Non-canonical antagonism of PI3K by the kinase Itpkb delays thymocyte $\beta$-selection and renders it Notch-dependent

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Abstract $\beta$-selection is the most pivotal event determining $\alpha\beta$ T cell fate. Here, surface-expression of a pre-T cell receptor (pre-TCR) induces thymocyte metabolic activation, proliferation, survival and differentiation. Besides the pre-TCR, $\beta$-selection also requires co-stimulatory signals from Notch receptors - key cell fate determinants in eukaryotes. Here, we show that this Notch-dependence is established through antagonistic signaling by the pre-TCR/Notch effector, phosphoinositide 3-kinase (PI3K), and by inositol-trisphosphate 3-kinase B (Itpkb). Canonically, PI3K is counteracted by the lipid-phosphatases Pten and Inpp5d/SHIP-1. In contrast, Itpkb dampens pre-TCR induced PI3K/Akt signaling by producing IP$_4$, a soluble antagonist of the Akt-activating PI3K-product PIP$_3$. Itpkb$^{-/-}$ thymocytes are pre-TCR hyperresponsive, hyperactivate Akt, downstream mTOR and metabolism, undergo an accelerated $\beta$-selection and can develop to CD4$^+$CD8$^+$ cells without Notch. This is reversed by inhibition of Akt, mTOR or glucose metabolism. Thus, non-canonical PI3K-antagonism by Itpkb restricts pre-TCR induced metabolic activation to enforce coincidence-detection of pre-TCR expression and Notch-engagement.

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Introduction

To generate a diverse T cell repertoire reactive against many pathogens, the T cell receptor (TCR) $\alpha$ and $\beta$ chain genes somatically rearrange in developing thymocytes. TCR functionality is then assessed at various checkpoints. Thymocytes develop from bone marrow (BM) progenitors through successive CD4$^-$CD8$^-$ ‘double-negative’ CD44$^+$CD25$^+$ DN1, CD44$^+$CD25$^+$c-Kit$^+$ DN2, HSA(high)c-Kit$^+$CD44$^+$CD25$^+$DN3 and HSA(high)CD44$^+$CD25$^+$DN4 stages (Petrie and Zuniga-Pflucker, 2007; Xiong et al., 2011). Productive rearrangement of one TCR$\beta$-allele causes surface-expression of a pre-TCR comprised of TCR$\beta$, pre-TCR$\alpha$ and signal-transducing CD3 subunits on DN3 cells (Aifantis et al., 2006). At the first checkpoint, $\beta$-selection, ligand-independent pre-TCR signaling triggers DN3 cell metabolic activation, proliferation and survival. It also triggers allelic exclusion of the second TCR$\beta$ allele, initiation of TCR$\alpha$ gene-rearrangements and differentiation via CD8$^-$HSA(high)TCR$\beta$low immature single positive (ISP) precursors into CD4$^+$CD8$^-$ ‘double-positive’ (DP) cells (Petrie and Zuniga-Pflucker, 2007; Xiong et al., 2011). $\beta$-selection ensures that only DN3 cells

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T cells defend our body against cancer and infectious agents such as viruses. However, they can also cause rheumatoid arthritis and other autoimmune diseases by attacking healthy tissue. T cells recognize target cells via receptor proteins on their surface. To maximize the variety of infections and cancers our immune system can recognize, we generate millions of T cells with different T cell receptors every day.

To ensure T cells work correctly, T cell receptors are tested at various checkpoints. The first checkpoint involves a process called beta (β) selection, during which T cells produce their first T cell receptor – the so-called pre-T cell receptor. This receptor causes T cells to divide and mature, and sets their future identity or “fate”. To complete β-selection, T cells must also receive signals from another surface receptor – one that belongs to the Notch family, which determines cell fate in many different tissues.

The Notch receptor and the pre-T cell receptor both activate an enzyme called PI3K – a key mediator of β-selection. But the pre-T cell receptor also activates another enzyme called Itpkb that is required for T cell development. Westernberg, Conche et al. have now investigated how these different proteins and signaling processes work and interact during β-selection, using mice that lack several immune genes, including the gene that produces Itpkb.

The results of the experiments show that during β-selection, Itpkb limits the ability of PI3K to activate some of its key target proteins. This “dampened” PI3K signaling ensures that both the pre-T cell receptor and the Notch receptor must be activated to trigger T cell maturation. Without Itpkb, β-selection can occur in the absence of Notch signaling.

As Notch signaling is important for determining the fate of many different cell types, Westernberg, Conche et al.’s findings raise the possibility that Itpkb might also regulate cell fate determination in other tissues. Moreover, Itpkb may suppress tumor development, because excessive PI3K signaling drives many cancers.

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**expressing a functional TCRβ chain develop further. It is the major cell-fate determining event for αβ T cells. Defective β-selection causes a DN3 block and severe immunodeficiency (Juntilla and Koretzky, 2008; Aifantis et al., 2006).**

**pre-TCR signaling alone is insufficient for DN-to-DP cell differentiation without costimulation by thymic microenvironmental signals. In particular, ligand engagement of Notch on DN3/DN4 cells promotes nutrient receptor expression, glucose uptake, metabolism, growth, survival, proliferation and differentiation. But excessive Notch signaling causes thymocyte transformation and T cell acute lymphoblastic leukemia (T-ALL). This is augmented by pre-TCR signals (Ciofani et al., 2004; Ciofani and Zuniga-Pflucker, 2005; Campese et al., 2006; Fayard et al., 2010; Taghon et al., 2006; Aifantis et al., 2006; Tussiwand et al., 2011). So, pre-TCR/Notch costimulation needs to be limited and elucidating the underlying mechanisms is of great importance.**

**Both pre-TCR and Notch activate phosphatidylinositol 3-kinases (PI3K) (Ciofani and Zuniga-Pflucker, 2005; Juntilla and Koretzky, 2008; Fayard et al., 2010). PI3K phosphorylate the membrane lipid phosphatidylinositol(4,5)bisphosphate (PIP₂) into phosphatidylinositol(3,4,5)trisphosphate (PIP₃). PIP₃ recruits and activates Itk/Tec-, Pdk1-, and Akt-family kinases by binding to their PH domains. PI3K are essential and rate-limiting for β-selection by promoting metabolism, proliferation, survival and differentiation (Juntilla and Koretzky, 2008; Fayard et al., 2010). Itk promotes activation of phospholipase-Cγ1 (PLCγ1). PLCγ1 hydrolyzes PIP₂ into the second messengers inositol (1,4,5)trisphosphate (IP₃) and diacylglycerol (DAG), which then convey downstream signals (Aifantis et al., 2006). Itk loss only subtly impairs β-selection (Lucas et al., 2007). Pdk1 is required for DN3/DN4 cell differentiation mostly by activating Akt, and for thymocyte proliferation through other effectors (Kelly et al., 2007; Fayard et al., 2010). Akt kinases are required for β-selection by promoting DN3/DN4 cell glucose uptake, glycolysis, viability and differentiation (Juntilla et al., 2007; Fayard et al., 2007; Mao et al., 2007; Fayard et al., 2010). Recent studies suggest important roles for the Akt activator mTORC2 and possibly the Akt downstream-effector mTORC1 in β-selection (Lee et al., 2012; Tang et al., 2012; Chou et al., 2014).**
Canonically, PI3K function is limited through PIP<sub>3</sub>-removal by the lipid-phosphatases Inpp5d/SHIP1 and Pten (Juntilla and Koretzky, 2008; Fayard et al., 2010). Inpp5d/SHIP1<sup>−/−</sup> early thymocytes develop normally (Kashiwada et al., 2006). Conditionally Pten<sup>−/−</sup> DN cells show constitutively active Akt and accelerated development to DP cells. They can generate DP cells without pre-TCR or Notch-signaling (Hagenbeek et al., 2004; Kelly et al., 2007; Shiroki et al., 2007; Wong et al., 2012; Hagenbeek et al., 2014). Notch may promote DN3/DN4 cell survival and differentiation in part by repressing Pten (Wong et al., 2012). So, limiting PI3K signaling is required for β-selection and its dependence on both pre-TCR and Notch. But many details about how pre-TCR and Notch cross-talk via PI3K are controversial, and it remains unclear why pre-TCR signaling alone is insufficient for β-selection (Juntilla and Koretzky, 2008; Fayard et al., 2010; Hagenbeek et al., 2014).

IP<sub>3</sub> is well known to mobilize Ca<sup>2+</sup> but can also be phosphorylated into inositol(1,3,4,5)tetrakisphosphate (IP<sub>4</sub>) by four mammalian IP<sub>3</sub> 3-kinases (Sauer and Cooke, 2010). Among these, we and others have identified Itpkb as an essential TCR effector. Thymocyte development in Itpkb<sup>−/−</sup> mice is blocked at the DP stage due to defective positive selection (Huang et al., 2007; Pouillon et al., 2003; Wen et al., 2004). In thymocytes, TCR signaling activates Itpkb to produce IP<sub>4</sub>, a soluble analog of the PH domain binding moiety of PIP<sub>3</sub>. Itpkb<sup>−/−</sup> thymocytes have strongly reduced IP<sub>3</sub> 3-kinase activity and IP<sub>4</sub> levels, but normal IP<sub>3</sub> levels and Ca<sup>2+</sup> mobilization (Pouillon et al., 2003; Wen et al., 2004). IP<sub>4</sub> can bind to PH domains and control PIP<sub>3</sub> binding (Huang et al., 2007; Jia et al., 2007). In NK cells, myeloid cells and hematopoietic stem cells (HSC), IP<sub>4</sub> competitively limits PIP<sub>3</sub>-binding to, and activation of Akt (Jia et al., 2008; 2007; Sauer et al., 2013; Siegemund et al., 2015). Thus, besides PIP<sub>3</sub>-turnover by Inpp5d/SHIP1 and Pten, IP<sub>3</sub> 3-kinases can limit PI3K function through a non-canonical mechanism, IP<sub>4</sub> antagonism with PIP<sub>3</sub>

Here, we present data which suggest that this non-canonical mechanism restricts pre-TCR induced pro-metabolic PI3K/Akt signaling to limit the kinetics and enforce the Notch-dependence of β-selection. Itpkb<sup>−/−</sup> DN3 cells were pre-TCR hyperresponsive with Akt/mTOR hyperactivation and evidence for metabolic hyperactivity. They showed an accelerated and Notch independent, but pre-TCR dependent differentiation to the DP stage. Pharmacologic inhibition of Akt, mTOR or glucose metabolism restored wildtype (WT) developmental kinetics and Notch-dependence of Itpkb<sup>−/−</sup> DN3 cells.

**Results**

**Altered β-selection in Itpkb<sup>−/−</sup> mice**

DN3 cells from Itpkb<sup>+/−</sup>Rag2<sup>−/−</sup> but not Itpkb<sup>−/−</sup>Rag2<sup>−/−</sup> mice express Itpkb (Figure 1). To study if Itpkb is required for DN3 cell development, we analyzed DN cell subsets in Itpkb<sup>+/+</sup> (WT) vs. Itpkb<sup>−/−</sup> mice by flow-cytometry. For enhanced sensitivity, we gated out lineage-marker positive (Lin<sup>+</sup>) non-T cells, γδ T cells and HSA<sup>low</sup> mature DN αβ T cells (Bruno et al., 1996). Compared to controls, Itpkb<sup>−/−</sup> mice had increased DN3 and reduced DN4 cell proportions with a ~three-fold increased DN3:DN4 ratio (Figure 2A,B). Blocked β-selection usually increases this ratio via accumulation of pre-selection DN3 cells and loss of DN4 cells and descendants, ultimately reducing thymic cellularity (Michie and Zuniga-Pflucker, 2002). Surprisingly, Itpkb<sup>−/−</sup> mice had WT-like total thymic cellularity and numbers of DN3 cells and CD25<sup>high</sup> ‘DN3-4’ intermediates between DN3 and DN4 cells (Xiong et al., 2011) (Figure 2C).

Itpkb<sup>−/−</sup> DN3, DN3-4 and DN4 cells contained WT amounts of total TCRβ and CD3 protein (Figure 2D). Upon successful TCRβ-rearrangement, DN3 cells express intracellular TCRβ protein which is then transported to the cell surface (Aifantis et al., 2006). Due to constitutive endocytosis, only small surface TCRβ/CD3 amounts are detectable on DN3 and DN3-4 cells (Panigada et al., 2002). These were similar between genotypes (Figure 2D). In contrast, the proportion of Lin<sup>+</sup> HSA<sup>high</sup> DN4 cells expressing surface TCRβ/CD3 was reduced in Itpkb<sup>−/−</sup> vs. WT mice. These HSA<sup>high</sup>Lin<sup>+</sup> (including DX5) CD4<sup>+</sup>CD8<sup>−</sup>CD44<sup>+</sup>CD25<sup>+</sup> TCRβ<sup>+</sup> cells are not β-selection intermediates, but rather comprise post-selection mature precursor thymocytes of CD4<sup>+</sup>CD8<sup>−</sup> αβ T cells, found mainly in the gut epithelium (Pobezsinsky et al., 2012; Gangadharan et al., 2006) (and Hilde Cheroutre, personal communication). Their reduction reflects the mature T cell efficiency in Itpkb<sup>−/−</sup> mice (Huang et al., 2007; Pouillon et al., 2003; Wen et al., 2004). Surface TCRβ<sup>+</sup> DN4 cells have all hallmarks of ‘true’ DN4 cells: They proliferate highly, are metabolically active and efficiently generate DP cells in
vitro (Petrie et al., 1990; Levelt et al., 1993; Panigada et al., 2002; Kelly et al., 2007; Yuan et al., 2011). So, we used TCRβ-DN4 cells to further characterize DN4 cell phenotypes. Compared to WT mice, Itpkb<sup>-/-</sup> mice had significantly less TCRβ-DN4 and ISP cells, but similar DP cell numbers and an increased (DN3 + DN3-4):TCRβ-DN4 cell ratio (Figure 2C).

pre-TCR expression correlates with upregulated surface CD2, pre-TCR signaling upregulates surface CD5, and surface CD27 upregulation is one of the earliest markers of β-selection (Taghon et al., 2006; Patra et al., 2006). Compared to WT controls, Itpkb<sup>-/-</sup> DN3-4 cells and later stages had increased CD2 levels and normal to increased surface levels of CD5, CD27 and CD71 (Figure 3A). Also, Itpkb<sup>-/-</sup> DN3-4 and DN4 cells had elevated surface-levels of the costimulatory chemokine-receptor and Notch-target CXCR4 (Trampont et al., 2010; Xie et al., 2013). In contrast, Itpkb<sup>-/-</sup> and WT DN3, DN3-4 and TCRβ-DN4 cells each expressed comparable surface CD28, CD127 and CD98. Normal to elevated activation markers, and normal DN3 cell and total thymocyte numbers argue against a pre-TCR signaling defect and β-selection block in Itpkb<sup>-/-</sup> mice but might rather suggest pre-TCR hyper-responsiveness. To further test this, we bred our mice to Nr4a1/Nur77-GFP transgenics. Here, Nr4a1/Nur77-GFP expression is a highly sensitive readout for TCR signal intensity (Moran et al., 2011). Supporting pre-TCR hyper-responsiveness, DN3 and later stages of thymocyte development expressed more Nr4a1/Nur77-GFP in Itpkb<sup>-/-</sup> than WT mice (Figure 3B).

We next analyzed whether the reduced DN4 and ISP cell numbers in Itpkb<sup>-/-</sup> mice might reflect reduced proliferation or viability. But similar Ki67-staining and <i>in vivo</i> BrdU incorporation suggest comparable steady-state proliferation of all thymocyte subsets between genotypes (Figure 3C). Similar AnnexinV staining suggests comparable viability (Figure 3D).

**Itpkb-loss cell-intrinsically alters β-selection**

To explore if the altered β-selection is caused by thymocyte-intrinsic Itpkb-loss, we injected a 1:1 mix of mature T/B cell-depleted CD45.1 WT and CD45.2 Itpkb<sup>-/-</sup> BM into lethally irradiated CD45.1/CD45.2-congenic hosts and analyzed reconstituted thymocyte subsets 6–8 weeks later. We distinguished WT vs. Itpkb<sup>-/-</sup> donor-derived cells by CD45 allelic expression. Compared to WT controls, Itpkb<sup>-/-</sup> donor-derived thymocytes reproduced the published cell-intrinsic block at the DP stage (Wen et al., 2004) and the increased DN3 and reduced DN4 cell proportions, partial loss of TCRβ-DN4 and ISP cells, and increased (DN3 + DN3-4):TCRβ-DN4 cell ratio of Itpkb<sup>-/-</sup> mice (Figure 4A–C). Moreover, Itpkb<sup>-/-</sup> vs. WT donor-derived DN3-4 and DN4 cells overexpressed CD2 and tended
to upregulate CXCR4 (Figure 4D). Ki67 staining was again similar between genotypes (Figure 4E). Thus, Itpkb−/− thymocytes show a cell-intrinsically altered β-selection not rescued by a WT environment and the presence of WT thymocytes.
Itpkb−/−DN3 cells develop faster to DP cells

The above data suggest that a developmental block, hypoproliferation or increased death do not cause the loss of DN4 and ISP cells in Itpkb−/− mice. Another possibility is that these subsets are depleted by accelerated development to DP cells. Indeed, mathematical modeling suggests that a two fold or larger increase in the rate constants for the successive transitions from DN3 to DN4 to ISP to DP cells with unaltered rates for progenitor development to DN3 cells, and for thymocyte turnover due to proliferation and death, can cause similarly reduced DN4 cell and ISP numbers but normal DN3 and DP cell numbers as seen in Itpkb−/− vs. WT mice (Figure 5A,B).
Consistent with faster thymocyte development, Itpkb−/− fetal thymi had reduced overall DN and DN4 cell proportions but higher DP cell proportions and total numbers than WT controls on embryonic day 16.5 (E16.5) where DP cells are first detectable in WT mice, and on E17.5 despite ‘catching up’ WT DP cells (Figure 5C, Figure 5—figure supplement 1).

To corroborate these findings in an in vivo system that allows one to kinetically study β-selection of a synchronized DN3 cell population, we generated Itpkb−/− Rag2−/− mice. Rag2-loss causes a developmental arrest at the DN3 stage due to blocked TCRβ expression. Injected anti-CD3 antibodies (a-CD3) can crosslink CD3ε on Rag−/− DN3 cells and trigger their differentiation to DP cells.
Our mathematical model predicted immediately increasing DN4 cell numbers, and DP cell accumulation at ≥2 days post α-CD3 injection in Rag2−/− mice (Figure 6A,B). Simulating over two-fold faster DN3-to-DP development in Rag2−/−Itpkb−/− mice predicted transiently increased DN4 cell accumulation resulting in earlier DP cell accumulation. Experiments confirmed the predictions: Thymocyte development was blocked at the DN3 stage in Itpkb−/−Rag2−/− and Rag2−/− control mice (Figure 6C,D). α-CD3 injection triggered progressive accumulation of DN4, ISP, and DP cells 2 and 3 days later in both genotypes (Figure 6C–F). However, Itpkb−/−Rag2−/− mice accumulated larger proportions of DN4 (33% vs. 18%), ISP (3% vs. 1%) and DP cells (20% vs. 1%) than Rag2−/− mice on day 2 when Rag2−/− mice barely had any DP cells. Itpkb−/− DN4 and ISP cell numbers tended to be increased on day 2 but this was not statistically significant (Figure 6E). Itpkb−/−Rag2−/− mice continued to accumulate more DP cells towards an ~four fold excess over Rag2−/− controls on day 3 (Figure 6F). This resulted mostly from faster development, as both genotypes showed similar thymocyte subset viability and proliferation post α-CD3 injection (Figure 6G,H).
Further supporting accelerated development, Itpkb−/− sorted DN3 cells generated larger proportions of DP cells than WT DN3 cells after 4-day co-culture on OP9DL1 stroma cells (Ciofani and Zuniga-Pflucker, 2005; Ciofani et al., 2004) (Figure 7A,B, Figure 9—figure supplement 1). Finally, Itpkb−/− E15.5 fetal thymic organ cultures (FTOC) produced more DP cells than WT controls (Figure 7C,D).

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Increased pre-TCR signaling via PI3K/Akt/mTOR in *Itpkb*−/− DN3 cells

Activation marker upregulation and faster DN-to-DP cell development suggest increased pre-TCR signaling in *Itpkb*−/− mice. To confirm this, we analyzed the α-CD3 induced upregulation of pre-TCR activation markers in the *Rag2*−/− mouse system. Compared to un.injected *Rag2*−/− controls, α-CD3 injection upregulated surface CD2, CD5, CD71 and CXCR4 on DN3 cells, and, more pronounced, on DN4 cells in *Rag2*−/− and *Itpkb*−/− *Rag2*−/− mice 2 days later (Figure 6I). All markers reached higher surface-levels on *Itpkb*−/− *Rag2*−/− than *Rag2*−/− DN3 and DN4 cells. Thus, *Itpkb*−/− DN3/DN4 cells are hyper-responsive to CD3-crosslinking. To determine if signaling is increased, we injected α-CD3 into Nr4a1/
Nur77-GFP transgenic Rag2−/− and Itpkb−/−Rag2−/− mice. Higher Nr4a1/Nur77-GFP induction in Itpkb−/−Rag2−/− than Rag2−/− mice confirmed increased CD3 signaling (Figure 6f).

Conditionally Pten−/− DN cells show constitutively active Akt and accelerated development to DP cells (Hagenbeek et al., 2004; Kelly et al., 2007; Shiroki et al., 2007; Wong et al., 2012). Itpkb can limit receptor-mediated Akt activation through IP4/PIP3 antagonism (Sauer and Cooke, 2010). To test if this mechanism restricts pre-TCR responses, we compared signaling via PI3K, Akt and downstream mTOR in Itpkb−/− and WT thymocyte subsets. Compared to negative controls and positive controls treated with the phosphatase-inhibitor Calyculin A (Pozuelo-Rubio et al., 2010), WT and Itpkb−/− TCRβ+ pre-selection DN3 cells had significant but similar basal amounts of phosphorylated active Akt, mTOR and downstream S6 protein (Figure 8A). TCRβ+ DN3 cells undergoing β-selection upregulated phospho-Akt, -mTOR, -S6 and control -Erk, indicating pre-TCR mediated activation. Importantly, and contrasting with a WT-like total Akt protein content in Itpkb−/− cells, phospho-Akt, -mTOR and -S6 but not -Erk levels were higher in Itpkb−/− than WT TCRβ+ DN3 and DN3-4 cells (Figure 8A). This genotype difference disappeared as signaling was downregulated in later developmental stages or in mature TCRβ+ ‘DN4-phenotype’ gut T cell precursors.

Akt/mTOR-activation by the pre-TCR and Notch promotes DN3/DN4 cell metabolism in part by increasing nutrient import through upregulation or activation of the glucose-transporter Glut1, the L-amino acid transporter CD98 and the transferrin receptor CD71 on the cell surface. This results in an increased cell size (Ciofani and Zuniga-Pflucker, 2005; Kelly et al., 2007; Fayard et al., 2010; Juntilla and Koretzky, 2008; Janas et al., 2010). Metabolic activation appeared increased in Itpkb−/− TCRβ+ DN3 and DN3-4 cells, because they showed Glut-1 hyperinduction and increased large cell proportions over WT controls (Figure 8A,B). Moreover, α-CD3 injection hyperinduced surface CD71 on DN3 and DN4 cells in Itpkb−/−Rag2−/− versus Rag2−/− mice (Figure 6f). Taken together, these data suggest that Itpkb limits PI3K/Akt/mTOR signaling in, and metabolic activation of surface TCRβ+ DN3 and DN3-4 cells.

**Itpkb restricts pre-TCR signaling to delay β-selection and render it Notch-dependent**

To determine if the Akt/mTOR and metabolic hyperactivation of Itpkb−/− pre-TCR+ DN3 cells causes their accelerated development to DP cells, we next studied if treatment with inhibitors of Akt (Akt-inhibitor VIII, mTOR (rapamycin) or glucose-metabolism (2-deoxy-D-glucose, 2DG) could reverse the increased DP cell generation from equal numbers of sorted Itpkb−/− versus Itpkb−/+ DN3 cells on OP9DL1 stroma. Strikingly, all three treatments yielded Itpkb−/− DP cell proportions similar to those of untreated or carrier-treated WT controls after 4-day co-culture (Figure 9A, Figure 9—figure supplement 1A,C,E). As expected, the treatments also reduced WT DP cell generation below untreated controls. Their reduced efficacy towards Itpkb−/− DN cells is expected, as these had increased amounts of the respective active inhibitor-targets (Figure 8A). Similarly complete rapamycin reversal of the accelerated DN-to-DP development in Itpkb−/− versus Itpkb+/+ FTOC confirmed these important findings in a less reductionist system (Figure 7C,D). These data suggest that the hyperactive Akt, mTOR and glucose metabolism of Itpkb−/− DN3 cells contribute to their accelerated DN-to-DP development.

Constitutive Akt activity promotes glucose metabolism and allows DN3-to-DP cell maturation without Notch signaling (Ciofani and Zuniga-Pflucker, 2005; Lee et al., 2012; Fayard et al., 2010; Juntilla and Koretzky, 2008). To test if the Akt/mTOR hyperactivity in Itpkb−/− TCRβ+ DN3 cells has the same effect, we co-cultured sorted Itpkb−/− or WT DN3 cells with OP9 stroma lacking Notch ligands (Figures 7A,B, 9B, Figure 9—figure supplement 1B,D,F). As previously described (Ciofani and Zuniga-Pflucker, 2005; Xiong et al., 2011), WT DN3 cells much less efficiently generated DP cells without than with Notch (Figures 7A,B, 9A,B). Inhibition of Akt, mTOR or Glucose-metabolism further reduced WT DP cell production consistent with known Akt, mTOR and glycolysis requirements for DN thymocyte survival and differentiation (Lee et al., 2012; Fayard et al., 2010; Juntilla and Koretzky, 2008). In striking contrast, Itpkb−/− DN3 cells efficiently generated DP cells on OP9 stroma (Figures 7A,B, 9B, Figure 9—figure supplement 1B,D,F). Inhibition of Akt, mTOR or glycolysis strongly reduced DP cell output. Thus, Itpkb-loss renders the DN3-to-DP transition Notch-independent in an at least partially Akt, mTOR and glycolysis-dependent manner.

Notch signaling depends on its cleavage by cellular γ-secretases (Wong et al., 2004). To corroborate our findings in vivo, we thus analyzed DN3 cell maturation in Itpkb−/−Rag2−/− versus Rag2−/− mice
treated p.o. with vehicle or the γ-secretase inhibitor LY-411,575 for two days post α-CD3 injection. P.o. administered 10 mg/kg LY-411,575 potently inhibited γ-secretase function in mice and impaired DN thymocyte maturation into αβ T cells with a particularly profound loss of DP cells (Wong et al., 2004). We found that LY-411,575 strongly impaired the α-CD3 induced DN cell development into
ISP and DP cells in Rag2^-/- but not Itpkb^-/- Rag2^-/- mice (Figure 10). Hence, Itpkb-loss reduces the Notch-dependence of DN thymocyte development to DP cells both in vitro and in vivo.

Discussion

Here, we identify Itpkb as a novel pre-TCR effector which restricts the kinetics of β-selection and establishes its Notch-dependence. Itpkb^-/- mice show a cell-autonomously accelerated and Notch independent, but pre-TCR dependent DN3-to-DP cell differentiation associated with DN3 cell pre-TCR hyperresponsiveness, Akt/mTOR hyperactivation and evidence for metabolic hyperactivity. Pharmacologic inhibition of Akt, mTOR or glucose metabolism restored WT kinetics and Notch-dependence of Itpkb^-/- DN3-to-DP cell development.

In thymocytes, TCR engagement activates Itpkb to produce IP_3. Itpkb^-/- thymocytes had strongly reduced IP_3-kinase activity and IP_3 levels, but normal IP_3 levels and Ca^{2+} mobilization (Huang et al., 2007; Pouillon et al., 2003; Wen et al., 2004). IP_3 competitively limits PIP_3-binding to the Akt PH domain and Akt activation in NK cells, myeloid cells and HSC (Jia et al., 2008;
Thus, we propose that pre-TCR induced IP$_{4}$/PIP$_{3}$ antagonism governs $\beta$-selection by restricting PI3K/Akt/mTOR signaling and metabolic activation. We derive a model where Itpkb controls pre-TCR/Notch crosstalk through combined restriction of pre-TCR induced and Notch induced PI3K signaling via Akt (Figure 11). Itpkb enforced coincidence detection of pre-TCR surface expression and Notch-engagement ensures that Akt is only

![Figure 10. Itpkb-loss reduces the Notch-dependence of DN thymocyte development to DP cells in vivo. Shown are (A) CD4/CD8 expression on total thymocytes and (B) HSA/TCR$\beta$ expression on CD4$^-$CD8$^+$ thymocytes from Rag2$^{-/-}$ and Rag2$^{-/-}$Itpkb$^{-/-}$ mice two days post $\alpha$-CD3 antibody injection. Starting 3–4 hr before $\alpha$-CD3 injection, the mice were treated once daily with orally administered $\gamma$-secretase inhibitor LY-411,575 or vehicle (Wong et al., 2004). Numbers indicate % cells per respective gate. The gates in (B) denote CD8$^+$HSA$^{high}$ ISP (Petrie and Zuniga-Pflucker, 2007; Xiong et al., 2011). Representative of two independent experiments (n = 3). DOI: 10.7554/eLife.10786.014]
activated to the degree needed for b-selection and only in an appropriate context, pre-TCR+ DN3 cells interacting with Notch-ligand expressing subcapsular stromal cells (Petrie and Zuniga-Pflucker, 2007). This prevents premature differentiation. Similarly accelerated DN-to-DP cell development of Itpkb−/− and Pten−/− thymocytes (Hagenbeek et al., 2004; Shiroki et al., 2007) and enhanced DP cell production from DN3 cells expressing a dominant-active mutant version of the class I PI3K regulatory subunit p85α/Pik3r1 (p65PI3K transgenic mice) (Rodriguez-Borlado et al., 2003) or dominant-active, myristoylated Akt1 (myr-Akt transgenic mice) (Lee et al., 2012) highlight the importance of restricting PI3K signaling via Akt for proper b-selection kinetics, even though the specific purpose of delaying DP cell maturation remains unknown.

The increased DP cell production from Itpkb−/− versus WT DN3 thymocytes in several different in vivo and in vitro models without concomitantly increased proliferation or viability suggests accelerated developmental kinetics, consistent with our mathematical simulations. We propose that this is caused by pre-TCR hyperresponsiveness based on the Akt/mTORC1 hyperactivation, increased Nr4a1/Nur77-GFP expression and hyperinduction of activation markers in Itpkb−/− versus WT DN3 and later stage thymocytes, and on the phenotype reversal by Akt/mTORC1 and metabolic inhibitors. Importantly, the Nr4a1/Nur77-GFP and activation marker hyperinduction in Itpkb−/− DN3 cells indicate increased transcriptional responses. This might accelerate development by inducing required amounts of cell fate determinants earlier in Itpkb−/− DN3 cells than in WT cells. Alternatively or in addition, Itpkb-loss might increase the number of DN3 cells responding to pre-TCR signals or developmental cues present even in the reductionist OP9-DL1 cell co-culture system. Our present data do not allow us to discern the relative contributions of accelerated kinetics of cellular signaling.

**Figure 11.** Antagonistic signaling by PI3K and Itpkb controls the kinetics and Notch-dependence of b-selection. (A) We propose a model in which pre-TCR and Notch signaling both activate PI3K to produce PIP3 in DN3/DN3-4 cells. PIP3 then recruits and activates Akt to increase glucose metabolism via the Akt/mTOR pathway. This is required for DN3-to-DP cell differentiation. However, pre-TCR signaling also activates Itpkb to produce IP4, which competes with PIP3 for Akt PH domain binding and limits Akt recruitment, Akt and mTOR activation in pre-TCR expressing DN3/DN3-4 cells. IP4 may have additional effectors, indicated by the question mark. By limiting downstream glucose metabolism, this “IP4 brake” delays the kinetics of b-selection and renders this process dependent on Notch costimulation. (B) Without Itpkb, IP4 no more dampens Akt activation and pre-TCR signaling alone sufficiently activates Akt/mTOR signaling to trigger DP cell development in the absence of Notch engagement. (C) In the presence of Notch-signals, Akt is now hyperactivated and causes an accelerated DN3-to-DP cell differentiation.

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events versus increased proportions of responding cells due to lowered pre-TCR signaling thresholds or enhanced sensitivity to developmental cues upon Itpkb-loss. Distinguishing between these possibilities will require future detailed studies of the effects of Itpkb-loss on the sizes (amplitudes and proportions of cells responding), kinetics (rate constants) and shapes (analog, digital) of pre-TCR signaling events, transcriptional and functional responses in populations of individually analyzed DN3 cells, combined with mathematical simulations.

Pre-TCR and Notch signaling both promote DN3 cell proliferation, survival and differentiation in part by activating PI3K/Akt/mTOR (Janas et al., 2010; Ciofani et al., 2004; Ciofani and Zuniga-Pflucker, 2005; Taghon et al., 2006; Lee et al., 2012; Kelly et al., 2007). The dependence of β-selection on pre-TCR and Notch signaling relies on dampened PI3K/Akt signaling (Juntilla and Koretzky, 2008; Fayard et al., 2010). Conditionally Pten−/− DN cells have constitutively active Akt and generate DP cells without pre-TCR or Notch-signaling (Hagenbeek et al., 2004; Kelly et al., 2007, Shiroki et al., 2007; Wong et al., 2012). And constitutive Akt activity can substitute for Notch or mTORC2 to promote DN cell glycolysis, survival and differentiation. It allows DN3-to-DP cell development without pre-TCR or Notch-signaling, but not without both (Mao et al., 2007; Ciofani and Zuniga-Pflucker, 2005; Kelly et al., 2007; Lee et al., 2012). Although many details of how Notch and PI3K intersect remain unclear, Notch may promote β-selection in part by inducing HES1 to repress Pten, and c-Myc to promote proliferation (Wong et al., 2012).

Contrasting with Pten-loss, Notch or Akt hyperactivity, Itpkb-loss accelerates DN3 cell differentiation without significant effects on proliferation and viability, and overcomes the Notch dependence but not the pre-TCR dependence of β-selection. This is evidenced by the lack of accumulating intracellular TCRβ DP cells in Itpkb−/− mice, and of post-DN3 cells in Itpkb−/−Rag2−/− mice (Figures 2D, 6C, D). We speculate that this reflects the need for TCR signals to activate Itpkb and produce IP3 (Chamberlain et al., 2005; Wu et al., 2004; Pouillon et al., 2003). By abrogating pre-TCR induced IP3-inhibition of pre-TCR and Notch induced Akt/mTOR signaling, Itpkb-loss mimics the effects of Pten-loss or dominant active Akt1 expression. This overcomes Notch-requirements and accelerates differentiation but not proliferation, because Notch-induction of c-Myc is PI3K-independent (Wong et al., 2012). The surprising lack of increased DN3/DN4 cell viability in Itpkb−/− mice might reflect differing degrees of Akt/mTOR hyperactivation in Pten−/−, dominant active Akt1-expressing and Itpkb−/− DN3/DN4 cells, consistent with unaltered development of Inpp5d/SHIP1−/− thymocytes (Kashihwada et al., 2006). Altogether, the largely restored developmental kinetics and Notch-dependence of Itpkb−/− DN3 cells by treatment with Akt, mTORC1 or metabolic inhibitors support contributing roles for the Akt/mTOR-hyperactivity. Future studies with sub-optimal Akt/mTOR inhibitor concentrations not affecting WT thymocytes but still reversing the Itpkb−/− phenotype, with complex genetic models and with inhibitors of β-selection effectors unaffected by Itpkb will be needed to more conclusively distinguish between specific causative roles for the Akt/mTORC1 and metabolic hyperactivity and mere remaining sensitivity of Itpkb−/− DN thymocytes to inhibition of this particular pathway. Such studies can also address whether additional mechanisms contribute to the β-selection phenotype of Itpkb−/− mice.

Contrasting with dominant active Akt1 expression or loss of Pten, which has high constitutive PI3K-phosphatase activity (Leslie and Foti, 2011), Itpkb-loss cannot replace pre-TCR signals because Itpkb is inactive without them, so its loss has no further effect. Itpkb-loss might also reduce less essential positive Itpkb roles in pre-TCR signaling, such as augmenting PLCγ1/Erk activation by Itk (Huang et al., 2007). Indeed, TCRβ+ DN3 cells from Itpkb−/− vs. WT mice tended to have mildly reduced Erk activity (Figure 8A). Erk signaling is required for DN cell proliferation and differentiation (Kortum et al., 2013). The mild defects in Itpkb−/− mice are consistent with the only minor role of Itk in β-selection (Lucas et al., 2007) and the unaltered DN cell proliferation.

Hyper-upregulation of Glut1, CD71 and cell-size in Itpkb−/− TCRβ+ DN3 cells and reversal of their accelerated, Notch-independent differentiation by the glycolytic inhibitor 2DG suggest that Itpkb controls β-selection by ultimately restricting DN3 cell metabolic activation. Similar Akt-inhibitor and rapamycin effects indicate a causative role for Akt/mTOR hyperactivation. Akt promotes metabolism by increasing Glut1 expression and activity, regulating enzymes in glucose and lipid metabolism and promoting mTOR-dependent protein translation (Juntilla et al., 2007). In DN cells, Pdk1/Akt/mTORC1 also upregulate surface CD71 and CD98 downstream of pre-TCR and Notch (Kelly et al., 2007; Fayard et al., 2010). Thus, upregulated iron uptake, glucose and amino acid metabolism and
protein biosynthesis might all contribute to the accelerated, Notch-independent development of Itpkb\(^{-/-}\) DN3 cells.

Excessive Notch signaling causes thymocyte transformation and T-ALL. This is augmented by pre-TCR signals (Campese et al., 2006; Fayard et al., 2010). Excessive Akt activity in thymocytes due to PI3K hyperactivity, Pten inactivation or dominant-active Akt expression causes leukemia/lymphoma (Aifantis et al., 2006; Fayard et al., 2010). The intermediate β-selection phenotype of Itpkb-loss between those of Pten-loss (Hagenbeek et al., 2004; Kelly et al., 2007; Shiroki et al., 2007; Wong et al., 2012) and Inpp5d/SHIP1-loss (Kashiwada et al., 2006) raises the possibility that IP\(_3\) 3-kinases could have tumor suppressor functions by limiting Akt signaling. But we have not seen thymocyte neoplasia or accumulation of intracellular TCR\(\beta\) DP cells in Itpkb\(^{-/-}\) mice. One possible explanation consistent with low residual IP\(_3\) 3-kinase activity and IP\(_4\)-production in Itpkb\(^{-/-}\) thymocytes (Wen et al., 2004; Pouillon et al., 2003) is partial Itpkb redundancy with other IP\(_3\) 3-kinases. Moreover, their premature lethality due to infections (Pouillon et al., 2003) and anemia (Siegemund et al., 2015) limits aging studies with Itpkb\(^{-/-}\) mice. It will be important to re-assess in a germ-free vivarium whether conditional Itpkb-disruption in thymocytes causes T-ALL as the mice age, or on a sensitized Trp53\(^{-/-}\) background as seen for p65\(^{pI3K}\) transgenics (Borlado et al., 2000). Then again, IP\(_4\)-loss might simply not augment PIP\(_3\) cellular activity sufficiently to transform thymocytes, reminiscent of Inpp5d/SHIP1\(^{-/-}\)-loss (Kashiwada et al., 2006). Also, the potential reduction of required Akt-unrelated IP\(_4\) functions such as promoting Itk/Erk signaling (Huang et al., 2007) might prevent thymocyte transformation in Itpkb\(^{-/-}\) mice. Clearly, more studies are needed to assess the tumor suppressor potential of Itpks.

By unveiling Itpkb antagonism with PI3K/Akt/mTOR signaling as a key determinant of the kinetics and Notch-dependence of thymocyte β-selection, our findings expand our limited knowledge about physiological IP\(_3\) 3-kinase functions (Sauer and Cooke, 2010). They unveil a novel molecular mechanism that integrates pre-TCR signaling with costimulatory Notch signaling to specifically restrict DN3 cell differentiation uncoupled from proliferation and survival. Broad expression of IP\(_3\) 3-kinases and PI3Ks, IP\(_4\) detection in multiple tissues (Sauer and Cooke, 2010) and common PI3K implication in costimulation raise the possibility that ‘metabokinetic’ control and costimulation-enforcement through non-canonical PI3K antagonism by IP\(_3\) 3-kinases are broadly relevant.

**Materials and methods**

**Mice**

Our C57BL/6 Itpkb\(^{-/-}\) mice were described in (Sauer et al., 2013). All animal studies were approved by the Scripps Research Institute animal care and use committee and conform to all relevant regulatory standards. Mixed bone marrow chimeras were generated as in (Sauer et al., 2013). For in vivo induction of Rag2\(^{-/-}\) DN3 cell differentiation, 10 μg anti-CD3 antibodies (BD Biosciences, San Jose, CA, clone 145-2C11) were injected i.p. 1–3 days later, the mice were euthanized and analyzed. Where indicated, the mice were treated orally once daily with 10 mg/kg LY-411,575 (Wong et al., 2004) or vehicle (5% polyethylene glycol, 3% propylene glycol, 1% ethanol, 0.4% methylcellulose). The first dose was administered 3–4 hr prior to α-CD3 injection.

For BrdU incorporation assays, we injected mice i.p. with 100 μl BrdU [10 mg/ml] and analyzed thymi 4 hr later. For preparation of thymocyte suspensions, thymi were placed in M199 medium/2% FCS/1x penicillin/streptomycin/glutamate at room temperature and single-cell suspensions prepared by passage through a 40 μm mesh (BD Biosciences).

**Flow cytometry and cell sorting**

Thymocytes were stained with fluorochrome-conjugated antibodies against CD2 (clone RM2-5), CD3 (145-2C11, eBiosciences), CD4 (SK1.5), CD5 (53–7.3), CD8α (53–6.7), CD24/HSA (M1/69), CD25 (3C7), CD27 (LG.3A10), CD44 (IM7), CD71 (R17217), CD98 (RL388, eBiosciences), CD127 (A7R34), TCR\(\beta\) (H57-597) or CXCR4 (2B11, eBiosciences). Our lineage (Lin) cocktail included biotinylated antibodies against CD11b (M1/70), CD11c (N418), CD19 (6D5), B220 (30-F11), CD49b (DX5), Gr-1 (RB6-8C5), Ly-76/Ter119 (TER-119) and TCR\(\gamma/\delta\) (GL3). Unless indicated otherwise, all antibodies were from BioLegend. For intracellular staining, cells were permeabilized with BD Cytofix/Cytoperm kits or 0.3% Triton X100 and stained with antibodies against Ki-67 (B56, BD Biosciences), phospho-Akt T\(_{308}\)
(C31E5E, Cell Signaling Technology), phospho-mTORC1 S2481 (poly6517, Biolegend) (Soliman et al., 2010), phospho-ribosomal protein S6 S235/S236 (DS7.2.2E, Cell Signaling Technology), Glut1 (Fitzgerald Industries), phospho-Erk T202/Y204 (D13.14.4E, Cell Signaling Technology), Akt (Cell Signaling Technology) or isotype controls (Cell Signaling Technology) followed by anti-rabbit IgG secondary antibodies (Cell Signaling Technology) if needed. Calyculin A (Cell Signaling) was used according to the manufacturer’s protocol. Annexin V staining was performed using eBioscience Annexin V apoptosis detection kits. BrdU incorporation was assayed with BD Biosciences BrdU-FITC kits, using 0.8 μl anti-BrdU-FITC antibodies per 10^6 cells. All data were acquired on an LSRII flow cytometer (BD Biosciences) and analyzed using FlowJo software.

For DN3 cell purification, thymocytes were first immunomagnetically depleted of CD3, CD4 or CD8α positive cells using biotinylated antibodies against CD3 (145-2C11, Biolegend), CD4 (GK1.5, eBioscience) and CD8α (53–6.7, eBioscience), anti-biotin microbeads and LS columns (Miltenyi Biotech) according to the manufacturer’s protocol, and then stained with anti-CD44-FITC (IM7, eBiosciences), anti-CD25-PerCp-Cy5.5 (3C7, Biolegend) and Streptavidin-APC (Life Technologies). CD44^-CD25^+SA^-DN3 cells were sorted on a BD FACS Aria cell sorter. CD53^-DP thymocytes were purified as in (Huang et al., 2007).

**Fetal thymic organ cultures**

Different lobes from the same thymus of an embryonic day 15.5 (E15.5) Itpkb^+/+ or Itpkb^-/- embryo were cultured on gelfoam sponges in complete DMEM-10 with vehicle (100% ethanol) or 20 μM rapamycin in ethanol (BIOTANG/TSZCHEM). New vehicle or rapamycin were added on culture days 1, 2 and 3. The lobes were analyzed on day 4.

**Op9 cell co-cultures**

OP9 or OP9DL1 cells (Ciofani and Zuniga-Pflucker, 2005; Ciofani et al., 2004) were seeded at 8000 cells per well and incubated for 24 hr in OP9 Culture Media (alpha-MEM, Life Technology/15% FCS/1x Penicillin and Streptomycin), followed by addition of 70,000–100,000 sorted DN3 cells per well together with 1 ng/ml recombinant mouse IL-7 (PeproTech) and once on day 0 (rapamycin, Akt-I) or once-daily (2DG) addition of carrier, 500 nM Akt-inhibitor VIII (Sauer et al., 2013) (Akt-I, Calbiochem) in DMSO, 4 μM rapamycin (BIOTANG/TSZCHEM) in ethanol or 500 μM 2-deoxy-D-glucose (Wang et al., 2011) (2DG, SIGMA) in PBS (all concentrations final).

**Biochemistry**

Thymocytes were lysed as previously described (Huang et al., 2007) in 1% Triton X-100/60 mM octylglucoside/150 mM NaCl/25 mM Tris-Cl, pH 7.5/1 mM EDTA containing Roche Complete Mini Protease Inhibitor and PhosSTOP Phosphatase Inhibitor Cocktails. Lysates were incubated for 20 min at 4°C, then cleared by centrifugation at 14,000 g for 10 min at 4°C. For immunoprecipitations, pre-cleared lysates were incubated for 1.5 hr with anti-Itpkb antibodies (G-20, Santa-Cruz Biotechnology) followed by incubation with Protein G-conjugated beads for 1.5 hr. Beads were washed 3 times with 1x lysis buffer, denaturated in 1x sample buffer at 99°C for 10 min and analyzed via SDS-PAGE/immunoblot. For immunoblot analysis, nitrocellulose membranes were incubated overnight at 4°C with anti-Itpkb (#AP8167b, Abgent) or anti-PLCγ1 (#2822, Cell Signaling Technology) antibodies and then for 45 min with anti-rabbit-HRP secondary antibodies (Bio-Rad Laboratories) in TBS. Bound antibodies were detected by enhanced chemiluminescence (ECL kit, GE Healthcare).

**Mathematical modeling**

The kinetics of DN thymocyte differentiation were modeled by a set of linear ordinary differential equations (ODE). In these, the rate constants for DN3 cell generation and for thymocyte subset turnover were similar between genotypes. The rate constants for DN3-to-DP cell differentiation were increased over two-fold for Itpkb^-/- cells. The ODE were solved by pen and paper calculations and results verified using BIONETGEN.
Modeling steady-state thymocyte developmental kinetics in WT vs. Itpkb−/− mice

We have built a linear ordinary differential equation (ODE) based model for the kinetics of β-selection in the presence or absence of Itpkb. We approximated the arrival of hematopoietic progenitors followed by their successive maturation via DN1 to DN3 cells as a constant influx rate $K$ (Figure 5A). DN3 cells transit to the DN4 stage with a differentiation rate $K_1$ or turn over at a turnover rate $K_{d1}$. Similarly, cells at the DN4 stage can further differentiate into ISP at a rate $K_2$ or turn over at a rate $K_{d2}$. ISP cells further mature into DP cells at a rate $K_3$ or turn over at a rate $K_{d3}$. Most DP cells turn over through death by neglect at a rate $K_{d4}$. Only very few DP cells mature to SP thymocytes ($\text{Starr et al., } 2003$); these are ignored. In the ODE, we denote the numbers of DN3, DN4, ISP and DP cells as $C_1$, $C_2$, $C_3$ and $C_4$, respectively.

\[
\begin{align*}
\frac{dC_1}{dt} &= K - K_1C_1 - K_{d1}C_1 \\
\frac{dC_2}{dt} &= K_1C_1 - K_2C_2 - K_{d2}C_2 \\
\frac{dC_3}{dt} &= K_2C_2 - K_3C_3 - K_{d3}C_3 \\
\frac{dC_4}{dt} &= K_3C_3 - K_{d4}C_4
\end{align*}
\]

(S1)

It is reasonable to assume that the thymocyte subset population sizes represent steady state solutions of the above ODEs. The ODEs reach the steady state at time scales much longer than the times associated with the kinetic rate constants. At the steady state, $\frac{dC_1}{dt}=\frac{dC_2}{dt}=\frac{dC_3}{dt}=\frac{dC_4}{dt}=0$. This implies that the influx of cells to a particular stage due to differentiation is balanced by outflux due to both differentiation into the next stage and cell death. The steady state solutions of the ODEs are then given by

\[
\begin{align*}
C_1 &= \frac{K}{K_1 + K_{d1}} \\
C_2 &= \frac{K_1K_2}{(K_1 + K_{d1})(K_2 + K_{d2})} \\
C_3 &= \frac{K_1K_3}{(K_1 + K_{d1})(K_2 + K_{d2})(K_3 + K_{d3})} \\
C_4 &= \frac{K_1K_3}{K_{d4}(K_1 + K_{d1})(K_2 + K_{d2})(K_3 + K_{d3})}
\end{align*}
\]

(S2)

This implies that regardless of the initial values of the population sizes, the system will reach the above concentrations at long times. DN4 cells and ISP are highly proliferative compared to DN3 and DP cells but show similar overall viability as those, resulting in lower turnover rates (Figure 3C,D). The DN4 cell and ISP cell turnover rates ($K_{d2}, K_{d3}$) are thus small compared to the respective differentiation rates ($K_2, K_3$), such that $K_2/(K_{d2}+K_2) \approx 1$ and $K_3/(K_{d3}+K_3) \approx 1$. In this case, equation (S2) can be further simplified as

\[
\begin{align*}
C_1 &= \frac{K}{K_1 + K_{d1}} \\
C_2 &= \frac{K_1K_2}{K_2(K_1 + K_{d1})} \\
C_3 &= \frac{K_1K_3}{K_1(K_1 + K_{d1})} \\
C_4 &= \frac{K_1K_3}{K_{d4}(K_1 + K_{d1})}
\end{align*}
\]

(S3)

From equation (S3), we observe that when differentiation rates $K_1$ (DN3 to DN4 cells), $K_2$ (DN4 cells to ISP) and $K_3$ (ISP to DP cells) increase while progenitor influx rate $K$ is constant, then the
numbers of DN3 cells (\(C_3\)) and DP cells (\(C_4\)) remain relatively unaffected, while the numbers of DN4 cells (\(C_2\)) and ISP (\(C_1\)) decrease. Indeed, increasing \(K_r\), \(K_2\) and \(K_3\) to the values in Table 1 modeled the steady state DN cell subset number distribution in WT vs. \(Itpkb^{+/−}\) (increased \(K_{1/2/3}\)) mice (Figure 2C, Figure 5B).

Modeling thymocyte developmental kinetics in α-CD3 antibody injected Rag2\(^{−−}\) mice

In Rag2\(^{−−}\)/Itpkb\(^{+/−}\) or Rag2\(^{−−}\)/Itpkb\(^{+/−}\) mice, essentially all thymocytes are arrested at the DN3 stage. α-CD3 antibody injection triggers their synchronized maturation to DP cells with high proliferation and viability of all developmental stages (Figure 6C–H). Progenitor influx within 3 days is negligible. Therefore, we set the progenitor influx rate (\(K\)) to 0. We started our calculation with \(C(0) = 3 \times 10^6\) DN3 cells and infinitesimally low numbers of DN4, ISP and DP cells, respectively. Otherwise, we used the same differentiation rates and turnover rates as above (Table 1). The kinetics of the thymocyte population sizes can then be described by

\[
\begin{align*}
\frac{dC_1}{dt} &= -K_1C_1 - K_{d1}C_1 \\
\frac{dC_2}{dt} &= K_1C_1 - K_2C_2 \\
\frac{dC_3}{dt} &= K_2C_2 - K_3C_3 \\
\frac{dC_4}{dt} &= K_3C_3 - K_{d4}C_4
\end{align*}
\]  

(S4)

Equation (S4) is solved analytically. To express the solution compactly, we use the variable \(\tilde{K}_1 = K_1 + K_{d1}\):

\[
\begin{align*}
C_1(t) &= C_1(0)\exp(-\tilde{K}_1 t) \\
C_2(t) &= \frac{K_1C_1(0)}{K_2 - \tilde{K}_1}\left[\exp(-\tilde{K}_1 t) - \exp(-K_2 t)\right] \\
C_3(t) &= \frac{K_1K_2C_1(0)}{K_3 - K_2}\left[\exp(-K_2 t) - \exp(-K_3 t)\right] + \frac{K_2 - \tilde{K}_1}{K_3 - K_1}\exp(-K_3 t) \\
C_4(t) &= \frac{K_1K_2K_3C_1(0)}{K_2 - K_1}\left[\exp(-\tilde{K}_1 t) - \exp(-K_2 t)\right] + \frac{K_2 - \tilde{K}_1}{K_3 - K_1}\left[\exp(-K_3 t) - \exp(-K_{d4} t)\right] \\
&\quad - \frac{K_1K_2K_3C_1(0)}{K_2 - K_1}\left[\exp(-K_2 t) - \exp(-K_{d4} t)\right]
\end{align*}
\]  

(S5)

In equation (S5), \(t\) denotes the time post α-CD3 antibody injection in hr. The ODE were solved by pen and spaper calculations and results verified using BIONETGEN (Hlavacek et al., 2006). Results of a simulation with 3 hr time increments for 3 days are shown in Figure 6A,B.

Table 1. Parameters used in our mathematical models.

| Genotype          | \(K\) (cells/day) | \(K_1\) (days\(^{-1}\)) | \(K_{d1}\) (days\(^{-1}\)) | \(K_2\) (days\(^{-1}\)) | \(K_3\) (days\(^{-1}\)) | \(K_{d4}\) (days\(^{-1}\)) |
|-------------------|-------------------|------------------------|------------------------|------------------------|------------------------|------------------------|
| WT                | \(15.4 \times 10^4\) | 0.1                    | 0.1                    | 0.162                  | 0.07                   | 0.00058                |
| Itpkb\(^{−−}\)    | \(15.4 \times 10^4\) | 0.2                    | 0.1                    | 0.486                  | 0.21                   | 0.00058                |
| Rag2\(^{−−}\)/Itpkb\(^{+/−}\) | 0          | 0.1                    | 0.1                    | 0.162                  | 0.07                   | 0.00058                |
| Rag2\(^{−−}\)/Itpkb\(^{+/−}\) | 0          | 0.2                    | 0.1                    | 0.486                  | 0.21                   | 0.00058                |

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Statistical analyses

Aggregated results are shown as mean ± SEM. p values for the indicated comparisons were calculated by two-tailed unpaired Student’s t-test. Group sizes are described in the figure legends. Significant p values are denoted by asterisks: *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001. All statistical analyses were performed in Prism.

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Ethics

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