PKCθ is necessary for efficient activation of NFκB, NFAT, and AP-1 during positive selection of thymocytes

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ABSTRACT

While it has been shown in several publications that the serine-threonine kinase PKCθ is required for efficient activation of mature T lymphocytes, the role of PKCθ in T cell development in the thymus is somewhat controversial. In this study, using knockout mice, we show that PKCθ is important in positive selection. The thymus of PKCθ−/− animals contains significantly less mature single positive T cells compared to wild-type controls. Biochemically, PKCθ deficient thymocytes show defective activation of the transcription factors AP-1, NFAT and NFκB as well as impaired phosphorylation of the MAP kinase ERK after T cell receptor stimulation in vitro. Together, these results reveal a crucial role of PKCθ in positive selection of thymocytes in a pathway leading to the activation of ERK, AP-1, NFAT, and NFκB.

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1. Introduction

T cells represent a major component of the adaptive immune system that allows the organism to fight various pathogens. Activation and subsequent differentiation of T lymphocytes crucially depend on the interaction of the T cell receptor (TCR) with a cognate antigen-MHC-complex which is provided on the surface of antigen presenting cells (APCs). This dependence upon self-MHC molecules is known as “MHC restriction”.

The formation of a TCR during T cell maturation is the consequence of random rearrangements of the TCR loci giving rise to a great diversity of T lymphocytes with distinct antigen specificities. In a current model of T cell development, thymocytes undergo a twofold selection process [1]: in the thymic cortex, CD4−/CD8+ double positive (DP) thymocytes undergo a twofold selection process [1]: in the thymic cortex, CD4−/CD8+ double positive (DP) thymocytes interact with epithelial cells expressing MHC class I and MHC class II on their surface. DP cells bearing TCRs with low to moderate affinity to MHC/antigen complexes receive a survival signal in a process called positive selection. However, the great majority (around 90%) of DP thymocytes without proper TCRs die by neglect. Positively selected DP cells then migrate towards the corticomedullary junction, where negative selection occurs: high-affinity interaction of TCRs with self-MHC/self-peptide complexes destines the thymocytes for apoptosis. Subsequently, selected thymocytes downregulate either CD4 or CD8 and go through several rounds of division in the medulla before they leave the thymus as fully matured peripheral T cells.

Since signaling through the TCR can have completely different outcomes (positive vs. negative selection), the biochemical events following TCR engagement on thymocytes are of special interest. A number of effector molecules that drive T cell maturation have been defined so far. It was shown that sustained signaling through the MAP kinase ERK is required for positive selection, whereas strong but transient ERK activation promotes negative selection [2,3]. Negative selection is also regulated by Bim, a proapoptotic member of the BH3-only Bcl-2 family [4]. Interestingly, it was demonstrated that ERK can mediate the phosphorylation of Bim [5] which leads to its ubiquitination and proteasomal degradation [6,7]. In several studies, the calcium-dependent phosphatase calcineurin has been shown to be important for positive selection of thymocytes [8–11]. This result suggests that the downstream transcriptional effector of calcineurin, i.e. NFAT (nuclear factor of activated T cells) may be involved in the transcriptional program required for positive selection. Indeed, loss of function of nfatc3 leads to a defect in thymocyte selection which resembles the phenotype of calcineurinA/B knockout mice [8,12]. Most recently, PKCθ has been linked to the maturation of thymocytes during positive selection [13]. This serine-threonine kinase was shown to be crucial for productive NFAT activation in peripheral T cells [14–17]. In the present study we provide evidence that PKCθ indeed plays an important role in the signaling events leading to positive selection of thymocytes. The numbers of mature single positive T cells in the thymus were reduced in PKCθ−/− mice. Biochemically, phosphorylation of the MAP kinase ERK1/2 was impaired in thymocytes of

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PKCδ knockout animals compared to wild-type controls. PKCδ deficient thymocytes also showed a severe activation defect, reflected in decreased proliferation and IL-2 secretion. Most importantly, the present work for the first time demonstrates that PKCδ is crucial for the activation of the transcription factors NFAT, AP-1 and NFκB in immature thymocytes.

2. Materials and methods

2.1. Mice and reagents

PKCδ knockout mice were described elsewhere [15]. γ<sup>32P</sup>-ATP was purchased from Amersham. The antibodies used for T cell stimulation were anti-CD28 monoclonal antibody (mAb) (clone 28.2) and the CD3-specific mAb 2C11 (mouse). The anti-hamster immunoglobulin G1 antibody (clone HIG-632) was used for crosslinking the CD3 antibodies during short-term stimulation.

2.2. Analysis of proliferative responses and cytokine release

For anti-CD3-mediated stimulation, 5 × 10<sup>5</sup> thymocytes in 200 μl of proliferation medium [RPMI supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, and 50 units/ml penicillin/streptomycin] were added in duplicates to 96-well plates precoated with 10 μg/ml anti-CD3 antibody. Soluble anti-CD28 antibody (1 μg/ml) was also added where indicated. Cells were harvested at 64 h after a 16 h pulse with 1 μCi [3H]-thymidine per well; [3H]-thymidine incorporation was measured with a Matrix 150 direct β counter system. IL-2 produced from the cultures was measured via the IL-2-dependent indicator cell line CTLL-2. Supernatants of cultures were harvested at 2C11 (mouse) titer 3 days after stimulation. The presence of surface markers was analyzed with a FACS Calibur™ cytometer (BD Bioscience) and CellQuest™ software according to standard protocols. Antibodies against murine CD4 and CD8 were obtained from CalTag Laboratories. Antibodies against CD5 and CD69 were obtained from BD Pharmingen.

2.5. Western blotting analysis of ERK activation

Thymocytes were stimulated with 1 μg/ml of hamster anti-CD3 antibody and 1 μg/ml of hamster anti-CD28 antibody together with 2 μg/ml of anti-hamster crosslinking antibody at 37°C for indicated time periods. Cells were lysed in ice-cold lysis buffer (5 mM NaPiP, 5 mM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 5 mM EDTA, 50 mM NaCl, 50 mM Tris, pH 7.3, 1% NP-40, 50 μg/ml each of aprotinin and leupeptin) and centrifuged at 15,000 × g for 15 min at 4°C. Protein lysates were subjected to Western blotting analysis with antibodies against phospho-ERK1/2 (Thr202/Tyr204) and ERK1/2 (Cell Signaling Technology).

3. Results

3.1. PKCδ<sup>−/−</sup> thymocytes show diminished positive selection

To examine whether PKCδ is of importance in the maturation of thymocytes, we compared the numbers of single positive T cells in the thymi of wild-type and PKCδ deficient mice. The loss of PKCδ led to a strong reduction of CD4 single positive T cells in the thymus (4.9% vs. 11.1%) (Fig. 1A). The number of CD8 positive T cells was also reduced, albeit less pronounced (1.2% vs. 1.9%) (Fig. 1B). Consequently, the double positive fraction was enhanced from 79% to 85%, whereas total thymocyte numbers were comparable between wild-type and PKCδ<sup>−/−</sup> mice (not shown). The numbers of mature T lymphocytes in lymph nodes and spleens were also not altered between genotypes (not shown), indicating that migration of T cells from the thymus into the periphery is not influenced by the loss of PKCδ. To investigate whether the reduction of mature T cells in the thymi of PKCδ<sup>−/−</sup> mice was due to a suboptimal positive selection, we analyzed the expression of CD69 and CD5. The upregulation of these surface proteins at the double positive stage has been associated with positive selection of developing thymocytes and can therefore serve as marker molecules [18, 19]. Interestingly, CD69<sup>+</sup> double positive thymocytes were diminished by 50% in the PKCδ knockout mice (7.9% vs. 16.4%) (Fig. 1C), and CD5 expression was reduced by one third (23.7% vs. 36.8%) (Fig. 1D). These results strongly indicate that PKCδ is important for efficient positive selection of thymocytes that occurs during the transition from CD4<sup>+</sup>CD8<sup>+</sup> double positive to either CD4<sup>+</sup> or CD8<sup>+</sup> single positive T cells.

3.2. H-Y TCR transgenic PKCδ<sup>−/−</sup> thymocytes show diminished positive selection

As the CD8<sup>+</sup> single positive subset comprises only about 2% of whole thymocytes, a defect in the maturation of this lineage is difficult to detect. In order to investigate whether PKCδ is important in the positive selection of the CD4<sup>+</sup>CD8<sup>+</sup> subset, we introduced the H-Y T cell receptor transgene into mice deficient in PKCδ. Consistent with the results obtained with TCR non-transgenic mice, PKCδ deficient H-Y TCR transgenic mice showed a clear defect in the maturation of CD8<sup>+</sup> single positive thymocytes (11.7% vs. 18.6%) (Fig. 1E). This developmental defect is most probably due to a suboptimal positive selection as the number of CD69<sup>+</sup> double positive thymocytes was decreased by 70% in PKCδ deficient compared to wild-type mice (9.6% vs. 32%) (Fig. 1F). Total thymocyte numbers were not altered between genotypes, and male H-Y TCR mice deficient in PKCδ exhibited the same subset distribution as...
Fig. 1. PKCθ deficiency leads to impaired maturation of thymocytes. Wild-type and PKCθ−/− thymocytes from non-transgenic (A–D) or HY TCR transgenic (E–F) mice were stained with PE-anti-CD4, APC-anti-CD8, and FITC-anti-CD5 or FITC-anti-CD69. Percentages of indicated subsets are shown as boxplots. \( p < 0.01 \) (A–E); \( p = 0.015 \) (F). \( p \) values were calculated with Student's \( t \)-test.

3.3. PKCθ deficient thymocytes lack significant ERK signaling

Since ERK activation was suggested to be required for positive selection [3,20], we investigated whether PKCθ deficiency influenced ERK activation, quantified as phosphorylation of ERK1/2 on Thr202 and Tyr204. After CD3/CD28 stimulation of wild-type thymocytes, ERK phosphorylation was readily detected and prolonged over at least 40 min. By contrast, in PKCθ−/− thymocytes virtually no ERK phosphorylation was detectable over a time period of 60 min (Fig. 2).

3.4. PKCθ−/− thymocytes exhibit diminished proliferation and IL-2 responses

It was shown previously that proliferation and IL-2 secretion of mature T cells crucially depend on PKCθ [14,15,21]. To assess whether this holds also true for immature T cells, we stimulated thymocytes with anti-CD3 and anti-CD28 antibodies and measured proliferative and IL-2 responses. PKCθ deficient thymocytes showed profound defects in proliferation as well as IL-2 secretion.
3.5. PKC\textsuperscript{-/–} thymocytes show defective activation of the transcription factors NFAT, AP-1, and NFk\textsubscript{B}

IL-2 expression after TCR/CD28 engagement is contingent on a number of gene regulatory proteins, the most prominent being NFAT, AP-1, and NFk\textsubscript{B} [22,23]. The activation of these transcription factors in mature peripheral T cells is crucially dependent on PKC\textsuperscript{δ} [14,15,21]. In light of these findings and considering the IL-2 secretion defect of PKC\textsuperscript{δ} deficient thymocytes, we examined whether the activation of these transcription factors is also PKC\textsuperscript{δ}-dependent in developing immature T cells. Wild-type and PKC\textsuperscript{-/–} thymocytes were stimulated with anti-CD3 and anti-CD28 antibodies and nuclear extracts were analyzed in band shift assays. The activation of NFAT and AP-1 was strongly diminished in PKC\textsuperscript{-/–} thymocytes (Fig. 3C). Interestingly and in contrast to previous reports [13,21], NFk\textsubscript{B} activation was also severely impaired in PKC\textsuperscript{-/–} thymocytes (Fig. 3C). This defect in NFk\textsubscript{B} activation was reproducibly observed and confirmed by analyzing the nuclear translocation of p50, a NFk\textsubscript{B} subunit (Fig. 3D). To exclude that this differing result was simply due to different stimulation conditions, we treated thymocytes in accordance with Sun et al. either with anti-CD3 alone or with phorbol 12,13-dibutyrate (PdBu) alone for 8 h. Still, PKC\textsuperscript{δ} deficient thymocytes showed a severe defect in NFk\textsubscript{B} activation (Fig. 3E).

4. Discussion

It has been shown in several publications that PKC\textsuperscript{δ} is required for efficient activation of mature T lymphocytes [14–17,21]. Particularly, PKC\textsuperscript{δ} is a positive regulator of pathways that activate the transcription factors NFAT, AP-1, and NFk\textsubscript{B} and ultimately converge at the induction of IL-2 expression in peripheral T cells. PKC\textsuperscript{δ} also mediates LFA-1/ICAM-1 adhesion and stabilizes the immunological synapse [24]. We could demonstrate recently that activation of PKC\textsuperscript{δ} leads to the degradation of the negative regulator Cbl-b, thereby contributing to full T cell activation [14]. While the significance of PKC\textsuperscript{δ} in the activation of mature peripheral T lymphocytes
is well established [25], the role of PKCθ in T cell development in the thymus is somewhat controversial [13,15,21]. Our study now confirms a previous one in which PKCθ was shown to be required for efficient positive selection [13]. Albeit inconsistent with an earlier publication by our laboratory which concentrated on mature peripheral T cells [15], a more thorough investigation now revealed clearly that the loss of PKCθ led to a strong reduction of mature T cells in the thymus and a concomitant decrease of markers for positive selection, i.e. CD5 and CD69 (Fig. 1). However, while Morley et al. used CD4-lacking TCR transgenic mice, we employed the CD8-lacking H-Y specific TCR, thereby complementing previous data (Fig. 1E and F). The H-Y specific and MHC class I restricted thymocytes expressing the H-Y TCR are positively selected in female H-2b mice and negatively selected in male H-2b mice [26]. Therefore, in female mice, the thymocyte selection of PKCθ impaired ERK activity could contribute to the defective positive selection (Fig. 2). Thus, as opposed to the situation in mature T cells [15], the ERK1/2 was strongly impaired in PKCθ deficient thymocytes: The calcineurin inhibitors cyclosporin A and FK506 inhibit the thymus to a somewhat controversial [13,15,21]. Our study now confirms a previous one in which PKCθ [26]−/− deficient thymocytes exhibited a more severe positive selection, no complete block in thymocyte maturation [13,21]. None of these double-deficient mouse lines showed profound defects in proliferation as well as IL-2 production (Fig. 3C), it is likely that the defective positive selection of PKCθ deficient thymocytes is at least in part due to impaired NFAT and/or AP-1 activation.

Surprisingly, in spite of contrasting to previous reports [13,21], NFκB activation was also reproducibly affected by the loss of PKCθ (Fig. 3C–E). The reasons for these conflicting results are not known, but the different mouse strains used in the studies are one possible explanation for the varying outcomes. Whether our observed NFκB activation defect contributes to impaired positive selection is not known so far. At least one publication stated that NFκB was dispensable for positive selection [34], whereas two groups reported that it plays a role in apoptosis of double positive thymocytes [35,36].

Collectively, we could demonstrate that in our system PKCθ is required for full activation of NFAT, AP-1, and NFκB in thymocytes. In our hands, PKCθ proved to be upstream of key transcription factors not only in mature [15] but also in immature T cells. Despite the fundamental role PKCθ obviously plays in positive selection, no complete block in thymocyte maturation was observed. To examine whether other PKC isoforms partially compensate for the loss of PKCθ, we generated mice that were additionally deficient in either PKCα, beta or epsilon (PKCθ−/−α/−, PKCθ−/−β/−, PKCθ−/−ε/−). None of these double-deficient mouse lines exhibited a more severe positive selection defect than did the PKCθ-deficient alone (not shown). This indicates that there is no functional redundancy between these PKC isoforms during the maturation of thymocytes.

Conflict of interest
The authors have no financial conflict of interest.

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