LKB1 Mediates the Development of Conventional and Innate T Cells via AMP-Dependent Kinase Autonomous Pathways

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Abstract

The present study has examined the role of the serine/threonine kinase LKB1 in the survival and differentiation of CD4/8 double positive thymocytes. LKB1-null DPs can respond to signals from the mature α/β T-cell-antigen receptor and initiate positive selection. However, in the absence of LKB1, thymocytes fail to mature to conventional single positive cells causing severe lymphopenia in the peripheral lymphoid tissues. LKB1 thus appears to be dispensable for positive selection but important for the maturation of positively selected thymocytes. LKB1 also strikingly prevented the development of invariant Vα14 NKT cells and innate TCR αβ gut lymphocytes. Previous studies with gain of function mutants have suggested that the role of LKB1 in T cell development is mediated by its substrate the AMP-activated protein kinase (AMPK). The present study now analyses the impact of AMPK deletion in DP thymocytes and shows that the role of LKB1 during the development of both conventional and innate T cells is mediated by AMPK-independent pathways.

Introduction

The adaptive immune response is mediated by T cells that express T cell antigen receptor complexes comprising of highly variable TCRα and β subunits [1]. These T cells can be subdivided into cells that express CD8, the receptor for major histocompatibility antigen complex I (MHC class I), and cells that express CD4, the receptor for MHC class II molecules. CD4 positive T cells can be further subdivided into conventional CD4 T cells, regulatory T cells (Tregs) and Natural Killer T (NKT) cells [2]. Conventional CD4 and CD8 T cells express α/β TCR complexes that recognize peptide/MHC complexes whereas NKT cells express an invariant Vα14 T cell receptor that recognize glycolipid/CD1d antigen complexes (NKTs) and play a role in immune surveillance and immune homeostasis [3]. CD8 T cells can also be subdivided into conventional CD8 cells that express a CD8 αβ heterodimer and CD8 T cell populations that express a CD8αα homodimer [4]. TCRαβ+ CD8αβ+ conventional T cells recirculate between the blood, secondary lymphoid tissue and the lymphatics and respond to immune activation and differentiate to produce cytolytic effector cells. TCRαβ+ CD8αα+ T cells are typically found in the epithelial layer in the gut and play a role in regulating inflammatory immune responses in the gut [5].

The balanced production of different T cell subpopulations, each with unique functions, during thymus development is essential to ensure the function and the homeostasis of the peripheral immune system. Hence, understanding the nature of the signals required for the development of different T cell subpopulations is important. All T cells that express αβ TCR complexes develop in the thymus from progenitors that lack expression of CD4 and CD8, hence termed double negative (DN) thymocytes. At the DN stage of thymocyte development T cell progenitors undergo genetic rearrangement of the TCRβ locus, which leads to the expression of a pre-TCR complex. This immature TCR complex drives DNs to proliferate and differentiate into CD4/8 double positive (DP) thymocytes. DP thymocytes that have successfully re-arranged their TCRα chain will undergo a selection process and differentiate to conventional TCR αβ CD4+ or CD8+ T cells, NKT cells or TCRαβ+ CD8αα+ gut lymphocytes.

In this context, there is currently considerable interest in understanding the signalling pathways that control metabolic checkpoints in T lymphocytes. It is thus relevant that recent studies have shown that the serine/threonine kinase LKB1 (Liver kinase B1 also known as serine/threonine kinase 11 - STK11) is important in controlling metabolic homeostasis in early T cell progenitors in the thymus [6,7]. There is also evidence that LKB1 is important in CD4/CD8 DPs. LKB1 null DPs thus appear to be unable to develop into conventional TCRαβ CD4+ and CD8+ T cells [8,9]. However, there are a number of important unanswered questions about LKB1 and its role in thymus development. For example, is LKB1 required for DP thymocyte survival and does this explain why LKB1 null DPs cannot produce mature SP T cells? To date most studies of LKB1 in DP thymocytes have studied the few DPs that survive LKB1 deletion at the thymocyte progenitor stage and have not looked at the immediate impact of LKB1 loss in DPs. One question is whether LKB1 is important in non-conventional T cells, i.e. TCRαβ+ CD8αα+ IELs...
or TCRαβ+ CD4+ iNKTs? In this respect it is evident that LKB1 is not essential for all T cells. For example, LKB1 has an obligatory role to control survival of T cell progenitors [6,7] but is not essential for the metabolic control of quiescent naïve T cells in the periphery [6]. One other fundamental question is how does LKB1 control T cell development? One proposal is that LKB1 controls thymocyte development via regulation of the adenosine monophosphate (AMP)-activated protein kinase α1 (AMPKα1) [7]. This kinase is phosphorylated and activated by LKB1 in response to cellular energy stresses that cause increases in cellular AMP:ATP ratios [10]. It is a candidate to mediate the role of LKB1 in thymocyte development because in many cell lineages AMPKα1 acts to restore cellular energy balance by terminating ATP consuming processes and stimulating ATP generating pathways [10]. However, the evidence supporting a role for AMPKα1 in thymocyte development stems solely from experiments where overexpression of a constitutively active AMPKα1 construct could promote survival of LKB1 null DP thymocytes [7]. This gain of function strategy does not inform whether AMPKα1 is essential for thymus development. It is thus relevant that mice homozygous for deleted AMPKα1 alleles appear to undergo normal thymocyte development [8,11]. The caveat of these studies is that AMPKα1 null mice on a mixed genetic background are not born at normal Mendelian frequency and indeed global deletion of AMPK results in embryonic lethality on a C57Bl/6 background [11]. The studies to date about the role of AMPKα1 in T cells have thus been on the few mice that can compensate AMPKα1 loss in early embryo development. Accordingly, to directly compare the impact of AMPKα1 deletion and LKB1 deletion on thymus development there is a requirement to compare the consequences of selective deletion of either of these kinases at a defined stage of thymus development. We have therefore used a CD4Cre transgene to delete LKB1 or AMPKα1 floxed alleles at the DP stage of thymocyte development. We found that LKB1 does not regulate survival of DP thymocytes although these cells fail to differentiate to conventional TCRαβ SP cell populations and are also defective in the development of iNKT cells and TCRαβ CD8αα IEIs. In contrast, AMPKα1 null DP cells produce normal numbers of both conventional and innate TCR αβ peripheral T cells. LKB1 is thus essential for the development of both conventional and innate TCR αβ T cells in the thymus but its mode of action is not through the activation of AMPK.

Results

DPs survive without LKB1

To explore the role of LKB1 in DP thymocytes, we backcrossed LKB1fl/fl mice to mice that express Cre recombinase under the control of the CD4 promoter. In this model, cre recombinase is expressed during the transition of DN to DP and this ensures deletion of LKB1 in DP thymocytes (Figure 1A). We noted that there appeared to be some residual LKB1 protein in DP thymocytes probably reflecting some asynchrony of LKB1 loss as thymocytes make the DN to DP transition. LKB1 controls the survival of DN thymocytes [6–8]. It was also suggested that LKB1 null DP cells had survival defects [7]. However, our results indicate that the direct deletion of LKB1 in DP thymocytes did not cause cell death of DPs in vivo. LKB1fl/fl CD4Creαβ mice thus have normal numbers of DPs and there was no evidence for increased apoptosis of these LKB1 null DPs (Figure 1B). Normal DP thymocytes undergo apoptosis if removed from the thymus and cultured in vitro in the absence of thymic stroma. The deletion of LKB1 did not increase the rate at which DP cells die when removed from the thymus (Figure 1C). LKB1 is thus not essential for survival of DP thymocytes in vivo or ex vivo. A further indication of the viability of LKB1 null DP thymocytes comes from analysis of their ability to respond normally to chemotactic stimuli. DP thymocytes express the chemokine receptor CXCR4 and can chemotax on an integrin matrix in response to CXCL12 (SDF-1) [12]. The data show that LKB1-deficient thymocytes migrated normally on fibronectin-coated transwells (Figure 1D). These data indicate that the CXCL12/CXCR4 signalling axis and integrin-dependent adhesion do not require LKB1. They also confirm the viability of LKB1 null DP thymocytes.

Deletion of LKB1 impairs the production of mature αβ T cells.

LKB1fl/fl CD4Creαβ mice had normal numbers of DP thymocytes but produced fewer TCRβα high mature CD4 and CD8 SP thymocytes (Figure 2A–C). The impact of LKB1 loss on the production of CD8 SP thymocytes appeared more severe than the impact on CD4 T cells (Figure 2A and C). LKB1fl/fl CD4Creαβ mice also lacked the normal complement of mature αβ CD4 and CD8 SP cells in secondary lymphoid organs such as the spleen and lymph nodes (Figure 2D). They also did not have a normal frequency of αβ TCR intraepithelial T cells in the small intestine (Figure 2E).

The transition of DPs to SPs can be staged by expression of the cell surface antigen CD69 and by the levels of TCR αβ complex expression [13]. DP thymocytes thus express low level of TCRβ chains and no CD69. If they undergo a successful rearrangement of their TCR alpha locus and express an αβ TCR complex that recognises self peptide MHC complexes in the surface of thymic epithelial cells they are positively selected and either down-regulate CD4 or CD8 molecules and differentiate to SPs. The first indication of successful TCR engagement in DPs is up-regulation of CD69. Cells undergoing selection then up-regulate expression of αβ TCR complexes [13]. The expression of CD69 is then down-regulated while TCRβ expression remains high on the most mature SPs [13]. The analysis of CD69 and TCR levels on thymocytes from the LKB11/0 CD4Creαβ mice shows that LKB1 null DP thymocytes respond to TCR triggering to up-regulate CD69 expression (Figure 3A). However, thymocytes co-expressing high levels of both TCRβ and CD69 are reduced approximately by 50% in LKB11/0 CD4Creαβ thymi. Mature SP thymocytes down-regulate expression of CD24 but increase expression of the adhesion molecule CD62L (L-selectin). In this context, CD24low CD62Lhigh SP cells were almost undetectable in LKB11/0 CD4Creαβ thymi (Figure 3B). These data show that LKB1 is not required for the TCR mediated signalling events that initiate positive selection but LKB1 null thymocytes cannot complete positive selection to produce mature αβ TCR SP thymocytes. LKB11/0 CD4Creαβ thymocytes thus show defective maturation of positively selected SPs rather than a defect in positive selection per se. This explains why LKB11/0 CD4Creαβ mice lack mature αβ T cells in peripheral tissues.

LKB1 is required for NKT cell development

DP thymocytes also differentiate to produce CD4+ NKT cells that have an invariant Vα14 T cell receptor that recognises glycolipid antigens presented by the MHC-like molecule CD1d. In this context, there is evidence that there are different signalling requirements for the differentiation of iNKT cells and CD4 or CD8 SP mature T cells. For example, DP thymocytes lacking expression of Phospholipid-dependent kinase 1 (PDK1) fail to produce iNKT cells despite normal development of conventional
T cell development is independent of AMPK.

LKB1 phosphorylates and activates AMPK [6]. We therefore interrogated whether the thymic and peripheral T cell phenotype of LKB1fl/fl CD4Cre pos mice was dependent on LKB1-mediated regulation of AMPK. T cells exclusively express the AMPKα1 catalytic subunit and we therefore examined thymus development in AMPKα1 fl/fl CD4Cre pos mice. Western blot analysis confirmed that the DP thymocytes that develop in AMPKα1 fl/fl CD4Cre pos mice had deleted AMPKα1 (Figure 5A). However, thymocyte numbers, the production of mature CD4 and CD8 SP T cells in the thymus and the peripheral T cell compartment was normal in AMPKα1 fl/fl CD4Cre pos mice (Figure 5B and C). We also found that the frequencies of iNKT cells in AMPKα1 fl/fl CD4Cre pos mice were comparable to littermate controls (Figure 5D). The intraepithelial T cell compartment was also normal in AMPKα1 fl/fl CD4Cre pos mice (Figure 5E).

To explore more precisely the role of AMPK in thymocyte positive selection we backcrossed AMPKα1 fl/fl CD4Cre pos mice to mice expressing the defined OT1 αβ TCR transgene that select for class I restricted CD8 T cells. The data show that AMPK loss had no impact on the selection of thymocytes expressing the OT1 αβ TCR complex (Figure 6A). It has been described that peripheral T cells from the whole body AMPKα1 null mice make higher levels of interferon γ (IFNγ) compared to wild type T cells [8]. The data in Figure 6B compare IFNγ production by naïve wild type and AMPKα1 null OT1 TCR transgenic T cells. These data show

Figure 1. Double positive thymocytes survive without LKB1. (A) CD4 pos thymocytes purified from LKB1 fl/fl CD4Cre neg or CD4Cre pos thymi using MACS were lysed at 3 x 10^7 cells mL^-1 in lysis buffer. Proteins extracted from cells and denatured were resolved on NuPAGE Bis-Tris 4–12% gels under reducing conditions and subsequent immunobots were probed for indicated proteins, showing that LKB1 protein was efficiently deleted. GSK3β and β-catenin were used as loading controls for equal loading. Data are representative of two independent experiments. (B) Freshly isolated thymi from LKB1 fl/fl CD4Cre pos or LKB1 fl/fl CD4Cre pos mice were mashed to single cell suspensions. Cell number of double positive (DP) thymocytes was determined from a given volume using calibrated counting beads and from the frequency of cells co-stained for the MHC-receptors CD4 and CD8 of total number of thymocytes. Data are summary of four to six mice, where each symbol represents one mouse. (C) Single cell suspensions of freshly isolated thymi from LKB1 fl/fl CD4Cre pos or littermate controls were seeded at a cell density of 5–10 x 10^6 cells mL^-1 in complete culture medium for 24 h. Frequency of live DP thymocytes was determined by staining for surface co-expression of CD4 and CD8 and exclusion of cells positive for the DNA binding dye DAPI. Statistical analysis using the Mann-Whitney test showed comparable frequencies of DAPI+ DP cells between LKB1 fl/fl CD4Cre pos and controls. Data summarise three independent experiments. (D) Thymocytes were isolated and 1 x 10^6 cells placed into the fibronectin-coated upper chamber of the transwell plate. Cells were left to migrate into the lower chamber containing medium only or 500 ng mL^-1 CXCL12 for three hours. Cells from the lower chamber were collected and counted using counting beads using flow cytometry and the frequency of cells migrated was determined against the input that was used as putatively maximal migration capacity. Data summarise three independent experiments showing mean ± SEM.

doi:10.1371/journal.pone.0060217.g001
Figure 2. LKB1 required for accumulation of T cells in thymus, lymphoid organs and the small intestine. LKB1\(^{fl/fl}\) CD4\(^{Cre^{pos}}\) and CD4\(^{Cre^{neg}}\) thymi were isolated and analysed for (A) co-expression of CD4 and CD8 and (B) TCR\(\beta\) expression. (B) Total number of TCR\(\beta^{\text{high}}\) expressing thymocytes was quantified. (C) Ratio and total cell number of CD4\(^{\text{pos}}\) and CD8\(^{\text{pos}}\) TCR\(\beta^{\text{high}}\) (SP) cells. (D) Analysis of lymphocyte populations in secondary lymphoid organs (spleen and lymph nodes). The frequency of B cells and T cells was determined by flow cytometric analysis for the expression of B220 and TCR\(\beta\), respectively. Quantification of total lymphocytes and TCR\(\beta^{\text{high}}\) lymphocytes is shown to the right showing that T cells were significantly reduced in secondary lymphoid tissues in LKB1\(^{fl/fl}\) CD4\(^{Cre^{+}}\) mice. Flow cytometric histograms and plots are representative of four experiments. Dot plots and bar graphs summarise data from at least four independent experiments. (E) Bi-parametric histogram shows frequency of TCR\(\beta^{\text{pos}}\) and TCR\(\gamma\delta^{\text{pos}}\) T cells isolated from the epithelial layer of small intestines from LKB1\(^{fl/fl}\) CD4\(^{Cre^{neg}}\) and CD4\(^{Cre^{pos}}\) mice followed by flow cytometric analysis. Data shown were gated on DAPI\(^{neg}\) cells to identify live cells that remained intact following extraction and staining procedures. Dot plot summarises the frequencies of TCR\(\beta^{\text{pos}}\) intraepithelial lymphocytes (IEL) from three mice per genotype. Statistical differences as indicated were determined using the Mann-Whitney test, where *\(p<0.05\) and **\(p<0.01\).

doi:10.1371/journal.pone.0060217.g002
that deletion of AMPKα1 does cause enhanced production of IFNγ by CD8 T cells.

Discussion

Previous studies have shown that LKB1 controls the survival of T cell progenitors at the DN stage of development. In these studies LKB1 was deleted at the DN2/3 stage of thymocyte development using the LckCre recombinase model. In LckCre LKB1fl/fl mice a few DP thymocytes survive the early deletion of LKB1 and become DP thymocytes [6]. These DP thymocytes had a reduced survival capacity but it was unclear whether these results really inform as to whether LKB1 is directly required for DP survival. We have addressed this issue by using a CD4 promoter Cre recombinase to delete LKB1 directly in DP thymocytes. Our studies of LKB1 null DP thymocytes from the LKB1 fl/fl CD4Cre mice found no evidence that LKB1 was directly required for DP survival. LKB1 was however required for DPs to differentiate to become iNKT cells and for the production of conventional αβ T cells.

The role for LKB1 in the development of conventional αβ TCR T cells has been previously reported [6-9]. However, the present study now shows that LKB1 is also essential for the development of innate lymphoid populations such as iNKT cells and gut intraepithelial T cells. Moreover, the present study has more precisely delineated that LKB1 is not involved in the initial phase of positive selection of mature T cells. LKB1 null DPs can thus respond to TCR signals to up-regulate expression of CD69. However, they fail to complete positive selection to produce SP T cells in the thymus. Why would DPs fail to make iNKT cells? One explanation is that iNKT cell progenitors undergo a very robust proliferative expansion at the DP stage of thymocyte development. The basis for the failed iNKT cell development of LKB1 null DPs must thus reflect that LKB1 has an essential role for the proliferative burst of iNKT cells that accompanies the positive selection of these cells. This is reminiscent of the LKB1 requirement for the proliferative expansion of DN T cell progenitors and mature conventional T cells [6]. In this respect, previous studies [17] have shown that T cells undergoing positive selection undergo proliferative expansion once they up-regulate expression of CD69. These results are thus consistent with a model that LKB1 is necessary for T cells whenever there is a metabolic demand on the cells imposed by a phase of rapid proliferation. It is also noteworthy that a previous study has indicated that LKB1 might control the recruitment of phospholipase C-gamma 1 (PLCγ1) to the T cell membrane and hence directly control T cell antigen receptor signal transduction [9]. The failure of LKB1 null DP thymocytes to differentiate to iNKT cells could thus also reflect a failure of antigen receptor signalling in DP thymocytes. However, the ability of LKB1 null DPs to initiate positive selection and up-regulate CD69 expression is not consistent with a global defect in TCR-mediated signal transduction. Similarly, LKB1 null DPs express normal levels of CD5 (data not shown) and it is well established that CD5 expression in thymocytes is controlled by the strength of TCR signalling.
Figure 5. Development and accumulation of T cells does not require AMPKα1. (A) CD4<sup>pos</sup> thymocytes from AMPK<sup>α1<sup>fl/fl</sup></sup> CD4C<sup>cre<sup>neg</sup></sup> or CD4C<sup>cre<sup>pos</sup></sup> thymi were purified and lysed as described in Figure 1. Immunoblots were probed for AMPK<sup>α1</sup> and Smc1 as control for equal loading. Data are representative of at least two independent experiments. (B and C) Thymi and secondary lymphoid organs were isolated from AMPK<sup>α1<sup>fl/fl</sup></sup> CD4C<sup>cre<sup>neg</sup></sup> and CD4C<sup>cre<sup>pos</sup></sup> mice and analysed as described in Figure 1. Quantification of cell numbers in thymi (B) and spleens (C) are also shown. Histograms are representative for at least three mice in panels B and C. Numeric dot plots and bar graphs summarise data from at least five to six mice. Mice were analysed between 60–80 days of age. (D) iNKT cells were identified as CD24<sup>low</sup> CD1d-tGalCer<sup>pos</sup> thymocytes isolated from AMPK<sup>α1<sup>fl/fl</sup></sup> CD4C<sup>cre<sup>neg</sup></sup> and CD4C<sup>cre<sup>pos</sup></sup> thymi. Flow cytometric histograms and frequencies shown are representative of at least three independent experiments. (E) TCR<sup>b</sup> and TCR<sup>cd</sup> T cells were identified from cell preparations isolated from the epithelial layer of small intestines from AMPK<sup>α1<sup>fl/fl</sup></sup> CD4C<sup>cre<sup>neg</sup></sup> and CD4C<sup>cre<sup>pos</sup></sup> mice followed by flow cytometric analysis as described in Figure 2. Dot plot summarises frequencies of TCR<sup>b</sup> IEL cells from four different mice. Histograms are representative of four independent analyses.

doi:10.1371/journal.pone.0060217.g005
It has been proposed that LKB1 controls thymus development via its substrate AMPK [7,8]. This model was first proposed because a constitutively active AMPK construct could ‘rescue’ the survival defects of LKB1 null thymocytes [7]. However, gain-of-function strategies do not always inform about the physiological role of a kinase and there has always been further more the exception of ear and blood biopsies for genotyping purposes, mice then authorised by a project licence under the UK Home Office Animals (Scientific Procedures) Act 1986 issued by the Home Office on 14th April 2008.

Mice

LKB1<sup>fl/fl</sup> mice were generated and bred as previously described by Sakamoto et al [21]. LKB1<sup>fl/fl</sup> mice were back-crossed to transgenic mice expressing Cre recombinase under the control of the human cd4 promoter. AMPK<sup>fl/fl</sup> mice were obtained from Benoit Viollet (Institut Cochin, INSERM, Université Paris Descartes, Paris) and bred to OT1-TCR transgenic mice and/or CD4Cre<sup>+</sup> mice. Genotypes of bred mice were determined and confirmed by polymerase chain reaction of genomic DNA extracted from ear snips of weaned mice and expression of TCR transgene was confirmed by flow cytometric analysis of blood biopsies from the mouse tail veins. For tissue isolations with the exception of ear and blood biopsies for genotyping purposes, mice were sacrificed by increasing concentrations of carbon dioxide in compliance with the project licence.

Cell Culture

Single cell suspensions of freshly isolated thymi were maintained at 5–10<sup>6</sup> cells mL<sup>-1</sup> in DMEM containing 10% heat-inactivated foetal calf serum (FCS) (Life Technologies, UK), 50 μM 2-mercaptoethanol (Sigma-Aldrich, Germany), 100 U mL<sup>-1</sup> penicillin and 100 μg mL<sup>-1</sup> streptomycin (Life Technologies, UK). Lymph nodes and spleens were gently disaggregated. Disaggregated spleens were also treated for lysis of red blood cells. Lymph nodes and spleens were re-suspended in 5–10<sup>6</sup> cells mL<sup>-1</sup> in RPMI-1640 containing L-glutamine and supplemented with 10% heat-inactivated FCS, 50 μM 2-mercaptoethanol, 100 U mL<sup>-1</sup> penicillin and 100 μg mL<sup>-1</sup> streptomycin. Primary CD8<sup>+</sup> T cells (4×10<sup>6</sup> cells mL<sup>-1</sup>) from OT1-TCR transgenic
mice were activated with 0.5 μM soluble ovalbumin-derived SIINFEKL peptide for 10 h in 96-well plates and supernatants were collected followed by cytokine secretion assays. IFNγ secretion was determined by enzyme-linked immunosorbent assay (ELISA) using a commercially available kit from eBiosciences.

MACS Purification of double positive (DP) Thymocytes
Thymocytes (1×10^6 cells) were labelled with biotinylated anti-CD4 (BD Pharmingen) and CD4+ thymocytes were isolated using streptavidin-coated magnetic beads by autoMACS (Miltenyi Biotec, Germany). The positive fraction collected was then lysed for immunoblotting.

Immunoblotting
Thymocytes (3×10^7 cells) were lysed in 1 mL of F buffer [10 mM Tris-HCl pH 7.05, 50 mM NaCl, 30 mM Na-pyrophosphate, 50 mM NaF, 5 μM ZnCl2, 10% Glycerol, 1% NP-40, 1 mM DTT] supplemented with 50 nM calyculin A for 15 min on ice and centrifuged for 20 min at 1.32×10^3 rpm. Lysates were mixed and boiled with NuPAGE LDS sample buffer (Life Technologies) supplemented with 100 mM DTT. Samples were separated on NuPAGE Bis-Tris 4–12% gradient gels (Life Technologies) at 200 V for up to 60 min under reducing conditions. Separated proteins were transferred onto Hybond™-C Super nitrocellulose membrane (Amersham Biosciences, UK) at 30 V for 150 min in Novex XCell II Modules (Invitrogen, UK) at 4°C. Membranes were blocked with 5% dry milk/PBS supplemented with 0.5% Tween-20 (Sigma) and probed for indicated pan-proteins. Anti-AMPKα1 was a kind gift of Grahame Hardie, University of Dundee. Anti-Smc1 was obtained from Bethyl Laboratories Inc. All other antibodies for immunoblotting were obtained from Cell Signaling Technology.

Isolation of intraepithelial gut lymphocytes
Freshly isolated small intestines were freed from mesenteric lymph nodes, Peyer’s patches, debris, adipose and connective tissues. Digested food was removed mechanically and the intestinal lumen was cleaned using PBS. Intestines were opened longitudinally and then cut into 1-cm-pieces, which were incubated in Ca^2+/Mg^2+-free PBS (Sigma) supplemented with 10% filtered heat-inactivated FCS, 1 mM Na pyruvate, 20 mM HEPES pH 8.0, 10 mM EDTA pH 8.0 and 10 μg mL^{-1} Polymyxin B for 30 min at 230 rpm and 37°C. Tissue suspensions were filtered using a 70-μm filter cell strainer (BD Falcon) and cells were collected by centrifugation. Cells were re-suspended in 37.5% isotonic percoll (Sigma) and collected by centrifugation (without break). Following careful recovery of the cell pellet, cells were washed, re-suspended in complete RPMI-1640 culture medium, filtered through a 40-μm filter (BD Falcon) and stained for flow cytometric analysis.

Flow cytometry
Accurate cell counts of lymphocyte cultures were taken by using AccuCheck counting beads (Life Technologies, UK). One to two million cells of freshly disaggregated secondary lymphoid organs or extracted from small intestines were incubated with FITC Block (BD Pharmingen) for 10 min at 4°C in RPMI-1640 or PBS supplemented with 1% FCS (FACS buffer). FITC Block was omitted for thymocyte suspensions. Cells were labelled with saturating concentrations of antibody in FACS buffer. Antibodies used were conjugated to fluorescein-isothiocyanate, phycoerythrin (PE), peridinin-chlorophyll protein (PerCP-Cy5.5, PE-Cy7, allophycocyanin (APC), APC-Cy7 or –eFluor®780, Horizon V450 or V500, Alexa Fluor®647 as obtained from BD Pharmingen or eBiosciences: anti-CD4 (L5T4), anti-CD8α (53-6.7), anti-CD8β (H35-17.2), anti-CD44 (IM7), anti-CD69 (H1.2.F3), anti-CD2 (B20.1), anti-VD5.1/5.2 TCR (MR9-4), anti-TCRβ (H57-597), anti-TCRγδ (GL3) and anti-CD24 (M1/69). Staining for Vγ14 TCR to detect NKT cells was performed as described previously [9,14]. Where mentioned, DAPI was used at a concentration of 1 μg mL^{-1} for live cell determination. Following incubation with antibodies, cells were washed and resuspended in FACS buffer. Samples were analysed using a FACScalibur, LSR II or Fortessa (Becton Dickinson). A minimum of 1×10^6 ungated events were acquired and stored. Data files were processed using the latest version of FlowJo software V9.6 (Treestar) for Mac OS. Live cells were gated according to their forward and side scatters and exclusion of DAPI, where indicated.

Transwell migration assay
Migration assays were performed using Transwell chemotaxis plates (Corning). Membrane inserts of transwell plates were coated with 5 μg mL^{-1} fibronectin at 4°C over night. Membranes were then blocked with 2% heat-inactivated FCS/PBS for one hour at 37°C. Freshly isolated thymocytes (1×10^6 cells in 100 μL of complete DMEM medium) were placed in the upper chamber of the transwell plate in triplicate. Culture medium without or with CXCL12 (500 ng mL^{-1}) in 600 μL was placed in the lower chamber. After 3 h of incubation at 37°C in 5% CO₂, the percentage of cells against the input control was determined using flow cytometry.

Statistical Analysis
Quantified data were evaluated using non-parametric Mann-Whitney test, where experimental numbers were not sufficient to prove normal distribution. Bar graphs are shown as mean ± standard deviation unless otherwise stated. GraphPad Prism 4.0c or later for Mac OS X was used for statistical evaluation and generation of bar graphs and dot plots of quantified data.

Acknowledgments
We would specifically like to thank Arlene Whigham for her assistance in preparing and performing the flow cytometric analysis of gut lymphocytes. We would like to thank the Cantrell lab in the College of Life Sciences, University of Dundee, for critical comments and reading of the manuscript. We thank Elizabeth Emmslie for her help, Grahame Hardie and Benoit Viollet for providing reagents and AMPKfl/fl mice, respectively. We would specifically like to thank the Wellcome Trust Flow Cytometry Facility of the University of Dundee for technical support and advice, and the Biological Resource Unit for the animal care.

Author Contributions
Conceived and designed the experiments: MZ, JR, DAC. Performed the experiments: MZ, JR. Analyzed the data: MZ, DAC. Wrote the paper: MZ, DAC.

References
1. Carpenter AC, Bosseut R (2010) Decision checkpoints in the thymus. Nature Immunology 11: 666–673. doi:10.1038/ni.1887.
2. Xiong Y, Bosseut R (2012) CD4–CD8 differentiation in the thymus: connecting circuits and building memories. Current Opinion in Immunology 24: 139–145. doi:10.1016/j.coi.2012.02.002.
3. Godfrey DI, Stankovic S, Baxter AG (2010) Raising the NKT cell family. Nature Immunology 11: 197–206. doi:10.1038/ni.1941.
4. Gangadhara N, Lambolez F, Antinger A, Wang-Zhu Y, Sullivan BA, et al. (2006) Identification of Pre- and Postselection TCRαβ+ Intraepithelial Lymphocyte Precursors in the Thymus. Immunity 23: 631–641. doi:10.1016/j.immuni.2006.08.018.
5. Cheroutre H, Lambolez F, Mucida D (2011) The light and dark sides of intestinal intraepithelial lymphocytes. Nature Reviews Immunology 11: 445–456. doi:10.1038/ni.2007.
6. Tamai P, Macintyre A, Finlay D, Clarke R, Feijoo Carnero C, et al. (2009) LKB1 is essential for the proliferation of T-cell progenitors and mature peripheral T cells. Eur J Immunol 40: 242–253. doi:10.1002/eji.200939677.
7. Cao Y, Li H, Liu H, Zheng C, Li B, et al. (2009) The serine/threonine kinase LKB1 controls thymocyte survival through regulation of AMPK activation and Bcl-XL expression. Cell Research: 99–108. doi:10.1038/cr.2009.141.
8. MacEver NJ, Blagh J, Saucillo DC, Tonelli L, Griss T, et al. (2011) The Liver Kinase B1 Is a Central Regulator of T Cell Development, Activation, and Metabolism. The Journal of Immunology 187: 4187–471. doi:10.1049/jimmunol.1100367.
9. Cao Y, Li H, Liu H, Zhang M, Hua Z, et al. (2011) LKB1 regulates TCR-mediated PLCγ1 activation and thymocyte positive selection. The EMBO Journal: 1–11. doi:10.1038/embj.2011.116.
10. Hardie DG, Ross FA, Hawley SA (2012) AMPK: a nutrient and energy sensor that maintains energy homeostasis. Nature Publishing Group: 13: 251–262. doi:10.1038/nrm3311.
11. Mayer A, Deanglaire S, Viollet B, Leo O, Andris F (2008) AMP-activated protein kinase regulates lymphocyte responses to metabolic stress but is largely dispensable for immune cell development and function. Eur J Immunol 38: 948–956. doi:10.1002/eji.200739045.
12. Love PE, Bhandoola A (2011) Signal integration and crosstalk during thymocyte migration and emigration. Nature Reviews Immunology 11: 469–477. doi:10.1038/nri2899.
13. Lesourne R, Uchara S, Lee J, Song K-D, Li L, et al. (2009) Themis, a T cell-specific protein important for late thymocyte development. Nature Immunology 10: 840–847. doi:10.1038/ni.1768.
14. Finlay DK, Kelly AP, Clarke R, Sinclair LV, Deak M, et al. (2010) Temporal Differences in the Dependency on Phosphoinositide-Dependent Kinase 1 Distinguish the Development of Invariant V14 NKT Cells and Conventional T Cells. 185: 5973–5982. Available: http://www.jimmunol.org/cgi/doi/10.4049/jimmunol.1000237.
15. Jang W, Ferrero I, Laurenti E, Trumpf A, MacDonald HR (2010) c-Myc controls the development of CD8 TCR intestinal intraepithelial lymphocytes from thymic precursors by regulating IL-15-dependent survival. Blood 115: 4431–4438. doi:10.1182/blood-2009-11-256498.
16. Doe M, Steckman BP, Han J, Berdeneyer AL, Bendelac A, et al. (2009) Intrathymic proliferation wave essential for Vα14+ natural killer T cell development depends on c-Myc. Proc Natl Acad Sci USA 106: 8641–8646. doi:10.1073/pnas.0812253106.
17. Wilkinson RW, Anderson G, Owen JJ, Jenkinson EJ (1995) Positive selection of thymocytes involves sustained interactions with the thymic microenvironment. J Immunol 155: 5234–5240.
18. Lazzaro JM, Goransson O, Toth R, Deak M, Morrice NA, et al. (2004) LKB1 is a master kinase that activates 13 kinases of the AMPK subfamily, including MARK/PAR-1. The EMBO Journal 23: 833–843. doi:10.1038/sj.emboj.7600110.
19. Zagorska A, Deak M, Campbell DG, Banerjee S, Hirano M, et al. (2010) New Roles for the LKB1-NUAK Pathway in Controlling Myosin Phosphatase Complexes and Cell Adhesion. Science Signaling: 3: ra25–ra25. doi:10.1126/scisignal.2000616.
20. Lin L, Ulrike J, Muller J, Wustefeld T, Aebertard L, et al. (2013) Deregulated MYC expression induces dependence upon AMPK-related kinase 5. Nature 483: 608–612. doi:10.1038/nature10927.
21. Sakamoto K, McCarthy A, Smith D, Green KA, Grahame Hardie D, et al. (2005) Deficiency of LKB1 in skeletal muscle prevents AMPK activation and glucose uptake during contraction. The EMBO Journal 24: 1810–1820. Available: http://emboj.embj.org/cgi/doi=embj2004061810.