FEBS Letters 580 (2006) 2135–2140

Phosphoinositide-dependent kinase 1 (PDK1) haplo-insufficiency inhibits production of alpha/beta (α/β) but not gamma delta (γ/δ) T lymphocytes

April P. Kelly[a], Heather J. Hinton[b], Rosemary G. Clarke[a], Doreen A. Cantrell[a,*]

[a] *Division of Cell Biology and Immunology, School of Life Sciences, WTB Complex, University of Dundee, Dow Street, Dundee DD1 5EH, UK*
[b] *Cytos Biotechnology AG, Wagistrasse 25, 8952 Zürich-Schlieren, Switzerland*

Received 3 January 2006; revised 20 February 2006; accepted 7 March 2006

Available online 15 March 2006

Edited by Ned Mantei

Abstract In the present study, we have explored the impact of deleting a single allele of PDK1 in T cell progenitors on α/β and γ/δ T cell development. The data show that deleting a single allele of PDK1 allows differentiation of α/β T cells but prevents their proliferative expansion in the thymus. Accordingly, mice with T cells that are haplo-insufficient for PDK1 have reduced numbers of thymocytes and α/β peripheral T cells. T cell progenitors also give rise to γ/δ T cells but in contrast to the loss of α/β T cells in T-PDK1 null and haplo-insufficient mice, there were increased numbers of γ/δ T cells. The production of α/β T cells is dependent on the proliferative expansion of thymocytes and is determined by a balance between the frequency with which cells enter the proliferative phase of the cell cycle and rates of cell death. Herein, we show that PDK1 haplo-insufficient thymocytes have no defects in their ability to enter the cell cycle but show increased apoptosis. PDK1 thus plays a determining role in the development of α/β T lymphocytes but does not limit γ/δ T cell development.

Keywords: PDK1; Gamma delta T lymphocytes; Haplo-insufficiency

1. Introduction

Phosphoinositide-dependent kinase 1 (PDK1) phosphorylates a key threonine within the catalytic domain of a number 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 or protein kinase B (PKB) and the 70-kilodalton ribosomal S6 kinase 1 (S6K1) [1–4]. PDK1 phosphorylation of AGC kinases is essential for their activation and, accordingly, deletion of PDK1 by homologous recombination removes one of the rate-limiting regulators of multiple serine kinases and causes embryonic lethality [5]. To circumvent this prenatal death and explore the role of PDK1 in different tissues, mice expressing PDK1 alleles flanked with the *loxP* Cre excision sequence ($PDK1^{fl\Delta neo/fl\Delta neo}$ mice) have been generated [5]. Thus, the impact of tissue-specific deletion of PDK1 has been investigated in cardiac muscle, where severe defects in cardiac function caused embryonic lethality [6], and in liver which led to glucose intolerance [7]. The importance of PDK1 in T lymphocyte biology has also been explored by analysing T cell development by deleting PDK1 in T cell progenitors in the thymus using Cre expression driven by the proximal $p56^{Lck}$ promoter [8,9]. There are two main lineages of T cells that direct the adaptive immune response: alpha/beta (α/β) T cells that recognise foreign antigen in the context of major histo-compatibility (MHC) molecules and non-MHC restricted gamma delta (γ/δ) T cells (reviewed in [10,11]). Deletion of PDK1 in T cell progenitors in the thymus results in a block in the differentiation of T cells that express α/β TCR complexes [8]. The fate of γ/δ T cells following deletion of PDK1 in T cell progenitors has not been examined. Since the signalling mechanisms that control the development of α/β and γ/δ are divergent [11], we cannot assume that the deletion of PDK1 in T cell progenitors will equally impact α/β and γ/δ T cells.

The signalling mechanisms that control α/β T cell development in the thymus are recapitulated in mature T cells as they respond to foreign antigen. Accordingly, the impact of PDK1 deletion on α/β T cell differentiation indicates that inhibitors of this molecule might be useful immunosuppressive drugs. However, gene deletion studies are of limited use as predictors of the in vivo consequences of pharmacological inhibitors because most inhibitors will reduce rather than totally abrogate enzyme function. It is thus more relevant to probe the cellular impact of reduced expression of PDK1 because this will more faithfully model the in vivo impact of a pharmacological inhibitor of PDK1. In this context, mice expressing hypomorphic PDK1 alleles have been studied [5,12]. These have 10% of normal levels of PDK1 and are viable, although they are born with reduced Mendelian frequency. PDK1 hypomorphic mice have multiple defects including drastically reduced body mass due to cellular growth defects. They also have defects in thymocyte development; α/β T cell differentiation proceeds in PDK1 hypomorphic mice but there is impaired proliferative expansion of pre-T cells, which causes a reduction in numbers of thymocytes and α/β T cells [8].

In PDK1 hypomorphic mice all cells of the body have reduced expression of PDK1 and there are multiple tissue defects [5], which makes it impossible to be sure that the α/β T cell defects are T cell autonomous. An attempt to circumvent this

*Corresponding author. Fax: +44 1382 385783/20 7269 3479.
E-mail address: d.a.cantrell@dundee.ac.uk (D.A. Cantrell).

Abbreviations: DAPI; 4′,6-diamidino-2-phenylindole; DN, double negative; DP, double positive; FSC, forward scatter; MHC, major histocompatibility complex; NLC, normal littermate control; PDK-1, 3′-phosphoinositide-dependent protein kinase 1; PI, propidium iodine; SP, single positive

0014-5793/$32.00
doi:10.1016/j.febslet.2006.03.022

complication was made by performing adoptive transfer experiments with PDK1 hypomorphic bone marrow [8]. These experiments showed that the α/β T cell defects in PDK1 hypomorphic mice could be transferred with PDK1 hypomorphic bone marrow arguing that the effect was autonomous to haematopoietic cells. However, to explore whether there are specifically T cell autonomous effects of reduced PDK1 expression it is necessary to look at T cell function in mice with T cell-specific reduction in PDK1 expression. Accordingly, the present study explores the impact of T cell autonomous PDK1 haplo-insufficiency on thymocyte development. Selective deletion of a single allele of PDK1 in T cell progenitors was achieved by generating *Lck*Cre^{+}PDK1$^{+/flΔneo}$ (T-PDK1$^{+/-}$) mice. The data show that PDK1 haplo-insufficiency impairs thymocyte development and the production of peripheral α/β T lymphocytes. This is T cell autonomous and associated with increased apoptosis of PDK1 haplo-insufficient thymocytes. Interestingly, numbers of γ/δ T cells are increased in mice with a T cell specific deletion of either one or both PDK1 alleles. These results show that α/β but not γ/δ T cell development is sensitive to PDK1 loss.

2. Methods

2.1. Transgenic mice

As described previously, T-PDK1$^{-/-}$ mice were generated by crossing mice whose PDK1 exons 3 and 4 were floxed on both alleles (PDK1$^{flΔneo/flΔneo}$) [5] with mice expressing *Cre* recombinase, driven by the T cell-specific p56^{Lck} promoter, resulting in ablated PDK1 expression in T cells [5,8]. In the current study, PDK1$^{+/flΔneo}$ mice were crossed with mice expressing Cre recombinase under the control of the T cell-specific proximal p56^{Lck} promoter to generate *Lck*Cre^{+}PDK1$^{+/flΔneo}$ (T-PDK1$^{+/-}$) mice, which have reduced PDK1 expression in T cells. All mice used in these experiments were between 5 and 7 weeks of age. Control mice used for analyses of T-PDK1$^{+/-}$ mice were age-matched wild-type littermates.

Mice were bred and maintained in pathogen-free conditions at Wellcome Trust Biocentre, Dundee. Animal experimentation was approved by Home Office Project License PPL60/3116.

2.2. Flow cytometric analysis

Antibodies conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), allophoycocyanin (APC) and biotin were obtained from Pharmingen (San Diego, CA, USA). TriColour conjugated antibodies were obtained from Caltag (Burlingame, CA, USA). Cells were stained for surface expression of the following markers using the antibodies in parentheses: CD4 (RM4-5), CD8 (53-6.7), CD25 (7D4), CD44 (IM7), Thy1.2 (53-2.1), TCRβ (H57-597), CD3e (145-2C11), B220 (RA3-6B2), TCR γ/δ (GL3) and pan-NK (Dx5). Cells were stained with saturating concentrations of antibody in reduced serum (2%) growth media and data acquired on either a Calibur Fluorescence-Activated Cell Sorter (FACS Calibur: Becton Dickinson, Franklin Lakes, NJ, USA) or a LSR1 (Becton Dickinson). 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 (FSC) and side-scatter (SSC) profiles.

CD4 and CD8 DN subsets were gated by lineage exclusion of all CD4, CD8 double positive (DP) and single positive (SP) cells, as well as cells of non-T cell lineages, using a panel of biotinylated antibodies (to CD4, CD8, CD3, TCR γ/δ, B220 and Dx5) [13]. Mature SP thymocytes were defined as Thy-1^{+}, TCRβ^{hi} and SP for CD4 or CD8.

Death of T lymphocytes was analysed by staining with annexin V and 4′,6-diamidino-2-phenylindole (DAPI). Thymocytes were gently harvested and stained in media containing 2.5 mM $CaCl_2$ with CD4, CD8 and Thy-1 fluorochrome-conjugated antibodies to identify thymocyte subsets and annexin V–FITC and DAPI were included to identify dead and dying populations.

2.3. Cell cycle analysis

The cellular DNA content of DN3 and DN4 thymocytes was analysed on live, saponin-permeabilised cells using 4,6-diamidino-2-phenylindole dihydrochloride hydrate (DAPI) staining. Thymocytes (5×10^6) were surface stained for CD25, Thy-1 and lineage markers (including CD44). Cells were incubated for 15 min on ice in 800 μl DAPI staining solution (5 μg/ml DAPI, 0.12% saponin in phosphate buffered saline supplemented with 10 mM HEPES, pH 7.4, and 5% heat inactivated foetal bovine serum) immediately prior to analysis. Events were collected on either a FACS Calibur or an LSR1 with CellQuest software and analysed with a doublet discrimination module, using either CellQuest or FlowJo software.

3. Results

To investigate the role of PDK1 in T lymphocytes, mice with PDK1 null (T-PDK1$^{-/-}$) and PDK1 deficient (T-PDK1$^{+/-}$) T cells were generated. T-PDK1$^{-/-}$ mice have been described previously [8]. These mice have a very small thymus with reduced numbers of thymocytes and almost no mature α/β TCR positive T cells in the periphery. The data in Fig. 1 show that deletion of a single PDK1 allele in pre-T cells also had a severe impact on T cell development. T-PDK1$^{+/-}$ mice thus had fewer thymocytes in total and produced markedly fewer (55%) α/β TCR positive T lymphocytes in the periphery than normal (Fig. 1A and 1C).

The thymus also gives rise to γ/δ T cells and we observed a seven and threefold increase in γ/δ T cells in T-PDK1$^{-/-}$ and T-PDK1$^{+/-}$ thymi, respectively (Fig. 1B). To examine the impact of PDK1 loss on the development of the γ/δ T cell compartment we analysed the spleens of T-PDK1$^{-/-}$ and T-PDK1$^{+/-}$ mice for the presence of γ/δ T cell populations. Increased numbers of γ/δ T cells in the spleens of these mice was observed (Fig. 1D). Numbers of splenic B lymphocytes were not significantly different (Fig. 1E).

A reduction in numbers of α/β T cells can result from impaired thymocyte differentiation or impaired thymocyte proliferation [14,15] and reviewed in [16,17]. T cell differentiation can be staged by expression of the co-receptors for major histocompatibility molecules, CD4 and CD8 [18]. The earliest intra-thymic progenitors are defined by the absence of mature T lineage markers (CD4/8 double negative (DN)) (reviewed in [19]). Following productive rearrangement of the TCRβ locus, the expression of a functional pre-TCR induces a proliferation and differentiation program known as β-selection which can occur just prior to the onset of CD4 and CD8 expression in immature thymocytes [20]. These CD4^{+}CD8^{+} double positive, DP thymocytes rearrange their TCRα locus [21] and, following the expression of a mature TCR α/β complex, these cells are selected to either the CD4^{+} or the CD8^{+} single positive (SP) lineage (reviewed in [22,23]). T-PDK1$^{-/-}$ mice lack the normal complement of CD4/8 DPs and comprise almost entirely of DNs (Fig. 2A and [8]). In T-PDK1$^{-/+}$ mice DPs and SPs were present but consideration of cell numbers showed that DN numbers were moderately increased in T-PDK1$^{+/-}$ mice; normal littermate control (NLC) mice typically had approximately 4×10^6 to 7×10^6 DN cells, whereas T-PDK1$^{+/-}$ mice had 6×10^6 to 10×10^6 DN cells (Fig. 2B, left). In contrast, DP cell numbers were reduced by at least a third in T-PDK1$^{+/-}$ mice and absent in T-PDK1$^{-/-}$ mice (Fig. 2B, right). DP thymocytes normally express a mature cell surface α/β TCR and are selected to either CD4 or CD8 SP T cells (reviewed in [24]). T-PDK1$^{+/-}$ mice had approximately 50% fewer CD4 and CD8