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Protein kinase D2 has a restricted but critical role in T-cell antigen receptor signalling in mature T-cells

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PKD (protein kinase D) 2 is a serine/threonine kinase activated by diacylglycerol in response to engagement of antigen receptors in lymphocytes. To explore PKD2 regulation and function in TCR (T-cell antigen receptor) signal transduction we expressed TCR complexes with fixed affinity for self antigens in the T-cells of PKD2-null mice or mice deficient in PKD2 catalytic activity. We also developed a single cell assay to quantify PKD2 activation as T-cells respond to developmental stimuli or engagement of α/β TCR complexes *in vivo*. Strikingly, PKD2 loss caused increases in thymic output, lymphadenopathy and splenomegaly in TCR transgenic mice. The precise magnitude and timing of PKD2 activation during T-cell development is thus critical to regulate thymic homoeostasis. PKD2-null T-cells that exit the thymus have a normal transcriptome, but show a limited and abnormal transcriptional response to antigen. Transcriptional profiling reveals the full consequences of PKD2 loss and maps in detail the selective, but critical, function for PKD2 in signalling by α/β mature TCR complexes in peripheral T-cells.

Key words: cytokine, lymphocyte, pre-cell antigen receptor, protein kinase D (PKD), T-cell antigen receptor (TCR), thymus.

INTRODUCTION

The TCR (T-cell antigen receptor) controls the function of peripheral T-lymphocytes during adaptive immune responses. Antigen receptors also control T-cell development in the thymus and ensure that thymocytes follow a differentiation program in the thymus with two final objectives: the acquisition of MHC-restricted specific functions, that will assure T-cell function during immune responses; and deletion of potentially auto-reactive cells, crucial for central tolerance. Antigen receptors signal by activating cytosolic tyrosine kinases including the Src family kinases Lck/Fyn and the Syk family kinases ZAP70 [ζ-chain (TCR)-associated protein kinase 70 kDa] and Syk. These then phosphorylate adapter molecules such as LAT (linker of activated T cells) and SLP76 [SH2 (Src homology 2) domain-containing protein of 76 kDa] [1]. The key signalling 'second messengers' that mediate TCR function include calcium, PI(3,4,5)P_3 [phosphatidylinositol (3,4,5)-triphosphate] and polyunsaturated DAGs (diacylglycerols) [2]. A major research challenge is to understand the effector pathways for these different signalling molecules that co-ordinate T-cell development in the thymus and T-cell function in the periphery.

DAG signalling is known to be important for both thymocyte and peripheral T-cell function as judged by the T-cell developmental defects caused by loss of DGKs (DAG kinases). For example, thymocytes that lack both DGKα and DGKζ fail to produce normal peripheral T-cells [3]. Moreover, deficiency of either DGKα or DGKζ boosts peripheral T-cell activation and prevents anergy induction [4,5]. Hence controlling the strength and timing of DAG signalling is essential for normal T-cell homoeostasis in the thymus and periphery. In this context, thymocytes and peripheral T-cells express high levels of PKD (protein kinase D) 2, a DAG-binding serine/threonine kinase [6]. PKD2 activation by antigen receptors requires PKC (protein kinase C)-mediated phosphorylation of two serine residues within the activation loop of the catalytic domain, but is also initiated by DAG binding to the N-terminus of PKD [7,8]. PKD2 is thus positioned to control DAG-mediated signalling in the context of TCR signal transduction. To explore the role of PKD2 as a DAG effector in T-cells we recently generated PKD2-null mice and mice deficient in PKD2 enzymatic activity via homozygous expression of PKD2 S707A/S711A knockin alleles (PKD2$^{SSAA/SSAA}$) [6]. These PKD2 mutant mice have normal numbers and frequencies of thymocyte subpopulations. There are also normal numbers of peripheral T-cells in PKD2-null mice, although these cells appear anergic and fail to produce IL (interleukin)-2 or IFN (interferon)-γ when activated via their TCR. Moreover, PKD2 is critical for optimal T-cell-dependent antibody responses *in vivo* [6]. One interpretation of these data is that PKD2 is not required for antigen receptor function in the thymus, but has a unique role to mediate antigen receptor function in peripheral T-cells. However, it is also possible that the peripheral T-cell defects observed in PKD2-null mice reflect that PKD2 loss results in abnormal thymocyte selection. PKD2 deficiency may thus select for the development of 'anergic' or unresponsive T-cells. It must be emphasized that developmental deficiencies in the thymus can be compensated and masked by changes in the TCR repertoire and by changes in the expression of regulatory co-receptors.

The complex process of thymocyte selection is difficult to explore in mice with a polyclonal T-cell repertoire and is more readily dissected by working with thymi that express a TCR complex with known specificity and hence a fixed repertoire.

Abbreviations used: APC, allophycocyanin; Ccl, chemokine ligand; Cy7, indotricarbocyanine; DAG, diacylglycerol; DAVID, database for annotation, visualization and integrated discovery; DN, double negative; DGK, DAG kinase; DP, double positive; E15, embryonic day 15; IFN-γ, interferon γ; IL, interleukin; FBS, fetal bovine serum; GFP, green fluorescent protein; HDAC7, histone deacetylase 7; LCMV, lymphocytic choriomeningitis virus; NF-κB, nuclear factor κB; NP-40, Nonidet P40; Nr4a, nuclear receptor subfamily 4A; PE, phycoerythrin; PKD, protein kinase D; SP, single positive; TCR, T-cell antigen receptor; TNF, tumour necrosis factor.

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The array data reported will appear in the NCBI Gene Expression Omnibus Database under the accession code GSE33942.

Accordingly, to explore in detail the role of PKD2 in thymocyte development we backcrossed mice expressing defined α/β TCRs to either PKD2 null mice or mice deficient in PKD2 catalytic activity. We also developed a single cell assay to quantify PKD2 activation as thymocytes respond to developmental stimuli or expression of α/β TCR complexes *in vivo*. The salient finding is that the precise magnitude and timing of PKD2 activation during T-cell development is critical. Premature activation of PKD2 is shown to suppress pre-T-cell proliferation and constrain thymic output. These results thus reveal an unexpected role for PKD2 as a sentinel to control abnormal proliferative responses *in vivo*. PKD2 also controls a restricted, but crucial, subset of the α/β TCR-mediated transcriptional program in peripheral T-cells.

MATERIALS AND METHODS

Mice

PKD2-null and PKD2^{SSAA} (PKD2$^{S701A/S711A}$) knockin mice (described previously in [6]), P14, OTI and OTII TCR transgenic mice were bred and maintained under specific pathogen-free conditions in the Wellcome Trust Biocentre at the University of Dundee (Dundee, U.K.) in compliance with U.K. Home Office Animals (Scientific Procedures) Act 1986 guidelines as described previously [6,9,10].

Cell preparation and culture

Spleens, thymi and lymph nodes were removed from 2–4-month-old mice, disaggregated and red blood cells were lysed as required before the lymphocytes were suspended in RPMI 1640 medium containing L-glutamine (Invitrogen) with 10 % (v/v) heat-inactivated FBS (fetal bovine serum), 50 units/ml penicillin, 50 μg/ml streptomycin and 50 μM 2-mercaptoethanol. Total cell numbers were assessed by flow cytometry using AccuCheck Counting Beads (Invitrogen). For primary T-cell activation, OTI-TCR transgenic lymph node cells were activated with the ovalbumin-derived peptide SIINFEKL (2 μM) and P14-TCR transgenic cells were activated with the LCMV (lymphocytic choriomeningitis virus)-derived peptide gp33-41 (KAVYNFATM; 10 μM) for the indicated times at a density of $\sim 3–4 \times 10^6$ cells/ml.

Western blot analysis

Protein expression and phosphorylation was assessed using standard Western blotting protocols. Briefly, cell lysates (3×10^7 cells per ml of lysis buffer) were prepared on ice using NP-40 (Nonidet P40) lysis buffer [50 mM Hepes (pH 7.4), 75 mM sodium chloride, 1 % NP-40, 10 mM sodium fluoride, 10 mM iodoacetamide, 1 mM EDTA, 40 mM 2-glycerophosphate, protease inhibitors and 1 mM PMSF], and then centrifuged at 16 000 ***g*** for 10 min at 4 °C. Protein samples were separated by SDS/PAGE (4–12 % Bis/Tris gels; Invitrogen), transferred on to PVDF membranes and blocked with 5 % (w/v) non-fat dried skimmed milk powder in PBS containing 0.05 % Tween 20. Blots were probed with antibodies recognizing phosphorylated and non-phosphorylated PKD2, as indicated. Generation and characterization of pan and phosphospecific antibodies for PKD has been described previously [6,11].

Flow cytometry

Cells were stained with saturating concentrations of antibody in accordance with the manufacturer's instructions for 20 min at 4 °C in RPMI medium containing 1 % FBS. Antibodies conjugated with FITC, PE (phycoerythrin), APC (allophycocyanin), PE-Cy7 and APC-Cy7 were obtained from BD Pharmingen. Cells were stained for surface expression of the following markers using the antibody clones in parentheses: CD4 (RM4-5), CD8 (53-6.7), CD25 (PC61), CD44 (IM7), CD62L (MEL-14), CD69 (H1.2F3), Thy1.2 (53-2.1), TCR β (H57-597), TCR γ/δ (GL3), TCRβVβ8 (F23.1) and TCRαVα2 (B20.1). Data were acquired on either a FACS Calibur or an LSR2 flow cytometer using CellQuest software or a LSR Fortessa using DIVA software (Becton Dickinson) and were analysed using FlowJo (Treestar) software. Viable cells were gated according to their forward- and side-scatter profiles. The different thymocyte populations were electronically gated based on the following markers: for TCR-transgenic cells, the DN (double negative) population was gated as TCRαVα2$^+$CD4$^-$CD8$^-$ and the DP (double positive) population as TCRαVα2$^+$CD4$^+$CD8$^+$; in non-TCR transgenic mice, the total DN population was gated as Thy1.2$^+$CD4$^-$CD8$^-$TCRγ/δ^- with DN3 and DN4 cells further defined as CD25$^+$CD44$^-$ and CD25$^-$CD44$^-$ respectively; DP cells were gated as Thy$^+$CD4$^+$CD8$^+$; CD4SP (single positive) as TCRβ^{hi}CD4$^+$CD8$^-$; and CD8SP as TCRβ^{hi}CD4$^-$CD8$^+$. In lymph nodes, TCR-transgenic naïve T-cells were gated as TCRαVα2$^+$CD8$^+$. Cells were surface stained with different markers and fixed with Fixation Buffer (eBiosciences) prior to intracellular staining. For assessment of phospho-PKD2 levels, intracellular staining with anti-phospho-PKD2 Ser^{873} antisera was performed in permeabilization buffer (eBiosciences), followed by detection with an anti-(rabbit PE) antibody (Jackson Immunoresearch). Intracellular staining with anti-(phospho-S6 Ser$^{235/236}$) (Cell Signaling Technology) was performed after permeabilization in 90 % methanol (v/v) for 5 min at -20 °C followed by detection with an anti-(rabbit PE) antibody. For cell-cycle analysis, cells were incubated in RPMI medium containing 1 % (v/v) FBS and 5 μg/ml Hoechst (Invitrogen) for 30 min at 37 °C prior to standard cell-surface staining.

OP9 cultures, retroviral production and cell transduction

OP9 bone marrow stromal cells expressing OP9-DL1 [12] were a gift from Professor Juan Carlos Zúñiga-Pflücker (Department of Immunology, University of Toronto, Toronto, Canada). OP9-DL1 cells were maintained in αMEM (α mimimal essential medium; Invitrogen) supplemented with 50 μM 2-mercaptoethanol, 100 units/ml penicillin, 1 mg/ml streptomycin and 20 % heat-inactivated FBS. The total DN subset was obtained by depleting thymic populations of CD4- and CD8-expressing cells using CD4 and CD8 isolation kits and an AutoMacs magnetic cell sorter (Miltenyi), and co-cultured on OP9-DL1 cell monolayers in the presence of 5 ng/ml of IL-7 (Peprotech) for the indicated times. On the day of harvest thymocytes were filtered through 50 μm filters to remove the OP9-DL1 cells before developmental progression of T-cell lineage and cell numbers were assessed by flow cytometry. Numbers were normalized to 10^4 DN cells seeded on OP9-DL1 cells at day 0 of the culture. A constitutively active PKD construct comprising the PKD catalytic domain has been described previously [13]. This construct was subcloned into the pBMN-Z retroviral vector (Addgene). Retrovirus production and cell infection was performed as described previously [14]. DN thymocytes were infected with the retrovirus prior to co-culture on OP9-DL1 cell monolayers as described above. Numbers were normalized to 10^4 total GFP (green fluorescent protein)-positive cells at day 2 of co-culture.

Fetal thymic organ culture

Thymic lobes from E15 (embryonic day 15) fetuses were harvested and cultured on filters (Whatman) in DMEM (Dulbecco's modified Eagle's medium) supplemented with 50 μM 2-mercaptoethanol, 100 units/ml penicillin, 1 mg/ml streptomycin and 10 % heat-inactivated FBS. On the indicated day, single lobes were harvested, disaggregated and analysed by flow cytometry to determine developmental progression of the T-cell lineage.

Affimetrix GeneChip mouse genome array

Lymph nodes from P14 TCR transgenic PKD2 wild-type and PKD2-null mice were harvested, disaggregated and cultured *in vitro* for 4 h in presence or absence of the LCMV gp33 peptide (3 μM). RNA was extracted using the RNeasy Mini kit (Qiagen) and microarray analysis from quiescent and TCR-triggered cells from three independent replicate samples was performed by the Finnish DNA Microarray Centre at the Centre for Biotechnology, Turku, Finland, using a GeneChip mouse genome 430_2.0 array (Affymetrix). Affymetrix Expression Console v1.1 (Affymetrix) was used to normalize the data. Normalization with MAS5 was used to select the probes present in at least one of the microarray samples, and RMA (robust multichip average) was used for normalization of the data. Multiple Experiment Viewer v4.3 was used for further statistical analysis. Three different sets of comparisons were made: $PKD2^{-/-}$ quiescent relative to $PKD2^{+/+}$ quiescent cells; $PKD2^{+/+}$ TCR-triggered relative to $PKD2^{+/+}$ quiescent cells; and $PKD2^{-/-}$ TCR-triggered relative to $PKD2^{-/-}$ quiescent cells. Probes that showed statistically significant ($P < 0.05$) differences of two-fold or more were identified with the SAM algorithm [15] with the 90th percentile false discovery rate set to 1 %. DAVID [database for annotation, visualization and integrated discovery (v6.7)] was used for identification of annotated genes and for functional pathway analysis. The data discussed in the present paper have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE33942.

Quantitative real-time PCR

mRNA extraction and quantitative real-time PCR was performed as described previously [14] using primers for the Nr4a (nuclear receptor subfamily 4A) family of transcription factors described previously [16].

RESULTS AND DISCUSSION

PKD2 loss causes lymphadenopathy and splenomegaly in TCR transgenic mice

To explore more precisely the role of PKD2 in thymocyte development we backcrossed PKD2-null mice [6] to mice expressing defined α/β TCRs: the OTI and P14 transgenic TCR models that select for class I-restricted CD8 T-cells and the OTII TCR transgenic TCR model which selects class II-restricted CD4 T-cells. The data (Figure 1) show a striking effect of PKD2 loss on T-cell development in the TCR transgenic models. PKD2 loss thus caused a marked increased in total thymic cellularity of OTI and P14 TCR transgenic mice compared with wild-type TCR transgenic mice (Figure 1a) and in particular increased numbers of DP and SP thymocytes (Figures 1b and 1c; see Figure 4a for a schematic representation of T-cell development). PKD2 deletion also increased the numbers of peripheral TCR transgenic T-cells in secondary lymphoid tissue as judged by the increased numbers of PKD2-null TCR transgenic T-cells in the peripheral lymph nodes of OTI and P14 TCR transgenic mice (Figure 1d). This hypercellularity was pronounced and caused a striking splenomegaly and lymphadenopathy in OTI TCR transgenic mice (Figure 1e). The ability of PKD2 deletion to increase the numbers of TCR transgenic thymocytes was not restricted to CD8 T-cells, but was also seen when PKD2-null mice were backcrossed to mice expressing the OTII TCR transgene which efficiently selects class II-restricted CD4 T-cells (Figure 1f).

To assess whether the impact of PKD2 loss on the hyperproduction of TCR transgenic T-cells was caused by the loss of PKD2 catalytic activity, we backcrossed TCR transgenic mice to mice expressing PKD2 S707A/S711A alleles ($PKD2^{SSAA/SSAA}$)[6]. The phosphorylation of Ser^{707} and Ser^{711} is essential for PKD2 catalytic activity [6]. Hence studies of $PKD2^{SSAA/SSAA}$ mutant mice allow an assessment of the importance of PKD2 catalytic activity *in vivo* while bypassing any impact of removing the scaffold function of PKD2. Figure 1(g) shows that TCR transgenic mice that express the $PKD2^{SSAA/SSAA}$ catalytically inactive PKD2 alleles also show a striking hypercellularity of the thymus, the lymph nodes and the spleen compared with the wild-type transgenic mice.

PKD2 is not essential for positive selection, but has a critical role in antigen receptor signal transduction in peripheral T-cells

During their development in the thymus, only those T-cells that recognize self peptide–MHC complexes with intermediate affinity will be selected to continue their differentiation and become SP cells (see Figure 4a). This process, known as positive selection, is controlled by the TCR [17]. As demonstrated above, loss of PKD2 expression or catalytic activity greatly increases the production of TCR transgenic T-cells. However, a key question is whether the peripheral T-cells that develop in PKD2-null TCR transgenic mice are normal. Figure 2(a) shows that PKD2-null P14 TCR transgenic T-cells express normal levels of CD8 and P14 TCR complexes. They also express high levels of CD62L and low levels of CD44 (Figure 2a), a characteristic phenotype of naïve T-cells. We also used Affymetrix microarray analysis to transcriptionally profile peripheral PKD2-null TCR transgenic peripheral T-cells. This would allow us to be sure that there was no compensation for loss of PKD2 and no selection of abnormal T-cells in PKD2-null mice during T-cell development. Approximately 14500 annotated genes were expressed in quiescent TCR transgenic T-cells that are wild-type for PKD2. The microarray analysis confirmed that PKD2-null T-cells lacked mRNA encoding PKD2 (Figure 2b). However, T-cells that develop in the absence of PKD2 had no other significant differences in their patterns of gene expression compared with control naïve cells (Figure 2b and Supplementary Table S1 at http://www.BiochemJ.org/bj/442/b4420649add.htm). The normal transcriptional profile of PKD2-null TCR transgenic T-cells argues that the loss of PKD2 expression increases the quantity of TCR transgenic peripheral T-cells produced, but does not affect their quality. This excludes any essential role for PKD2 in positive selection: the impact of PKD2 loss is thus quantitative, but not qualitative.

We have shown previously that PKD2-null peripheral T-cells have a defect in their ability to produce the inflammatory cytokines IL-2 and IFN-γ in response to triggering of their TCR complexes [6]. The present results now show that this defect is not caused by selection of transcriptionally abnormal T-cells in PKD2-null mice, suggesting a crucial role of PKD2 in TCR-mediated responses in T-cells. But how extensive is the role of PKD2 in TCR-mediated signal transduction? One unbiased way to address this question is to compare the impact of TCR activation on the transcriptional profiles of wild-type and

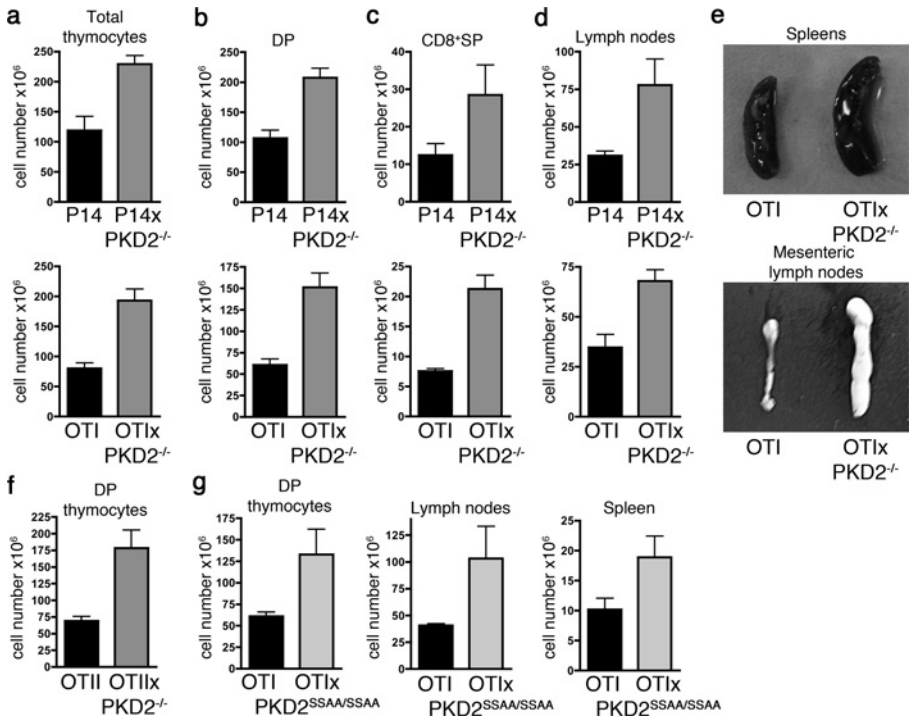

Figure 1 PKD2 regulates thymic output

The data show the number of T-cells in different tissues obtained from wild-type ($PKD2^{+/+}$) or PKD2-null mice ($PKD2^{-/-}$) backcrossed to different TCR transgenic mice, as an averaged value ± S.E.M. (**a**) Total thymocytes in P14 and OTI wild-type or PKD2-null mice ($PKD2^{-/-}$) ($n = 7$–14 mice). (**b**) DP thymocytes in P14 and OTI wild-type or PKD2-null mice ($PKD2^{-/-}$) ($n = 7$–14 mice). (**c**) SP thymocytes ($CD8^+$ SP) in P14 and OTI wild-type or PKD2-null mice ($PKD2^{-/-}$) ($n = 7$–14 mice). (**d**) Naïve T-cells in pooled lymph nodes in P14 and OTI wild-type or PKD2-null mice ($PKD2^{-/-}$) ($n = 3$–12). (**e**) Images depict spleens (upper panel) and mesenteric lymph nodes (lower panel) from age- and sex-matched OTI wild-type and PKD2-null mice ($PKD2^{-/-}$), representative of 7 experiments. (**f**) DP thymocytes from OTII wild-type and PKD2-null mice ($PKD2^{-/-}$) ($n = 5$). (**g**) DP thymocytes, naïve T-cells in spleens and pooled lymph nodes from OTI wild-type and $PKD2^{SSAA/SSAA}$ mutant ($n = 3$–7).

PKD2-null TCR transgenic T-cells. In this respect, TCR triggering with antigen–MHC complexes is known to trigger an extensive change in the T-cell transcriptional program [18]. To address the question of how normal the TCR-mediated response of PKD2-null P14 TCR cells is, we used Affymetrix microarray analysis to compare the transcriptomes of wild-type or PKD2-null P14 TCR transgenic T-cells activated by triggering the TCR with cognate peptide. Figure 2(c) shows that triggering of the TCR complex caused marked changes in the T-cell transcriptional profile of wild-type P14 TCR transgenic cells, increasing the expression of 1292 genes and decreasing the expression of 1405 genes. Strikingly, engagement of TCR complexes on PKD2-null P14 TCR transgenic T-cells also regulated expression of multiple genes and many of the key TCR-mediated changes in gene expression were normal (Figure 2d and Supplementary Table S2 at http://www.BiochemJ.org/bj/442/b4420649add.htm). For example, PKD2-null T-cells could still up-regulate expression of many TCR-induced immediate early genes such as the early growth response genes and Jun family transcription factors (Supplementary Table S2). PKD2 loss also did not influence the ability of TCR triggering to down-regulate expression of genes controlled by the FoxO family transcription factors such as the IL-7 receptor or the transcription factor KLF2 (Krüppel-like transcription factor 2) (Table 1 and Supplementary Table S2), which regulates the expression of naïve T-cell homing receptors such as L-selectin and CCR7 (chemokine receptor 7) [19]. TCR-induced down-regulation of naïve T-cell cytokine and homing receptors is critical for effector T-cell differentiation. The observation that TCR-induced down-regulation of all of these genes occurred normally in PKD2-null T-cells thus indicates that PKD2 is not required for this facet of TCR function (Table 1). In this context, PKD has been suggested to regulate expression of nuclear orphan receptor Nur77 (Nr4a1) via control of HDAC7 (histone deacetylase 7) [20]. Much of this work is based on experiments in lymphocyte cell lines in culture. However, a recent study failed to find a role for HDAC7 in Nur77 regulation during thymocyte selection [21]. Moreover, the microarray data indicated that PKD2-null cells respond to TCR triggering to normally up-regulate Nur77 and other members of the NR4A subfamily of nuclear orphan receptors. To examine this issue more closely we used quantitative PCR analysis. These data (Supplementary Figure S1 at http://www.BiochemJ.org/bj/442/b4420649add.htm) confirm that PKD2 is not essential for TCR-mediated up-regulation of the expression of NR4A family transcription factors.

There was, however, a marked PKD2 requirement for TCR induction of mRNAs encoding multiple cytokines and chemokines, like IL-2, -3 and -6, IFN-γ, Ccl (chemokine ligand) 3 or Ccl4 (Table 1 and Supplementary Table S2). In this context, unbiased pathway analysis of TCR-regulated genes affected by the loss of PKD2 (DAVID Bioinformatics Resources) implicated PKD2 in the regulation of cytokine activity and cytokine–receptor interactions, as well as chemokine activity and chemokine–receptor binding (Table 1 and Supplementary Figure S2 at http://www.BiochemJ.org/bj/442/b4420649add.htm). Here it is important to note that PKD2 loss did not always abrogate cytokine gene induction, but more frequently reduced the magnitude of the TCR-induced increases. Since the differentiation of naïve T-cells is dictated by qualitative and quantitative differences in the strength of antigenic and inflammatory signals during activation [22–25], the reduced magnitude of TCR response observed in

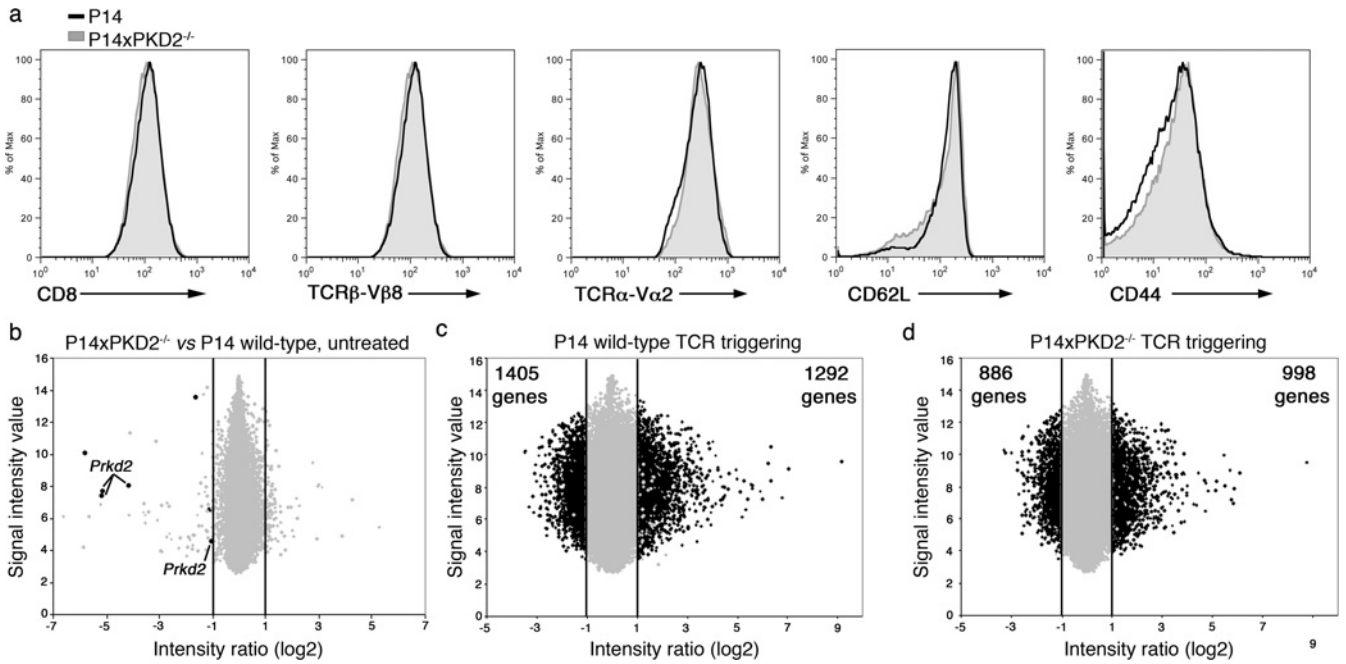

Figure 2 Microarray analysis of P14 PKD2-null T-cells

(**a**) The data show flow cytometric analysis of the expression of the different surface markers in P14 wild-type and P14xPKD2$^{-/-}$ naïve T-cells, and are representative of three independent experiments. The data in (**b**), (**c**) and (**d**) show gene expression profiles of lymph node cells from P14 and P14 PKD2-null mice unstimulated or stimulated for 4 h with LCMV gp33 peptide (TCR triggering) obtained from microarray analysis of the different samples. Data are presented as the distribution of the intensity ratio ($\log_2$-fold change) plotted by the average of the normalized intensity values for probes identified as being present in at least one sample; black dots indicate probes with a significant change ($P < 0.05$) of 2-fold or greater, and grey dots indicate probes with no significant change or a change below 2-fold. Data are representative of a single microarray analysis performed with triplicate samples. (**b**) Comparison of gene expression profiles of untreated lymph node cells of P14 PKD2-null (PKD2$^{-/-}$) relative to P14 wild-type. Lines within the graph indicate probes corresponding to the PKD2 gene (*Prkd2*). (**c**) Comparison of gene expression profiles of P14 wild-type TCR triggered relative to P14 wild-type untreated samples. (**d**) Comparison of gene expression profiles of P14 PKD2-null (PKD2$^{-/-}$) TCR triggered relative to untreated P14 PKD2-null (PKD2$^{-/-}$) samples.

PKD2-null cells anticipates an essential role for PKD2 in T-cell effector function.

Collectively these data show that loss of PKD2 expression increases the thymic output of TCR transgenic mice. The increased numbers of PKD2-null TCR transgenic T-cells that exit the thymus have the transcriptional profile of normal naïve T-cells, but they are unable to correctly reprogram their transcriptome in response to TCR engagement. The role of PKD2 in mediating the TCR-induced transcriptional program is selective, but includes the control of key cytokines and chemokines known to be essential for T-cell effector function. PKD2 thus has a restricted but vital role in antigen receptor signal transduction.

PKD2 activity during T-cell development

The lymphadenopathy and splenomegaly that we observed in PKD2-null TCR transgenic mice was striking (Figure 1e). In this respect, it is known that the thymic output of most TCR transgenic mice is constrained because premature expression of α/β TCR transgenes impairs the proliferative expansion of early T-cell progenitors, resulting in abnormally small thymi in many TCR transgenic mice [26–30]. During normal thymocyte development there is a huge proliferative expansion of cells following the process known as TCRβ selection. This selection process occurs in thymocytes that lack expression of the major histocompatibility co-receptors, CD4 and CD8, and are hence referred to as DN thymocytes. During this stage of thymic development re-arrangement of TCRβ alleles occurs. If the re-arrangement is productive the nascent TCRβ chain will associate with the invariant pre-TCRα chain and the CD3 subunits to form the pre-TCR complex. The expression of the pre-TCR complex at the plasma membrane then initiates the TCRβ selection process whereby pre-TCR-initiated signalling pathways ensure cell survival, exponential proliferation and the transition of DNs to DP thymocytes that now co-express CD4 and CD8 [30–32] (see Figure 4a for a schematic representation of T-cell development).

The observation that premature expression of mature α/β TCR transgenes impairs the proliferative expansion of early T-cell progenitors is thought to reflect that ligand-engaged mature α/β TCR complexes induce a 'strong' signal compared with the 'weak' ligand-independent signal delivered by the pre-TCR [26–31]. The molecular details behind the pre-TCR 'weak' signal and the mature α/β TCR 'strong' signal are not understood. However, the fact that loss of PKD2 expression or catalytic activity massively increases the production of TCR transgenic T-cells raises the possibility that differences in the strength of PKD2 activation underpin the anti-proliferative effects of mature α/β TCR complexes in early T-cell progenitors. To test this hypothesis we examined whether expression of TCR transgenes causes abnormal activation of PKD2 in DN thymocytes. However, to understand the impact of TCR transgene expression on PKD2 activity it was first crucial to understand the normal patterns of PKD2 activation as thymocytes progress through development. One effective way to do this is to use flow cytometric based assays to assess PKD2 activity at the single cell level. A well-characterized marker of endogenous PKD2 catalytic activity is the phosphorylation of Ser^{873}: a conserved autophosphorylation site in the C-terminus of the molecule [11]. A specific antisera selectively reactive with phospho-Ser^{873} has been used extensively in Western blot analysis to monitor cellular PKD2 activity [6].

Table 1 TCR-induced expression of different genes in P14 wild-type and PKD2-null (PKD2$^{-/-}$) T-cells

Expression of selected genes (from microarray analysis) presented as fold change (as unlogged value) of TCR-triggered samples relative to untreated samples in both P14 wild-type and PKD2-null (PKD2$^{-/-}$) cells.

Gene title	Gene symbol	P14 fold change	P14×PKD2$^{-/-}$ fold change
Interleukin 7 receptor	*Il7r*	−5.00	−5.88
Krüppel-like factor 2 (lung)	*Klf2*	−5.14	−5.03
Selectin, lymphocyte	*Sell*	−2.33	−2.78
Chemokine (C-C motif) receptor 7	*Ccr7*	−2.33	−2.33
Nuclear receptor subfamily 4, group A, member 1	*Nr4a1*	6.99	7.81
Nuclear receptor subfamily 4, group A, member 2	*Nr4a2*	9.61	9.23
Nuclear receptor subfamily 4, group A, member 3	*Nr4a3*	8.84	6.72
IL-2	*Il2*	111.99	56.62
IL-2 receptor, α chain	*Il2ra*	21.95	14.23
IL-3	*Il3*	6.96	1.3
IL-6	*Il6*	2.83	1.58
IFN-γ	*Ifng*	42.63	12.7
Chemokine (C-C motif) ligand 3	*Ccl3*	133.05	36.56
Chemokine (C-C motif) ligand 4	*Ccl4*	80.72	44.55
Chemokine (C-X-C motif) ligand 10	*Cxcl10*	30.9	16.21
Chemokine (C motif) ligand 1	*Xcl1*	572.12	440.68
Chemokine (C-X-C motif) ligand 9	*Cxcl9*	6.74	5.46
Chemokine (C-X-C motif) ligand 1	*Cxcl1*	6.52	2.89
Chemokine (C-X-C motif) ligand 2	*Cxcl2*	2.7	1.38
Chemokine (C-X-C motif) receptor 3	*Cxcr3*	2.52	1.78
Chemokine (C-X-C motif) receptor 6	*Cxcr6*	2.25	2.72
TNF	*Tnf*	20.52	21.94
TNF (ligand) superfamily, member 11	*Tnfsf11*	20.28	5.12
Lymphotoxin A	*Lta*	11.93	14.43
Leukaemia inhibitory factor	*Lif*	20.78	6.36
CD69 antigen	*Cd69*	4.74	5.18
B-cell leukaemia/lymphoma 2	*Bcl2*	2.44	2.35
B-cell leukaemia/lymphoma 2-related protein A1a	*Bcl2a1a*	6.65	4.08
Myelocytomatosis oncogene	*Myc*	16.2	11.37
Fas ligand (TNF superfamily, member 6)	*Fasl*	2.21	3.05
Cytotoxic and regulatory T-cell molecule	*Crtam*	81.16	52.38
T-box 21	*Tbx21*	48.9	35.17
Eomesodermin homologue (*Xenopus laevis*)	*Eomes*	2.19	2
Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1	*Nfatc1*	6.38	7.02

The P14 and OTI transgenic TCRs recognise the LCMV peptide gp33–41 in the context of the MHC class I molecule H2^{D} and the ovalbumin peptide SIINFEKL presented by the MHC class I molecule H-2K^{b} respectively. As shown in Figure 3(a), in P14 and OTI TCR transgenic T-cells stimulated via TCR with their cognate peptide, the phospho-Ser^{873} antisera efficiently detect active auto-phosphorylated PKD2 in lysates prepared for Western blotting. The data in Figure 3 show that this antisera can also be used as a probe in flow cytometry to monitor intracellular phospho-PKD2 levels in α/β TCR-triggered peripheral T-cells. In these experiments, peripheral CD8 T-lymphocytes from lymph nodes of transgenic mice expressing either the P14 or the OTI TCR transgenes were TCR triggered with cognate peptide and processed for analysis by flow cytometry. Figures 3(b) and 3(c) (left-hand panels) show that TCR triggering induces strong PKD2 phospho-Ser^{873} immunoreactivity. This response was not observed in PKD2-null T-cells, which proves the specificity of the PKD2 phospho-Ser^{873} antisera (Figures 3b and 3c, right-hand panels), although these latter cells were able to respond to TCR triggering and induce phosphorylation of Ser$^{235/236}$ of the S6 ribosomal subunit in a manner equivalent to that seen in wild-type T-cells (Figure 3d). In additional control experiments to assess the specificity of the antisera as a flow cytometry probe, we examined the PKD2 phospho-Ser^{873} immunoreactivity of T-cells deficient in PKD2 enzymatic activity. For these latter experiments we used T-cells homozygous for expression of PKD2 S707A/S711A knockin alleles. Phosphorylation of Ser^{707} and Ser^{711} are thus critical for PKD2 catalytic activity and PKD2$^{SSAA/SSAA}$ mutant mice cannot autophosphorylate on Ser^{873} [6]. These T-cells thus allow an assessment of the role of PKD2 catalytic activity while maintaining PKD2 protein levels. Figure 3(e, upper panel) shows that peripheral OTI TCR transgenic T-cells that lack PKD2 catalytic activity do not increase PKD2 phospho-Ser^{873} immunoreactivity in response to cognate peptide, although pS6 phosphorylation is effectively induced in these cells (Figure 3e, lower panel). Collectively, the data in Figure 3 show that phospho-Ser^{873} antisera can be used to monitor PKD2 activity by flow cytometry.

The next question we wished to address was whether thymocytes normally activate PKD2 as they progress through T-cell development (see Figure 4a for a schematic representation of T-cell development). The pre-TCR complex is first expressed in a subpopulation of CD4^{-}CD8^{-} DN thymocytes termed DN3 that have successfully rearranged the TCRβ locus, and typically represents approximately 15 % of the total DN3 population. As the pre-TCR expression initiates β selection, cells proceed to the next developmental stage, the DN4 population, in which 100 % of the population express a successfully rearranged TCRβ chain and thus the pre-TCR. We therefore used the flow cytometric-based assays to monitor PKD2 Ser^{873} phosphorylation in *ex vivo* DN3 and DN4 thymocytes from wild-type C57/B6 mice. If the pre-TCR induces PKD2 activation, we expected to detect an increase in PKD2 Ser^{873} phosphorylation in pre-TCR-expressing thymocytes (15 % of DN3 cells and 100 % of DN4 cells). However, there

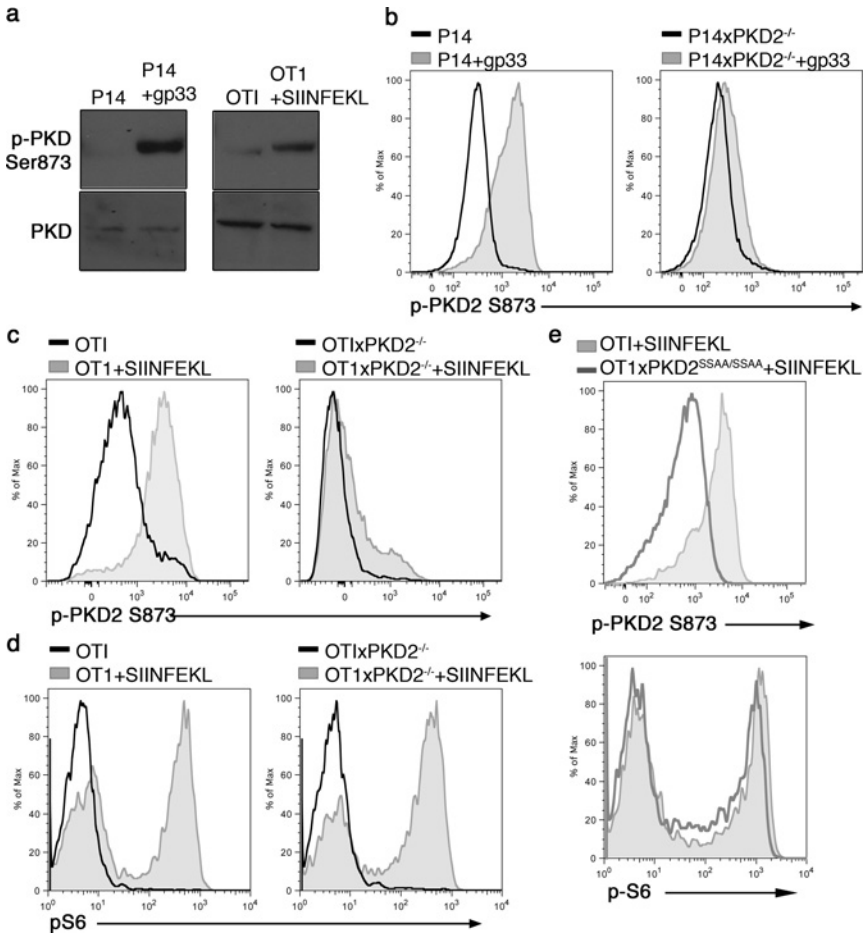

Figure 3 Single cell analysis of PKD2 phosphorylation in naïve T cells

(**a**) Western blot analysis of PKD2 phosphorylation in lymph node cells from P14 (left-hand panels) and OTI (right-hand panels) TCR transgenic mice unstimulated or stimulated with specific peptides (gp33 or SIINFEKL respectively) for 30 min, assessed using phospho-PKD2 Ser^{873} and pan PKD antisera. Western blot are representative of at least five experiments. In (**b**), (**c**), (**d**) and (**e**), data show the level of PKD2 or S6 phosphorylation in unstimulated or TCR-stimulated T-cells cultured for 4 h with specific peptide (gp33 for P14-TCR and SIINFEKL for OTI-TCR), assessed by flow cytometric analysis of intracellular staining with anti-phospho-PKD2 Ser^{873}. (**b**) Histograms compare the level of PKD2 phosphorylation in unstimulated or TCR-stimulated cells in P14 wild-type (left-hand panel) and P14 × PKD2$^{-/-}$ (right-hand panel). Representative of two experiments. (**c** and **d**) Histograms compare the level of PKD2 phosphorylation (**c**) and S6 phosphorylation (**d**) in unstimulated or TCR-stimulated OTI wild-type (left-hand panels) and OTIxPKD2$^{-/-}$ (right-hand panels) cells. Representative of four experiments. (**e**) Histograms compare the level of PKD2 phosphorylation (upper panel) and S6 phosphorylation (lower panel) in unstimulated or TCR-stimulated OTI wild-type and PKD2 $^{SSAA/SSAA}$ cells. Representative of two experiments. P, phospho-.

was no discernable population of DN3 thymocytes selectively immune reactive with the PKD2 phospho-Ser^{873} antisera that could correlate with the subset of DN3 that express the pre-TCR (Figure 4b). In addition, there was also only a very weak phospho-PKD2 Ser^{873} immunoreactivity in the pre-TCR-expressing DN4 thymocytes (Figure 4c). Following TCRβ selection, CD4$^+$CD8$^+$ DP thymocytes undergo TCRα locus rearrangements to produce a TCRα chain that pairs with pre-existing TCRβ and CD3 subunits to form the conventional mature α/β TCR complex. These α/β TCR-expressing DP cells are then positively selected to weakly recognise self-peptide–MHC complexes. Figure 4(d) shows that there was an increase in phospho-PKD2 levels in DP thymocytes and a further increase in phospho-PKD2 levels as cells progressed to the SP stage. Moreover, Figure 4(e) shows that TCRβ^{hi}CD69^{hi} DPs, which represent cells that have received positive signals through their α/β TCR, have increased levels of active autophosphorylated PKD2 compared with preselected DP cells (TCRβ^{int}CD69^{low}). Collectively, these results argue that PKD2 activity is not discernibly induced in response to expression of the pre-TCR, but is increased as thymocytes and peripheral T-cells respond to triggering of their α/β TCR complexes. PKD2 is thus activated by signals emanating from the mature α/β TCR, but is not activated by the pre-TCR.

What impact does expression of mature α/β TCR transgenes have on PKD2 activity in the thymus? In this respect, experiments in transgenic mice that express constitutively active PKD in developing thymocytes revealed that constitutively active PKD could mimic the effects of expressing mature α/β TCR complexes and drive the DN to DP transition of recombinase gene-null T-cell progenitors [13]. Figure 5(a) show that expression of mature α/β TCRs induces premature PKD2 activity in DN thymocytes compared with non-TCR transgenic thymocytes that express the pre-TCR, in which very weak PKD2 activity is detected. This premature activation of PKD2 in DNs occurred in OTI, P14 and OTII TCR transgenic mice (Figure 5a).

Premature activation of PKD2 underpins the anti-proliferative actions of mature α/β TCR complexes in DN cells

Figure 5(a) shows that expression of mature α/β TCR transgenes causes premature activation of PKD2. We therefore wondered

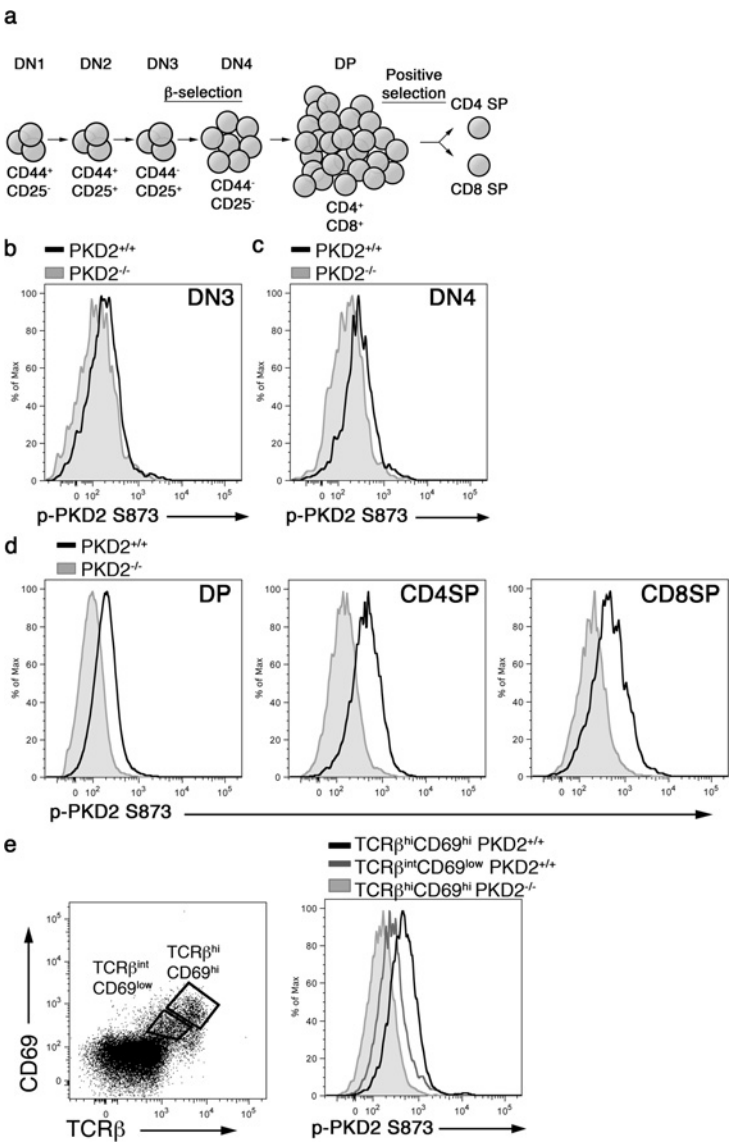

Figure 4 Single cell analysis of PKD2 phosphorylation during thymus development

(**a**) Schematic representation of T-cell development. In (**b**), (**c**), (**d**) and (**e**), the data show the level of PKD2 phosphorylation in the different thymocyte subsets assessed by flow cytometric analysis of intracellular staining with anti-phospho-PKD2 Ser^{873}. $PKD2^{-/-}$ subpopulations were used as controls for background staining. Histograms are representative of four independent experiments. (**b**) Histogram shows the level of PKD2 phosphorylation in DN3 thymocytes. (**c**) Histogram shows the level of PKD2 phosphorylation in DN4 thymocytes. (**d**) Histograms show levels of PKD2 phosphorylation in DP, CD4SP and CD8SP thymocytes. (**e**) Histogram shows the level of PKD2 phosphorylation in pre-selected ($TCR\beta^{int}CD69^{low}$) and post-selected ($TCR\beta^{hi}CD69^{hi}$) thymocytes. Populations were electronically gated as depicted in the dot plot.

whether premature PKD2 activity was responsible for the limited proliferation of TCR transgenic DN thymocytes. To examine this issue we first compared the ability of wild-type and PKD2-null DN cells to proliferate and differentiate into DP cells *in vitro*, using an OP9-DL1 stromal cell co-culture system [12]. Figure 5(b) shows that loss of PKD2 expression does not affect the ability of non-TCR transgenic DN cells to undergo a massive proliferative expansion as they differentiate into DP cells. Taken together with the modest induction of PKD2 activity by the pre-TCR (Figures 4b, 4c and 5a), these data argue that PKD2 is not essential for pre-TCR-induced proliferation or generation of DP thymocytes (Figure 5b). We next used the OP9-DL1 co-culture system to quantitatively analyse the proliferation and differentiation of non-TCR and TCR transgenic DN thymocytes. Figures 5(c) and 5(d) demonstrate the powerful suppressive effect of mature α/β TCRs on DN thymocyte proliferation: P14 and OTI TCR transgenic DNs thus transit to DPs, but fail to undergo proliferative expansion compared with wild-type non-TCR transgenic control DNs. Strikingly, Figure 5(d) shows that PKD2-null or PKD2 catalytically inactive TCR transgenic DNs differentiate to DP, but also proliferate robustly. It is noteworthy that the increase in generation of DP cells in TCR transgenic PKD2-null mice was detected early on during embryonic development. Thymic lobes were isolated from OTI TCR transgenic wild-type and PKD2-null E15 fetuses and cultured *in vitro* in fetal thymic organ culture. PKD2-null OTI TCR transgenic DN thymocytes showed an accelerated transition into the DP stage and more extensive proliferation compared with wild-type OTI DN cells that express active PKD2 (Figure 5e).

The rate of cell proliferation is a balance between the rate of cell-cycle entry compared with the rate of cell death. In this context, TCR transgenic DN thymocytes show no evidence of

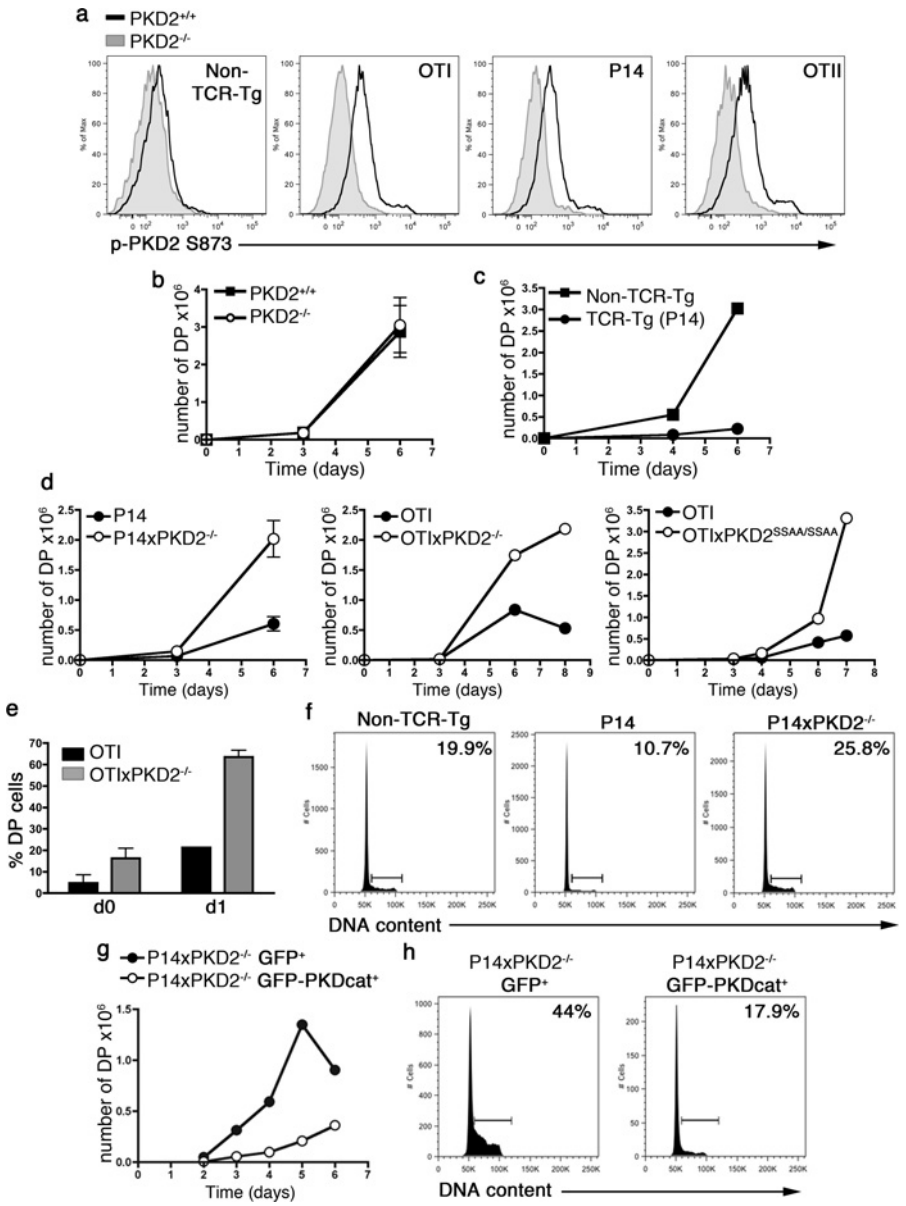

Figure 5 Role of PKD2 in thymus development of TCR transgenic mice

(**a**) The data show the level of PKD2 phosphorylation in the DN thymocyte subset of non-TCR transgenic (Non-TCR-Tg), OTI, P14 and OTII TCR transgenic mice, assessed by flow cytometric analysis of intracellular staining with anti-phospho-PKD2 Ser^{873}. $PKD2^{-/-}$ subpopulations were used as controls for background staining. Histograms are representative of three experiments. (**b**) Number of DP cells generated after co-culture of DN thymocytes from non-TCR transgenic wild-type ($PKD2^{+/+}$) and PKD2-null ($PKD2^{-/-}$) mice with OP9-DL1 stroma. Numbers are an averaged value of three independent experiments $\pm$ S.E.M. (**c**) Number of DP cells generated after co-culture of DN thymocytes from non-TCR transgenic (Non-TCR-Tg) and P14 TCR transgenic (TCR-Tg) mice with OP9-DL1 stroma. Representative of two experiments performed in parallel. (**d**) The data show the number of DP cells generated after co-culture of DN thymocytes from different TCR transgenic mice with OP9-DL1 stroma. Left-hand panel, thymocytes from P14 wild-type and PKD2-null ($PKD2^{-/-}$) mice. Numbers are an averaged value of three independent experiments $\pm$ S.E.M. Middle panel, thymocytes from OTI wild-type and PKD2-null ($PKD2^{-/-}$) mice. Representative of two experiments. Right-hand panel, thymocytes from OTI wild-type and PKD2 S707A/S711A mutant ($PKD2^{SSAA/SSAA}$) mice. Representative of two experiments. (**e**) Percentage of DP cells in E15 fetal thymic lobes from OTI wild-type or PKD2-null ($PKD2^{-/-}$) mice cultured *in vitro*, as an averaged value $\pm$ S.E.M. ($n = 4$ experiments). (**f**) Histograms show flow cytometric analysis of DNA content in *ex vivo* isolated DN thymocytes from non-TCR transgenic (Non-TCR-Tg), P14 wild-type (P14) and P14 PKD2-null (P14xPKD2$^{-/-}$) mice. Numbers within histograms represent the percentages of cells in S/G_2-phase. (**g**) Number of DP cells generated after co-culture of DN thymocytes from P14 PKD2-null ($PKD2^{-/-}$) mice infected with a retroviral vector encoding either GFP alone or GFP–PKDcat with OP9-DL1 stroma. Graph shows number of GFP^+ DP cells and is representative of three independent experiments. (**h**) Histograms show flow cytometric analysis DNA content of DN thymocytes from P14 PKD2-null ($PKD2^{-/-}$) mice infected with a retroviral vector encoding either GFP alone or GFP–PKDcat after 4 days of co-culture with OP9-DL1 stroma. Numbers within histograms represent the percentages of cells in S/G_2-phase.

increased cell death compared with the control DNs (results not shown), but are clearly defective in their ability to enter the cell cycle. Figure 5(f) shows that *ex vivo*-isolated P14 DNs have very few cells in the proliferative (S/G_2) phases of the cell cycle compared with non-TCR transgenic control DNs. However, PKD2-null DNs that express mature TCR complexes show a high frequency of cells in the S/G_2-phases of the cell cycle. Furthermore, the increased cell-cycle entry and increased proliferation of PKD2-null TCR transgenic thymocytes can be reversed by expression of active GFP-tagged PKD as judged by the impact of retroviral expression of a GFP-tagged catalytically active PKD on DP numbers and the cellular DNA profile of DNs from P14 $PKD2^{-/-}$ TCR transgenic mice (Figures 5g and 5h). Figure 5 demonstrates that the suppressive effect of mature

α/β TCR transgenes on DN cell proliferation is mediated by the abnormal activation of PKD2 catalytic activity. This explains why T-cell numbers are increased in TCR transgenic PKD-null mice, both in the thymus and in the secondary lymphoid organs (Figure 1). Collectively these results show that PKD2 mediates the anti-proliferative effect of mature α/β TCR complexes in DN thymocytes.

Conclusions

The present study reveals that controlling the strength and timing of PKD2 activity is fundamental for T-cell homoeostasis in the thymus. Previous studies had shown PKD2-null T-cells did not make IL-2 and IFN-γ in response to antigen receptor engagement [6]. However, a key question was whether any defects in PKD2-null peripheral T-cells were a consequence of abnormal thymocyte development and the selection of anergic unresponsive T-cells in PKD2-null mice. To address this issue we expressed TCR complexes with fixed affinity for self antigens in the thymocytes of PKD2-null mice or mice deficient in PKD2 catalytic activity. Strikingly, PKD2 loss caused a massive increase in thymic output of TCR transgenic mice without affecting the T-cell-selection process. Importantly, PKD2-null TCR transgenic T-cells exit the thymus with the transcriptional program of a normal naïve CD8 T-cell, but they are unable to respond to antigen and switch their transcriptional program to an effector T-cell state in response to TCR triggering. PKD2 expression or catalytic activity is thus not essential for the ability of α/β TCR complexes to select the TCR repertoire. The TCR signalling defects in PKD2-null peripheral T-cells are therefore cell intrinsic and are not caused by abnormal selection of anergic peripheral T-cells.

One salient observation is that PKD2 is not essential for all α/β TCR functions in peripheral T-cells, but rather controls a restricted, but crucial, subset of the α/β TCR-mediated transcriptional program. In this respect, many roles for PKD2 have been described [33]. Most of this work is *in vitro* and uses transformed cell lines [34–36]. The value of the present data is that they explore PKD2 function *in vivo* during normal physiological T-cell responses. The selectivity of the role of PKD2 in the control of gene expression in TCR-activated T-cells was informative and indeed was a valuable way to exclude some of the previously described functions of PKD2 as being relevant to the *in vivo* role of PKD2 in T-cells.

For example, PKD has been implicated in the activation of NF-κB (nuclear factor κB) during oxidative stress signalling promoting cell survival in HeLa cells [36]. In our analysis of the transcriptional program induced by TCR triggering, some of the genes with a reduced induction in PKD2-null cells, like IL-2 or Cxcl10 [chemokine (C-X-C motif) ligand 10], are NF-κB target genes [37,38]. However, PKD2 loss had little or no impact on TCR induction of other NF-κB-controlled genes like TNFα (tumour necrosis factor α), lymphotoxin A, CD69, Bcl2 or c-Myc. Hence, PKD2 loss does globally impair NF-κB function in T-cells. It has also been described that PKD regulates actin-remodelling processes that control cell motility [39,40]. However, PKD2-null mature T-cells undergo normal positive selection and exit the thymus and populate peripheral lymphoid tissue normally, and this would not be possible if they have any intrinsic abnormalities with cell motility or adhesion [41]. We also considered whether PKD2-null T-cells might show abnormalities in the actin reorganisation required to initiate and sustain the adhesive contacts between T-cells and antigen-presenting cells that are required for T-cell activation. However, PKD2-null T-cells showed normal integrin-mediated cell adhesion (S.A. Matthews, unpublished work). As well, any such defects in the ability to sustain and/or disengage cell contacts and cell adhesion would manifest and impact on thymus selection and would have had a wider impact on the TCR-regulated transcriptional program.

One particularly striking observation on the present study was that loss of PKD2 increases thymic output of TCR transgenic mice resulting in lymphoid hyperplasia and splenomegaly. In this context, TCR transgenic mice are widely used and are invaluable for immunological research. However, it has been known for many years that the expression of transgenic α/β TCR complexes inhibits the proliferative expansion of progenitor T-cells [26–28,30,31]. The molecular basis for this effect is unknown, but is thought to stem from the fact that thymocyte proliferation is normally driven by the pre-TCR complex and that expression of α/β TCR complexes somehow delivers a different signal to these cells. This concept has been vague, although it is generally considered that strength of signal is the key: α/β TCRs are thus proposed to negatively regulate DN progenitors because they signal with greater strength than the pre-TCR [27,42]. What has been unknown is whether α/β TCRs increase the strength of a single or multiple signalling module(s). The present results resolve this issue and show that the pre-TCR and α/β TCRs differ in the magnitude of PKD2 activity they induce. PKD2 is not detectably activated by the pre-TCR and is not essential for the function of the pre-TCR in thymus development. In contrast, PKD2 is activated by α/β TCR complexes in the thymus, but is not required to select the TCR repertoire. However, the ability of α/β TCRs to constrain thymic output is strictly PKD2 dependent. In this respect there have been several studies in tumour cell lines *in vitro* that have suggested that PKD2 may be important in controlling the proliferation of transformed cells [43,44]. The present results thus reveal a role for PKD2 as a negative regulator of cell proliferation and unveil an unexpected role for PKD2 as a sentinel to control abnormal proliferative responses *in vivo*.

AUTHOR CONTRIBUTION

Maria Navarro, Linda Sinclair, Carmen Feijoo-Carnero, Rosemary Clarke and Sharon Matthews performed the experiments; Maria Navarro and Doreen Cantrell designed the experiments and analysed the results; and Maria Navarro and Doreen Cantrell wrote the paper.

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SUPPLEMENTARY ONLINE DATA

Protein kinase D2 has a restricted but critical role in T-cell antigen receptor signalling in mature T-cells

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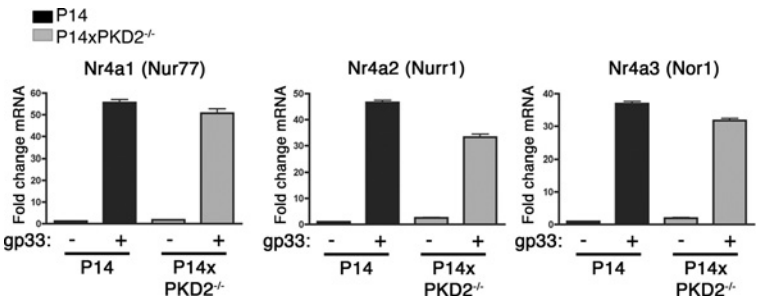

Figure S1 mRNA expression of Nr4a family transcription factors

Histograms show mRNA expression of Nr4a1 (Nur77), Nr4a2 (Nurr1) and Nr4a3 (Nor1) in P14 wild-type and PKD2-null (PKD2$^{-/-}$) naïve T-cells stimulated for 4 h with LCMV gp33 peptide as the fold change compared to unstimulated P14 cells (set as 1). The data were obtained by quantitative real-time PCR.

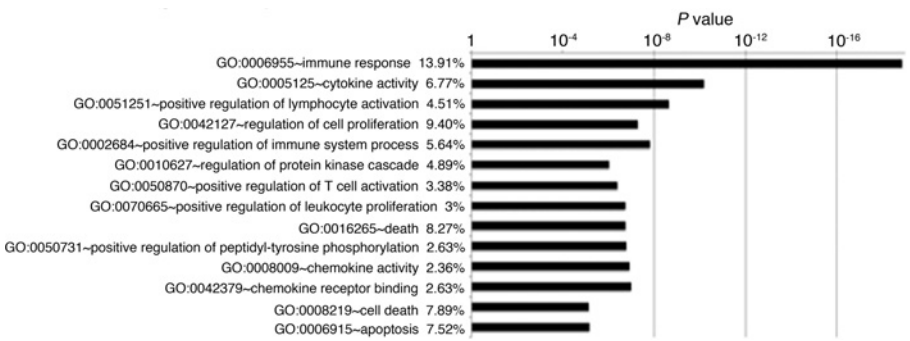

Figure S2 Functional pathway analysis of genes differentially induced by TCR triggering in P14 wild-type and PKD2-null T-cells

To select these genes, we calculated the ratio of the TCR induced fold change in wild-type relative to PKD2-null cells, and selected genes that are differentially expressed between wild-type and PKD2-null cells (1.5-fold expression). This selection yielded a list of 347 probes corresponding to 266 gene IDs that were subjected to functional pathway analysis using DAVID Bioinformatics Resources. The Figure depicts the most significant molecular function and biological processes, indicating *P* value and GO term. Numbers in GO pathways represent the percentage of genes involved in each particular pathway (relative to the 266 genes subjected to analysis).

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Table S1 Microarray analysis data

Full list of genes with a fold change of 2-fold or more in P14 PKD2-null (PKD2$^{-/-}$) cells relative to P14 wild-type untreated cells. The Table includes all probes with a significant change ($P < 0.05$) of two-fold or greater, presented as probe set ID (GeneChip mouse genome 430_2.0 array, Affymetrix), gene symbol, gene name and fold change (as unlogged value) in P14 PKD2-null untreated relative to P14 untreated samples.

Probe set ID	Gene name	Gene symbol	Fold change (unlogged)
1437509_x_at	Protein kinase D2	*Prkd2*	− 2.0611248
1445895_at	T-cell receptor β, variable 8.2	*Tcrb-V8.2*	− 2.1419686
1417184_s_at	Hemoglobin, β adult major chain///hemoglobin, beta adult minor chain	*Hbb-b1///Hbb-b2*	− 3.0955721
1434334_at	Protein kinase D2	*Prkd2*	− 17.799937
1436589_x_at	Protein kinase D2	*Prkd2*	− 35.004105
1434333_a_at	Protein kinase D2	*Prkd2*	− 36.119353
1447456_x_at	Demilune cell and parotid protein 3	*Dcpp3*	− 55.520763

Table S2 Microarray analysis data

Full list of genes with a fold change of 2-fold or more in TCR-stimulated P14 wild-type and PKD2-null (PKD2$^{-/-}$) T-cells. Microarray analysis of the expression profiles of the different samples was compared (P14 wild-type TCR stimulated relative to P14 wild-type untreated samples, and P14 PKD2-null TCR-triggered stimulated relative to untreated P14 PKD2-null samples). The list includes all probes with a significant change ($P < 0.05$) of two-fold or greater found in stimulated P14, P14xPKD2$^{-/-}$ or both, presented as probe set ID (GeneChip mouse genome 430_2.0 array, Affymetrix), gene symbol, gene name and fold change (as unlogged value) in P14 and P14xPKD2$^{-/-}$, relative to their respective untreated samples. Green coloured excel cells represent significant genes ($P < 0.05$) of 2-fold or greater, whereas not coloured cells represent non-significant changes. This Table can be accessed at http://www.BiochemJ.org/bj/442/bj4420649add.htm.

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