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Antigen receptor regulation of phosphoinositide-dependent kinase 1 pathways during thymocyte development

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Abstract Phosphoinositide-dependent kinase 1 (PDK1) is essential for T cell development but little is know about the stimuli that regulate PDK1 signaling in vivo. The thymus contains a heterogeneous mixture of cells at different stages of development making it difficult to use biochemical techniques to examine the activity of PDK1 pathways as thymocytes develop in situ. Herein, we use a single cell assay to quantify activation of the PDK1 target kinase ribosomal S6 kinase 1 (S6K1) in different murine thymocyte subsets immediately ex vivo. This technique allows an assessment of S6K1 activation as thymocytes respond to developmental stimuli in vivo. These studies reveal that only a small percentage of thymocytes show evidence for activation of PDK1 mediated signaling in situ. The thymic subpopulations that contain active PDK1/S6K1 are those known to be responding to signaling by the pre T cell receptor and the mature alpha/beta T cell antigen receptor (TCR). Moreover, loss of antigen receptor signaling in T cell progenitors that cannot rearrange their TCR beta locus prevents in vivo activation of S6K1. The present data identifying antigen receptor signaling as a key activator of PDK1 mediated signaling afford a molecular explanation for the important role of this molecule in T cells.

Keywords: Thymocyte; Kinase; Signal transduction; T cell receptor; Pre T cell receptor; PDK1; S6K1; Thymus; TCR signaling

1. Introduction

T lymphocyte differentiation, proliferation and maturation in the thymus are fundamental processes in the formation of the adaptive immune system. Thymocyte development is controlled by cytokines, chemokines, the pre T cell antigen receptor (preTCR) and the mature T cell antigen receptor (TCR) complex which engage signal transduction pathways mediated by tyrosine kinases, adapters and GTPases [1–4]. Serine kinases are also crucial for T cell development as judged by the impact of deleting phosphoinositide-dependent kinase 1 (PDK1) in T cell precursors [5]. PDK1 phosphorylates a key 'T' loop site within the catalytic domain of AGC kinases including diacylglycerol regulated kinases of the protein kinase C (PKC) family and phosphatidyl inositol-3 kinase (PI3K)-controlled serine kinases such as Akt (protein kinase B, PKB) and the 70 kDa ribosomal S6 kinase 1 (S6K1) [6,7]. A key step in T cell development is characterised by the co-expression of the receptors for major histocompatibility complex (MHC) molecules, CD4 and CD8. Conditional gene deletion of PDK1 in T cell precursors prevents T cell differentiation to the CD4/8 double positive stage of T cell development and also has a role in regulating the size or mass of T cell progenitors [5].

Despite the importance of PDK1 in the thymus, nothing is known about the regulation of PDK1 signaling during thymocyte development. In this respect, in vitro studies in mature peripheral T cells have revealed that PDK1-mediated signal transduction pathways can be triggered by a variety of stimuli including antigen receptors and costimulators, as well as chemokines and cytokines [8–10]. Although thymocyte development is regulated by the antigen receptor and signals from stromal cells stimulated via Notch and/or cytokines such as Interleukin 7 (IL-7) [11–13], little is known about the relative contribution of these signals to the activation of PDK1-mediated pathways in thymocytes in vivo. However, it has recently been reported that expression of a constitutively active PKB can overcome the requirement for Notch in the survival and differentiation signals associated with β-selection [14].

The thymus contains a heterogeneous mixture of cells at different stages of development and thus the analysis of signal transduction pathways induced in situ in these different subsets requires a sensitive and quantitative single cell assay. One such assay has been described that uses flow cytometry to quantify phosphorylation of the ribosomal S6 subunit on S235/236 in single cells [5]. The phosphorylation of S6 is mediated by S6K1 which must itself be phosphorylated by PDK1 at its T loop site to become activated [15–17]. S6K1 has an additional requirement for PDK1 function as its activation is dependent on PKB, another PDK1 substrate, that regulates S6K1 via modulation of Tsc-1/2 function [18–20]. S6 phosphorylation thus requires the coordinate activation of two PDK1 controlled serine kinases and provides a very sensitive measure for PDK1 function because it quantifies phosphorylation of a downstream target of this pathway at a point where there has been considerable signal amplification. Importantly, the ability of S6 phosphorylation to report PDK1 activity has been verified in PDK1 null embryonic stem cells and thymocytes [5,16].

The present results establish that that there is dynamic regulation of S6 phosphorylation in developing thymocytes and that the preTCR and the mature TCR are potent in vivo regulators of this signal response during thymocyte development.

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2. Results

2.1. Activation of PDK1/S6K1signaling by the preTCR

The progression of T cells through the different stages of intrathymic differentiation may be tracked by the sequential pattern of expression of a number of surface markers including CD44, CD25, CD4 and CD8 [2,21]. Early T cell progenitors are negative for both CD4 and CD8 (termed double negative, DN). The earliest of these DN thymocytes are $CD44^{+}CD25^{-}$ (termed DN1); they then go on to acquire CD25 (DN2) where they become committed to the T-cell lineage and begin to rearrange T-cell receptor β-loci. The cells then downregulate the expression of CD44 to become DN3 thymocytes and continue β-chain rearrangements to completion. At the DN3 pre-T cell stage, cells that have successfully rearranged their TCRβ-locus express a functional receptor complex known as the preTCR complex, which comprises the preTCRα chain, the TCRβ subunit and the CD3 antigen. Relocation of the preTCR to the plasma membrane induces DN3s to proliferate rapidly, downregulate CD25 and differentiate into DN4 cells [22].

To explore the role of the preTCR in regulating PDK1 signaling a flow cytometric based assay was used to quantify S6 phosphorylation in ex vivo DN3 and DN4 thymocytes from wild type C57/B6 mice. Immediately ex vivo DN3 cells were heterogeneous for phosphoS6, the majority of cells were phosphoS6^{low} but a significant percentage of cells, approximately 20%, were phosphoS6^{high}. DN4 thymocytes were primarily phosphoS6^{high} (Fig. 1a). Treatment of cells with rapamycin, which inhibits the activity of mTOR, is known to rapidly reverse S6 phosphorylation. The data in Fig. 1a show that phosphoS6 staining in DN3 and DN4 thymocytes was lost when cells were treated with rapamycin. To estimate the maximal potential for S6 phosphorylation, thymocytes were activated pharmacologically with phorbol 12,13-dibutyrate (PDBu), which activates S6K1 via stimulation of Protein Kinase C serine kinases. Stimulation for 30 min with PDBu induced S6 phosphorylation in the phosphoS6^{low} population (Fig. 1a). Moreover, levels of S6 phosphorylation in phosphoS6^{high} cells were similar to those in PDBu activated cells. The requirement of PDK1 for S6K1 activation has been reported previously [5,16]. Fig. 1b shows that DN4 thymocytes from mice with a T cell specific gene deletion of PDK1($LckCre^{+}Pdpk1^{-/-}$) had no basal level of S6 phosphorylation, although these cells were able to respond to pharmacological stimuli and induce S6 phosphorylation in response to PDBu.

The DN3 thymocyte subpopulation can be subdivided into cells that have not yet completed TCRβ locus rearrangements and those that express a functional TCRβ subunit that allows surface expression and signaling of the preTCR complex [23]. Only cells with a functional preTCR transit to the DN4 stage. To examine if preTCR signaling plays a role in regulating S6 phosphorylation in DN3 thymocytes we determined if those DN3 cells with high phosphoS6 correspond to those that express TCRβ subunits. The most sensitive way to assess TCRβ expression in DN subpopulations is to use intracellular (ic) staining protocols. Analysis of icTCRβ expression revealed a small but discernable subpopulation of DN3 cells that expressed icTCRβ, while the majority of DN4 cells expressed icTCRβ (Fig. 2a). Simultaneous analysis of icTCRβ expression and S6 phosphorylation showed that DN3 thymocytes that express icTCRβ were generally phosphoS6^{high} whereas DN3 thymocytes that were icTCRβ null were uniformly phosphoS6^{low} (Fig. 2b). Thus there was a correlation between the expression of the TCRβ and the induction of S6 phosphorylation. To test

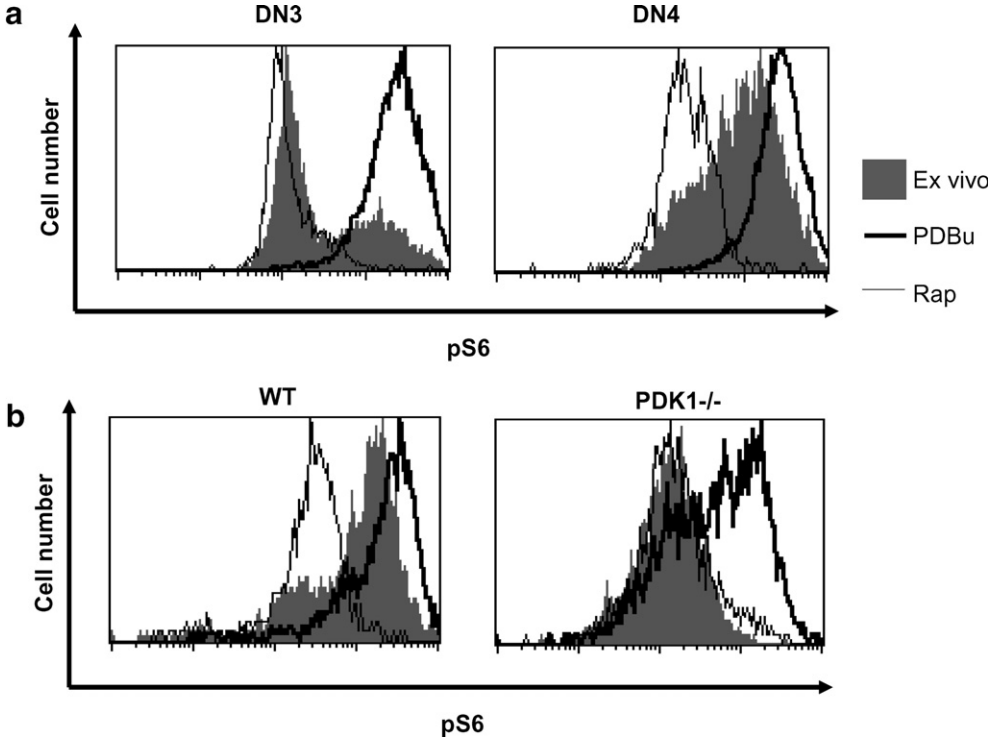

Fig. 1. S6 phosphorylation in DN subsets. Histograms show S6 phosphorylation in (a) WT DN3 (left) and DN4 (right) thymocytes and (b) DN4 thymocytes from WT (left) and $LckCre^{+}Pdpk1^{-/-}$ (right) mice. S6 phosphorylation was measured either immediately ex vivo (filled) or after treatment for 30 min with 20 nM Rapamycin (fine line) or 4 nM PDBu (bold line).

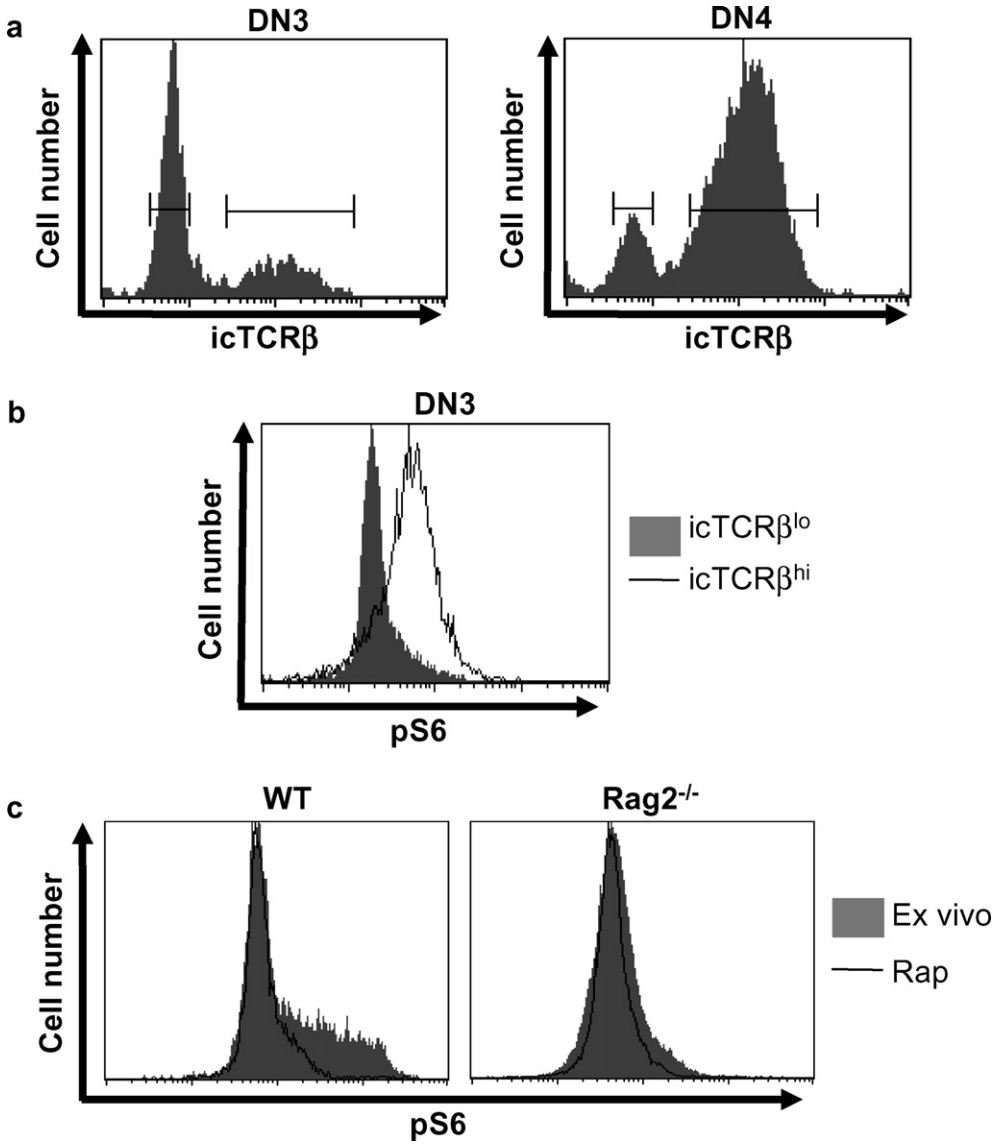

Fig. 2. preTCR signaling is required for S6 phoshorylation. (a) Histograms show intracellular (ic) TCRβ expression in DN3 (left) and DN4 (right) thymocytes. Gates mark icTCRβ^{high} and icTCRβ^{low} populations. (b) S6 phosphorylation in the icTCRβ^{low} (filled) and icTCRβ^{high} (line) populations. (c) Histograms show S6 phosphorylation in DN3 thymocytes from WT (left) and Rag2$^{-/-}$ (right) mice either ex vivo (filled) or treated for 30 min with 20 nM rapamycin (line).

whether the expression of TCRβ was necessary for S6 phosphorylation we assessed phosphoS6 levels in DN3 cells from recombinase gene 2 (Rag2) null mice. Rag2$^{-/-}$ mice are defective in antigen receptor rearrangements and therefore do not express TCRβ. Consequently, thymocytes in Rag2$^{-/-}$ mice are blocked at the DN3 stage of thymocyte development [24]. The Rag2$^{-/-}$ DN3 subset did not contain phosphoS6^{high} cells and levels of S6 phosphorylation corresponded to those seen in rapamycin treated DN3 cells (Fig. 2c). Collectively these results indicate that the induction of S6 phosphorylation in DN3 thymocytes was regulated by the preTCR, revealing a role for the preTCR in the control of PDK1 signalling pathways in thymocytes.

2.2. Inability of pre T cells to sustain S6K1 activity in vitro

Given that γ_c cytokines such as IL-7 control the survival of DN3 thymocytes [25,26], it was striking that DN3 thymocytes lacking TCRβ subunits showed no evidence of S6 phosphorylation. In the absence of a preTCR signal (for example in Rag or preTα null mice) T cell progenitors are blocked at the DN3 stage of development [24,27,28]. If the γ_c cytokine receptor and the preTα are simultaneously removed, DN3 cells do not develop and a DN2 thymic block occurs [25]. However, the absence of S6 phosphorylation in TCRβ null DN3 thymocytes argues that γ_c cytokine signaling or other signals from thymic stroma were not sufficient to induce PDK1-mediated signaling pathways: These pathways were only activated following TCRβ locus rearrangements and preTCR expression.

DN4 thymocytes were homogeneously phosphoS6^{high} (Fig. 1) and express preTCR complexes that regulate their proliferation and differentiation [23]. In situ in the thymus DN4 cells will also be in contact with thymic stroma and respond to signals produced by their stromal environment. This raises the question of whether preTCR signaling alone is sufficient

to induce and sustain S6 phosphorylation or whether other stromal signals regulate PDK1 activity and S6 phosphorylation in T cell progenitors. To assess any contribution of stromal signals to the regulation of S6 phosphorylation we compared levels of phosphoS6 in DN4 thymocytes immediately following removal from the thymus, and after subsequent culture in vitro in the absence of thymic stroma. The data in Fig. 3 show that DN4 thymocytes analysed immediately ex vivo had high levels of S6 phosphorylation but these levels reduced to those seen in rapamycin treated controls after culture in vitro for 2 h. These results indicate that DN4 cells cannot autonomously sustain S6 phosphorylation and signals received from thymic stromal cells contribute to the activation of S6 phosphorylation in this thymocyte subset.

2.3. Activation of PDK1 signaling by the mature TCR during positive selection

Signaling by the preTCR induces thymocytes to co-express receptors for major histocompatibility complex (MHC) molecules, CD4 and CD8 and undergo TCR α-chain rearrangements [29,30]. The fate of these cells (termed CD4/CD8 double positive; DP) is determined by signaling triggered by self peptides presented by MHC molecules on thymic stroma. Insufficient signaling via α/β TCRs results in cell death by neglect; whereas signaling that is too strong induces cell death leading to 'negative' selection of self-reactive T cells. Signaling in the optimal range allows positive selection which leads to survival, proliferation and lineage commitment to mature T cells that are single positive (SP) for either CD4 or CD8 [29,30].

Analysis of phosphoS6 staining in DP cells revealed that the majority of these cells were phosphoS6^{low} (Fig. 4a). Similarly, CD4 and CD8 SP cells were phosphoS6^{low}. There was however a small subset of phosphoS6^{high} cells within the DP population. The majority of DP thymocytes are cells that have failed selection and are destined to die by neglect. However, the DP subset does contain a small percentage of cells in which the TCR is being triggered by MHC/self peptides and these can be identified because they express the CD69 activation marker [31,32]. Analysis of phosphoS6 staining in CD69^{high} and CD69^{low} DP cells revealed that CD69^{high} cells, which are

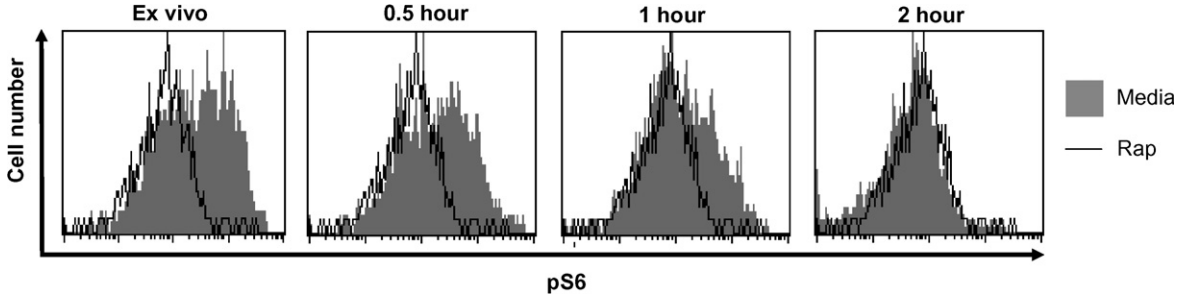

Fig. 3. preTCR signaling is not sufficient for S6 phoshorylation. WT thymocytes were cultured in vitro for the indicated periods. Histograms show S6 phosphorylation in DN4 thymocytes (filled) overlayed with DN4 thymocytes treated for 30 min with 20 nM Rapamycin immediately ex vivo (line) as a negative control.

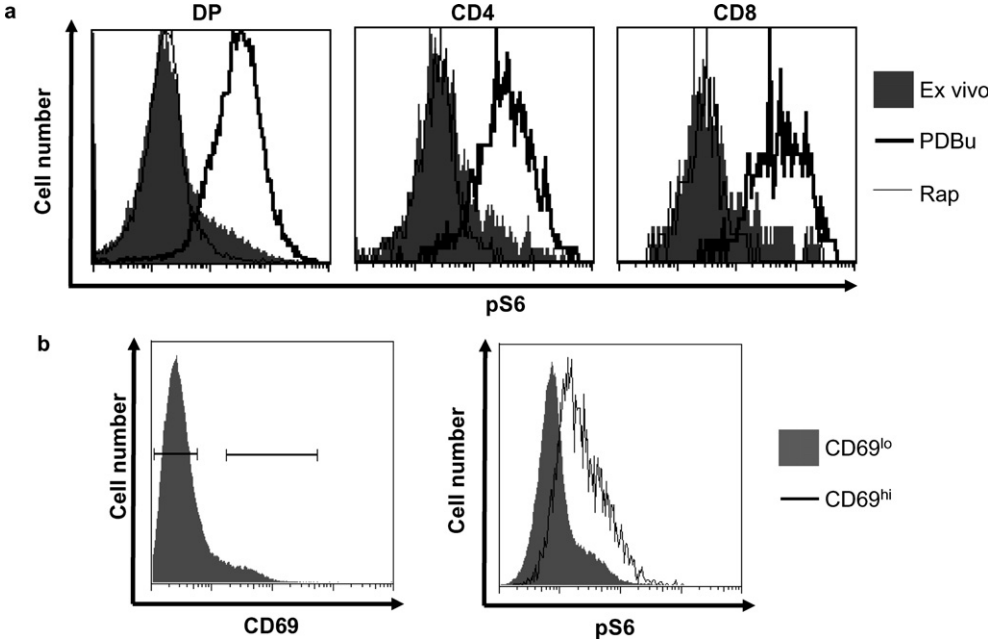

Fig. 4. S6 phosphorylation in DP and SP cell subsets. (a) Histograms show S6 phosphorylation in DP (left), CD4 SP (middle) and CD8 SP (right) thymocytes ex vivo (filled) or treated for 30 min with 20 nM Rapamycin (fine line) or 4 nM PDBu (bold line). (b) Left histogram shows CD69 expression in DP thymocytes. Gates mark CD69^{high} and CD69^{low} populations. Right histogram shows S6 phosphorylation in the CD69^{low} (filled) and CD69^{high} (line) populations.

known to correspond to thymocytes that are TCR activated, stained positive for phosphoS6 while the majority of CD69^{low} cells were negative for phosphoS6 (Fig. 4b). This correlation between CD69 expression and S6 phosphorylation reveals that triggering of the mature α/βTCR regulates PDK1 signaling pathways during thymocyte selection.

3. Discussion

The present results show that in the heterogeneous mixture of cells that comprise an adult thymus a small percentage of cells have activated the PDK1/S6K pathway in situ. These correspond to thymic subpopulations known to be responding to signaling by the preTCR and by the mature α/βTCR. Thymocytes known to be responding to preTCR signals include the DN4 cell subset and a minor subset of DN3 cells that have rearranged their TCRβ locus and we show herein that these have induced S6 phosphorylation in vivo. Strikingly, DN3 cells that have not rearranged their TCRβ locus show no evidence for in situ S6 phosphorylation. The importance of antigen receptor signaling for the induction of S6 phosphorylation is emphasised by the loss of this pathway in T cell progenitors that cannot rearrange their TCRβ locus. Hence, Rag2$^{-/-}$ DN3 cells that cannot undergo TCRβ chain rearrangements, and which fail to express a functional preTCR complex, show no induction of S6 phosphorylation in situ. This latter result was unexpected because previous studies have shown that the DN3 cell subset in preTα$^{-/-}$ mice, which also cannot express the preTCR is maintained by signaling from the γ_c cytokine IL-7 [25]. This conclusion stems from observations that the simultaneous deletion of the γ_c subunit and preTα genes results in the loss of the DN3 subset. IL-7 is known to activate PDK1-mediated serine kinases in mature T cells [33,34]. However, the failure to see S6 phosphorylation in Rag2$^{-/-}$ DN3 cells argues that IL-7 is not a potent activator of this pathway in pre T cells. Nevertheless, the present study does provide an indication that pre T cells cannot autonomously sustain PDK1 signaling when removed from their thymic microenvironment. Hence DN4 cells do not maintain high levels of S6 phosphorylation in vitro. Whether this reflects a requirement for direct cell contact between pre T cells and thymic stroma or the requirement for a soluble factor produced by thymic stroma is not established. However, DN3 cells from Rag2$^{-/-}$ mice grown ex vivo on the BM stromal cell line OP9 require notch signaling for PKB phosphorylation at Ser473 which is required for its full activation [14]. Given that PKB modulates S6K1 activity through the Tsc1/2 complex it is possible that notch signaling from the thymic stroma is required to maintain high levels of S6 phosphorylation in DN4 cells.

T cells that reach the CD4/8 DP stage of thymocyte development can only differentiate to mature T cells if positively selected by self peptides that trigger mature α/βTCR complexes. Thymocytes responding to self peptides can be identified because they transiently upregulate surface expression of the activation marker CD69. The present data show that these CD69^{high} DP cells have also induced S6 phosphorylation. This is a clear indication that the mature TCR triggers PDK1 signaling pathways in situ.

The importance of PDK1 for thymocyte development has been shown previously [5]. Accordingly, deletion of PDK1 in the DN4 thymocyte subset causes a block at this stage of thymocyte development. The failure of PDK1 null thymocytes to differentiate to DP cells precludes analysis of the role of PDK1 in mature α/βTCR signaling. However, the present data establishing the importance of the preTCR for in vivo activation of PDK1/S6K1 afford a molecular explanation for why PDK1 null DN4 thymocytes cannot develop further as differentiation from DN4 cells is dependent on the preTCR.

4. Methods

4.1. Mice

WT C57/B6 and Rag2$^{-/-}$ mice were breed under specific pathogen free conditions. Experiments were approved by the University of Dundee Animal Ethics Committee.

4.2. Flow cytometric analysis

Antibodies conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), allophoycocyanin (APC) and biotin were obtained from Pharmingen (San Diego, CA, USA). TriColour and APC-Cy7 conjugated antibodies were obtained from Caltag (Burlingame, CA, USA). Cells were stained for surface expression of the following markers using the antibodies given in brackets: CD4 (RM4-5), CD8 (53-6.7), CD25 (7D4), CD44 (IM7), CD69 (H1.2F3), Thy1.2 (53-2.1), TCRβ (H57-597), CD3ε (145-2C11), B220 (RA3-6B2), Gr1 (RB6-8C5), CD11b (M1/70), TCRγδ (GL3), and pan-NK (DX5). Cells were stained with saturating concentrations of antibody and data acquired on either a FACS Calibur or LSR1 flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). Events were collected and stored ungated using CellQuest software. Data were analysed using either CellQuest (Becton Dickinson) or FlowJo (Treestar, San Carlos, CA, USA) software. Live cells were gated according to their forward-scatter (FCS) and side-scatter (SSC) profiles.

4.3. Extracellular staining

DN3 and DN4 cells were identified using a panel of biotinylated antibodies to lineage exclude cells expressing CD4, CD8 and CD44 as well as cells of non-T cell lineages (TCRγδ, CD11b, Gr1, B220, and NK). DN3 and DN4 subsets were further distinguished on the basis of CD25 expression [35].

Thymocyte subsets were also identified on the basis of Thy1.2, CD4 and CD8 surface expression. DP thymocytes undergoing positive selection were further identified based on the expression of CD69.

4.4. Intracellular phospho S6 staining

Thymocytes were treated with 20 nM rapamycin or 4 nM PDBu at 37 °C for 30 min, unless otherwise stated. Treatment of cells with rapamycin inhibits the activity of mTor and rapidly reverses S6 phosphorylation [9,36]. PhosphoS6 staining of rapamycin treated cells thus provides an internal negative control as a standard for each sample. Cells were washed and stained for surface markers to define thymocytes subsets, then fixed in 0.5% PFA for 15 min at 37 °C, followed by 15 min in 90% methanol on ice. Following fixation, cells were washed twice in BSA buffer (0.5% bovine serum albumin in PBS) then blocked for 10 min at RT in BSA buffer. Cells were incubated with rabbit anti-phosphoS6 antibody (Cell Signaling

Technologies, Beverly, MA) in BSA buffer for 30 min at RT, washed and incubated with FITC conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch) for 30 min at RT. Samples were washed in BSA buffer and analysed.

4.5. Combined intracellular TCRβ and phosphoS6 staining

Thymocytes were first stained for cell surface markers to define DN3 and DN4 subsets, including biotinylated TCRβ antibody in the lineage exclusion to block cell surface TCRβ binding sites. After fixation in 2% paraformaldehyde for 10 min at room temperature (RT), cells were washed in PBS and permeabilized in saponin buffer (0.5% w/v saponin, 5% FBS, 10 mM Hepes pH7.4 in PBS) for 10 min at RT. Permeabilised cells were incubated with PE-conjugated anti-TCRβ and anti-phosphoS6 antibodies for 45 min in saponin buffer, washed in saponin buffer and subsequently stained with FITC conjugated donkey anti-rabbit IgG. After washing in saponin buffer, cells were analysed.

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