Deletion of PKBα/Akt1 Affects Thymic Development

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Background. The thymus constitutes the primary lymphoid organ for the majority of T cells. The phosphatidyl-inositol 3 kinase (PI3K) signaling pathway is involved in lymphoid development. Defects in single components of this pathway prevent thymocytes from progressing beyond early T cell developmental stages. Protein kinase B (PKB) is the main effector of the PI3K pathway. Methodology/Principal Findings. To determine whether PKB mediates PI3K signaling in the thymus, we characterized PKB knockout thymi. Our results reveal a significant thymic hypocellularity in PKBα−/− neonates and an accumulation of early thymocyte subsets in PKBα−/− adult mice. Using thymic grafting and fetal liver cell transfer experiments, the latter finding was specifically attributed to the lack of PKB within the lymphoid component of the thymus. Microarray analyses show that the absence of PKBα in early thymocyte subsets modifies the expression of genes known to be involved in pre-TCR signaling, in T cell activation, and in the transduction of interferon-mediated signals. Conclusions/Significance. This report highlights the specific requirements of PKBα for thymic development and opens up new prospects as to the mechanism downstream of PKBα in early thymocytes.

INTRODUCTION

The thymus constitutes the primary lymphoid organ for the majority of T cells as its microenvironments provide an exclusive combination of different stromal type cells critical for the generation and selection of thymocytes to mature T cells [1]. During their thymic development, T lineage committed precursors progress through an ordered sequence of differentiation events [2]. These events reflect the complex progression from immature progenitors to post-selection T cells, which are tolerant to self but recognize foreign antigens in the context of self-MHC molecules. Immature intrathymic precursors are characterized by the absence of CD4 and CD8 cell surface expression and are hence designated double negative (DN) thymocytes. Based on the expression of CD25 and CD44, DN thymocytes are further distinguished into four sequentially evolving subpopulations (DN1-DN4) [3]. Early during maturation, the productive rearrangement of the T cell antigen receptor β (TCRβ) locus allows for the expression of a nascent TCRβ chain that, together with the expression of the pre-TCR (pTCR) chain and the CD3 complex, forms the pre-TCR complex [4]. This particular stage represents a critical checkpoint in T cell development that is known as β-selection. Signaling via a functional pre-TCR allows for the further differentiation of thymocytes and initiates the surface expression of both CD4 and CD8. Developing T cells concurrently expressing CD4 and CD8 (designated double positive, DP, thymocytes) rearrange their TCRα locus, which enables the cell surface expression of a mature TCRαβ complex. Subsequently, the events of positive and negative TCR selection take place giving rise to single CD4- or CD8-positive (SP) mature T cells that are eventually released into the periphery [5]. Changes in the thymic stromal compartment and alterations of key signaling pathways in thymocytes result in an aberrant development and the lack of regular T cells.

The phosphatidyl-inositol 3 kinase (PI3K) signaling pathway has been reported to be involved in lymphoid development as impaired PI3K signaling results in immunodeficiency, while unrestrained signaling contributes to lymphoma formation and autoimmunity [6]. The function of PI3K is to convert at the plasma membrane phosphatidyl-inositol-(4,5)-bisphosphate (PIP2) to the second messenger phosphatidyl-inositol-(3,4,5)-trisphosphate (PIP3). The 3′-phosphate lipid phosphatase PTEN antagonizes the generation of PIP3 [7]. PIP3 acts as a binding site for various intracellular enzymes that contain a pleckstrin homology (PH) domain, such as the serine/threonine kinases phosphoinositide-dependent kinase 1 (PDK1) and protein kinase B (PKB). Hence, PIP3 promotes the translocation of the corresponding proteins from the cytoplasm to the plasma membrane. Recruited at the membrane, PDK1 phosphorylates a key residue within the catalytic domain of one of its substrates, PKB [8], which is the most important effector of the PI3K pathway. To be fully active, PKB needs to be phosphorylated at a second key residue located in the hydrophobic motif within the regulatory domain [9]. For this to occur, a number of upstream kinase candidates have been identified, including DNA-dependent protein kinase (DNA-PK) [10] or the rictor-mTOR complex [11]. Once activated, PKB phosphorylates numerous substrates influencing diverse cellular and physiological processes attributed to the PI3K pathway [12].

Mice genetically impaired for single components of the PI3K signaling pathway display distinct deficiencies in the development and function of the immune system. For instance, severe combined immunodeficiency (SCID) in mice correlates with a nonsense
PKBz, Thymus and T Cell

mutation within the gene of the DNA-PK catalytic subunit (DNA-PKcs) [13–15]. Moreover, mice deficient for DNA-PKcs exhibit a severe immunodeficiency partly associated with a block in T cell development due to impaired variable/diversity/joining (V(D)J) rearrangements at the DN3 stage [16]. Furthermore, deletion of PDK1 in T cell precursors prevents T cell differentiation at the DN to DP transition and downregulates the cell size of immature thymocytes [17], suggesting that signals downstream of PDK1 and/or DNA-PK are essential for T cell development. On the other hand, heterozygous deletion of PTEV and T cell-specific PTEV-null mutation in mice lead to increased thymic cellularity and the development of not only lymphoid hyperplasia, which progresses to T cell lymphoma, but also autoimmunity likely due to impaired Fas signaling [18–21]. Mutations in PTEV allow unrestrained PIP3 production, which results in constitutive PKB activation. Correspondingly, mice engineered to express a constitutively active form of PKB in T cells display a phenotype similar to that of PTEV-mutant mice [22–24].

Three PKB isoforms encoded by separate genes and of identical structural organization have been described for mammalian cells: PKBz, PKBβ, and PKBγ [25]. While PKBz is ubiquitously detected, PKBβ and PKBγ tend to be expressed in a tissue-specific pattern. Targeted disruption of each of these isoforms in mice has helped to elucidate the physiological in vivo relevance of the PKB isoforms, revealing both specific and redundant functions [26–34]. However, specific immunological defects have not been reported for single mutant mice.

To characterize the specific contribution of distinct PKB isoforms within the PI3K signaling pathway for thymic development, we investigated mice deficient for each of the isoforms of PKB. Our results reveal a significant thymic hypocellularity in PKBz−/− neonates and an accumulation of early thymocyte subsets at the DN to DP transition during adult T cell development in PKBz−/− mice due to cell-autonomous effects. Moreover, in early thymocytes PKBz regulates genes known to respond to pre-TCR, TCR, or interferon signaling. This report uncovers the specific requirements of PKBz for thymic development.

RESULTS

The deletion of PKBα leads to a hypocellular thymus in mouse neonates

To determine the potential impact of PKB on thymic development, we analyzed the thymus of PKBα mutant mice. The dissection of neonates revealed that the size of PKBz−/− thymi was reduced to less than half that of wild-type controls (Figure 1A, top panel). We and others had previously reported that genetic ablation of PKBz leads to a decreased body weight [26,28,34], suggesting a general but proportional reduction in the size of any organ. To confirm this, we compared the weight of the thymus in relation to the body weight. In neonatal mice deficient for PKBz, the thymus weight was reduced to 60% of wild-type controls when normalized to the body weight (Figure 1A, bottom panel). This finding was specific since the weight of other organs, such as the kidney, was reduced in proportion to the reduction of body weight (Figure 1A and data not shown). In contrast to the results in neonatal mice, the relative weight of the thymus was not diminished in adult animals deficient for PKBz (Figure S1A), a result that is consistent with our previous findings [34]. Western-blot analyses showed that all three PKB isoforms were present within the thymus of wild-type neonates (Figure 1B, top panel), rendering it possible that a deletion of either PKBβ or PKBγ could also affect thymic size. Mice deficient for either of these isoforms demonstrated, however, a normal thymus weight (Figure 1C). Moreover, the loss of one of the PKB isoforms was not compensated by an upregulation in the expression of any of the other isoforms (Figure 1B, bottom panel). Taken together, these data indicate that the loss of expression of a PKB isoform is not off set by higher expression levels of another isoform and that PKBz is necessary for the normal size of the neonatal thymus.

The organ size is determined by the number and/or the volume of its cells. While the size of thymocytes was not affected by the loss of PKBz (Figure 2A), the number of PKBz−/− thymocytes was significantly reduced in newborns (but not in adults) when compared to that of wild-type littermates (Figure 2B, left panel, and S1B). Hence, a lower thymocyte cellularity accounted, in neonatal mice, for the diminished tissue weight and also correlated with a decrease in peripheral T cells (Figure 2B, right panel). To determine whether the decreased thymic cellularity of neonatal mice was caused by an increase in programmed cell death, we performed TUNEL assay on thymus tissue sections as well as annexin V/propium iodide staining of thymocytes. The frequency of apoptotic cells within the thymus was similar for control and PKBz−/− neonates, excluding the possibility of increased programmed cell death to account for the noted hypocellularity (Figure 2C and 2D).

The lack of PKBα leads to an accumulation of thymocyte subsets at an early checkpoint during T cell development

To address whether a partial or complete block in T cell development could explain the hypocellularity observed in the thymus of neonates deficient for PKBz, we analyzed in PKBz+/− and PKBz−/− mice the major thymocyte subsets. Using flow cytometry, the main subsets of mutant mice displayed similar relative frequencies when compared to age-matched wild-type controls in both neonatal and adult mice (data not shown and Figure S2A). We therefore excluded that a block in T cell development would account for thymic hypocellularity in PKBz−/− neonates. However, a refined phenotypic analysis of adult thymocytes revealed an accumulation at early developmental stages, suggesting that, in addition to its effect on neonatal thymic cellularity, the deletion of PKBz also affected T cell development. Even though CD25+CD44− thymic subset (designated DN1) appeared to be reduced in PKBz−/− mice (Figure 3A), when analyzed for surface expression of c-kit, T cell precursors (CD25+CD44+c-kit+) were only slightly affected (data not shown). On the other hand, while CD25+CD44+ (designated DN2) cell subset was unchanged, a subpopulation of thymocytes that express CD25 but lack CD44 at the cell surface (defined as DN3) was increased in the adult PKBz−/− thymus in comparison to wild-type controls (Figure 3A). These DN3 thymocytes are at a developmental stage immediately prior to the β-selection checkpoint. DN3 thymocytes with a productively rearranged TCRβ locus and a successful expression of the pre-TCR complex pass the β selection checkpoint, downregulate CD25, and develop into thymocytes with a DN4 phenotype (CD25+CD44−). In view of an accumulation of DN3 cells in PKBz−/− mice, we investigated whether it could be associated with a defect in TCRβ expression. We measured intracellular TCRβ protein using flow cytometry and found the expression of this receptor subunit in DN3 thymocytes at comparable levels in both PKBz−/− and control mice (Figure 3B). Furthermore, PKBz+/− and PKBz−/− DN4 thymocytes expressed intracellularly the TCRβ proteins (Figure S2B). These results suggest that the PKBz deletion does not impair the rearrangement or the expression of the TCRβ chain and that PKBz is not directly involved in the process of pre-TCR
formation. However, the cell surface expression of the α chain of the interleukin-2 receptor (CD25) was increased among DN3 cells of PKBα−/− mice when compared to the equivalent subpopulation of wild-type mice, suggesting a role of PKBα in cell signaling at this stage of early thymocyte development (Figure 3C). Moreover, a population of immature thymocytes expressing CD8, but still lacking the cell surface expression of both CD4 and CD3, and displaying intracellular TCRβ proteins, accumulated in the thymus of PKBα−/− mutant mice (Figure 3D and 3E). These thymocytes represent a stage immediately prior to that of DP cells and are hence designated immature single CD8⁺ thymocytes (ISP8) [35]. However, no apparent differences in thymocyte

Figure 1. The deletion of PKBα leads to a reduced thymic size in mouse neonates. A: The weight of freshly dissected thymi was measured in PKBα++ and PKBα−/− neonates (top panel) and expressed as ratio to body weight (bottom panel). The kidney was used as a control. Error bars represent standard error of the mean; n=13. B: Western-blot analysis of 50 µg protein extracts from wild-type neonatal thymus using PKB isoform specific antibodies (top panel). Western-blot analysis of 50 µg protein extracts from PKBα−/−, PKBα++, PKBγ−/−, and PKBγ++ neonatal thymus using PKB isoform specific antibodies (bottom panel). Actin was used as a loading control. C: The weight of freshly dissected thymi was measured in PKBβ++, PKBβ−/−, PKBγ−/−, and PKBγ++ neonates (top panels) and expressed as ratio to body weight (bottom panels). The kidney was used as a control. Error bars represent standard error of the mean. n=7 (n = number of mice analyzed per genotype).

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The deletion of PKBα leads to a reduced number of thymocytes in neonatal mice. A: Thymocytes were isolated from neonatal PKBα+/+ and PKBα−/− littermates and their size compared by flow cytometry using the forward scatter (FSC) parameter. The histogram is representative of 3 litters. B: (left panel) Thymocytes were isolated and counted from PKBα+/+ and PKBα−/− neonatal mice. (right panel) Lymphocytes isolated from the spleen of PKBα+/+ and PKBα−/− neonates were stained with anti-CD19 and anti-CD3 antibodies. The number of T cells (CD3+CD19+) is shown. n=3. Error bars represent standard error of the mean. C: TUNEL assay on neonatal thymus sections from PKBα+/+ and PKBα−/− littermates. The graph represents the quantification of TUNEL-positive cells from 5 fields on 3 sections. The result shown is representative of 3 independent experiments. The bar shown on the pictures represents 200 μm. Error bars represent standard error of the mean. D: Thymocytes were isolated from PKBα+/+ and PKBα−/− neonates and stained with annexin V and propidium iodide (PI). Histograms show results that are representative of 2 independent experiments; n=3 (n = number of mice per genotype within the same experiment).

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proliferation, apoptosis, or size were detected when comparing PKBα+/+ and PKBα−/− specific thymocyte subsets (Figure S2C and S2D and data not shown). Overall, our data reveal a critical role for PKBα in the transition from a DN to DP phenotype with a partial accumulation of DN3 and ISP8 thymocytes in mice deficient for PKBα expression.

The accumulation of thymocyte subsets at the DN to DP transition in early T cell development originates from the absence of PKBα in hematopoietic precursors

The thymus is composed of a heterogeneous population of cells, including thymocytes at various developmental stages and different stromal cells that are either hematopoietic, mesenchymal, or epithelial in origin. In thymocytes, PKBα was the main isoform located downstream of PDK1 since PKBα−/− thymocytes showed only minimally phosphorylated PKB levels at the PDK1 dependent-Thr308 residue (Figure 4A). PKBα expression was also observed in thymic epithelial cells (JG and GAH, unpublished), which are the most abundant component of the stromal compartment. Therefore, ablation of PKBα expression in either of these compartments could potentially account for the impairment in the transition from DN to DP thymocytes. To determine whether the observed phenotype was due to a lack of PKBα in non-hematopoietic stromal and/or in blood-borne cells, we next performed thymic grafting and fetal liver cell transfer experiments, respectively. In the first instance, we assessed the ability of PKBα−/− thymic stroma to support T cell development. For this purpose, embryonic day E15.5 thymi were isolated from both PKBα−/− and wild-type embryos. The fetal lobes were treated in vitro with deoxyguanosine for 6 days to deplete lymphoid cells, and then grafted under the kidney capsule of wild-type recipient mice. Four weeks post transplantation, the number of wild-type host-derived thymocytes developing within the PKBα−/− grafted thymic stroma was significantly reduced when compared to control tissue but regular thymocyte development was not affected (Figure 4B and 4C). In a second series of experiments, we evaluated the capacity of fetal liver derived-hematopoietic stem cells (HSC) from wild-type and PKBα−/− embryonic day E15.5 donors (CD45.2) to recapitulate normal thymopoiesis in wild-type thymic stromal environment of lethally-irradiated congenic
PKBα, Thymus and T Cell

Figure 3. The lack of PKBα leads to an accumulation of DN3 and ISP8 early thymocyte subsets. Flow cytometric analysis of early thymocytes at the transition from DN to DP. A: Density plots show thymocytes from PKBα+/+ and PKBα−/− mice that were stained with cell surface markers for identification of lineage-negative thymocytes DN1 (CD25+CD44−), DN2 (CD25−CD44−), DN3 (CD25−CD44+), and DN4 (CD25+CD44−). B: Histograms show the intracellular protein expression of TCRβ (iTCRβ) in DN3 thymocytes from PKBα+/+ and PKBα−/− mice. C: Histograms show the surface expression of CD25 on lineage-negative PKBα+/+ and PKBα−/− thymocytes. MFI: mean fluorescence intensity. D: Density plots and histograms show thymocytes from PKBα+/+ and PKBα−/− mice that were labeled with cell surface markers for identification of ISP8 (CD4−CD8+CD3+) thymocytes. E: Histograms show the intracellular protein expression of TCRβ (iTCRβ) in ISP8 thymocytes from PKBα+/+ and PKBα−/− mice. The results shown are representative of 3 independent experiments on 4 to 6 week-old mice. n=4 (n = number of mice per genotype within the same experiment). doi:10.1371/journal.pone.0000992.g003

(CD15.1) mice. Five weeks after reconstitution, the bone marrow chimeras had similar overall numbers of thymocytes and peripheral lymphocytes, irrespective whether they were derived from PKBα+/− or wild-type fetal liver cells (Figure 4B). Flow cytometric analyses further showed that PKBα+/− HSC were able to give rise to all thymocyte subsets (DN, DP, SP CD4+, and SP CD8+), but again both DN3 and ISP8 cells accumulated to the same extent as what had been observed in unmanipulated PKBα−/− mice (Figure 4D). Taken together, these data indicate that the accumulation of thymocytes during early T cell development observed in PKBα-deficient mice is the specific consequence of a lack of PKBα in lymphoid cells.

The absence of PKBα in early thymocytes affects the expression of genes known to be regulated in thymocyte and T cell response processes, and in interferon signaling

As the developmental changes at early stages of thymocyte maturation appeared to be a cell-autonomous effect caused by the loss of PKBα expression, we next determined the gene expression profile in DN3 and ISP8 cells using Affymetrix microarrays. Expression data analysis of specific transcripts in wild-type DN3 and ISP8 sorted cells revealed that while PKBα was the main isoform in both of these thymocyte populations, PKBβ was expressed at a significantly lower level and PKBγ was present in an even lesser abundance (Figure 5A). These results suggest that PKBα is the main isoform expressed in DN3 and ISP8 thymocytes. Analyses of microarray data revealed that DN3 and ISP8 thymocytes were differently affected in their gene expression profiles by the absence of PKBα with only 5 genes being differentially expressed in both subpopulations (Tables 1 and 2). In the DN3 subset, the absence of PKBα resulted for example in a down-regulation of the chemokine (C-C motif) receptor 9 (CCR9), whose expression is known to be induced upon pre-TCR signaling [36]. This result suggests that the absence of PKBα potentially affects pre-TCR signaling in DN3. Moreover, the integrin alpha E epithelial-associated (Igα or CD103) gene, that is known to be expressed in DN and whose product interacts with E-cadherin on thymic epithelial cells, was downregulated in the absence of PKBα. Furthermore, 8 genes whose expression was modified in PKBα−/− DN3 are typically induced by interferon and were systematically downregulated in cells lacking PKBα. These genes constituted 50% of all the genes whose expression was downregulated as a consequence of PKBα ablation in DN3 cells. In the ISP8 subset, several genes known to be induced in their expression upon TCR activation or involved in T cell activation were found to be downregulated in the absence of PKBα: the cell membrane glycoprotein CD53 antigen, the lymphocyte antigen 6 complex locus A (Ly6a), the lymphocyte antigen 6 complex locus C (Ly6c), the T-cell specific GTPase (TGFp), or the MHC class II antigen (H2-Ab). In contrast, transcripts for other gene products...
known to act as negative regulators in TCR signaling, or in other pathways involved in T cell activation, were upregulated in the absence of PKBα, including the suppressor of cytokine signaling 3 (SOCS3), the cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), or the immunoglobulin superfamily member Igsf3. Furthermore, some genes whose expression was upregulated in PKBα−/− ISP8, such as PTEN, Notch3, and one of its target genes Dcx1, have previously been shown to be involved in the transition from DN to DP thymocytes [37,38]. Finally, 6 genes differentially expressed in PKBα−/− ISP8 are interferon-inducible in their expression and were systematically downregulated in cells lacking PKBα. These genes constituted 29% of all the genes whose expression was downregulated in PKBα−/− ISP8 cells.

**DISCUSSION**

The deletion of PKBα leads to a reduced size of the thymus in mouse neonates, which is attributed to hypocellularity

The regulation of both cell number and volume contributes to the establishment of organ size. A number of studies have implicated the PI3K signaling pathway, and more specifically PKB, in determination of cell, organ, and body size. Tissue-specific activation of this pathway, either by expressing active PI3K or PKB or by deleting PTEN, results in an increased organ weight, a finding often associated with enlarged cell volume [39–41]. In contrast, the ablation of a single PKB isoform causes a reduction in the size of the
animal and/or specific organs. For instance, deletion of PKBz leads to a 30% reduction of body weight [26,28,34], while ablation of PKBb specifically causes a significant reduction in brain tissue due to reduced cell number and size [29,32]. In this study, we report a disproportionately reduced thymic size in PKBz−/− neonates that consistently show reduced thymic cellularity, the extent of which was somewhat variable. This decrease was not due to an increase in thymocyte apoptosis. Contrary to the latter result, previous study reported an increase in spontaneous apoptosis among PKBz−/− thymic cells of adult mice [26] yet, this observation was not linked to any reduced organ size. This apparent discrepancy between the two studies may possibly arise from a variation in the age of the mice analyzed and/or from differences in the genetic background; while the genetic background of the PKBz−/− mice in our study was statistically above 90% C57Bl/6, in the study reported by Chen et al. it was an equal mix of C57Bl/6 and 129 R1.

The lymphoid component of the thymus is not self-renewing and must be continually reseeded by fetal liver or adult bone marrow derived thymic progenitor cells. As such, the decrease in thymocyte numbers observed in PKBz−/− neonates could be lymphoid cell autonomous and relate to a reduction in either the absolute number or the efficiency of thymic progenitor cells. Alternatively, or additionally, the thymic cellularity could be affected by a defective thymic microenvironment in PKBz−/− neonates. Indeed, PKBz-deficient thymic grafts displayed a decrease in thymocyte number, which was not associated with impaired T cell development. In addition, in some of the PKBz−/− neonates, thymic sections analyzed using hematoxylin and eosin staining as well as immunohistology displayed disorganized cortical/medullary epithelial cell compartment (Figure S3). However, neither cellularity nor morphology was abnormal in thymi of adult PKBz−/− mice nor in PKBb−/− and PKBγ−/− neonatal thymi. We speculate that the hypocellularity observed in PKBz−/− neonatal thymi could be due to a delay in thymic development, possibly and partly originating from a defective microenvironment within the thymus at early stages.

The lack of PKBz in lymphoid cells leads to an accumulation of thymocyte subsets at the DN to DP transition in early T cell development

Alteration in specific components of the PI3K signaling pathway, such as PDK1, leads to an impaired transition from DN to DP thymocytes, suggesting an essential role of factors downstream of PDK1 in T cell development. PKB is the most important mediator of the PI3K signaling and, from our data, PKBz is the main functional PKB isoform positioned downstream of PDK1 in thymocytes. Our study highlights an accumulation of PKBz−/− DN3 and ISP8 thymocyte subsets. We attribute this accumulation to a cell-autonomous lack of PKBz within the T lymphoid component of the thymus and concurrently exclude a contribution by PKBγ-deficient thymic stroma to this finding. While the deletion of PKBz does not prevent further maturation to the SP stages, our results indicate that PKBz is important in the transition from DN to DP. This effect is not due to impaired TCRβ chain expression, even though we observed downregulated expression of one of the numerous TCRβ-V segments (Vβ13) in PKBz−/− DN3 thymocytes. Furthermore, the surface expression of the z chain of the interleukin-2 receptor (CD25) was increased in the PKBz−/− DN3 subset. While with our current knowledge, we cannot relate this observation to the phenotype observed, this increased CD25 surface expression has also been reported in DN3 cells lacking PDK1 [17].

Our data suggest that the z isoform of PKB is an important effector of PDK1 in the transition from DN to DP subsets, which constitutes a critical step during T cell development. Interestingly, in view of the reduced percentage of CD25+CD44+c-kit− thymocytes in PKBz−/− thymi, PKBz could also affect a subpopulation of cells within the thymus that is positive for CD44 surface expression but not (yet) committed to the T cell lineage.

While one could hypothesize that the distinct phenotypes reported in PKBz, PKBb, and PKBγ mutant mice are due to specific and distinct functions of the PKB isoforms, it could be equally well argued that these differences are merely due to a loss of an abundant isoform, which leads in a specific tissue to a reduction of total PKB below a critical level. Based on our data concerning differential expression levels of PKBz, PKBb, and PKBγ in early thymocyte subsets, we predict that a combined deletion of PKBz and PKBγ would lead to a more extensive block during early T cell development compromising thymocyte maturation further. Mice lacking both PKBz and PKBγ, however, die at birth with multiple defects [31]. Moreover, while complete deletion of PDK1 in early thymocytes arrests its progression to mature T cells, reduced PDK1 expression to 10% of normal levels still allows T cell development [17]. Therefore, the residual PKB activity present in PKBz−/− thymocytes might be sufficient to

Figure 5. PKBz is the main isoform in DN3 and ISP8 thymocyte subsets. A: mRNA levels of PKBz, PKBb, and PKBγ isoforms in DN3 and ISP8 thymocyte subsets. The expression data obtained following microarray analysis were corrected for GC-bias within oligos, allowing gene expression signals to be expressed on the same scale; this permits a semi-quantitative comparison of the expression of different genes. B: Proposed model of PKBz mediating PI3K1 signaling at the transition from DN to DP thymocyte subsets. TCRβ and TCR refer to intracellular and surface expression of TCRβ, respectively.
Table 1. Genes with altered expression in PKB-α−/− FACS sorted DN3 thymocyte subset compared to PKB-α+/+ cells.

| Gene name | Gene symbol | Affymetrix identification | GenBank accession number | Regulation (a) |
|-----------|-------------|---------------------------|--------------------------|----------------|
| pre-TCR signaling | chemokine (C-C motif) receptor 9 | Ccr9 | 1427419_x_at | NM_009913 | down regulated (×1.6) |
| | T cell receptor beta chain, clone library HK8.3-6F8 | TCrb-V13 | 1444088_at | BE447255 (UniGene) | down regulated (×2.5) |
| T cell response/development process | integrin, alpha E, epithelial-associated | Itgae, CD103 | 1447541_s_at | NM_008399, NM_172944 | down regulated (×1.6) |
| | phosphodiesterase 2A, cGMP-stimulated | Pde2a | 1440707_s_at | NM_001408548 | up regulated (×1.9) |
| Interferon-inducible | eukaryotic translation initiation factor 2-alpha kinase 2 | Ifi2ak2, PKR | 1422006_at | NM_011163 | down regulated (×1.7) |
| | interferon-induced protein with tetratricopeptide repeats 1 | Ifit1, garg16 | 1450783_at | NM_008331 | down regulated (×3.3) |
| | interferon inducible GTPase 1 | Igp1 | 1419042_at, 1419043_a_at | NM_021792 | up regulated (×2.9) |
| | 2′-5′ oligoadenylate synthetase 1A | Oas1a | 1424775_at | NM_145211 | down regulated (×2.2) |
| | 2′-5′ oligoadenylate synthetase-like 2 | Oas2 | 1435196_a_at | NM_011854 | down regulated (×2.0) |
| | radical S-adenosyl methionine domain containing 2 (*) | Rsd2, vig1, cig5, viperin | 143058_at | NM_021384 | down regulated (×3.6) |
| | Receptor transporter protein 4 (*) | Rtp4, Ifg28 | 1418580_at | NM_023386 | down regulated (×1.6) |
| | ubiquitin specific peptidase 18 | Usp18, UBP43, Ubp15 | 1418191_at | NM_011909 | down regulated (×2.4) |
| Inflammation | scavenger receptor cysteine-rich type 1 protein CD163c-alpha precursor (*) | E430002D04Rik | 1440808_x_at, 1455527_at | NM_172909 | up regulated (×3.0) |
| | MAD homolog 7 (Drosophila) | Smad7 | 1423389_at | NM_001042660 | up regulated (×1.6) |
| Signal transduction | G protein-coupled receptor, family C, group 5, member B | Grpr5b, Raig-2 | 1424613_at | NM_022420 | up regulated (×2.3) |
| Transcription | RIKEN cDNA 111005B16 gene | 111005B16Rik | 1445710_x_at | NM_183389 | down regulated (×2.1) |
| | predicted gene, EG622175 | EG622175 | 1440202_at | XM_898168, XM_911074 | up regulated (×1.7) |
| | myeloid leukemia factor 1 interacting protein | Mif11p | 1428518_at | NM_027973 | up regulated (×1.7) |
| | RAD54 homolog B (S. cerevisiae) | Rad54b | 1434734_at | NM_001039556 | up regulated (×1.7) |
| Other | cyclin-dependent kinase inhibitor 1A (P21) | Cdkn1a | 1421679_a_at | NM_007669 | down regulated (×1.7) |
| | plasma glutamate carboxypeptidase | Pgc1p, Hs2, Lai-1 | 1416441_at | NM_176073 | down regulated (×1.6) |
| | aldo-keto reductase family 1, members C12 and C13 | Akr1c12, Akr1c13 | 1422000_at | NM_013757 | up regulated (×4.6) |
| | adaptor-related protein complex 3, mu 1 subunit | Ap3m1 | 1416374_at | NM_018829 | up regulated (×1.7) |
| | heat repeat containing 1 | Hear1 | 1437965_at | NM_144835 | up regulated (×1.8) |
| | ribonucleotide reductase M2 | Rrm2 | 1448226_at | NM_009104 | up regulated (×1.9) |
| | synaptotagmin-like 4 | Syt4 | 1417336_a_at | NM_013757 | up regulated (×2.0) |
| | testis derived transcript | Tes | 1424246_a_at | NM_011570, NM_207176 | up regulated (×1.8) |
| | TCCD-inducible poly(ADP-ribose) polymerase | Tiparp | 1452161_at | NM_178929 | up regulated (×2.2) |
| | vitamin K epoxide reductase complex subunit 1-like 1 | Vkor111 | 1429092_at | NM_00100327, NM_027121 | up regulated (×1.7) |
| Unknown | PREDICTED: hypothetical protein LOC77994 | 2810055G20Rik | 1445363_at, 1456787_at | BB451286 (UniGene) | down regulated (×2.4) |
| | cDNA sequence | BCO13672 | 1439114_at, 1451777_at | NM_001081215 | down regulated (×2.3) |
| | RIKEN cDNA 583043A10 gene | 583043A10Rik | 1436491_at | XR_002313 | up regulated (×2.2) |
| | RIKEN cDNA A630023P12 gene (*) | A630023P12Rik | 1452161_at | NM_178929 | up regulated (×2.2) |
| | RIKEN cDNA B230342M21 gene | B230342M21Rik (LOC100637) | 1444143_at | NM_133898 | up regulated (×1.8) |
| | Mus musculus, clone IMAGE:3983821 (*) | 1427820_at | BC021831 | up regulated (×2.3) |
| | gb:BB337926 | 1440400_at | BB337926 | up regulated (×1.7) |

(a) P<0.05, significant changes of ≥1.5-fold. (*) genes modified in both DN3 and ISP8 subsets. doi:10.1371/journal.pone.0000992.t001
| Gene name                                      | Gene symbol | Affymetrix identification | GenBank accession number | Regulation (a) |
|-----------------------------------------------|-------------|---------------------------|--------------------------|----------------|
| TCR signaling                                 | CD53        | 1446617_at                | NM_007651                | down regulation (×1.8) |
| suppressor of cytokine signaling 3            | Socs3       | 1453899_x_at              | NM_007707                | up regulation (×2.0)  |
| T cell response/development process           | Gap1, connexin43 | 1415800_at, 1437992_x_at | NM_010288                | down regulation (×1.9) |
| cytotoxic T-lymphocyte-associated protein 4   | Ctla4, Cd152, Ly-56, Ctla-4 | 1419334_at                | NM_009843                | up regulation (×4.6)  |
| deltex 1 homolog (Drosophila)                 | Dtx1        | 1425822_a_at              | NM_008052                | up regulation (×1.9)  |
| immunoglobulin superfamily, member 3          | Igfs3, V7, Cd101, Igfsf2 | 1455049_at                | NM_207205                | up regulation (×1.9)  |
| phospholipase C, beta 2, similar to phospholipase C, beta 2 | LOC545451, Plcb2 | 1452481_at                | NM_177568                | up regulation (×1.9)  |
| Notch gene homolog 3 (Drosophila)             | Notch3      | 1421964_at                | NM_008716                | up regulation (×1.9)  |
| phosphatidylinositol 3-kinase catalytic delta polypeptide | Plk3cd, p110i | 1453281_at                | NM_01029837, NM_008840   | up regulation (×1.6)  |
| Phosphatase and tensin homolog                | Pten        | 1441593_at                | NM_008960                | up regulation (×2.2)  |
| Interferon-inducible                          | H2-Aa       | 1438858_x_at              | NM_010378                | down regulation (×1.9) |
| lymphocyte antigen 6 complex, locus A         | Ly6a, TAP, Scare1, Ly-6A,a, Ly-6A-E, Ly-6e.1 | 1417185_at                | NM_010738                | down regulation (×1.8) |
| lymphocyte antigen 6 complex, locus C         | Ly6c        | 1421571_a_at              | NM_015274                | down regulation (×2.2) |
| radical 5-adenosyl methionine domain containing 2 (†) | Rdad2, vlg1, csg5, vipherin | 1436058_at                | NM_021384                | down regulation (×2.9) |
| Receptor transporter protein 4 (†)            | Rbp4, Ifrg28 | 1418580_at                | NM_023386                | down regulation (×2.3) |
| T-cell specific GTPase                        | Tgtp, Gtp2, Mg21 | 1440909_at                | NM_017579                | down regulation (×1.8) |
| Inflammation                                  | E4300202D04Rik | 1455257_at                | NM_172909                | up regulation (×2.2)  |
| guanine nucleotide binding protein (G protein), gamma 12 | Gng12 | 1421947_at                | NM_025278                | down regulation (×1.6) |
| tubulin, beta 3                               | Tubb3       | 1415978_at                | NM_023279                | down regulation (×1.8) |
| BMP and activin membrane-bound inhibitor, pseudogene (Xenopus laevis) | Bambi-ps1 | 1456178_at                | BF730112 (Unigene)       | up regulation (×3.4)  |
| Transcription                                 | Nfix        | 1436364_x_at              | NM_010906                | down regulation (×1.8) |
| gb:BG073921                                   | AF4/FMR2 family, member 1, AF1, AF4, Rob, Mitf2h | 1444937_at                | NM_133919                | up regulation (×2.3)  |
| Other                                         | Acot6       | 1428803_at                | NM_172580                | down regulation (×1.6) |
| expressed sequence C85492                     | C85492, Ago61 | 1436499_x_at              | NM_153540                | down regulation (×2.6) |
| cyclin D2                                     | Ccnd2       | 1430127_a_at, 1434745_at  | NM_009829                | down regulation (×2.0) |
| cytoplasmic FMR1 interacting protein 1        | Cyfip1      | 1416329_at                | NM_013370                | down regulation (×2.2) |
| region containing histocompatibility 2, Q region locus 9 and locus 7 | LOC630509, LOC674192 | 1418536_at                | down regulation (×1.9)  |
| protein disulfide isomerase associated 5      | Pdia5       | 1424650_at                | NM_028295                | down regulation (×1.6) |
| solute carrier family 22 (organic cation transporter), member 3 | Scl2a3, EMT, EMTH; OCT3 | 1420444_at                | NM_011395                | down regulation (×2.0) |
| Gene name                                                                 | Gene symbol | Affymetrix identification | GenBank accession number       | Regulation (a) |
|--------------------------------------------------------------------------|-------------|----------------------------|-------------------------------|----------------|
| testis derived transcript                                               | Tes, TSS, Tes1, Tes2, testin2, D6Ertd352e          | 1460378_a_at                  | NM_011570, NM_207176         | down regulation (x1.7) |
| RIKEN cDNA 2310032D16 gene                                              | 2310032D16Rik, Prei4                               | 1458701_at                    | NM_001042671, NM_001042672, NM_028802 | up regulation (x2.2) |
| arginine-RNA-protein transferase 1                                      | Ate1        | 1420652_at                  | NM_001029885, NM_013799      | up regulation (x1.9) |
| RIKEN cDNA 5730405i09 gene, cyclin Y                                    | Ccny        | 1441283_at                  | NM_026484                    | up regulation (x3.6) |
| CDC14 cell division cycle 14 homolog B (S. cerevisiae)                  | Cdc14b      | 1437070_at                  | NM_172587                    | up regulation (x1.7) |
| cytidine monophospho-N-acetylneuraminic acid hydroxylase                | Cmah        | 1421214_at, 1447019_at      | NM_007717                    | up regulation (x2.1) |
| fibulin 1                                                                | Fbln1       | 1451119_a_at                | NM_010180                    | up regulation (x2.1) |
| hepatoma-derived growth factor related protein 3                         | Hdgfrp3, HRP-3                                    | 1423252_at                   | NM_013886                    | up regulation (x1.7) |
| similar to solute carrier family 28 (sodium-coupled nucleoside transporter), member 2 | LOC381417, Scl2a2, cnt2 | 1450539_at                 | NM_172890                    | up regulation (x2.2) |
| nudix (nucleoside diphosphate linked moiety X)-type motif 21             | Nudt21      | 1455966_s_at                | NM_026623                    | up regulation (x2.0) |
| palmitoyl-protein thioesterase 1                                         | Ppt1, PPT, CLN1, INCL                             | 1420015_s_at                 | NM_008917                    | up regulation (x1.8) |
| Sfi1 homolog, spindle assembly associated (yeast)                        | Sf1         | 1426787_at                  | NM_030207                    | up regulation (x2.1) |
| gb:BB201882                                                              |             | 1443353_at                  | NM_011767                    | up regulation (x2.7) |
| Unknown                                                                  | RIKEN cDNA 2610019E17 gene                         | 2610019E17Rik                | AK011460                     | down regulation (x1.5) |
| RIKEN cDNA 1700054N08 gene                                               | 1700054N08Rik                                     | 1424796_at                   | NM_028536                    | up regulation (x1.8) |
| RIKEN cDNA 5830416F10 gene                                               | 5830416F10Rik                                     | 1453244_at                   | AK017935                     | up regulation (x2.5) |
| RIKEN cDNA A130022J15 gene                                               | A130022J15Rik                                     | 1433671_at                   | NM_175313                    | up regulation (x1.6) |
| RIKEN cDNA A630023P12 gene (*)                                           | A630023P12Rik                                     | 1455370_at                   | NM_173766                    | up regulation (x2.7) |
| CDNA sequence BC031575, mRNA, RIKEN cDNA 4921513D23 gene                 | BC031575, 4921513D23Rik                           | 1447110_at                   | NM_153549                    | up regulation (x2.3) |
| gb:BG086638                                                              |             | 1420312_s_at                | BGO86638                     | up regulation (x4.0) |
| Mus musculus, clone IMAGE:3983821 (*)                                   | 1427820_at  | BC021831                    | up regulation (x2.1)         |
| gb:AW546720                                                              |             | 1446882_at                  | AW546720                     | up regulation (x1.7) |

(a) P ≤ 0.05, significant changes of ≥ 1.5-fold.
(*) genes modified in both DN3 and ISP8 subsets.
doi:10.1371/journal.pone.0000992.t002
permit thymocytes to progress to mature T cells despite accumulation of early thymocyte subsets at the DN to DP transition. Alternatively and in view of the potential role attributed to the serine/threonine kinase S6K downstream of PDK1 [42], we suspect that PKB and S6K could compensate for each other during thymocyte development. This contention is further supported by the finding that single S6K mutant mice fail to reveal a defect in T cell development [43,44].

The signal transduction pathways that control thymocytes are often recapitulated in mature T cells. From our data, a number of genes whose expression is modulated upon the loss of PKBz are known to be involved in pre-TCR and/or TCR signaling and T cell activation. The presented results hence suggest that the deletion of *PkBz* affects the pre-TCR signaling in early thymocytes. Interestingly, several recent reports show a significant role of the PI3K pathway in the pre-TCR controlled developmental transition of DN to DP thymocytes. For instance, TCRβ-deficient mice activated by anti-CD3 isoforms, plays a crucial role in thymic development and the fact that PKB during early T cell development and the potential role attributed to the serine/threonine kinase S6K downstream of PDK1 [42], we suspect that PKB and S6K could compensate for each other during thymocyte development. This contention is further supported by the finding that single S6K mutant mice fail to reveal a defect in T cell development [43,44].

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chemiluminescence reagents (Amersham Biosciences, Piscataway, NJ, USA). PKB isoform-specific antibodies obtained by immunizing rabbits with isoform-specific peptides have already been reported [34]. Antibodies against phospho Thr308-PKB (the PDK1 site) and pan-actin were purchased from Cell Signalling Technologies (Danvers, MA, USA) and NeoMarkers (Fremont, CA, USA), respectively.

TUNEL assay
Mouse thymi were fixed in formalin (10% v/v) for 16 hours at 4°C. After dehydration in ethanol, samples were embedded in paraffin, cut into 5 μm-thick sections, and treated with 20 μg/ml proteinase K for 10 minutes at 37°C. Endogenous peroxidase was inactivated with 3% H2O2 in methanol for 30 minutes at room temperature. The sections were incubated in terminal deoxynucleotidyl transferase (TdT) buffer for 15 minutes at room temperature and TdT and biotinylated dUTP for 1 hour at 37°C. Washing with 1X SSC (0.15 M NaCl, 0.015 M sodium citrate) was used to stop the reaction. The Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA) was used for color development as described by the manufacturer. For quantification, 5 fields in each of 3 sections were counted for TUNEL-positive cells.

Flow cytometric analysis and FACS sorting
Two million lymphocytes in suspension were stained at 4°C for 20 minutes in FACS buffer (PBS and 2% FCS) with fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)-, Cy5-, and/or biotin-conjugated antibodies to cell surface molecules. Biotinylated antibodies were visualized with streptavidin-Cy5. For labeling of thymocyte precursors, cells were stained with FITC-CD25, PE-CD4, and biotin-CD4, CD8, TCRβ, TCRγδ, CD19, B220, CD11b, CD11c, Gr-1, and NK1.1. Cy5-negative precursor cells, corresponding to lineage-negative cells, were analyzed for expression of CD25 and CD44. Cells were stained with FITC-CD3, PE-CD4, and Cy5-CD8 to label later stages. For labeling of peripheral lymphocytes, cells were isolated from the spleen, depleted of red blood cells, and stained with PE-Cy7-CD19 and Cy5-CD3. For intracellular staining, lymphocytes labeled with cell surface markers were incubated for 16 hours at 4°C in fixation buffer (BD Biosciences, San Jose, CA, USA) and processed in permeabilization buffer (BD Biosciences). For analysis of thymocyte apoptosis, 10^6 cells were stained at 4°C for 20 minutes in annexin binding buffer (Vybrant apoptosis assay kit #3, Molecular Probes, Eugene, OR, USA) with FITC-annexin V and propidium iodide (PI) according to the manufacturer’s instructions. For flow cytometric analysis, labeled thymocytes were washed with FACS buffer, permeabilization buffer (when intracellular staining), or annexin binding buffer (when annexin V-PI staining) and analyzed on a FACSCalibur (Becton Dickinson, Franklin Lakes, NJ, USA). Data were processed with Cell Quest Pro (BD Biosciences). For FACS sorting, labeled thymocytes were washed with FACS buffer, filtered on a 40 μm-nylon membrane, and sorted on the flow sorter MoFlo (DakoCytomation, Baar, Switzerland).

Bone marrow transplant and thymic grafting experiments
For bone marrow transplant experiments, fetal liver from PKBz+/+ and PKBz−/− E13.5 embryos (CD45.2) were dissected and disrupted to single cell suspension by passages through a G25-syringe. The resultant suspension was layered over Ficoll and spun down for 25 minutes at 2 000 g. After removing theuffy coat, the fetal liver cells were washed, counted, and resuspended at 5×10^6 cells/ml. Bone marrow chimeras were generated by intravenous injection of 10^6 fetal liver cells into lethally irradiated (2×5500 rad) 4-week-old congenic recipient mice (CD45.1) on a C57Bl/6 background (B6 Ly5.1). The donor-derived-lymphocyte populations were analyzed by flow cytometry 5 weeks post transplant. For grafting experiments, fetal thymic lobes from PKBz+/+ and PKBz−/− E13.5 embryos were dissected and depleted of thymocytes by 6 day-treatment with 1.35 mM deoxyguanosine. Donor thymic stroma were then subrethally engrafted into 4 week-old Fox8 rosa26 recipient mice. The grafts were analyzed by flow cytometry 4 weeks post grafting.

RNA extraction and microarray experiment
DN3 and ISP8 thymocyte subsets were sorted by FACS from PKBz+/−/− wild-type littermate pair. The same number of DN3 or ISP8 cells was sorted (7 000 to 25 000 cells) within a PKBz+/−/− wild-type pair. Total RNA was extracted using PicoPure™ RNA isolation kit (Arcturus, Sunnyvale, CA, USA) according to manufacturer’s instructions. RNA quality was controlled using the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Total RNA was amplified and labeled using the Affymetrix 2-cycle 3’ labeling kit according to manufacturer’s instructions. After fragmentation, 10 μg cRNA was hybridised to mouse genome 430 2.0 GeneChips (Affymetrix, Santa Clara, CA, USA). After scanning the Genechips in an Affymetrix 2500 scanner, transcript expression values were estimated using the GC-RMA function provided by Refiner 3.1 (Genedata, Basel, Switzerland) and statistical analysis was performed using Analyst 3.1 (Genedata). GeneData’s implementation of GC-RMA includes the generation of an Affymetrix detection P-value. A gene was considered to be reliably detected if it had a detection P-value<0.04 (Affymetrix default, marginal calls ignored) in at least 2/3 of the biological replicates of a condition. A power analysis of our experimental design showed we could expect to have a power of 0.8 to distinguish samples differing by 1.5-fold with a normalised standard deviation less than 0.461 and it could resolve differences of 1.5-fold (power of 0.8) when the normalised standard deviation was less than 0.613. We selected genes that were significantly (paired t-test P<0.05) modified by ≥1.5-fold between PKBz+/+ and the corresponding control in at least three of the four pairs. Only genes with expression data above 20 in at least one of the conditions within a pair and in at least 3 pairs are displayed. The microarray data have been deposited in the Gene Expression Omnibus of NCBI (accession number: GSE7875).

Statistical analysis
Data are provided as arithmetic mean±standard error of the mean and tested for significance using one-way analysis of variance (ANOVA). Only results with a P value of ≤0.05 (*) were considered statistically significant.

Note
Materials and Methods related to Figures in Supporting Information can be found in “Materials and Methods S1”.

SUPPORTING INFORMATION
Figure S1 The deletion of PKBz does not affect T cell number in adult mice. A: The weight of freshly dissected thymus was measured in PKBz+/+ and PKBz−/− adult mice and expressed as ratio to body weight. B: Thymocytes were isolated from PKBz+/+ and PKBz−/− adult mice and counted; cell number was expressed as
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