Protein kinase D regulates positive selection of CD4⁺ thymocytes through phosphorylation of SHP-1

Eri Ishikawa¹, Hidetaka Kosako², Tomoharu Yasuda³, Masaki Ohmuraya⁴, Kimi Araki⁴, Tomohiro Kurosaki³,⁵, Takashi Saito⁶,⁷ & Sho Yamasaki¹

Thymic selection shapes an appropriate T cell antigen receptor (TCR) repertoire during T cell development. Here, we show that a serine/threonine kinase, protein kinase D (PKD), is crucial for thymocyte positive selection. In T cell-specific PKD-deficient (PKD2/PKD3 double-deficient) mice, the generation of CD4 single positive thymocytes is abrogated. This defect is likely caused by attenuated TCR signalling during positive selection and incomplete CD4 lineage specification in PKD-deficient thymocytes; however, TCR-proximal tyrosine phosphorylation is not affected. PKD is activated in CD4⁺CD8⁺ double positive (DP) thymocytes on stimulation with positively selecting peptides. By phosphoproteomic analysis, we identify SH2-containing protein tyrosine phosphatase-1 (SHP-1) as a direct substrate of PKD. Substitution of wild-type SHP-1 by phosphorylation-defective mutant (SHP-1S557A) impairs generation of CD4⁺ thymocytes. These results suggest that the PKD-SHP-1 axis positively regulates TCR signalling to promote CD4⁺ T cell development.
A n appropriate αβ T cell receptor (TCR) repertoire is shaped in the thymus through multiple selection steps. In this process, transduction of signals through the TCR in CD4+CD8+ double positive (DP) thymocytes determines CD4/CD8 lineage specification and generates CD4+CD8− and CD4−CD8+ single positive (SP) thymocytes. At the DP stage, interaction of the TCR with self-peptides on major histocompatibility complex (MHC) molecules generates positive-selecting signals. DP thymocytes that undergo positive selection increase their surface expression of CD5, CD69 and TCR and differentiate into a CD4+CD8− transitional stage. At this stage, persistent TCR signalling promotes CD4 lineage specification through a series of transcriptional programs. However, the molecular mechanisms by which the signal duration is translated into specific responses have yet to be fully defined.

It is well established that sequential tyrosine phosphorylation events triggered by protein tyrosine kinases (PTKs), such as Src-, Syk- and Tec-family PTKs, orchestrate TCR signalling during T cell development. Serine/threonine kinases also regulate T cell development by controlling transcriptional and metabolic programs. Until now, loss of several serine/threonine kinases has been reported to result in defective T cell development. However, the crosstalk between serine/threonine kinase and tyrosine phosphorylation cascade is not clearly understood.

PKD, initially called PKCμ, is a serine/threonine kinase family now classified within the CaMK group and separated from the AGC group (named for PKA, PKG and PKC). The three isoforms, PKD1, PKD2 and PKD3, are encoded by different genes, prkd1, prkd2 and prkd3, respectively. All PKD isoforms have a highly conserved amino acid sequence with a diacylglycerol (DAG)-binding domain, pleckstrin homology (PH) domain and kinase domain. Among them, PKD2 is abundantly expressed in T cells and is phosphorylated on TCR stimulation. In addition, constitutively active PKD2 drives T cell development. However, thymic selection was not grossly disturbed in PKD2-deficient mice. Until now, no studies have addressed the role of PKD in thymocyte development using mice lacking all PKD isoforms, and the downstream substrates of PKD in thymocytes have not been fully elucidated.

Inhibitory signalling molecules, such as protein tyrosine phosphatases (PTPs), modulate TCR signalling and thus contribute to setting the threshold for thymocyte selection. In particular, SH2-containing protein tyrosine phosphatase-1 (SHP-1) is reported to regulate thymic selection through a variety of proposed mechanisms. The inhibitory activity of SHP-1 is regulated by binding to the phosphorylated targets via SH2 domains and by phosphorylation of its C-terminal tyrosines (Ty336 and Tyr564). However, the role of serine/threonine phosphorylation in the function of SHP-1 remains unclear.

In this study, we established mice lacking all PKD isoforms in T cells. The generation of CD4 SP thymocytes is impaired in these T cell-specific PKD-deficient mice. Using a phosphoproteomic approach, we identified SHP-1 as a direct substrate for PKD. The phosphorylation of SHP-1 by PKD positively regulates TCR signalling. Collectively, we demonstrate that the PKD–SHP-1 axis is required for CD4+ T cell development.

**Results**

**PKD is phosphorylated in preselection DP thymocytes.** We first investigated whether PKD is activated on TCR engagement in thymocytes by evaluating phosphorylation of its activation loop. To address this, ‘unengaged’ DP thymocytes that have never encountered self-peptides were prepared. To obtain preselection thymocytes bearing antigen-specific TCR, bone marrow (BM) cells from OT-I TCR Tg mice were transferred into transporter associated with antigen processing (TAP)-deficient mice, which lack functional MHC class I. The preselection thymocytes were stimulated with OVA peptides that differ in affinity such as OVA257–264 (SIINFEKL) (strong agonist), T4 (intermediate agonist) and G4 (weak agonist). To evaluate PKD activation, we established anti-phospho-PKD1/2/3 Abs that recognize phosphorylated activation loops containing a common sequence shared by all PKDs (Fig. 1a). Substantial PKD activation, as

**Figure 1 | Expression of PKD isoforms in the thymus.** (a) Characterization of anti-phospho-PKD1/2/3 Abs (anti-pPKDs). Reactivities of anti-pPKDs were evaluated by western blot analysis of lysates from HEK293 cells overexpressing WT PKD2 and mutants. Note that overexpressed PKD2 is constitutively phosphorylated. Anti-PKD1/2/3 (anti-PKDs) blotting was also performed as a control. (b) Preselection OT-I DP thymocytes, that are obtained from TAP-deficient mice transferred with OT-I Tg BM cells, were stimulated with a variety of OVA peptides (10 μM) for the indicated times and phosphorylation of PKD and Erk was analysed. PKD, Erk and β-actin were used as loading controls. (c) RT-PCR analysis of expression of PKD isoforms in the thymus, spleen, ovary and lung from C57BL/6 mice. mRNA expression of β-actin was analysed as a control. Threefold serial dilutions of cDNAs were used as templates. (d) RT-PCR analysis of PKD isoforms expressions in various cell subsets sorted by flow cytometry from WT thymocytes. Results are presented as expression relative to β-actin. ND, not detected. (e) mRNA copy number was evaluated by molecular indexing assay. The amount of RNA used in the experiment is indicated on the x axis. Data are representative of two independent experiments (a–c). Data are presented as mean ± s.d. of triplicate assays and representative of two independent experiments (d,e).
assessed by anti-phospho-PKDAs, was induced by low-affinity peptides (Fig. 1b), implying a role for PKD in positive selection.

We next examined the PKD isoforms expressed in thymocytes. Reverse transcription-PCR (RT-PCR) analyses confirmed that thymocytes express abundant PKD2, less PKD3 and undetectable PKD1 mRNA (Fig. 1c,d), as previously reported. The lack of PKD1 expression in thymocytes was further verified by evaluating mRNA copy number using a molecular indexing assay (Fig. 1e). The three PKD isoforms was expressed in PKD2/3ΔT TH mice using Lck-Cre Tg mice (PKD2/3ΔT) (Fig. 2a,b). We confirmed that none of the three PKD isoforms were expressed in PKD2/3ΔT thymocytes using Abs reacting with PKD1, PKD2 and PKD3 (Fig. 2c,d).

Defective T cell development in PKD2/3ΔT mice. In PKD2/3ΔT mice, the proportion and number of CD4 SP thymocytes were markedly lower compared with wild-type (WT) mice, whereas those from single-deficient (PKD2ΔT or PKD3ΔT) mice were not altered (Fig. 3a,b). These trends were more prominent in the TCRhi-gated mature CD4 SP population (Fig. 3a, lower panels).

Role of PKD in positive and negative selection. To examine the role of PKD in positive selection, we crossed PKD2/3ΔT mice with MHC class II-restricted OT-II TCR transgenic (Tg) mice. In OT-II × PKD2/3ΔT mice, the percentage of CD4 SP thymocytes was reduced to one-tenth that of PKD-sufficient OT-II mice (Fig. 4a), demonstrating that PKD is critically involved in positive selection for the CD4 lineage. In the MHC class I-restricted OT-I TCR background, the proportion of mature CD8 SP thymocytes in PKD2/3ΔT mice was reduced compared with that in control OT-I Tg mice (Fig. 4b), albeit less severe than in OT-II background. Furthermore, the expression of Tg-TCR was lower in PKD2/3ΔT mice (Fig. 4a, lower panels). Analysis of female H–Y TCR Tg mice supported this finding, as CD8 SP development was impaired in H–Y × PKD2/3ΔT mice (Fig. 4c). Thus, positive selection for both the CD4 and CD8 lineages is impaired in PKD2/3ΔT mice, particularly when the β2TCR is fixed by transgenes.

We next investigated the function of PKD in negative selection using two different models. In male H–Y Tg × PKD2/3ΔT mice, the percentage and number of DP thymocytes were significantly higher than in control male H–Y Tg mice, suggesting inefficient negative selection in PKD-deficient thymocytes (Fig. 4d). Strikingly, male H–Y Tg × PKD2/3ΔT mice had CD8 SP thymocytes bearing high levels of the H–Y TCR (Fig. 4e). Furthermore, the peripheral T cells in male H–Y Tg × PKD2/3ΔT mice expressed normal levels of CD8, unlike low-level expression in WT H–Y controls (Fig. 4f). These results suggest that the loss of PKD results in a shift of the normal positive selection window towards high self-reactivity due to impaired TCR signalling in thymocytes.

For the second model, we used a clonal deletion model triggered by endogenous superantigens. Thymocytes bearing Vβ5+ and Vβ11+ TCR are deleted in the presence of mtv-8 and mtv-9 on I–E (Fig. 4g, H-2Kb/d). This deletion persisted in PKD single-deficient mice; however, it was significantly blocked in PKD2/3ΔT (H-2Kb/d) mice (Fig. 4g, left panels). Irrelevant Vβ6+ cells were not affected by the absence of PKD (Fig. 4g, right panel). Thus, these two models reveal that PKD is involved in negative selection.

Collectively, our results indicate that PKD is critical for positive selection to the CD4 lineage and is also required for optimal positive selection to the CD8 lineage and negative selection.

Impaired CD4+CD8int thymocyte generation by loss of PKD. DP thymocytes stimulated with positive-selecting peptides express CD69 and then differentiate into CD4+CD8− intermediate cells, which are the precursors to both CD4 and CD8 SP thymocytes. Although the number of CD69+ DP cells was not altered in PKD2/3ΔT mice, the CD4+CD8− subpopulation was significantly decreased (Fig. 5a). The impaired generation of the CD4+CD8− population was also evident in OT-I and OT-II.
TCR Tg backgrounds (Fig. 5b), indicating that PKD is required for the transition from the DP to the CD4⁺CD8⁻ stage. Consistent with this, the CD4⁺CD8⁻ population in PKD2/3⁻/⁻ mice showed lower expression levels of CD5 than in WT mice, suggesting that loss of PKD led to the attenuation of TCR signalling during differentiation of DP to CD4⁺CD8⁻ stage (Fig. 5c).

To confirm this assumption, we conducted two-step differentiation assay in vitro. During recovery culture after cross-linking of TCR and co-receptor, DP thymocytes differentiate into CD4⁺CD8⁻ cells (Fig. 5d), as previously reported. However, PKD2/3⁻/⁻ DP thymocytes generated a much lower percentage of CD4⁺CD8⁻ cells than WT thymocytes after this treatment (Fig. 5d). Collectively, these results suggest that PKD regulates the TCR signals required for transition of DP to CD4⁺CD8⁻ thymocytes during positive selection.

**PKD mediates signals triggered by weak-affinity peptides.** To evaluate the strength of TCR signals, we performed quantitative assays using OVA peptide variants to stimulate thymocytes from TCR Tg mice. Ag peptide-induced co-receptor downregulation is a molecular indicator of the strength of TCR signalling in DP thymocytes. Indeed, stimulation of thymocytes from OT-II Tg mice resulted in co-receptor downregulation in accordance with the peptide affinity as seen using OVA232-339 (strong agonist), R9 (weak agonist) and F9 (very weak agonist) (Fig. 5e, open circles)²⁸. However, the responses were suppressed in PKD-deficient OT-II thymocytes, even on stimulation with the R9 variant (Fig. 5e, closed circles). Likewise, upregulation of CD69 and CD5 was also reduced in the absence of PKD (Fig. 5f).

To further investigate, we stimulated thymocytes bearing OT-I TCR. Again, the co-receptor downregulation faithfully reflected the peptide affinity: namely, OVA357-264 is the most potent, followed by T4 and G4 (Fig. 5g, open circles). We found that in the absence of PKD, the response was significantly attenuated when stimulated with a low-affinity variant, G4, which is known as a positive-selecting ligand (Fig. 5g, closed circles)²⁹. These findings suggest that PKD is required for TCR signalling triggered by weak-affinity ligands, which is consistent with the above observation that positive selection is severely affected in PKD2/3⁻/⁻ mice.

**PKD is involved in CD4 lineage specification.** As the generation of CD4 SP cells was more severely affected than CD8 SP cells in PKD2/3⁻/⁻ mice (Fig. 3a,b), we next investigated whether PKD influences CD4 lineage commitment. If PKD2/3⁻/⁻ thymocytes have an intrinsic defect in commitment to the CD4 lineage, they

![Image](image_url)
should give rise to CD8 SP cells even in the absence of MHC class I (in an MHC class II-dependent manner), which is called ‘CD8-redirection’ (refs 30,31). To address this possibility, we transferred BM cells from WT or PKD2/3ΔT mice to TAP-deficient mice. PKD-deficient BM cells, but not WT BM cells, gave rise to mature CD8 SP thymocytes even in TAP-deficient hosts lacking functional MHC class I (Fig. 6a), suggesting that PKD2/3ΔT cells are defective in lineage commitment to CD4 SP thymocytes.

In fact, expression of ThPOK, a critical regulator of CD4 lineage commitment 32, was decreased in PKD2/3ΔT CD4⁺CD8int cells, whereas Runx3, a transcription factor essential for CD8 lineage specification, was not decreased (Fig. 6b). Forced expression of ThPOK restored the percentage of CD4 SP thymocytes to nearly WT levels (Fig. 6c). However, the expression of the CD5 maturation marker in CD4⁺CD8int cells remained low even in the presence of ThPOK transgene (Fig. 6d). Thus, these results suggest that PKD regulates the development of

**Figure 4 | Impaired positive and negative selection in the absence of PKD.** (a,b) Thymocytes from WT and PKD2/3ΔT mice crossed with OT-II TCR Tg (a) or OT-I TCR Tg (b) were analysed for expression of CD4 and CD8 by flow cytometry. Numbers indicate the percentages of cells in each gate (upper panels). The percentage of Vα2⁺ cells is shown (lower panels). (c) Analysis of CD4 and CD8 expression (upper panels) and T3.70 expression (lower panels) of thymocytes from female (f) H–Y TCR Tg mice. (d) Analysis of male (m) H–Y TCR Tg mice (left panels). The average number of DP thymocytes is shown (bar graph). **P<0.01. (e) T3.70⁺ thymocytes from male H–Y Tg and H–Y Tg × PKD2/3ΔT mice were analysed for CD8 expression. Mean fluorescent intensities (MFIs) of gated population are 208.41 and 337.32, respectively. (g) Negative selection induced by endogenous superantigens. Percentage of Vβ5⁺, Vβ11⁺ and Vβ6⁺ cells among CD4 SP thymocytes from B6 (H-2b) WT, PKD2ΔT, PKD3ΔT and PKD2/3ΔT mice, backcrossed (H-2b/d) or not (H-2b/b) onto B10.D2 (H-2d) mice was analysed by flow cytometry. Each circle represents an individual mouse. Small horizontal lines indicate the mean. *P<0.05, **P<0.01. Data are presented as mean ± s.d. of eight mice (d, bar graph) and are representative of four independent experiments (a–f). Unpaired two-tailed Student’s t test is used to calculate P values.
CD4 SP cells through at least two mechanisms: (i) promotion of TCR signals in the DP stage that trigger transition to the CD4+CD8int stage; and (ii) specification of CD4 lineage development via a ThPOK-dependent process.

**SHP-1 is a PKD substrate in thymocytes.** The above results demonstrate that PKD promotes TCR signalling during thymic selection. However, proximal signalling events including Ca2+ influx and Erk activation following TCR ligation were comparable between WT and PKD2/3 thymocytes (Fig. 7a,b). Furthermore, in DP cells, TCR-induced tyrosine phosphorylation of proximal signalling molecules, such as CD3ζ and ZAP-70, as well as MAPKs, such as Erk, p38 and JNK, was unaffected by the loss of PKD (Fig. 7c).

On the other hand, expression of a truncated PKD2 transgene lacking the kinase domain inhibited the generation of CD4 SP thymocytes (Fig. 7d), suggesting that the PKD kinase activity is required for thymocyte development.

Hence, to clarify downstream signalling events, we performed an unbiased proteomic search for novel PKD substrates in thymocytes. To identify proteins that exhibit TCR-induced phosphorylation in WT but not PKD-deficient thymocytes, phosphoproteins from unstimulated WT, stimulated WT and stimulated PKD2/3 thymocytes were labelled with Cy2 (blue), Cy3 (red) and Cy5 (green) fluorescent markers, respectively, and analysed by two-dimensional fluorescence difference gel electrophoresis (2D-DIGE)35. We detected six ‘red’ spots representing proteins that were phosphorylated in a stimulation- and PKD-dependent manner (Fig. 8a). These spots were identified to be pro-IL-1β, SHP-1, NCK1 and Gads by mass spectrometry (MS) (Fig. 8a,b; Supplementary Data 1). SHP-1, NCK1 and Gads were reported to be involved in T cell development17,34,35. Phosphate-affinity SDS–PAGE (Phos-tag) western blotting33 showed that the migration of SHP-1, NCK1 and Gads was retarded (indicating phosphorylation) in stimulated-WT thymocytes (Fig. 8c; Supplementary Fig. 3 and Supplementary Data 2). Notably, these sites are similar in sequence to the PKD consensus phosphorylation motif, L/V/IxRxxS/T (Fig. 8d)37.

These substrates were also phosphorylated in OT-I thymocytes on stimulation with various OVA peptides as assessed by Phos-tag western blotting. Among these substrates, the phosphorylation of SHP-1 was largely dependent on PKD2/3 (Fig. 9a). In order to further examine SHP-1 phosphorylation in vivo, tryptic phosphopeptides were enriched using TiO2 from each

Figure 5 | Role of PKD in early differentiation of DP thymocytes.

(a) Average cell number of CD69+ DP and CD4+CD8int thymocytes from WT and PKD2/3 mice was analysed by flow cytometry. *P<0.05. (b) Average cell number of CD4+CD8int thymocytes from OT-I or OT-II Tg mice. **P<0.01. (c) CD5 expression on TCRβCD4+CD8int thymocytes from WT and PKD2/3 mice. (d) Average percentage of CD4+CD8int cells from recovery culture in two-step differentiation assay. **P<0.01. (e) Co-receptor downregulation (dulling) on OT-II DP thymocytes with OT-I Tg mice. (f) Percentage of CD69+ cells (upper panels, *P<0.0001) and CD5+ cells (lower panels, *P<0.00001) in DP thymocytes on OVA peptides stimulation. (g) Co-receptor downregulation (dulling) on preselection OT-I DP thymocytes with OVA peptide variants (OVA257–264, T4 and G4). The data are presented as mean ± s.d. of seven mice (a), five mice (b) and triplicate assays (d) and are representative of five (c), three (d) or two (e–g) independent experiments. Unpaired two-tailed Student’s t test is used to calculate P values.
The liquid chromatography (LC)–MS data revealed that a phosphorylated peptide corresponding to amino acid residues 555–570 was increased by TCR stimulation in a PKD2/3-dependent manner (Fig. 9b, Supplementary Fig. 4 and Supplementary Data 3). Tandem mass spectrometry (MS/MS) analysis confirmed phosphorylation at Ser557 of endogenous SHP-1 derived from stimulated-WT thymocytes (Fig. 9c). Finally, we developed an antibody that recognizes the phosphorylated Ser557 in SHP-1. TCR stimulation increased the levels of phospho-Ser557 SHP-1 in thymocytes from WT but not PKD2/3 knockout mice (Fig. 9d). These results indicate that PKD mediates TCR-induced phosphorylation of Ser557 in SHP-1. Importantly, the Ser557 amino acid is highly conserved across mammalian species (Fig. 9e).

**Mutant SHP-1 (SHP-1S557A) suppresses thymocyte development.** To further examine the role of the SHP-1 phosphorylation in T cell development, we turned to the DPK cell line, a pigeon cytochrome c (PCC)-specific DP cell line that retains the potential to undergo differentiation in vitro.28 Stimulation of DPK cells with cognate peptide on I-Ek resulted in downregulation of CD8 expression and differentiation into CD4 SP cells as previously reported28 (Fig. 10a, Mock). Forced expression of a phosphorylation-defective mutant of SHP-1 (SHP-1S557A) reduced the generation of CD4 SP cells (Fig. 10a). Likewise, SHP-1S557A also inhibited CD5 upregulation induced by peptide stimulation (Fig. 10b). These results suggest that SHP-1S557A can suppress TCR signalling in a dominant negative manner and that phosphorylation of Ser557 in SHP-1 promotes TCR signalling.

We finally examined the in vivo relevance of the phosphorylation of Ser557 in SHP-1 by establishing knock-in mice expressing mutant SHP-1 (SHP-1S557A/S557A mice) (Fig. 10c). Thymocytes from SHP-1S557A/S557A mice expressed comparable amount of SHP-1 to that of WT, whereas TCR-induced phosphorylation was impaired (Fig. 10d), indicating that Ser557 is a major phosphorylation site on TCR stimulation. To address the role of this site, we evaluated the ability of SHP-1S557A/S557A thymocytes to develop into SP cells in the context of competition with WT. CD3ε-deficient (CD3εΔ/Δ) mice lacking T cells were transferred with mixed BM cells from CD45.1+ WT and CD45.2+ SHP-1S557A/S557A mice and analysed after T cell reconstitution. SHP-1S557A/S557A thymocytes showed impaired development into CD4 SP population compared with WT thymocytes (Fig. 10e), indicating that Ser557 is required for the optimal generation of CD4 SP thymocytes. Collectively, these results suggest that the phosphorylation of Ser557 in SHP-1 can positively regulate TCR signalling in DP thymocytes to promote T cell development.

**Discussion**

In this study, we established T cell-specific PKD-deficient mice to characterize the role of PKD in T cell development. PKD appears to control the generation of CD4 SP thymocytes through promoting (i) TCR signalling during positive selection, and (ii) CD4 lineage specification. Since the decreased number of CD4+CD8int cells in PKD2/3 knockout mice was restored by Bcl-2 transgene (Fig. 3h), an important function of PKD during positive selection may be promoting survival that allows DP cells to differentiate into the CD4+CD8int stage. On the other hand, defective CD4 lineage specification on loss of PKD is likely due to the incomplete induction of ThPOK during positive selection, as CD4/CD8 ratio was restored by ThPOK transgene, but not by Bcl-2. Optimal ThPOK induction requires ‘duration’ of TCR signalling during positive selection.39 In contrast, the expression of Runx3, a transcription factor essential for CD8 lineage specification, is controlled by TCR-induced immediate upregulation of IL-7R and subsequent IL-7 signalling.40 As Runx3 induction occurred normally in PKD-deficient mice (Fig. 6b), PKD is apparently dispensable for the transient TCR signalling required for Runx3 induction. In this respect, we speculate that another critical role of PKD may be to maintain the ‘duration’ of TCR/co-receptor signalling after sensing a weak-affinity ligand. Consistent with this idea, PKD was efficiently activated by stimulation with weak-affinity peptide variants in preselection DP thymocytes (Supplementary Fig. 5).

PKD2 is abundantly expressed in thymocytes, while the amount of PKD3 is considerably lower. Nevertheless, the strikingly different phenotype of PKD2/3ΔT versus PKD2ΔT mice...
demonstrates that a limited amount of PKD3 is sufficient to support SP thymocyte generation. The redundant functions of PKD2 and PKD3 in T cell development suggest that their substrate specificities are likely to overlap. Indeed, all substrates tested in this study were phosphorylated by both PKD2 and PKD3 with similar efficiencies (Fig. 8d). The PKD consensus phosphorylation motif has been reported to be L/V/IxRxxS/T37. The new phosphorylation sites that we have identified herein are similar but not identical to this motif (Fig. 8f). This finding underscores the important contribution of unbiased phospho-proteomics33,41, in addition to motif-based approaches42, in identifying novel kinase substrates.

It has been suggested that SHP-1 dephosphorylates several key molecules involved in TCR signalling mainly through interactions with ITIM-containing transmembrane proteins17. Recently, Themis, a molecule critical for positive selection, was reported to recruit SHP-1 to antagonize TCR signalling, thereby preventing the apoptosis of DP cells that encountered weak agonists during positive selection43, although some debate exists44. Given that loss of Themis or PKD cause similar defects in T cell development, one might speculate that PKD also antagonizes TCR signalling to ensure positive selection. However, this possibility seems less likely, as the expression levels of CD5, CD69 (Fig. 5f) and Nur77 (Supplementary Fig. 6), which represent the summation of TCR signalling intensity45,46, were reduced in PKD-deficient cells. This finding is consistent with the fact that active PKD induces upregulation of CD5 in thymocytes15. In addition, a ‘selection shift’ from negative

Figure 7 | TCR downstream signalling in PKD-deficient thymocytes. (a) Ca2+ influx in freshly isolated DP thymocytes from WT and PKD2/3ΔT mice after TCR stimulation by CD3 cross-linking using indicated concentration of goat anti-hamster IgG (open arrowhead) followed by the addition of ionomycin (closed arrowhead) was assessed by flow cytometry. (b) OT-I preselection DP thymocytes were stimulated with OVA peptide variants for the indicated times and phosphorylation of PKD and Erk was analysed by immunoblotting. PKD and Erk were used as a loading control. (c) Phosphorylation of CD3ζ, ZAP-70, Erk, p38 and JNK in unstimulated (filled histograms) and anti-CD3-stimulated DP thymocytes (open histograms) was analysed by intracellular staining. (d) Average cell number of TCRbhi CD4 SP thymocytes from WT and truncated PKD2 Tg mice lacking the kinase domain (PKD2ΔKD Tg) was analysed by flow cytometry. Each circle represents an individual mouse. *P<0.01. Data are representative of three independent experiments (a–c). Data are presented as mean ± s.d. of four mice (d, bar graph). Unpaired two-tailed Student’s t test is used to calculate P values.
selection to positive selection has been reported in mice with defective TCR signalling, such as ZAP-70-mutated mice or CD3ζ−/− mice47,48, similar to what we observe herein for PKD2/3ΔT mice (Fig. 4e,f). In contrast, in Themis−/− mice, proximal TCR signalling is enhanced rather than attenuated49. Therefore, although PKD and Themis can both act on SHP-1, they appear to regulate TCR signalling in opposite directions. The generation of Themis−/− × PKD2/3ΔT triple-deficient mice will clarify this issue.

What is the molecular basis for the positive signalling resulting from the phosphorylation of Ser557 in SHP-1? There are at least three possibilities: First, the phosphorylation by PKD may constrain the inhibitory function, such as phosphatase activity, of SHP-1. Alternatively, the phosphorylation might alter the proper cellular localization of SHP-1 away from the area where efficient inhibition of TCR signalling takes place. Indeed, a 6-mer motif including Ser 557 in SHP-1 is reported to regulate its C-terminal phospho-Ser 557, and may recruit positive regulators into the vicinity of the TCR. In every case, it would
be extremely intriguing to identify the protein(s) that selectively bind to Ser557-phosphorylated SHP-1. Johnson et al.51 recently reported that T cell-specific SHP-1 deletion using Cd4-Cre Tg mice had no obvious effect on T cell development. This may be explained by the loss of both positive and negative regulation via SHP-1 in thymocytes lacking SHP-1 protein.

The observation that the defects found in SHP-1S557A/S557A mice are milder than that of PKD2/3ΔT mice could be explained by a possible contribution of other PKD substrates, such as Gads and NCK1. Indeed, co-transfection of phosphorylation-defective forms of three other PKD substrates (Gads, NCK1 and pro-IL-16) together with SHP-1S557A resulted in more severe impairment in CD5 upregulation (Supplementary Fig. 7). Thus, we cannot exclude the possibility that PKD might control T cell development by modulating multiple TCR signalling pathways through various substrates in addition to SHP-1.

Histone deacetylase 7 (HDAC7) is also reported to be a substrate for PKD52 and we confirmed this in vitro kinase assay (Fig. 8d), whereas we could not detect PKD-dependent phosphorylation of HDAC7 in thymocytes by 2D-DIGE.

Figure 9 | SHP-1 is phosphorylated by PKD2 and PKD3 at Ser557 in thymocytes. (a) Phosphorylation of SHP-1 on peptide stimulation. OT-I DP thymocytes were stimulated with the designated OVA peptides for the indicated times. Cell lysates were subjected to Phos-tag immunoblot analysis using anti-SHP-1. Arrowheads indicate phosphorylated SHP-1 that showed stimulation-dependent mobility shift. (b) The extracted ion chromatogram of m/z 496.23096 corresponds to the quadruply charged SHP-1 phosphopeptide (TSpS557KHKEEVYENVHSK), which was increased by TCR stimulation in tryptic phosphopeptides from WT thymocytes and was almost undetectable in those from stimulated PKD2/3ΔT thymocytes. The relative values of the peak area are indicated. Note that the hextuply charged ion peak at a retention time of 21.70 min was unrelated to this SHP-1 phosphopeptide. (c) Phosphorylation of Ser557 was demonstrated by the MS/MS spectrum of the m/z 496.23 ion at a retention time of 7.52 min in tryptic phosphopeptides from stimulated-WT thymocytes. (d) Phosphorylation of Ser557 of SHP-1 was analysed by immunoblotting. Total thymocytes were stimulated by CD3 cross-linking for 2 min and analysed by anti-phospho-SHP-1 (Ser557) Abs. Total SHP-1 was blotted as a control. (e) Schematic structure of SHP-1 and sequence alignment of the C-terminal region of SHP-1 that contains Ser residue corresponding to Ser557 of murine SHP-1 (shown in red). Data are representative of two independent experiments (a–d).
Mock

two-tailed Student’s t test is used to calculate P values.

In this regard, one can speculate that PKD0 and PKC\(\eta\) act as upstream regulators of PKD after TCR engagement in thymocytes. This hypothesis warrants further investigation.

In conclusion, we propose that the PKD represents one of the molecular switches that converts weak TCR engagement to positive selection and CD4 lineage specification. The critical roles of PTKs and PTPs in thymic selection have been clearly established. The present study brings to light the additional importance of serine/threonine kinases as key modulators of TCR signalling cascade regulated by tyrosine phosphorylation during thymic selection.

Methods

Mice. PKD2-floxed and PKD3-floxed mice were generated by homologous recombination-mediated gene targeting in embryonic stem (ES) cells of C57BL/6 and 129 genetic background, respectively. PKD3-floxed mice were backcrossed to C57BL/6 mice and floxed \(129\) genetic background, respectively. PKD3-floxed mice were backcrossed to C57BL/6 mice and floxed 129 genetic background, respectively. PKD3-floxed mice were backcrossed to C57BL/6 mice and floxed 129 genetic background, respectively. PKD3-floxed mice were backcrossed to C57BL/6 mice and floxed 129 genetic background, respectively. PKD3-floxed mice were backcrossed to C57BL/6 mice and floxed 129 genetic background, respectively. PKD3-floxed mice were backcrossed to C57BL/6 mice and floxed 129 genetic background, respectively. PKD3-floxed mice were backcrossed to C57BL/6 mice and floxed 129 genetic background, respectively.

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In this regard, one can speculate that PKD0 and PKC\(\eta\) act as upstream regulators of PKD after TCR engagement in thymocytes. This hypothesis warrants further investigation.

In conclusion, we propose that the PKD represents one of the molecular switches that converts weak TCR engagement to positive selection and CD4 lineage specification. The critical roles of PTKs and PTPs in thymic selection have been clearly established. The present study brings to light the additional importance of serine/threonine kinases as key modulators of TCR signalling cascade regulated by tyrosine phosphorylation during thymic selection.
**Cells and reagents.** DP thymocyte cell line, DPK cells were obtained from J. G. Kaye (The Scripps Research Institute, La Jolla, CA, USA) and maintained in RPMI1640 medium supplemented with 10% fetal bovine serum (FBS) (SIGMA), 100 U/ml penicillin, 100 μg/ml streptomycin and 50 μM 2-mercaptoethanol. Nonidet P-40, 1 mM PMSF, protease inhibitor cocktail, 10 mM NaF and 1 mM benzonase (Novagen) was added to the cultures before the addition of thymocytes (1.5 × 10^6 each of CD45.1 and CD45.2) to the wells coated with 5 × 10^5 thioglycollate-induced peritoneal cells used as APC and pulsed with 10 μM of Mtv-8 or Mtv-9 superantigen. In some wells, OVA peptides followed by centrifugation at 1,000 g at 4°C for 5 min were used as APC and pulsed with peptides before the addition of thymocytes (1.5 × 10^5–2 × 10^5) for 20 h in wells coated with 5 μg/ml anti-TCR and 10 μg/ml anti-CD2. Cells were washed and immediately analysed for CD4 and CD8 expression by flow cytometry (stimulatory culture) or transferred to a new plate, washed extensively, and cultured for another 20 h in same medium, followed by the analysis for the expression of CD4 and CD8 by flow cytometry (recovery culture).

**Co-receptor downregulation assay (Dal) (Supplementary Fig. 8–12).** Co-receptor downregulation was examined using OT-I thymocytes or preselection OT-I thymocytes^11. Overall, 1 × 10^5 thymocytes were suspended in phosphate-buffered saline (PBS) and 1 × 10^5 cells were injected into 8 Gy-irradiated TAP−/− mice. Then, 6 weeks after BMT, mice were dissected and thymocytes were analysed. For mixed BMT, CD8α−/− mice were transferred with 5 × 10^5 each of CD45.1+ WT and CD45.2+ SHP−/−S557A/S557A BM cells and analysed 4 weeks after BMT. The expression of CD4 and CD8 by flow cytometry was detected using FlowJo software (Tree Star).

**Ca2+ mobilization.** Thymocytes were freshly isolated and loaded with 1.2 μM Fluo-4 (Molecular Probes) in phenol red-free RPMI1640 containing 5% FBS for 30 min at 37°C. After washing, cells were stained with anti-CD3, phycoerythrin-conjugated anti-CD8 and PerCP-Cy5.5-conjugated anti-CD4 at the concentration described in the ‘Cells and reagents’ section for 30 min on ice. Cells were washed, suspended in PBS containing 5% FBS, pretreated to 37°C before analysis and were kept at 37°C during event collection on FACS Calibur (Becton-Dickinson). For cell stimulation, various concentrations of goat anti-hamster IgG were added to the cultures and 5 × 10^5 cells were added on MHC class I expressed on thymocytes themselves. The expression of CD4 and CD8 was analysed by flow cytometry after 16–20 h.

**Cell survival assay.** Thymocytes were cultured in medium at 37°C with 5% CO2. After incubation for different times, cells were stained with APC-conjugated anti-CD4, PE-conjugated anti-CD8, FITC-conjugated Annexin V in the concentration described in the ‘Cells and reagents’ section and propidium iodide at room temperature for 15 min, and immediately analysed by flow cytometry.
2D-DIGE. Thymocytes were freshly isolated and cultured for 16 h to upregulate TCR. Cells were then incubated with 10 μg/ml anti-CD3 mAb for 30 min on ice and stimulat- ed by cross-linking by 100 μg/ml anti-CD3 antibody IgG Ab for 2 min at 37 °C. The unstimulated WT, stimulated WT and stimulated PKD2/3T cells were lysed and phosphoproteins were enriched using PhosphoProtein Purification Kit (Qiagen). The interfering non-protein materials were subsequently removed by 2-D Clean Up Kit (GE Healthcare). Overall, 30 μg of the resultant phosphoproteins were minimally labelled with 400 pmol Cy2, Cy3 or Cy5 fluorescent dye (CyDye DIGE Fluors, GE Healthcare) for 30 min on ice. After quenching the labelling reaction with 10 μmol lysine, differentially labelled samples were mixed and subjected to first-dimension isoelectric focusing on immobilized pH gradient strips (24 cm; pH 4–7) using an Ettan IPIGphor II system. The second-dimensional SDS–PAGE was carried out on 10% acrylamide gel. The Cy2, Cy3, Cy5 and Cy7 signals were individually acquired with a Typhoon 9400 scanner (GE Healthcare).

Phos-tag western blotting. Lysates from thymocytes were subjected to PAGE clean up kit (Nalacal teaque) before phospho-affinity SDS–PAGE is performed using 6–7.5% acrylamide gels containing 25 or 50 μM Phos-tag acrylamide (Wako chemicals). After electrophoresis, gels were washed in Trans-Blot Turbo transfer buffer (Bio-Rad) added with 1 mM EDTA for 15 min and then incubated in the buffer without EDTA for 15 min. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes using Trans-Blot Turbo Transfer System (Bio-Rad) with the program for HIGH molecular weight protein (25 V, 10 min) and analysed by immunoblotting. For non-phosphorylated (NP) treatment, lysates were treated with λ-PP for 30 min at 30 °C prior to using PAGE clean up kit.

Preparation of GST fusion proteins. SHP-1, Gads and NCK1 cDNAs were cloned into pGEX-6P-1 vector (GE Healthcare). For the mutant GST fusion proteins, Ser57 and Ser591 of SHP-1, Ser186 of Gads and Ser57 of NCK1 were changed to alanine by site-directed mutagenesis. The vectors were transformed into E. coli BL21 and GST fusion proteins were purified using glutathione sepharose (GE Healthcare).

In vitro kinase assay. For in vitro kinase assay using [γ-32P]ATP, 2 μg GST-SHP-1, GST-Gads and GST-NCK1 were incubated with 50 ng active PKD2 or PKD3 (Carma Bioscience) and 100 μM [γ-32P]ATP (5 μCi) in 20 μl kinase buffer (5 mM MgCl2 and 20 mM Tris–HCl, pH 7.5) for 30 min at 30 °C. The reaction was stopped by addition of sample buffer followed by heating at 95 °C for 5 min. Half of the sample was subjected to 5–20% gradient SDS–PAGE and CBB staining. Phosphorylated proteins were visualized by autoradiography.

Identification of proteins and peptides by LC-MS/MS analysis. The 2D-DIGE gel was silver-stained (Thermo Scientific) and protein spots were excised and digested with trypsin (Promega). The obtained peptides were analysed by a capillary liquid chromatography system (Waters/Micromass) connected to a Q-TOF Ultra mass spectrometer (Waters/Micromass). Raw data were acquired and processed using Masslynx version 4.0 (Waters/Micromass) to generate a peak list file for MS/MS ion search. The peak list file were searched against the NCBI non-redundant protein database restricted Mus musculus list file for MS/MS ion search. The resultant MS/MS ion search on Mascot search engine. The false discovery rate was set to 0.01. To generate extracted ion chromatograms, the raw data were processed using Xcalibur software (Thermo Fisher Scientific).

Data availability. The mass spectrum proteomics have been deposited in the JPOST repository (https://repository.jpostdb.org) under the accession codes JPST000031, JPST000032 and JPST000033. All other data supporting the findings of this study are available within the article and Supplementary Information, and can also be obtained from the corresponding author upon request.

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Author contributions
E.I. performed experiments, analysed data and wrote the manuscript; H.K. performed experiments and analysed data; T.Y., M.O., K.A. and T.K. contributed to development of the mice; T.S. provided experimental mice and S.Y. designed the research, performed experiments and wrote the manuscript.

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