98059, and PP1 (all from Calbiochem) were added at the beginning of culture at concentrations as stated. On day 2, cells were pulsed with 1  $\mu$ Ci [<sup>3</sup>H]thymidine/well (ICN Pharmaceuticals, Asse-Relegem, Belgium) and harvested after 10–16 h.

### Flow cytometric analysis

Abs were obtained from BD PharMingen as FITC-, PE- or biotin-labeled conjugates or were prepared in our own laboratory (Institute of Virology and Immunobiology, University of Würzburg, Würzburg, Germany): CD4 (GK1.5), CD8 (YTS169.4), CD69 (H1.2F3), CD5 (53-7.3), TCR $\beta$  (H57-597), heat-stable Ag (HSA) (M1/69), CD44 (IM7), CD25 (7D4), V $\beta$ 5 (MR9-4), V $\beta$ 8 (F23.1), V $\beta$ 11 (RR3-15), V $\alpha$ 2 (B20.1). Biotinylated Abs were revealed with streptavidin-CyChrome (BD PharMingen). T3.70 mAb specific for the HY TCR transgenic V $\alpha$ -chain was a kind gift from Dr. T. Miyazaki (Center for Immunology, University of Texas Southwestern Medical Center, Dallas, TX). Cells were stained using standard procedure

and were analyzed on a FACScan or FACSCalibur (BD Biosciences, Mountain View, CA) using CellQuest software. For FACS analysis of double-negative (DN) cells, DP and mature single-positive (SP) cells were depleted by treatment with anti-CD4 (RL174.2) and anti-CD8 (3.168.1) supernatant and Low-Tox rabbit complement (Cedarlane Laboratories, Hornby, Ontario, Canada).

### Western blot and Lck immune complex kinase assay

Freshly isolated thymocytes ( $2-4 \times 10^6$ ) were lysed immediately or starved for 2 h in 1% FCS medium before stimulation with anti-CD3 mAb (145.2C11, 30  $\mu$ g/ml). Cells were lysed in buffer containing 20 mM HEPES, 2 mM EGTA, 50 mM  $\beta$ -glycerophosphate, 1% Triton X-100, 10% glycerol, 50 mM NaF, 0.04% azide, 1 mM DTT, 1 mM orthovanadate, 2  $\mu$ M leupeptin, 0.4 mM PMSF for 30 min on ice. Protein extracts from  $2-4 \times 10^6$  cells were separated on 8–12% SDS-PAGE and electroblotted to nitrocellulose membranes. Specific proteins were detected by Western blot analysis using the following primary Abs: anti-PKB, anti-phospho-PKB (Ser<sup>473</sup>), anti-phospho-Erk, anti-phospho-glycogen synthase kinase 3 (GSK3)  $\alpha\beta$ , anti-phosphotyrosine (4G10), anti-phospho-Lck (Tyr<sup>394</sup>), anti-phospho-c-Raf (Ser<sup>259</sup>) (New England Biolabs, Beverly, MA), anti-Lck (36), anti-Cbl-c, and anti-linker for activation of T cells (LAT) (Santa Cruz Biotechnology, Santa Cruz, CA). Primary Abs were detected by goat anti-rabbit (1/3000; Santa Cruz Biotechnology), goat anti-mouse (1/3000; Chemicon, Temecula, CA) or rabbit anti-goat (1/5000; Chemicon) Abs coupled with HRP and ECL (Pierce, Milwaukee, WI). Blots were reprobed with anti-actin Ab (Santa Cruz Biotechnology) to control protein loading. For Lck kinase assays,  $5 \times 10^7$  freshly isolated thymocytes were lysed immediately or starved for 2 h in 1% FCS medium before stimulation with anti-CD3 mAb (145.2C11, 30  $\mu$ g/ml) for the indicated time points. Cells were lysed in 500  $\mu$ l of Brij-98 lysis buffer (1% Brij-98 in TNE buffer: 25 mM Tris pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM Pefabloc, 5 mM iodoacetamide, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM NaF) and Lck was immunoprecipitated with anti-Lck Ab (36) and protein G-Sepharose (Amersham Biosciences, Freiburg, Germany). Lck precipitates were washed two times with lysis buffer and kinase assay buffer (0.1% Brij-98, 25 mM HEPES pH 7.4) before incubation with 30  $\mu$ l of assay buffer containing 10 mM MnCl<sub>2</sub>, 5  $\mu$ Ci [ $\gamma$ -<sup>32</sup>P]ATP (3000 Ci/mmol; Amersham Biosciences) and acidified enolase (5  $\mu$ g; Sigma-Aldrich, Taufkirchen, Germany) for 20 min at 30°C. After addition of 30  $\mu$ l of 2 $\times$  sample reducing buffer, Lck activation was detected by 10% SDS-PAGE and exposure to x-ray film.

### Preparation of glycolipid-enriched membrane microdomain fractions

Freshly isolated thymocytes ( $1 \times 10^8$ ) or purified CD4<sup>+</sup> T cells from lymph nodes in 200  $\mu$ l of PBS were treated with 200  $\mu$ l of cold Brij-98 lysis buffer (0.1% Brij-98 in TNE buffer: 25 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM Pefabloc, 5 mM iodoacetamide, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM NaF). The lysate was mixed with 400  $\mu$ l of 80% sucrose in TNE buffer. The solution was overlaid with 2.8 ml of 30% sucrose in TNE buffer followed by 400  $\mu$ l of TNE buffer and centrifuged at  $250,000 \times g$  for 22 h at 4°C. Proteins in the four harvested fractions were precipitated with acetone, and 50  $\mu$ l (fractions 1–3) or 250  $\mu$ l (fraction 4) of SDS-PAGE sample buffer were added to the precipitates. Twenty microliter samples were resolved by SDS-10% PAGE and Western blotted. Based on localization of LAT, the top fractions (1, 2) were designated the detergent insoluble raft microdomain fraction and the bottom fractions (3, 4), containing the majority of total protein, were designated detergent soluble. For activation of CD4<sup>+</sup> T cells,  $1 \times 10^8$  cells were stimulated with anti-CD3 mAb (145.2C11, 10  $\mu$ g) and anti-hamster IgG (10  $\mu$ g; Sigma-Aldrich) for 30 min at 37°C. Raft preparation of stimulated cells was as described for thymocytes.

### Measurement of apoptosis

Total thymocytes or purified lymph node (LN) CD4<sup>+</sup> T cells (each  $1 \times 10^6$ /ml) from wild-type and myr PKB mice were cultured in RPMI 1640 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 (BD PharMingen) and 7-amino-actinomycin D (7-AAD; Sigma-Aldrich) staining and the percentage of live 7-AAD- and annexin V-negative cells was determined. Apoptosis was induced with C2-ceramide, dexamethasone (both from Sigma-Aldrich), anti-fas mAb (Jo2; BD PharMingen), etoposide, or PMA (both Calbiochem). Apoptosis of thymocytes from OT1 and OT2 mice and crosses with myr PKB mice was studied by adding OVA peptide 257–264 (33) or 323–339 (34) to  $5 \times 10^5$  cells/well, respectively, in concentrations as indicated. In case of OT2 TCR



bearing mice irradiated splenic B cells ( $1 \times 10^6$  cells/well) were added as APCs.

## Results

### Generation of transgenic mice expressing myr PKB $\alpha$ in lymphocytes

To investigate the role of PKB in T cell development, we established transgenic mouse lines expressing a human myr PKB $\alpha$  cDNA (31) under the control of the human CD2 promoter and locus control region (Fig. 1A) (32). Of six transgenic founder lines, three lines (PKB 1, 2, and PKB 6) were crossed to the C57BL/6 background. Analysis of all three lines showed comparable results, with PKB 6 and PKB 2 being used throughout this study. Myr PKB expression in thymocytes and peripheral CD4 $^+$  and CD8 $^+$  T cells was determined by Western blots using anti-PKB and anti-phospho-PKB (Ser $^{473}$ ) Abs (Fig. 1, B and C). The kinase activity of myr PKB was verified for splenic CD4 $^+$  T cells in kinase assays as shown previously (37). All mice used in this study were heterozygous for myr PKB (PKB $^{+/-}$ ).

### Selective accumulation of CD4 $^+$ T cells in myr PKB mice

First, we examined the cellular composition of thymus, spleen, and LNs from myr PKB transgenic and wild-type mice of different ages (Fig. 2 and Table I). The composition of thymocyte subsets from young (4–12 wk of age) myr PKB transgenic mice was comparable to that of littermate control mice or showed a moderate increase in CD4 and CD8 SP T cells. A reduction in the number of DP thymocytes (to ~60% of wild-type mice) was prominent in aged mice (3–12 mo of age) and resulted in an overall reduction in

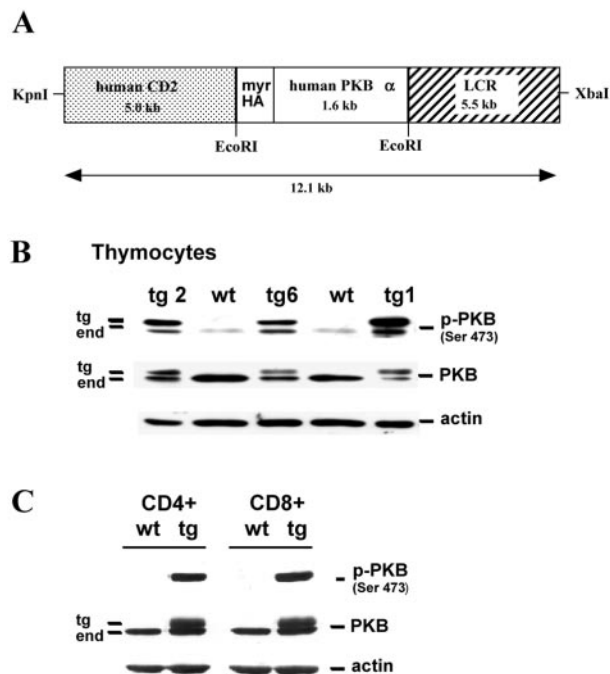
thymocyte cellularity. The development of DP thymocytes to mature SP thymocytes is associated with a series of phenotypic changes triggered by the TCR that can be defined using Abs reactive with the TCR, CD5, and CD69 Ags (38–40). Typically, a small percentage of DP thymocytes express higher levels of these markers which are putative indicators for cells that have received either positive (41–43) or negative selection (44) signals in response to TCR engagements. Although thymi from myr PKB mice were grossly normal in size and morphology, a more detailed analysis revealed that myr PKB DP thymocytes have a higher percentage of cells that have up-regulated TCR, CD5, and CD69 expression (Fig. 2A). This suggests that in PKB transgenic 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 PKB transgenic mice (Fig. 2B and Table I) showed that the CD4 compartment was selectively enlarged, with 2- to 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 ~5:1 in young mice (4–12 wk) and 11:1 in older mice (3–12 mo). 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.

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

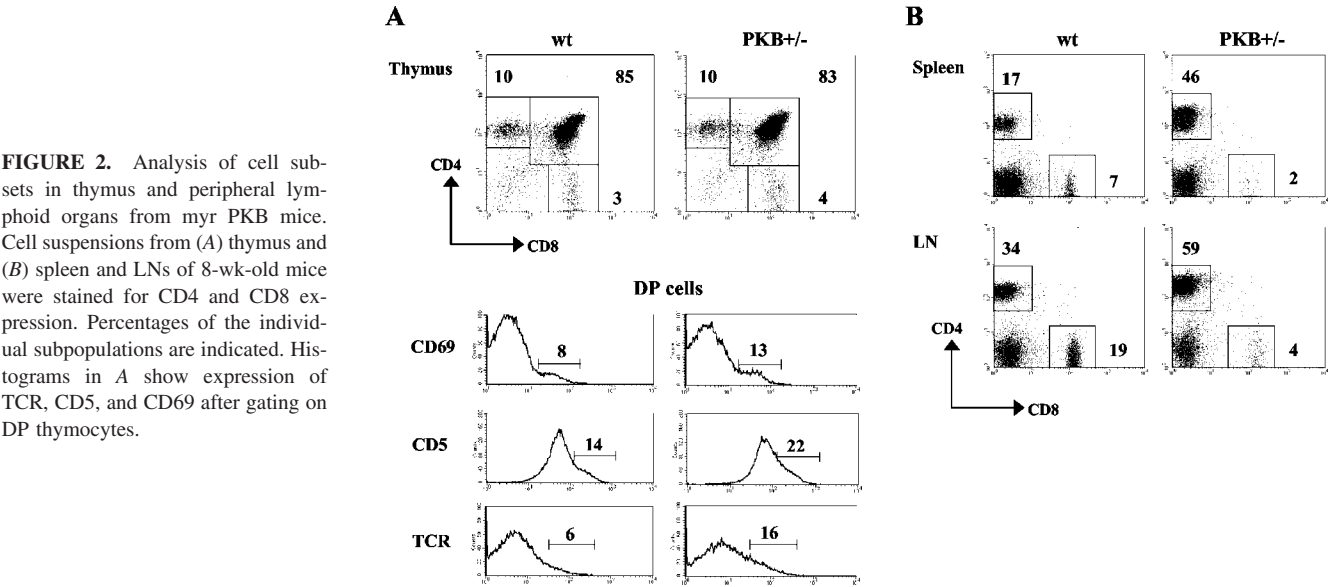
To correlate the change in the level of PKB activity to functional responses, we analyzed the proliferative potential of thymocytes in response to immobilized anti-CD3 mAb and to the pharmacological agents PMA and ionomycin, known to activate protein kinase C and to induce calcium flux, respectively. As shown in Fig. 3A, stimulation of thymocytes with different concentrations of anti-CD3 mAb resulted in higher [ $^3$ H]thymidine incorporation in myr PKB thymocytes. Astonishingly, thymocytes from myr PKB mice were capable to proliferate in response to PMA only, i.e., in the absence of the Ca $^{2+}$  fluxing agent ionomycin (Fig. 3B), whereas the two populations showed equivalent responses to stimulation with PMA plus ionomycin. Moreover, in the case of transgenic mice, the proliferative responses induced by anti-CD3 mAb, as well as those induced by PMA or PMA/ionomycin, were less sensitive to inhibition by the calcineurin inhibitors CsA or FK506 than those of wild-type thymocytes which were totally abolished (Fig. 3B). In addition, when TCR-induced signaling was blocked by mitogen-activated protein/Erk kinase (MEK) inhibitor PD98059 (Fig. 3C) or Src-kinase inhibitor PPI (Fig. 3D), myr PKB thymocytes again were more resistant to inhibition requiring 5- to 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. Similar results were obtained for peripheral CD4 $^+$  and CD8 $^+$  T cells (data not shown).

### 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 wk) was assayed after serum withdrawal or treatment with PMA, ionomycin, anti-CD3, or anti-fas mAbs, dexamethasone, etoposide, or UV irradiation (Fig. 4A). For most treatments, the survival of myr PKB thymocytes was only slightly enhanced or identical with wild-type cells. Survival was more significantly increased in the case of dexamethasone treatment. Titration of apoptosis-inducing reagents and measurement of survival on day 2



**FIGURE 1.** Generation of PKB transgenic mice. *A*, Schematic representation of the myr PKB transgenic construct. *B* and *C*, Expression of myr PKB in thymocytes and peripheral T cells. *B*, Thymocytes from wild-type (wt) and myr PKB transgenic lines (tg) were analyzed by Western blot using anti-PKB and anti-phospho-PKB (Ser $^{473}$ ) Abs. Results are shown for three different transgenic lines, PKB 2, 6, and 1. Upper bands correspond to transgenic myr PKB, lower bands to endogenous (end) PKB. Anti-actin Ab was used to control equal protein loading. *C*, Western blot analysis for phospho-PKB (Ser $^{473}$ ) expression in peripheral CD4 $^+$  and CD8 $^+$  T cells from wild-type and myr PKB transgenic mice.



**FIGURE 2.** Analysis of cell subsets in thymus and peripheral lymphoid organs from myr PKB mice. Cell suspensions from (A) thymus and (B) spleen and LNs of 8-wk-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.

(data not shown) reflected the differences observed as shown in Fig. 4A. When peripheral CD4<sup>+</sup> T cells were analyzed (Fig. 4B), survival and resistance to apoptosis after treatment with ceramide, dexamethasone, UV or gamma irradiation was distinctly higher in myr PKB CD4<sup>+</sup> T cells. This indicates that myr PKB activity delivers stronger survival signals for mature peripheral T cells than for thymocytes.

Because aged myr PKB mice showed a reduction of DP cells, we analyzed the apoptotic response of thymocytes from 7-mo-old mice (Fig. 4C). In contrast to young mice, DP thymocytes from aged myr PKB mice survived less well after serum withdrawal, anti-CD3 and anti-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<sup>+</sup> CD8<sup>+</sup> DN cells. Flow cytometric analysis of DN cells in aged myr PKB mice revealed that the percentage of CD25<sup>+</sup>CD44<sup>+</sup> (DN3) cells was reduced whereas the CD44<sup>+</sup>CD25<sup>+</sup> (DN1) population was increased compared with wild-type cells (Fig. 4D). The CD25<sup>+</sup>CD44<sup>+</sup> DN population of aged myr PKB mice contained a lower fraction of large cells as determined by forward side scatter analysis, indicating that they are less cycling. Altogether, these observations suggest that the

decrease in the DP thymocyte population in aged myr PKB mice could result from a combination of diminished survival of DP cells and a partial block in expansion/differentiation of DN cells.

*Myr PKB supports thymic selection of CD4<sup>+</sup> T cells*

To examine the role of myr PKB in positive selection of thymocytes with a single TCR specificity, we crossed myr PKB mice with OT2 or OT1 TCR transgenic mice. The majority of OT2 and OT1 TCR transgenic DP thymocytes bear TCRs specific for OVA peptides presented by MHC class II or class I molecules, such that they are selected into the CD4 or CD8 lineage, respectively (33, 34). In myr PKB OT2 double transgenic mice (Fig. 5), we detected a marked increase in the percentage and number of CD4 SP cells but not CD8 SP thymocytes compared with OT2 mice not harboring myr PKB. These CD4 SP thymocytes were fully mature as evidenced by the expression of the TCR transgenic Vα2 chain, CD69, CD5, and HSA, i.e., surface Ags that are up- or down-modulated when positively selected DP thymocytes mature to SP cells. Because staining profiles of OT2 myr PKB CD4 SP cells were comparable to those of OT2 CD4 SP cells, myr PKB enhances the efficiency of CD4 development.

The effect of myr PKB on positive selection of CD8 lineage cells was investigated in myr PKB OT1 double transgenic mice

Table I. Myr PKB mice show a reduction in thymus size, CD8 T cell numbers, and increased CD4:CD8 ratio with age<sup>a</sup>

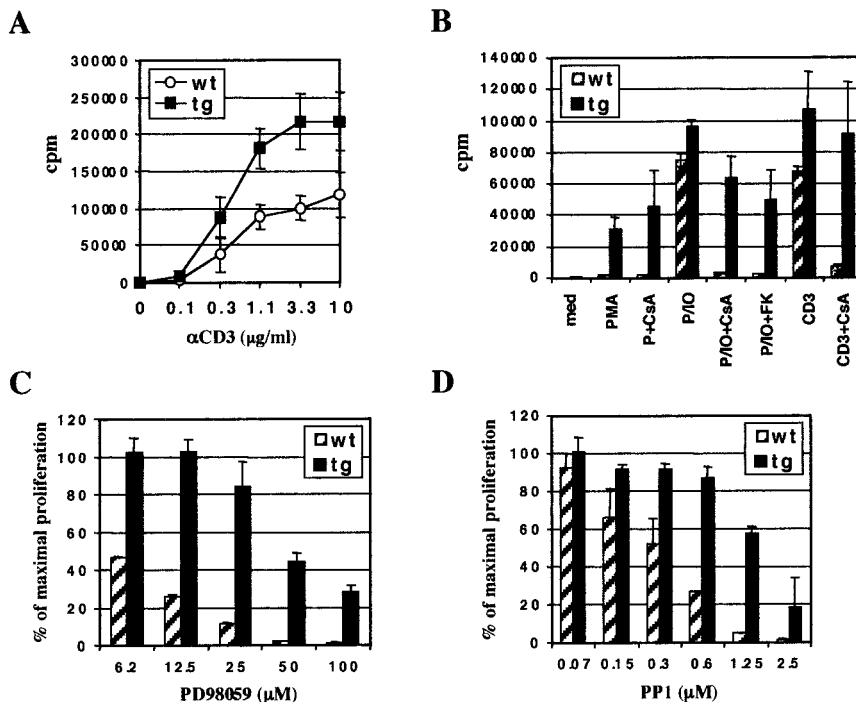
	Thymus				Spleen				CD4:CD8 ratio	
	Total	CD4	DP	CD8	Total	CD4	CD8		wt	PKB <sup>+/-</sup>
wt (cell no. × 10 <sup>7</sup> )										
4–12 wk	16.19 (±6.51)	1.26 (±0.79)	13.86 (±5.85)	0.45 (±0.30)	13.85 (±6.06)	2.38 <sup>b</sup> (±1.39)	1.10 (±0.79)			
3–12 mo	6.28 <sup>c</sup> (±2.92)	0.42 (±0.35)	5.67 <sup>c</sup> (±2.91)	0.11 (±0.10)	10.61 (±4.79)	1.84 <sup>b</sup> (±1.23)	0.77 <sup>c</sup> (±0.59)			
PKB tg										
4–12 wk	17.13 (±6.70)	1.47 (±0.71)	14.36 (±6.39)	0.67 (±0.35)	14.62 (±4.18)	4.67 <sup>b</sup> (±2.30)	0.94 (±0.61)	2.2	4.9	
3–12 mo	4.38 <sup>c</sup> (±2.16)	0.45 (±0.30)	3.77 <sup>c</sup> (±2.11)	0.13 (±0.08)	11.38 (±5.09)	4.51 <sup>b</sup> (±2.81)	0.40 <sup>c</sup> (±0.18)	2.4	11.3	

<sup>a</sup> Cell numbers from thymi (n = 28) and spleens (n = 29) from wild-type or myr PKB heterozygous mice aged either 4–12 wk or 3–12 mo were determined and cells were stained for CD4 and CD8 expression to detect T cells. Given are absolute cell numbers for the indicated subpopulations (×10<sup>7</sup>) and the CD4:CD8 ratios from pooled young or aged mice. wt, wild type; tg, transgenic.

<sup>b</sup> Student's *t* test, *p* < 0.001.

<sup>c</sup> Student's *t* test, *p* < 0.01.

**FIGURE 3.** Myr PKB 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 platebound anti-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/O, each 50 ng/ml), or anti-CD3 mAb (CD3, 5  $\mu$ g/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 anti-CD3 mAb (5  $\mu$ g/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 anti-CD3 stimulation alone, which was set as 100%. In **A–D** proliferation was measured after 48 h and data represent averages from triplicate cultures from two to three individual wild-type or myr PKB transgenic mice.



(Fig. 6, **A** and **B**). Selection of OT1 CD8 SP T cells was not enhanced or even reduced in myr PKB OT1 mice. Interestingly, in ~50% of myr PKB OT1 mice, a strong increase in the percentage and absolute cell number of OT1 CD4<sup>+</sup> T cells was observed. Thymi from OT1 myr PKB mice that did not show an overselection of CD4<sup>+</sup> T cells phenotypically looked like OT1 wild-type mice but DP cells showed enhanced reactivity to deleting stimuli (see Fig. 7C). OT1 CD4 SP cells were phenotypically mature as assessed by expression of TCR transgenic chains, CD69 and CD5 which were comparable to mature CD8 SP T cells from OT1 mice (Fig. 6A). In parallel, in spleens from OT1 myr PKB mice a 3- to 4-fold increase in OT1 V $\alpha$ 2 TCR<sup>high</sup>CD4<sup>+</sup> cells and a decrease in OT1 CD8<sup>+</sup> T cells was observed (Fig. 6B). Because the degree of CD4 overselection was variable, we analyzed two other myr PKB 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<sup>+</sup> OT1 T cells was also observed in a high percentage of, 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 toward the CD4 lineage.

#### Effects of myr PKB on negative selection

To investigate the role of myr PKB in negative selection, we crossed myr PKB mice with HY TCR transgenic mice. In these mice, thymocytes are positively selected in female, but negatively selected in male mice (35). 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. 7A). In myr PKB HY double transgenic female mice, cell numbers for HY-specific CD8 SP thymocytes, as determined with the T3.70 mAb (recognizing the transgenic V $\alpha$ -chain), were comparable to HY female mice but numbers of CD4<sup>+</sup>T3.70<sup>+</sup> cells were increased ~3- to 4-fold. This enhanced selection of CD4<sup>+</sup> T cells is similar to our observations in OT2 and OT1 TCR myr PKB mice. In negatively selecting male myr PKB

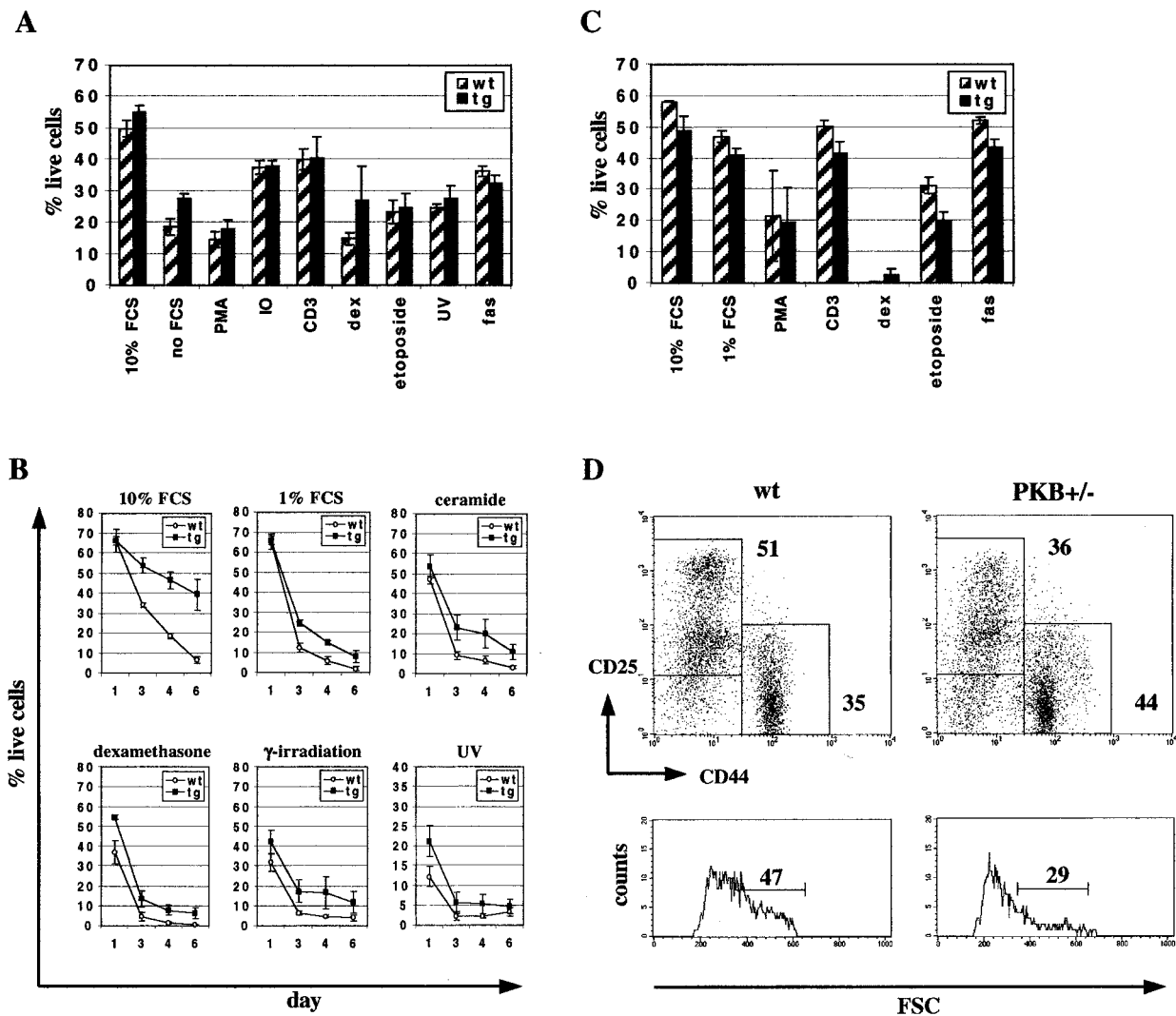
HY double transgenic mice, on an average a 2- to 3-fold increase in the numbers of HY-specific DP and CD8<sup>+</sup> T cells was detected as well as a 2- to 4-fold increase in peripheral CD4<sup>+</sup> and CD8<sup>+</sup> T cells expressing autoreactive TCRs (data not shown). Although total cell numbers in male myr PKB HY mice were still much lower than in female mice, myr PKB has a definite attenuating effect on negative selection.

Because 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 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 $\beta$ 5 and V $\beta$ 11 TCRs (45). In analyses as shown in Fig. 7B, we did not detect a myr PKB-dependent difference in the deletion of SAG-reactive V $\beta$ 5<sup>+</sup> or V $\beta$ 11<sup>+</sup> CD4<sup>+</sup> T cells in the thymus or periphery, suggesting that myr PKB does not alter *in vivo* negative selection of SAG-reactive T cells.

The effect of myr PKB on negative selection in OT1 and OT2 mice was tested by culturing thymocytes from 6- to 7-wk-old mice with TCR-specific agonistic peptides (46). As depicted in Fig. 7C, myr PKB distinctly enhanced negative selection induced by OVA peptide 257–264 in OT1 mice. In contrast, deletion of thymocytes from OT2 mice, induced by OVA 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.

#### 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 analyzed the activity of the 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 signaling in particular favor development of the CD4 lineage but are needed less so for differentiation to the



**FIGURE 4.** A–C, Effects of myr PKB on survival of thymocytes and peripheral CD4<sup>+</sup> T cells. A, Thymocytes from 6-wk-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), anti-CD3 mAb (10  $\mu$ g/ml), dexamethasone (dex, 1 nM), etoposide (1  $\mu$ M), UV irradiation (0.01 J/cm<sup>2</sup>), or anti-fas mAb (3  $\mu$ 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 CD4<sup>+</sup> T cells. CD4<sup>+</sup> T cells from wild-type and myr PKB mice were cultured in medium only containing 10% or 1% FCS or were treated with C2-ceramide (2  $\mu$ M), dexamethasone (1 nM), gamma irradiation (0.3 Gy), or UV irradiation (0.01 J/cm<sup>2</sup>) 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 mo) were cultured in medium with 10 or 1% FCS or were treated with PMA (50 ng/ml), anti-CD3 mAb (10  $\mu$ g/ml), dexamethasone (dex, 3 nM), etoposide (0.1  $\mu$ M), or anti-fas mAb (9  $\mu$ g/ml) and live cells were determined on day 1. D, CD44/CD25 profiles of DN thymocytes from 10-mo-old wild-type and myr PKB transgenic mice. Isolated DN cells were stained with anti-CD44 and anti-CD25 mAbs and analyzed by FACS. The percentage of cells present in each area is indicated. Histograms show cell size distribution within the CD25<sup>+</sup>CD44<sup>-</sup> population of wild-type and myr PKB transgenic mice as measured by FACS. Numbers give the percent of large cells.

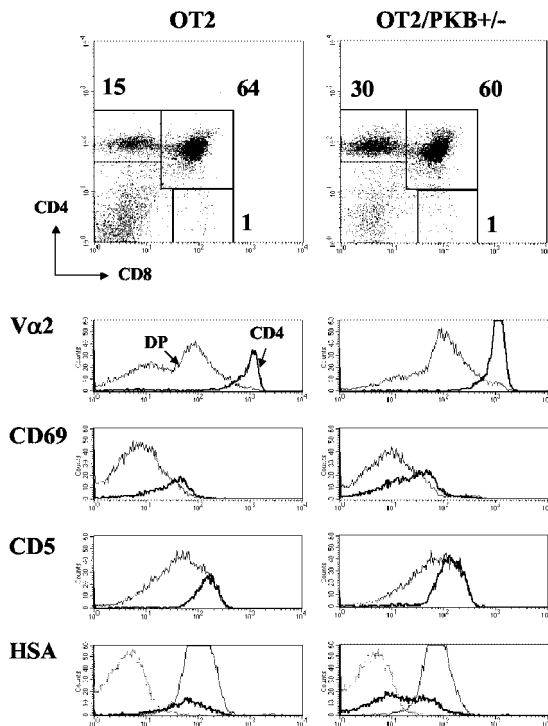
CD8 lineage (47). When freshly isolated thymocytes were analyzed for Erk activation (Fig. 8A, *top panel*), myr PKB thymocytes showed higher endogenous levels of phospho-Erk than wild-type thymocytes. Furthermore, stimulation of thymocytes with anti-CD3 mAbs (Fig. 8B) led to stronger and notably longer-lasting Erk activation in myr PKB transgenic 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 anti-phosphotyrosine mAb in Western blots and observed that in rested nonstimulated PKB transgenic thymocytes, tyrosine phosphorylation of Lck was as strong as in wild-type thymocytes after stimulation with anti-CD3 mAbs (Fig. 8B). Because phosphorylation can either activate or down-mod-

ulate Lck catalytic activity (48), the enhancement of Lck activity by myr PKB was confirmed by Lck kinase assay (Fig. 8B). As observed for Erk, increased Lck activity could also be detected in freshly isolated myr PKB thymocytes using the Lck kinase assay and Abs that detect the activating tyrosine 394 phosphorylation of Lck (Fig. 8A, *middle and bottom panels*). Thus, signals whose intensity and duration favor selection toward the CD4 lineage are clearly enhanced in myr PKB thymocytes and are likely to contribute to the observed increase in the selection of CD4 lineage cells found in myr PKB TCR transgenic 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





**FIGURE 5.** Enhanced positive selection of CD4<sup>+</sup> T cells in myr PKB OT2 TCR double-transgenic mice. Thymi from 8-wk-old myr PKB mice crossed with OT2 TCR transgenic mice were stained for CD4 and CD8 expression. Percentages of cells in each quadrant are given. Histograms show expression of the transgenic TCR Vα2 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 Vα2 chain were for OT2 littermate mice ( $n = 7$ , total  $9.27 \times 10^7 \pm 1.38$ , DP  $4.70 \times 10^7 \pm 1.2$ , CD4 SP  $1.90 \times 10^7 \pm 0.78$ , CD8 SP  $0.13 \times 10^7 \pm 0.10$ ) and for OT2 PKB transgenic mice ( $n = 9$ , total  $12.07 \times 10^7 \pm 2.97$ , DP  $6.35 \times 10^7 \pm 1.15$ , CD4 SP  $4.06 \times 10^7 \pm 1.49$ , CD8 SP  $0.17 \times 10^7 \pm 0.07$ ).

function, inducing the degradation of proteins by its ubiquitin ligase activity (49). As shown in Fig. 8B, TCR triggering of thymocytes induces phosphorylation of Cbl-c. Interestingly, myr PKB enhanced the phosphorylation of Cbl-c suggesting that one way in which PKB potentiates the Erk signaling cascade could be via activation of Cbl-c.

Increased activity of Lck, Erk, and possibly Cbl-c most likely contribute to the enhanced proliferation of myr PKB thymocytes. Because myr PKB T cells proliferated in the presence of the calcineurin inhibitors CsA or FK506 (Fig. 3), we analyzed phosphorylation and thus inactivation of GSK3, which has been shown to oppose nuclear NFAT localization (50). We found that TCR triggering in normal thymocytes leads to fast and transient phosphorylation of GSK3. However, in myr PKB thymocytes, phosphorylation of GSK3 is not only stronger but is also sustained over a much longer period (Fig. 8B). 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 thymocytes in the presence of these immunosuppressants.

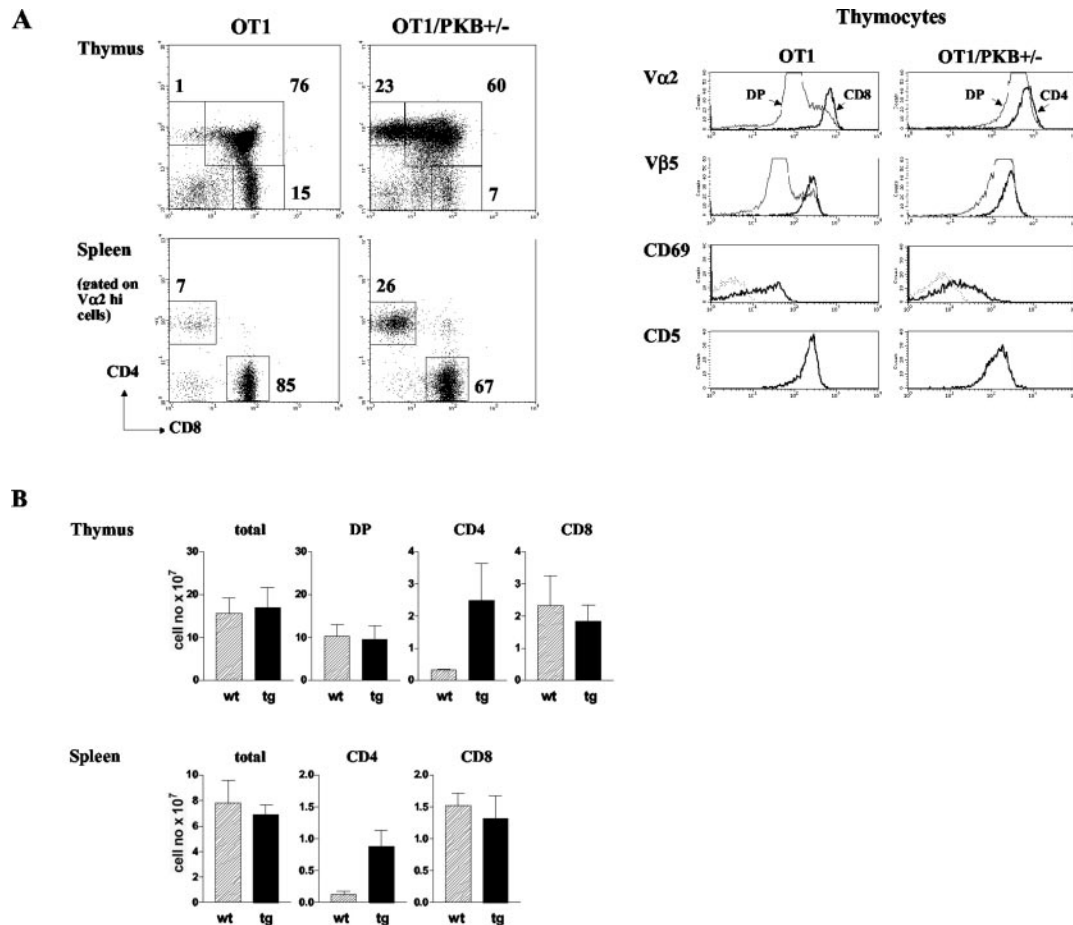
#### *PKB is recruited to membrane lipid rafts after TCR/CD3 stimulation*

Glycosphingolipid-enriched microdomains, 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 signaling (51). To define how

myr PKB could regulate the activation of Lck and Erk, we studied the membrane localization of the transgenic protein. Low density detergent insoluble fractions and heavy soluble fractions from freshly isolated thymocytes were separated by ultracentrifugation and analyzed by Western blotting (Fig. 8C). Whereas endogenous PKB in transgenic and wild-type thymocytes was found only in the soluble fractions, transgenic phosphorylated PKB was localized predominantly in the lipid raft fraction. The latter was identified by the presence of the transmembrane adapter proteins, phosphoprotein associated with glycosphingolipid-enriched microdomains (PAG) (52) and LAT (53). Similar results were obtained for peripheral CD4<sup>+</sup> T cells (Fig. 8D); however, in CD4<sup>+</sup> 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. 8D, lower two panels). Intriguingly, we found that after TCR stimulation, activated phosphorylated PKB is associated with lipid rafts in wild-type CD4<sup>+</sup> T cells similar to transgenic PKB. These results suggest a central role for PKB in TCR-induced formation of signaling complexes within lipid rafts.

#### **Discussion**

The purpose of this study was to explore how the serine threonine kinase PKB affects T cell development and T cell activation. Using transgenic mice, we find that membrane-targeted PKB influences positive and negative selection of thymocytes, survival, and activation/proliferation of T cells. Proteins that are involved in myr PKB-mediated signal transduction include Lck, Raf, and Erk as well as Cbl-c and GSK3, 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 signaling determine lineage commitment or efficiency of selection of CD4<sup>+</sup> or CD8<sup>+</sup> T cells (47, 54), whereby strong or long-lasting signals favor CD4 and weaker or short signals CD8 maturation. In particular, experiments using thymic organ culture (55, 56) and Lck transgenic mice (57, 58) have demonstrated that relatively small alterations in intrathymic Lck activity can significantly affect the CD4/CD8 lineage decision. Likewise, several studies using genetically modified mice and in vitro differentiation systems have shown that the strength/duration of Raf-Mek-Erk signaling regulates positive selection, lineage commitment, and negative selection (47). In this study, we observe in three different TCR transgenic systems that myr PKB promotes positive selection of CD4<sup>+</sup> T cells. In OT2 TCR transgenic mice, the positive cross-talk of myr PKB on Lck-Raf-Erk signaling 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<sup>+</sup> T cells. In view of the fact that myr PKB strengthens Lck-Erk activation, TCR signals might lie above the threshold levels required for maturation of CD8<sup>+</sup> T cells. In contrast, stronger Lck-Erk signals could drive a high proportion of DP cells from OT1 myr PKB double transgenic mice to develop into CD4<sup>+</sup> T cells, as we indeed observed in some mice. The variability in overselection of CD4<sup>+</sup> cells in OT1 mice, although the underlying mechanisms are unresolved, is most likely connected to myr PKB activity because 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<sup>+</sup> T cells is due to an initial switch of some DP cells to the CD4 lineage or rather a rescue of CD8 committed CD4<sup>+</sup>CD8<sup>low</sup> cells due to enhanced/prolonged Lck-Erk signaling. This also applies for the increased selection of CD4<sup>+</sup> T cells seen



**FIGURE 6.** Myr PKB supports selection of OT1 CD4<sup>+</sup> T cells. **A**, Thymi and spleens from 6- to 8-wk-old OT1 mice or myr PKB mice crossed with OT1 TCR transgenic mice were stained for CD4 and CD8 expression. Percentages for thymocyte and splenic subpopulations are given. Plots for spleens are gated on OT1 TCR Vα2<sup>hi</sup> cells. Histograms for thymocytes were gated on CD8 SP (OT1 mice) or CD4 SP cells (OT1/myr PKB<sup>+/-</sup> mice) and show expression of OT1 TCR-specific Vα2 and Vβ5 chains and of CD69 and CD5 markers (bold lines). Vα2 and Vβ5 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 mice ( $n = 12$ , data include CD4 overselecting mice and myr PKB OT1 mice looking phenotypically similar to wild-type OT1 mice) and OT1 mice ( $n = 12$ ) were determined and the absolute cell numbers for Vα2-positive (Vα2<sup>hi</sup> cells in case of mature cells) thymocyte and splenic subpopulations were calculated from percentages gained by FACS staining.

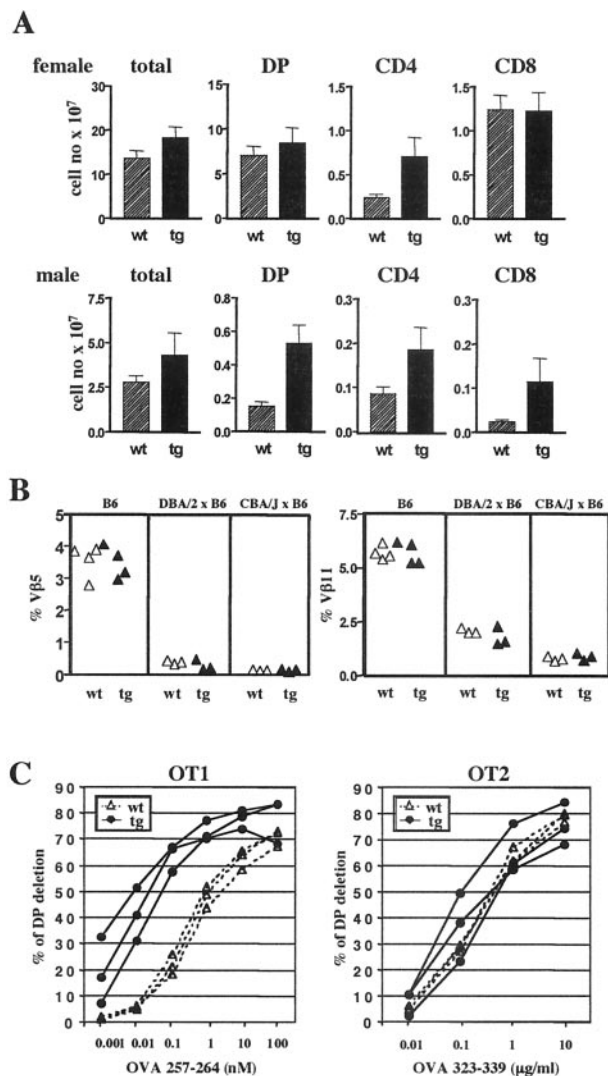
in OT2 and HY female mice expressing the transgene. In the case of OT1 mice, myr PKB clearly enhanced negative selection induced by peptide Ag, arguing for a higher sensitivity of OT1/myr PKB DP cells. Therefore, it is also imaginable that some DP cells that were destined for deletion might escape negative selection and mature instead to CD4<sup>+</sup> T cells. Negative selection not only operates at the DP stage but also at the level of semimature CD4<sup>+</sup>CD8<sup>low</sup> cells, detecting a role for fas-mediated deletion of these semimature cells depending on the Ag dose (59). Defects in fas-induced deletion of peripheral T cells have been detected in PTEN mutant (14, 15) and *gag*-PKB transgenic mice (60). 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<sup>+</sup>CD8<sup>low</sup> cells in OT1 mice cannot be totally excluded.

Maturation of DP cells is initiated by the ligation of the TCR and a number of coinducer/costimulatory receptors including CD28 (61) 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 coengagement of DP cells can either induce CD4 T cell maturation or negative selection, depending on the intensity of CD28 costimulation (62). Assuming that myr PKB reflects some aspects of CD28 signaling in thymo-

cytes, 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 activity of GSK3, a kinase identified in the regulation of nuclear export of NFATc (50), and supports T cell proliferation in the presence of calcineurin inhibitors or in the absence of significant calcium mobilization, it is also conceivable that differential regulation of NFAT proteins contributes to the altered phenotype in PKB transgenic mice. Various NFAT family members have been shown to be involved in thymocyte selection (63–65) 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<sup>+</sup> T cells (66), similar to what we observe in myr PKB transgenic mice. Because 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 c-Jun N-terminal kinase (67), p38 (68), or Notch (69) 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,



**FIGURE 7.** A–C, Effects of myr PKB on negative selection. **A**, Thymocytes from female and male HY and HY myr PKB littermate mice were analyzed by FACS for expression of CD4, CD8, and the HY TCR-specific  $V\alpha$ -chain using T3.70 mAb. The percentages for T3.70<sup>+</sup>CD4<sup>+</sup>, T3.70<sup>+</sup> DP and T3.70<sup>+</sup>CD8<sup>+</sup> cells were determined and absolute cell numbers for T3.70<sup>+</sup> cells were calculated from total cell numbers; HY male mice  $n = 12$  and HY female mice  $n = 7$  for wt and PKB transgenic mice. **B**, Effect of myr PKB on deletion of SAg-reactive T cells. CD4<sup>+</sup> T cells from wild-type and myr PKB transgenic mice on C57/BL6 (B6) background and myr PKB mice crossed to DBA/2 and CBA/J mice ( $F_1$  generation) were analyzed for V $\beta$ 5 and V $\beta$ 11 expression. Mice were assayed at 5 wk after birth. **C**, Influence of myr PKB on peptide Ag-induced deletion of OT1 and OT2 DP thymocytes. Thymocytes from OT1 and OT2 mice (wt, broken lines) and OT1/myr PKB or OT2/myr PKB (tg, full lines) mice aged 6–7 wk were cultured with the indicated concentrations of TCR-specific OVA peptide Ag (OVA). On day 1, 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 with cultures without Ag.

or had no effect on deletion. We conclude that the effect of myr PKB on negative selection is specific for each system analyzed depending on the timing, the Ag and TCR affinity/avidity, or whether Ag is presented by MHC class I or MHC class II molecules. Negative selection not only requires a high avidity TCR stimulus but also costimulatory signals from APCs which potentially can be provided by CD28 (70). For the deletion of different autoantigens a complex array of variable costimulators seems to be

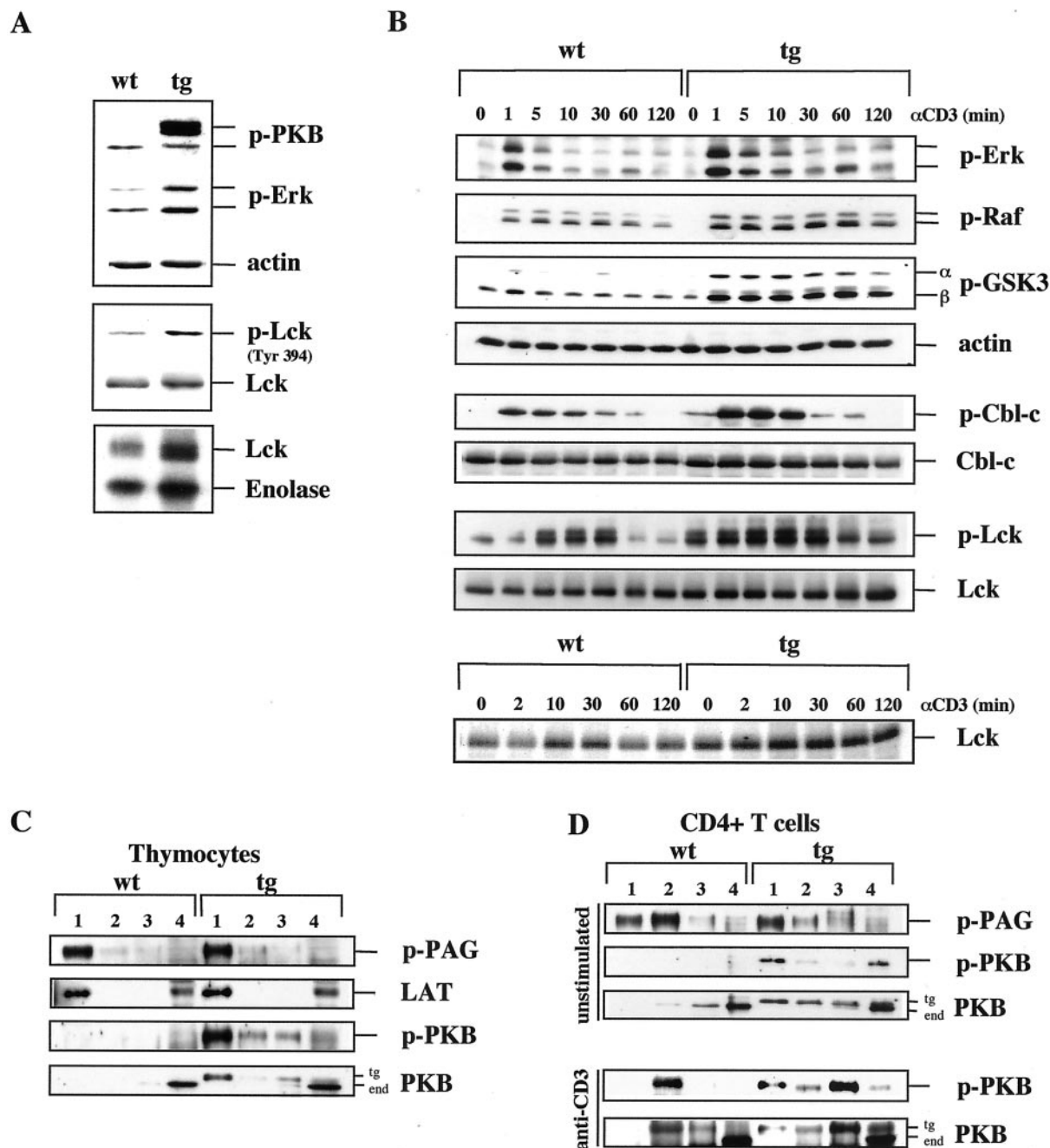
necessary (71). Because some of these molecules, like CD28 or Fas, signal via PKB, our results on negative selection in different model systems might reflect the complexity of molecules and their different downstream effector molecules as involved in negative selection.

In repeated *in vitro* experiments with thymocytes from 6- to 8-wk-old mice, we did not detect very strong differences in apoptosis/survival between wild-type and myr PKB thymocytes for most stimuli tested. In contrast, peripheral myr PKB T cells 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 mice. In a different approach, by generating Lck-crePtenflox<sup>-/-</sup> mice with T cell-specific deletion of the tumor suppressor gene PTEN, PKB activity was greatly enhanced in T cells (15). With regard to survival and negative selection, Lck-crePtenflox<sup>-/-</sup> mice had defects in negative selection *in vivo* using the HY TCR transgenic system but thymocyte apoptosis *in vitro* was also not affected when anti-CD3 or anti-fas mAbs were used. Thus, for certain stimuli in thymocyte apoptosis other mechanisms and molecules might be more critical.

In PKB transgenic mice generated using a *gag* PKB construct, Jones et al. (24), in contrast, 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 toward the CD4 lineage using the P14 TCR transgenic mouse model. This discrepancy most likely results from differences in expression levels or localization of transgenic PKB in addition to differences in the affinity/avidity of the transgenic TCR used (72, 73). As pointed out by these authors, the *gag* PKB transgenic protein detected in Western blots was much smaller than the expected *gag* PKB 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. In this study, 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 signaling such as Lck or LAT. In relation to these data, we favor the view that the different localization 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 transgenic T cells and that myr PKB in CD4<sup>+</sup> T cells was distributed in insoluble as well as soluble membrane fractions. Biochemical studies assessing the redistribution of signaling molecules into lipid rafts indicate that positive selection signals or TCR/CD3 stimulation alone can recruit TCR signaling components to the lipid raft fraction (51, 74). Recently Hill et al. (75) detected constitutively active PKB serine 473 kinase activity enriched in plasma membrane rafts. In this study, we show that TCR/CD3 stimulation of CD4<sup>+</sup> 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 signaling.

Collectively, our findings clearly show that PKB plays an important role in the signal transduction from TCR-initiated signaling complexes in lipid rafts by influencing the cross-talk between several important signaling molecules such as Lck, Erk, Cbl-c, or GSK3. By enhancing the strength/duration of Lck-Erk signals,





**FIGURE 8.** Active PKB localizes in membrane lipid rafts and enhances phosphorylation of Lck, Erk, Cbl-c, and GSK3. **A**, Freshly isolated thymocytes from control (wt) and myr PKB transgenic mice (tg) were lysed immediately and protein extracts were analyzed by Western blot for activation of PKB and Erk (*top panel*) and Lck (tyrosine 394, *middle panel*) using phosphospecific Abs. Blots were reprobed for actin and Lck to control protein loading. In the *bottom panel*, Lck tyrosine kinase activity from freshly isolated thymocytes was determined by immune complex kinase assay. In addition to auto-phosphorylation of Lck enolase was used as an indicator of transphosphorylation activity. **B**, Thymocytes from myr PKB and wild-type mice were rested for 2 h in 1% FCS medium before activation with anti-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 analyzed with anti-phosphotyrosine mAb. Protein content was controlled by reprobings blots with anti-Cbl-c, anti-Lck, and anti-actin Abs. In the *lowest panel*, Lck was immunoprecipitated and Lck activity was determined by kinase assay. **C**, 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 wild-type and myr PKB mice were detected by Western blot. In the anti-PKB blot, the lower band corresponds to endogenous (end) PKB, the upper band to transgenic (tg) PKB. **D**, PKB translocates into lipid rafts after TCR/CD3 stimulation. Proteins in lipid raft and detergent soluble fractions from LN CD4<sup>+</sup> T cells, either unstimulated (*upper three panels*) or stimulated for 30 min with anti-CD3 mAb (*lower two panels*), were detected as described in **C**. Note that after activation, endogenous PKB shows a mobility shift running at the same height as transgenic PKB.

PKB is vitally involved in the modulation of activation thresholds and selectional windows that govern T cell maturation and activation. Positive effects on Raf-Mek-Erk signaling and possibly differential activation of NFAT, via altered calcineurin and GSK3

activities, could also contribute to the development of lymphomas (Refs. 26, 27, and 76 and our own observations in PKB homozygous mice) and other cancer types, which are thought to result from overexpression of PKB isoforms.



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# CORRECTIONS

Na, S.-Y., A. Patra, Y. Scheuring, A. Marx, M. Tolaini, D. Kioussis, B. Hemmings, T. Hünig, and U. Bommhardt. 2003. Constitutively active protein kinase B enhances Lck and Erk activities and influences thymocyte selection and activation. *J. Immunol.* 171: 1285–1296.

The seventh author's middle initial was omitted. The correct name is Brian A. Hemmings.

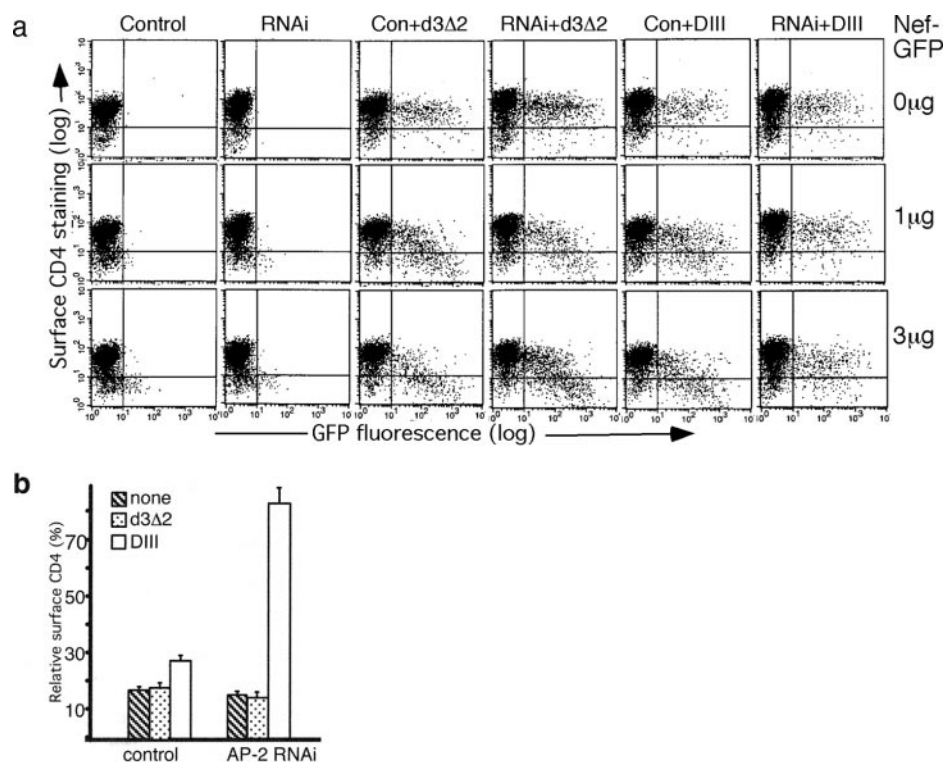
Wurfel, M. M., W. Y. Park, F. Radella, J. Ruzinski, A. Sandstrom, J. Strout, R. E. Bumgarner, and T. R. Martin. 2005. Identification of high and low responders to lipopolysaccharide in normal subjects: an unbiased approach to identify modulators of innate immunity. *J. Immunol.* 175: 2570–2578.

In **Materials and Methods**, the accession number should have been included in the paragraph under the heading *Gene expression profiling*. The omitted sentence is shown below.

The microarray data were deposited in the public Gene Expression Omnibus (GEO) database ([www.ncbi.nlm.nih.gov/geo/](http://www.ncbi.nlm.nih.gov/geo/)) under accession no. GSE3491.

Jin, Y.-J., C. Y. Cai, X. Zhang, H.-T. Zhang, J. A. Hirst, and S. J. Burakoff. 2005. HIV Nef-mediated CD4 down-regulation is adaptor protein complex 2 dependent. *J. Immunol.* 175: 3157–3164.

In **Discussion**, Figure 4b was inadvertently duplicated in Figure 6a. The corrected figure is shown below.



Chaudhry, A., S. R. Das, A. Hussain, S. Mayor, A. George, V. Bal, S. Jameel, and S. Rath. 2005. The Nef protein of HIV-1 induces loss of cell surface costimulatory molecules CD80 and CD86 in APCs. *J. Immunol.* 175: 4566–4574.

In **Results**, the first sentence of the third paragraph under the heading *Nef mutants, including a clinical isolate, can differentially affect down-modulation of MHCI vs CD80 and CD86* is incorrect. The corrected sentence is shown below. The error does not in any way change the essence of the work or the interpretation of the results.

The G2A and M20A mutants of F2-Nef behave like the corresponding mutants of NL4–3 Nef (Fig. 3C).

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Taubenheim, N., M. von Hornung, A. Durandy, K. Warnatz, L. Corcoran, H.-H. Peter, and H. Eibel. 2005. Defined blocks in terminal plasma cell differentiation of common variable immunodeficiency patients. *J. Immunol.* 175: 5498–5503.

In **Materials and Methods**, the ethics committee research protocol number in the second sentence of the second paragraph is incorrect. The corrected sentence is shown below.

Informed consent was obtained from all patients and controls using biopsy material and peripheral blood for scientific purposes according to a local ethics committee approved research protocol (no. 78/2001).

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Sharma, V., M. Delgado, and D. Ganea. 2006. Granzyme B, a new player in activation-induced cell death, is down-regulated by vasoactive intestinal peptide in Th2 but not Th1 effectors. *J. Immunol.* 176: 97–110.

In the **Abstract**, the year and volume are incorrect. The error has been corrected in the online version, which now differs from the print version as originally published. The corrected citation is shown above.

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Sugita, S., T. F. Ng, P. J. Lucas, R. E. Gress, and J. W. Streilein. 2006. B7<sup>+</sup> iris pigment epithelium induce CD8<sup>+</sup> T regulatory cells; both suppress CTLA-4<sup>+</sup> T cells. *J. Immunol.* 176: 118–127.

In the **Abstract**, the year and volume are incorrect. The error has been corrected in the online version, which now differs from the print version as originally published. The corrected citation is shown above.

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Matsue, H., J. Yao, K. Matsue, A. Nagasaka, H. Sugiyama, R. Aoki, M. Kitamura, and S. Shimada. 2006. Gap junction-mediated intercellular communication between dendritic cells (DCs) is required for effective activation of DCs. *J. Immunol.* 176: 181–190.

In the **Abstract**, the year and volume are incorrect. The error has been corrected in the online version, which now differs from the print version as originally published. The corrected citation is shown above.

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Debes, G. F., M. E. Dahl, A. J. Mahiny, K. Bonhagen, D. J. Campbell, K. Siegmund, K. J. Erb, D. B. Lewis, T. Kamradt, and A. Hamann. 2006. Chemotactic responses of IL-4-, IL-10-, and IFN- $\gamma$ -producing CD4<sup>+</sup> T cells depend on tissue origin and microbial stimulus. *J. Immunol.* 176: 557–566.

In the **Abstract**, the year and volume are incorrect. The error has been corrected in the online version, which now differs from the print version as originally published. The corrected citation is shown above.

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Han, S. H., J. H. Kim, H. S. Seo, M. H. Martin, G.-H. Chung, S. M. Michalek, and M. H. Nahm. 2006. Lipoteichoic acid-induced nitric oxide production depends on the activation of platelet-activating factor receptor and Jak2. *J. Immunol.* 176: 573–579.

In the **Abstract**, the year and volume are incorrect. The error has been corrected in the online version, which now differs from the print version as originally published. The corrected citation is shown above.

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Fu, Y., C. Xie, J. Chen, J. Zhu, H. Zhou, J. Thomas, X. J. Zhou, and C. Mohan. 2006. Innate stimuli accentuate end-organ damage by nephrotoxic antibodies via Fc receptor and TLR stimulation and IL-1/TNF- $\alpha$  production. *J. Immunol.* 176: 632–639.

In the **Abstract**, the year and volume are incorrect. The error has been corrected in the online version, which now differs from the print version as originally published. The corrected citation is shown above.