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ORIGINAL ARTICLE

A cell autonomous role for the Notch ligand Delta-like 3 in $\alpha\beta$ T-cell development

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Notch signalling is critical to help direct T-cell lineage commitment in early T-cell progenitors and in the development of $\alpha\beta$ T-cells. Epithelial and stromal cell populations in the thymus express the Notch DSL (Delta, Serrate and Lag2) ligands Delta-like 1 (Dll1), Delta-like 4 (Dll4), Jagged 1 and Jagged 2, and induce Notch signalling in thymocytes that express the Notch receptor. At present there is nothing known about the role of the Delta-like 3 (Dll3) ligand in the immune system. Here we describe a novel cell autonomous role for Dll3 in $\alpha\beta$ T-cell development. We show that Dll3 cannot activate Notch when expressed in *trans* but like other Notch ligands it can inhibit Notch signalling when expressed in *cis* with the receptor. The loss of Dll3 leads to an increase in *Hes5* expression in double positive thymocytes and their increased production of mature CD4⁺ and CD8⁺ T cells. Studies using competitive irradiation chimeras proved that Dll3 acts in a cell autonomous manner to regulate positive selection but not negative selection of autoreactive T cells. Our results indicate that Dll3 has a unique function during T-cell development that is distinct from the role played by the other DSL ligands of Notch and is in keeping with other recent studies indicating that Dll1 and Dll3 ligands have non-overlapping roles during embryonic development.

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The DSL (Delta, Serrate and Lag2) family of Notch ligands are single pass type I transmembrane proteins that contain an N-terminal cysteine rich DSL domain, epidermal growth factor-like repeats, a transmembrane domain and a C-terminal domain of variable length.¹ Notch signalling is transmitted through interaction between Notch ligands on one cell and Notch receptors expressed on a neighbouring cell. This interaction elicits a series of specific proteolytic cleavage events that culminate in the release of the intracellular domain of Notch (Notch IC).² Notch IC enters the nucleus where it interacts with the CBF1/Su(H)/Lag1 (CSL) DNA-binding protein to activate downstream genes such as those of the HES and HEY families of basic-loop-helix transcription factors.² In mammals, Notch signalling is mediated by the Delta-like (Dll1, Dll4) and Serrate-like (Jagged1, Jagged2) DSL ligands.^{3–6} In addition both classes of DSL ligands can inhibit Notch signalling when expressed in the same cell as Notch; this phenomenon is referred to as *cis* or cell autonomous inhibition of Notch signalling.^{7–9}

The investigation of DSL Notch ligands in T-cell development has largely focussed on their role in stromal cell populations and previous studies have identified that the Delta-like and Serrate-like ligands are critical to drive commitment to the T-cell lineage, but little is known about the role of Notch signalling beyond the double positive (DP) stage of thymocyte development following positive or negative selection.^{4–6,10,11} Originally it was thought that the Dll1 and Dll4 ligands had redundant roles in the thymus as deletion of Dll1 in thymic

stroma did not block T-cell development and stromal cells ectopically expressing either Dll1 or Dll4 were sufficient in supporting T-cell development *in vitro*.^{4,11} However, more recent studies have shown that Dll4 is the primary ligand expressed by thymic epithelial cells that direct T-cell differentiation in haematopoietic progenitors.^{12,13} At present there is nothing known about the role of the Delta-like 3 (Dll3) ligand in the thymus and whether or not it has a role in regulating T-cell development. This is an important issue as Dll3 is likely to function differently to the other DSL ligands in embryonic development.^{8,14} Here we show that Dll3 is unique amongst DSL ligands of Notch. It is expressed in thymocytes and is most abundant on mature T cells. We have examined T-cell development in *Dll3* deficient mice and find that in the absence of *Dll3* there is increased positive selection of DP thymocytes to both CD4⁺ and CD8⁺ T-cell lineages whereas negative selection of autoreactive T cells remains intact. The loss of *Dll3* leads to an increase in T-cell receptor (TCR) signalling in DP cells and an increase in *Hes5* expression. Thus Dll3 acts in a cell autonomous manner to regulate Notch signalling and this is important in regulating TCR signal strength.

RESULTS

Dll3 expression by thymocytes

We examined the expression of Notch1 and different DSL ligands using RNA prepared from fluorescence-activated cell sorting (FACS)

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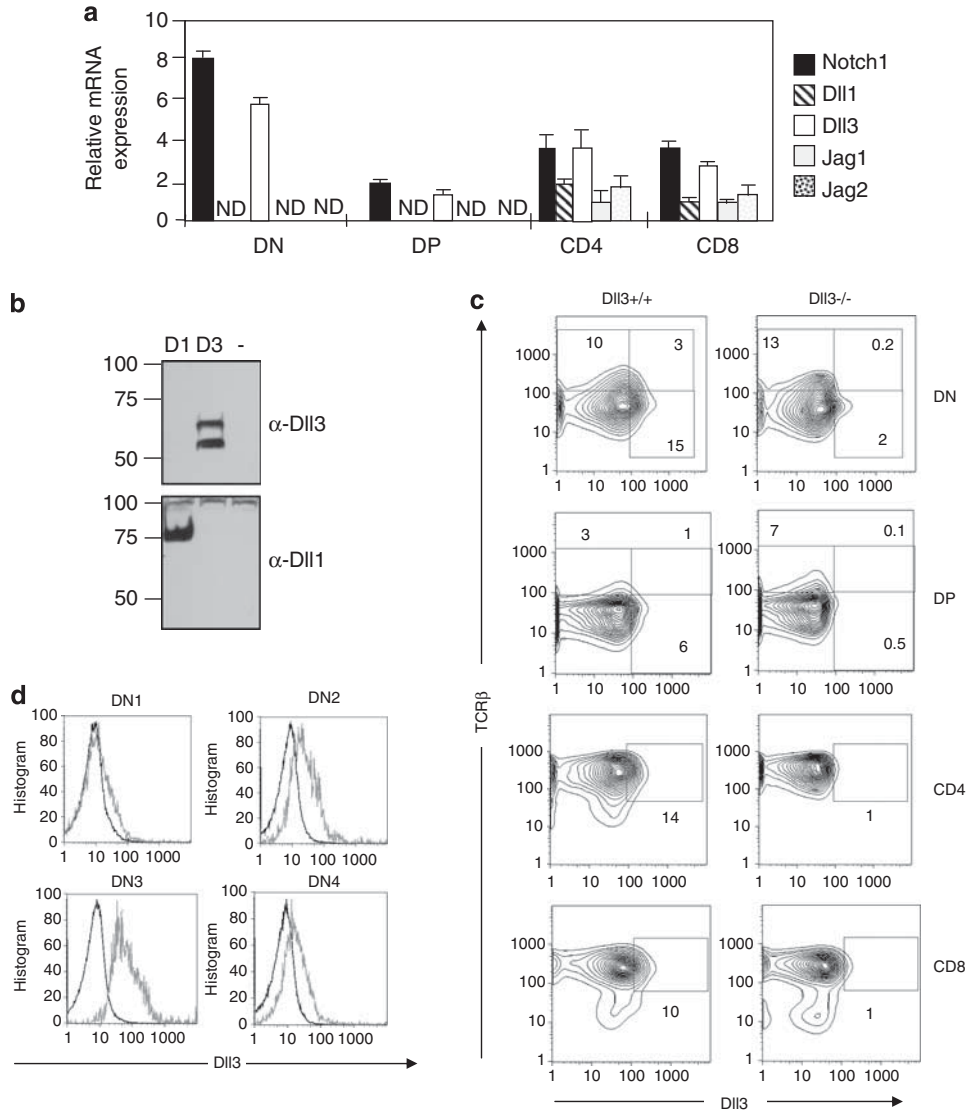


Figure 1 *Dll3* expression in T-cell development. **(a)** Real time PCR expression of *Notch1* (filled bars), *Dll1* (hatched bars), *Dll3* (open bars), *Jagged1* (grey bars) and *Jagged2* (stippled bars) in thymocytes at different stages of T-cell development. Data represent the average expression in three independent cell isolations. ND=not detected. **(b)** Western blot detection of Dll3 protein (using a novel anti-Dll3 antiserum) in lysates from C2C12 cells transfected with Dll3-HA (D3) but not in lysates from Dll1-HA transfected (D1) nor from untransfected cells (-). Dll1 expression was demonstrated on the same blot using an antibody against mouse Dll1. **(c)** Thymocytes were stained initially with anti-CD4, CD8, TCR-β and following intracellular permeabilisation were stained with anti-Dll3. Thymocytes were gated on DN (CD4⁻CD8⁻), DP (CD4⁺CD8⁺), CD4 or CD8 SP cells and the staining shows the expression of Dll3 versus TCR-β in the different cell populations for *Dll3*^{+/+} cells compared with *Dll3*^{-/-} mice, which serves as a negative control. The data are representative of one of four experiments performed with a total of 12 mice. **(d)** Lineage negative thymocytes were stained with anti-CD44 and anti-CD25 to identify the different DN cell subsets and FACS sorted and stained intracellularly with anti-Dll3. Data show representative histograms of Dll3 expression on DN1, DN2, DN3 and DN4 cells in the thymus of *Dll3*^{+/+} (grey line) and the *Dll3*^{-/-} (black line).

sorted double negative (DN), DP, CD4 single positive (SP) and CD8 SP thymocytes using real time PCR. The *Dll3* gene is expressed at all stages of T-cell development; expression was highest in DN cells, lowest in DP thymocytes and the predominant ligand in CD4 and CD8 SP cells (Figure 1a). Notably *Dll1*, *Jagged1* and *Jagged2* were absent from DN and DP cells, but were detected in CD4 and CD8 SP cells as previously published (Figure 1a).¹⁵ *Notch1* expression showed *Dll3* being high in DN cells, low in DP cells and higher in SP cells (Figure 1a).¹⁶ Next we examined the expression of the Dll3 protein in thymocytes using a polyclonal antibody raised to a peptide sequence on the C-terminus of the Dll3 protein that is highly divergent to other DSL ligands.¹⁴ The specificity of the Dll3 antibody was determined by

western blotting to lysates from Dll3- or Dll1-transfected C2C12 cells. As shown in Figure 1b the Dll3 antibody was specific for the Dll3 protein and does not cross react with the related Dll1 ligand (or Jagged1 (data not shown)), nor did it bind non-specifically to cell extracts. Next we examined the expression of the Dll3 ligand in *Dll3*^{+/+} thymocytes that were stained intracellularly and analysed by FACS, whereas *Dll3*^{-/-} thymocytes served as an effective negative control. The Dll3 protein was readily detected in a subset of mature CD4⁺ TCR-β^{hi} (14%) and CD8⁺ TCR-β^{hi} (10%) cells and was also detected in TCR-β^{lo} DP and DN thymocytes cells (Figure 1c). Lineage negative thymocytes were stained with CD44 and CD25 to identify the different DN cell types and following FACS sorting were analysed for

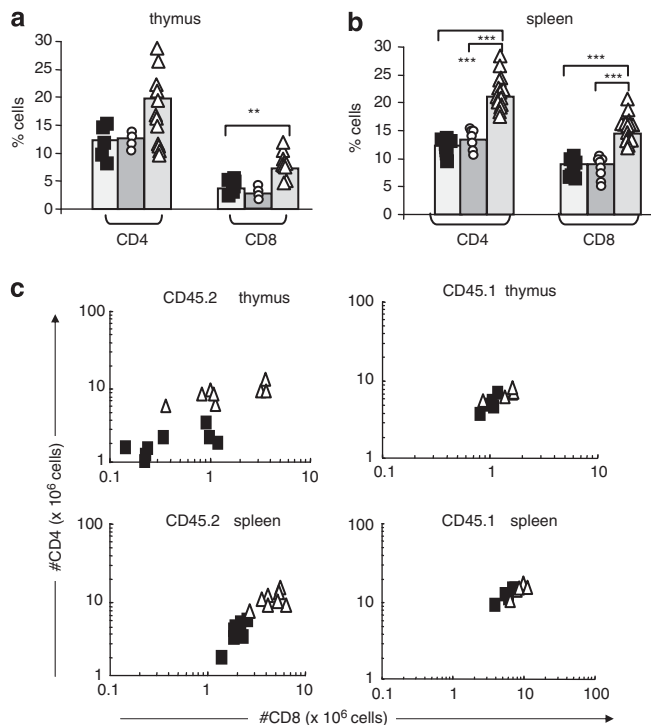


Figure 2 Loss of *Dll3* leads to an expansion of mature T cells in the thymus and spleen. Graphs display the percentage of (a) CD4⁺ and CD8⁺ thymocytes and (b) CD4⁺ and CD8⁺ splenocytes in *Dll3*^{+/+}, *Dll3*^{+/-} and *Dll3*^{-/-} mice. Mice were aged and sex matched; *Dll3*^{+/+} ($n=8$), *Dll3*^{+/-} ($n=4$) and *Dll3*^{-/-} ($n=12$). Bars represent the average for each group. Unpaired *t*-test was used to test for statistically significant differences between groups. ** $P<0.01$, *** $P<0.001$. (c) The absolute numbers of CD4⁺ and CD8⁺ T cells in the thymus and spleen of chimeric mice. The origin of the donor cells in each chimera was distinguished on the basis of CD45 allelic expression by FACS and the number of CD45.2 and CD45.1 derived CD4⁺ and CD8⁺ T cells was calculated. Cells from the *Dll3*^{+/+}/CD45.1 chimeras (filled squares), and *Dll3*^{-/-}/CD45.1 chimeras (open triangles) are shown.

Dll3 expression by FACS. *Dll3* was predominantly expressed by DN2 and DN3 cells and was lowest in DN1 and DN4 stage cells (Figure 1d). These results indicate that *Dll3* is expressed around the time of β -selection, which occurs in DN3 stage thymocytes.

Dll3 acts cell autonomously to regulate T-cell production *in vivo*

The generation of *Dll3*^{-/-} mice has been described.¹⁷ On a pure C57BL/6 background, *Dll3*^{-/-} mice die at birth but on a mixed (129/C57BL/6) F2 background, *Dll3*^{-/-} mice are viable and are born at the expected Mendelian frequencies. We examined T-cell development in the thymus of *Dll3*^{+/+}, *Dll3*^{+/-} and *Dll3*^{-/-} F2 mice by flow cytometry and each of the major T-cell subsets were present indicating that *Dll3* is not absolutely required for normal T-cell differentiation. However there was a significant increase in the frequency of CD8⁺ T cells in *Dll3*^{-/-} thymus compared with *Dll3*^{+/+} and *Dll3*^{+/-} mice ($P<0.01$) and a trend towards increased CD4⁺ cell frequency that just failed to reach statistical significance (Figure 2a). In the spleen there was a significant increase in the frequency of both CD4⁺ and CD8⁺ T cells in *Dll3*^{-/-} mice compared with *Dll3*^{+/+} and *Dll3*^{+/-} mice ($P<0.001$) (Figure 2b).

The changes in CD4⁺ and CD8⁺ T-cell frequencies in the thymus and spleen of *Dll3*^{-/-} mice prompted us to use mixed fetal liver cell chimeras to assess the contribution of the stromal compartment to the

changes in the observed T-cell frequencies. To avoid complications with the F2 mixed genetic background in the irradiation chimeras we chose to use fetal liver cells from *Dll3*^{-/-} and *Dll3*^{+/+} mice on a C57BL/6 background. The initial group of chimeras was generated by injecting an equal mix (50:50) of CD45.1 and *Dll3*^{-/-} (CD45.2) fetal liver cells into irradiated CD45.1 recipients or CD45.1 and *Dll3*^{+/+} (CD45.2) fetal liver cells into irradiated CD45.1 recipients. Thymus and spleen cells of the recipient mice were examined 12 weeks after the transfer. Cells were stained and analysed by flow cytometry and the donor-derived cells were distinguished on the basis of differences in CD45 allelic expression.

The *Dll3*^{-/-} progenitors were able to compete effectively with wild-type CD45.1 cells leading to normal CD4⁺ and CD8⁺ T-cell differentiation. It was noticeable that the *Dll3*^{-/-} progenitors gave rise to 3–4 fold more mature CD4⁺ and CD8⁺ T cells in the thymus compared with the *Dll3*^{+/+} CD45.2 progenitors ($P=0.01$) and this difference was also maintained in the spleen ($P=0.001$) (Figure 2c). In contrast there was no difference in the number of CD4⁺ and CD8⁺ T cells of CD45.1 origin in the thymus or spleen between the two sets of chimeras (Figure 2c). The results of the fetal liver chimeras indicates that the increase in mature T cells observed when *Dll3* is absent is due to a haemopoietic rather than a stromal defect and that *Dll3* has a non-redundant role in the regulation of $\alpha\beta$ -T-cell production in the thymus. To confirm this finding we set up reciprocal chimeras where bone marrow cells from CD45.1+ donor mice were transferred to irradiated *Dll3*^{+/+} or *Dll3*^{-/-} mice. Twelve weeks later we analysed the thymus and spleen and observed no difference in the number of DP cells or mature CD4⁺ and CD8⁺ T cells in the thymus or spleen between the different recipient mice (Supplementary Figure 1). This result confirms that the expression of *Dll3* in T cells is required for regulating T cell development in the thymus.

Dll3 regulates the expansion of T-cell progenitors

To confirm that *Dll3* acts in a cell autonomous manner in T-cell progenitors during TCR $\alpha\beta$ development, we made use of the OP9-Dll1 stromal line, which can support T-cell development *in vitro* along both TCR $\alpha\beta$ and TCR $\gamma\delta$ cell lineages.⁶ Fetal liver haematopoietic stem cells (HSCs) depleted of lineage committed cells (that is, Lin^{-ve}) derived from either *Dll3*^{+/+} or *Dll3*^{-/-} embryos (CD45.2) were combined at different starting ratios of 5:95, 25:75 and 50:50 with fetal liver HSCs purified from wild-type CD45.1 embryos. The HSCs were cultured with either control OP9 cells or OP9-Dll1 cells and were analysed by flow cytometry after 21 days. The HSCs cultured on OP9-Dll1 stromal cells expanded and underwent differentiation along the TCR $\alpha\beta$ and TCR $\gamma\delta$ lineages; however, the TCR $\alpha\beta$ lineage predominated for both *Dll3*^{+/+} and *Dll3*^{-/-} derived cell populations (Figure 3a). We examined the proportion of CD45.2 (*Dll3*^{+/+} or *Dll3*^{-/-}) cells within the final cell populations at the end of the culture period by flow cytometry. At all ratios the tested *Dll3*^{-/-} cells had expanded at a greater rate than the *Dll3*^{+/+} cells when placed in direct competition with CD45.1 wild-type cells (Figure 3b). Next we examined the proportion of CD45.2 cells within the DN and DP stages of development by flow cytometry. The *Dll3*^{+/+} cells remained at the same proportion as the starting ratio irrespective of the stage of T-cell development examined (Figure 3c). In contrast, *Dll3*^{-/-} cells always expanded well beyond the starting ratio, for example at the 5:95 ratio, *Dll3*^{-/-} cells represented on average 18% of the final DN and 12% of the DP cells (Figure 3c). At the 25:75 ratio the *Dll3*^{-/-} cells represented an average 50% of the DN and 40% of DP cells, whereas at the 50:50 ratio the *Dll3*^{-/-} cells comprised about 65% of the DN and 60% DP cells (Figure 3c).

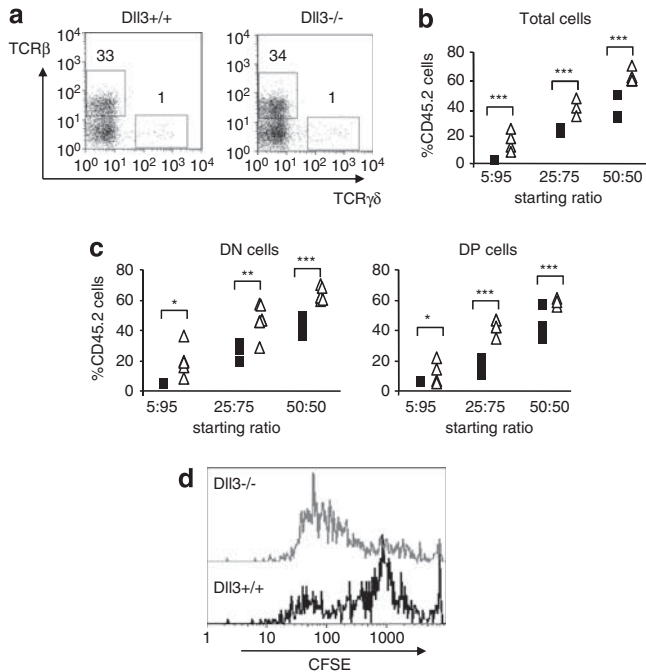


Figure 3 Loss of *Dll3* leads to increased production of TCR $\alpha\beta$ cells from fetal liver HSCs. Fetal liver from *Dll3*^{+/+} and *Dll3*^{-/-} HSCs were mixed at different ratios with E18 fetal liver HSCs from CD45.1+ embryos, these were cultured on OP9-Dll1 stromal cells *in vitro* and after 3 weeks cells were stained and analysed by FACS. (a) Representative FACS dot plots derived from *Dll3*^{+/+} and *Dll3*^{-/-} cells stained with anti-TCR β and anti-TCR δ antibodies. (b) Graph shows the percentage of CD45.2 derived cells in the total lymphocyte populations that were derived from the OP9-Dll1 stromal cell cultures. *** $P=0.0001$. (c) Graph shows the percentage of CD45.2-derived DN cells (left) * $P=0.029$, ** $P=0.001$, *** $P=0.0001$ and DP cells (right) * $P=0.019$, *** $P<0.001$ derived from the OP9-Dll1 stromal cell cultures. (d) FACS analysis of carboxyfluorescein diacetate succinimidyl ester (CFSE) dilution of *Dll3*^{+/+} (black line) and *Dll3*^{-/-} DN cells (grey line) grown on OP9-Dll1 cells for 3 days. Data show a representative overlay histogram comparing CFSE expression on Thy1+ lymphocytes growing in the cultures.

As the *Dll3*^{-/-} progenitors could not compete with the wild-type cells *in vitro* and *in vivo*, we examined the proliferative capacity of the *Dll3*^{+/+} and *Dll3*^{-/-} DN cells using carboxyfluorescein diacetate succinimidyl ester labelled purified Lin^{-ve} DN1 thymocytes. The cells were cultured in the presence of OP9-Dll1 cells and analysed by FACS after 3 days. The *Dll3*^{-/-} DN1 cells proliferated more extensively than the *Dll3*^{+/+} cells during the same culture period (Figure 3d). It should be noted that in this series of experiments the bone marrow HSCs from *Dll3*^{+/+} and *Dll3*^{-/-} mice that were cultured with the OP9 stromal line failed to expand *in vitro* over the culture period (data not shown). Therefore the *Dll3*^{+/+} and *Dll3*^{-/-} HSCs both rely on the delivery of an exogenous Notch ligand signal to support their growth and expansion on OP9-Dll1 stromal cells *in vitro* (discussed further below). Collectively these data obtained from the irradiation chimeras and the OP9-Dll1 assays, confirmed a cell autonomous function for Dll3 in regulating $\alpha\beta$ -T-cell development that begins with the early T cell progenitors.

Changes in TCR signalling detected in *Dll3*^{-/-} DP cells

We wanted to examine if the loss of Dll3 in thymocytes leads to any phenotypic changes during T cell development. Compared with the *Dll3*^{+/+} cells there was no difference in the cell surface expression of CD3, CD5 or CD69 on *Dll3*^{-/-} cells at any stage of T-cell

differentiation (Figure 4g and data not shown). Next we examined whether or not we could observe changes in TCR signalling within DP cells of the *Dll3*^{-/-} mice. One of the earliest events in TCR signalling is the dephosphorylation of the inhibitory tyrosine's on the non-receptor tyrosine kinase p56LCK.^{18,19} Thymocytes from newborn *Dll3*^{+/+} and *Dll3*^{-/-} mice were stained intracellularly and analysed for changes in pLckY505, pErk, Bcl-2 and pGSK3 β expression by flow cytometry. The *Dll3*^{-/-} DP cells showed decreased expression of pLckY505 and increased pErk staining compared with *Dll3*^{+/+} DP cells, but there was no difference in Bcl-2 or pGSK3 β staining (Figures 4a and b). These changes in TCR signalling were restricted to the DP cells, as mature CD4⁺ or CD8⁺ T cells in the thymus or spleen do not show any difference in staining of pLckY505 or pErk between *Dll3*^{+/+} and *Dll3*^{-/-} mice (Figures 4c and d and data not shown). Next we examined whether we could observe similar changes in pLckY505 expression in the thymus of the mixed chimaeric recipients previously described. DP cells from the thymus of the chimaeric recipients were stained intracellularly with a pLckY505 antibody, the *Dll3*^{-/-} DP cells showed a lower level of pLckY505 staining, which is indicative of active signalling through p56Lck compared with *Dll3*^{+/+} DP cells (Figure 4f). Comparing the mean intensity fluorescence of the pLckY505 in DP cells of individual chimaeric mice, it was evident that the *Dll3*^{-/-} DP cells had a significantly lower level of pLckY505 ($P<0.05$) compared with the *Dll3*^{+/+} cells in the thymus (Figure 4f). The *Dll3*^{-/-} DP cells also showed evidence of increased pErk staining (Figure 4f) but there was no difference in the expression of Bcl-2 between *Dll3*^{+/+} and *Dll3*^{-/-} DP cells (data not shown). Therefore the changes in TCR signalling observed in the mutant cells in the chimaeric recipients indicate that these changes are due to a cell intrinsic effect caused by the lack of *Dll3*.

Dll3 deficiency leads to an increase in positive selection of thymocytes

DP thymocytes will undergo either positive or negative selection depending on the affinity of the clonotypic TCR for peptide/major histocompatibility complex presented on the surface of epithelial cells. T cells with a high affinity TCR are signalled to die by apoptosis leading to clonal deletion, whereas DP cells that express a TCR with low to intermediate affinity for peptide/major histocompatibility complex will be positively selected and allowed to complete their maturation and differentiate as either CD8⁺ or CD4⁺ SP cells, respectively.

To examine the effect of the lack of *Dll3* has on positive and negative selection of antigen-specific CD4⁺ T cells we bred the *Dll3* null allele onto to the B10.BR 3A9 TCR transgenic background, which encodes a high affinity TCR specific for the immunodominant peptide of hen egg lysozyme (HEL) 46–63 presented in association with I-A^k.²⁰ An advantage of this model is that it is possible to track the development of the TCR transgenic cells throughout development in the thymus using a clonotype-specific monoclonal antibody.²¹ Negative selection of islet-specific CD4⁺ T cells in *TCR:insHel* mice is under the control of the autoimmune regulator gene, *Aire*.^{21,22} Promiscuous expression of the HEL protein by medullary thymic epithelial cells causes deletion of the majority of CD4⁺ T cells during the single positive stage of development. The *Dll3*^{-/-} TCR and *TCR:insHel* double transgenic mice do not survive beyond birth on a B10.BR background requiring us to examine irradiation chimeras using fetal liver cells from either *Dll3*^{+/+} or *Dll3*^{-/-} 3A9 TCR (CD45.2) embryos or wild-type 3A9 TCR CD45.1 embryos. The 50:50 mixtures of *Dll3*^{+/+}/CD45.1 or *Dll3*^{-/-}/CD45.1 were injected into irradiated B10.BR (non-transgenic) or B10.BR-*insHel* transgenic recipients that enabled us to examine the

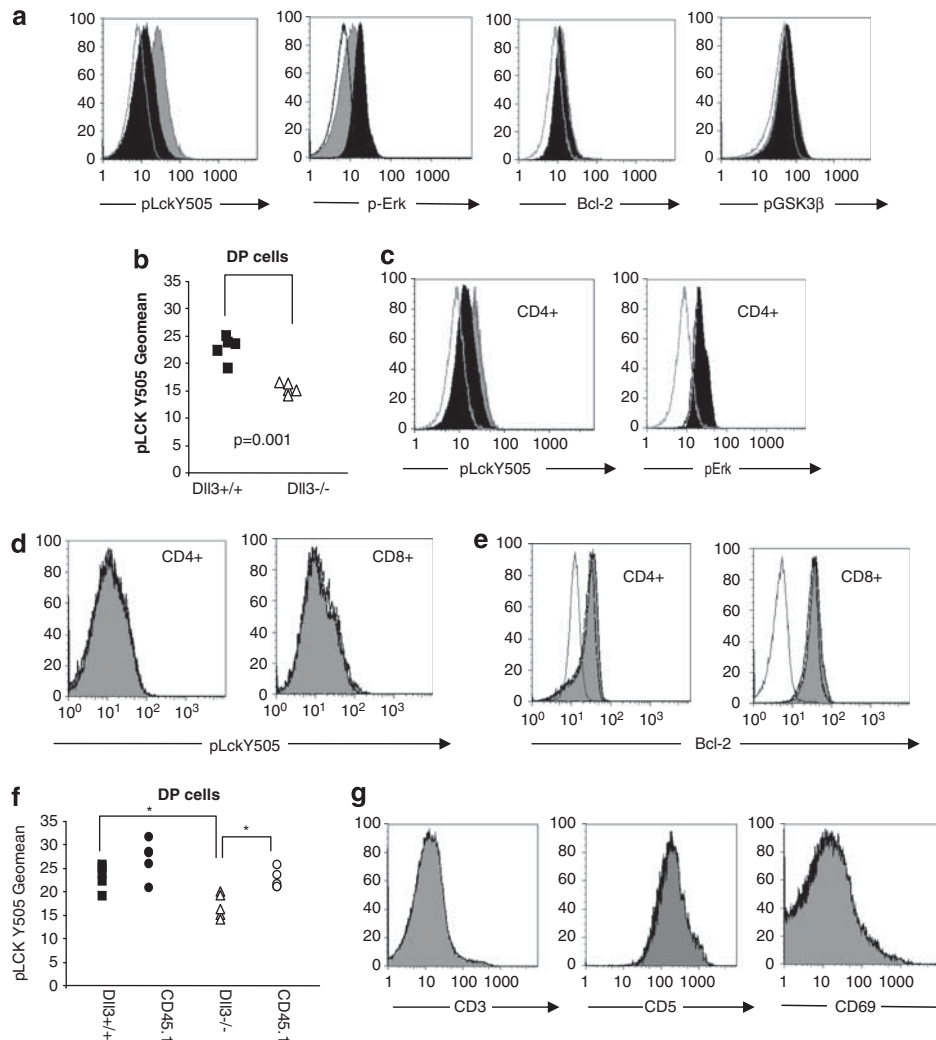


Figure 4 Loss of *Dll3* leads to a cell autonomous change in TCR signalling in DP thymocytes. (a) Representative histograms show overlay plots of *Dll3*^{+/+} (grey shade) and *Dll3*^{-/-} (black fill) DP cells stained with antibodies to pLCKY505, pERK, Bcl2 and pGSK3 β . (b) Graph shows the mean fluorescence intensity (MFI) values of pLCKY505 staining for DP cells from *Dll3*^{+/+} (filled squares) and *Dll3*^{-/-} mice (open triangles). The difference in mean is significant at $P=0.001$. Each symbol represents data from an individual mouse. (c) Staining of mature CD4⁺ thymocytes with pLCKY505 and pERK. (d) Overlay histograms comparing expression of pLCKY505 in spleen CD4⁺ and CD8⁺ T cells. The isotype control antibody is shown as a black line. (e) Overlay histograms comparing expression of Bcl-2 expression in spleen CD4⁺ and CD8⁺ T cells. (f) Graph shows the pLCKY505 geomean expression of CD45.2⁺ DP cells from individual chimaeric mice of *Dll3*^{+/+}/CD45.1 (filled shapes) and *Dll3*^{-/-}/CD45.1 chimaeric animals (open shapes). Significant differences are shown, $*P=0.05$ by one way analysis of variance. The genotype of the donor cells is shown on the x axis. Each symbol represents an individual recipient. (g) Representative histograms showing overlay plots of *Dll3*^{+/+} (gray shade) and *Dll3*^{-/-} (black fill) CD45.2⁺ DP cells stained with antibodies to CD3, CD5 and CD69, respectively.

effect of loss of *Dll3* on both positive and negative selection of 3A9T cells, respectively. Twelve weeks after the transfer the thymus and spleen of individual chimaeric mice were analysed by flow cytometry and the different donor cell populations could be distinguished on the basis of allelic expression of CD45 at the cell surface. In the *Dll3*^{+/+}/CD45.1 chimera the *Dll3*^{+/+}-derived 3A9 TCR transgenic cells represented just 40% of DP cells and 25% of the mature Hel-specific CD4⁺ 1G12⁺ cells (Figures 5a and b). In contrast, in the *Dll3*^{-/-}/CD45.1 chimera the *Dll3*^{-/-}-derived cells represented 60% of the thymocytes at both the DP and CD4⁺ 1G12⁺ stage (Figures 5a and b). In absolute terms the *Dll3*^{-/-} 3A9 progenitor cells gave rise to threefold more mature CD4⁺ 1G12⁺ T cells than the comparable *Dll3*^{+/+} 3A9 TCR population ($P<0.001$), whereas there were equivalent numbers of DP cells in both sets of chimeras (Figure 5c). These differences were also

maintained in the spleen (Supplementary Figure 2). In contrast the CD45.1-derived progenitor cells gave rise to equivalent numbers of DP cells and mature CD4⁺ 1G12⁺ T cells in the chimeras (Figure 5c). These results confirm that the loss of *Dll3* leads to increased positive selection of antigen-specific T cells confirming the cell autonomous role for *Dll3* in T-cell development. Flow cytometric analysis of the thymus and spleen of *insHel* recipients revealed that there was no defect in negative selection in the absence of *Dll3* as both sets of chimeras gave rise to equivalent numbers of DP and CD4⁺ 1G12⁺ T cells (Supplementary Figure 2). Furthermore none of the *insHel* recipients developed type 1 diabetes confirming that clonal deletion of the islet-reactive CD4⁺ T cells was sufficient to maintain tolerance to the islet-specific Hel antigen in the presence and absence of *Dll3*.

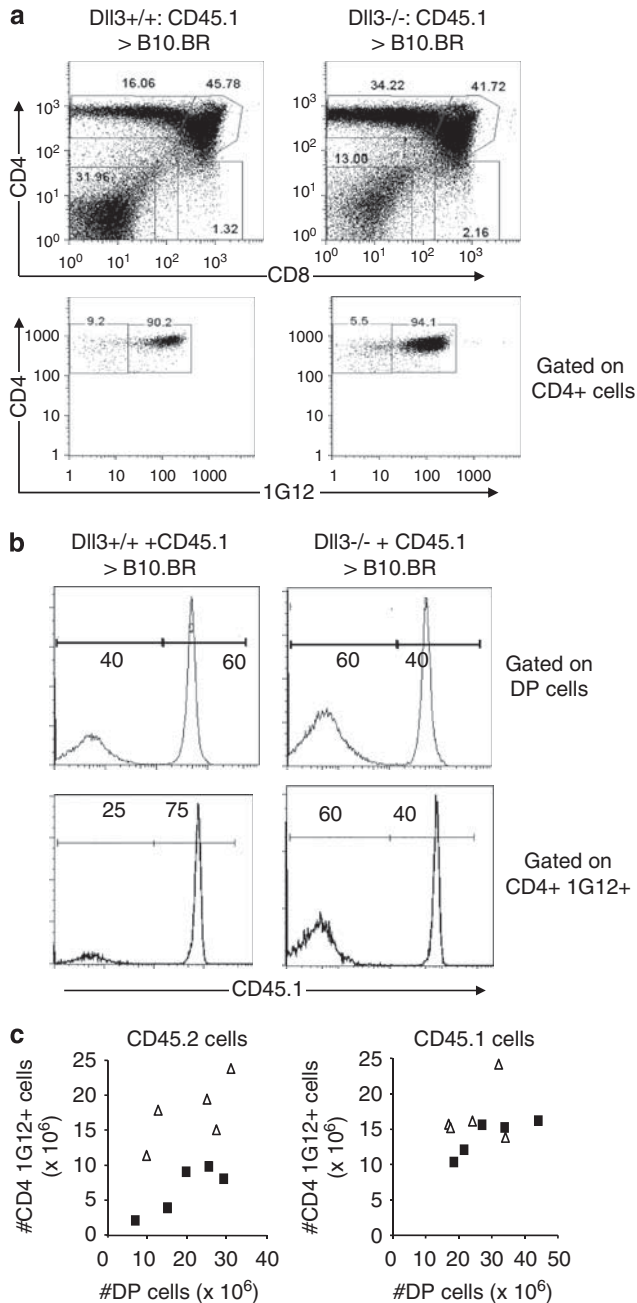


Figure 5 Enhanced positive selection of Hel-specific T cells *in vivo* in the absence of *Dll3*. **(a)** Representative FACS dot plots showing CD4 versus CD8 expression of total thymocytes from *Dll3*^{+/+} TCR/CD45.1+TCR+ > B10.Br chimaeras or *Dll3*^{-/-} TCR+/CD45.1+TCR+ > B10.Br chimaeras (top panel). Lower panel shows CD4 versus 1G12 staining on the CD4-gated thymocyte population. **(b)** Representative histograms showing the proportion of CD45.1+ and CD45.1- cells in the DP or CD4+ 1G12+ gates of representative chimaeras. **(c)** Graphs show the total cell numbers of DP and CD4+ 1G12+ cells in the thymus of individual chimaeric mice. Cells from the *Dll3*^{+/+} CD45.1 chimaeras (filled squares), and *Dll3*^{-/-} CD45.1 chimaeras (open triangles) are shown.

Dll3^{-/-} progenitors rely on a Notch signal for T-cell differentiation

We wanted to determine if the *Dll3*^{-/-} thymic progenitors were still dependent on ligand-induced Notch signalling during T-cell differentiation. FACS-purified *Dll3*^{+/+} and *Dll3*^{-/-} DN1 thymocytes were

cultured on OP9-Dll1 cells in the presence or absence of the γ -secretase inhibitor L-685,458 that inhibits the activity of both presenilin 1 and 2.^{6,10} L-685,458 potentially inhibited cell proliferation in these assays consistent with previous observations.^{10,23} The *Dll3*^{+/+} DN cells display a dose-dependent block in T-cell development in the presence of L-685,458 with a reduction in frequency of DP cells generated and there is a corresponding increase in the proportion of B220+ cells (>90%) in these cultures consistent with a block of Notch1 signalling in DN precursors (Supplementary Figure 3). The *Dll3*^{-/-} DN cells were also sensitive to the inhibitory effects of L-685,458 such that DP cell differentiation was reduced but a significant fraction of T-lineage-committed cells remained (~30%) in these cultures even at the highest concentration of inhibitor (1 μ M) whereas *Dll3*^{+/+} cells contained <5% T cells at the same concentration (Supplementary Figure 3). Treatment of *Dll3*^{-/-} DN cells with the inhibitor did increase the proportion of B cells in these cultures but they were less than those produced with *Dll3*^{+/+} cells (Supplementary Figure 3). The differentiation of *Dll3*^{+/+} or *Dll3*^{-/-} DN cells was not affected by the presence of a γ -secretase inhibitor that does not impair Notch cleavage (data not shown). We conclude that the *Dll3*^{-/-} thymocytes are still reliant on ligand-induced Notch1 signalling to drive lineage commitment and subsequent T-cell differentiation.

Next we wanted to determine if the loss of Dll3 lead to an increase in Notch signalling within Dll3 deficient thymocytes. To test this hypothesis we examined the expression of a range of Notch signalling target genes using RNA from FACS-purified DN and DP cells isolated from thymus of age and sex matched *Dll3*^{+/+} and *Dll3*^{-/-} mice. The data presented in Figure 6b shows the relative expression of the different target genes in *Dll3*^{-/-} DN or DP cells compared with the equivalent *Dll3*^{+/+} cell population, which was given an arbitrary value of 1. *Hes1* and *Hes5*, are direct target genes of Notch1 signalling in thymocytes.²⁴ There was a fourfold increase in *Hes5* in the *Dll3*^{-/-} DP cells but no change could be detected in *Hes1* expression. We conclude that at the level of Notch target gene regulation the loss of Dll3 is unlikely to lead to global dysregulation of Notch signalling in DN or DP cells.

To test whether Dll3 can activate Notch signalling in *trans*, we utilised a co-culture assay in which cell lines expressing Dll1 (3T3-D1), Dll3 (3T3-D3) or Jagged1 (3T3-J1) ligands were co-cultured with C2C12 cells that stably expressed the Notch1 receptor (C2C12-N1).^{25,26} The Dll1 and Jagged1 expressing cells could activate Notch1 signalling in *trans* and strongly induce transcription of the Notch responsive reporter gene. In contrast, presentation of Dll3-expressing cells in *trans* was unable to activate transcription of the Notch reporter gene (Figure 7a).

We further examined the cell autonomous function of Dll3 on Notch signalling by examining if the different Notch ligands were able to modulate Notch signalling when expressed in the same cell as the Notch1 receptor (*in cis*). Dll1 and Jag1 potentially inhibited *trans*-activation of Notch signalling induced by co-culturing with 3T3-D1 or 3T3-J1 cells (Figure 7b). Expression of Dll3 in the Notch-expressing cells also consistently inhibited signalling regardless of whether 3T3-D1 or 3T3-J1 cells were used to induce Notch signalling (Figure 7b). Interestingly, Dll3 did not inhibit signalling to the same extent as Dll1 or Jagged1 (Figure 7c). Residual Notch signalling was ~2–3-fold higher in cells expressing Dll3 in *cis* compared with those expressing Dll1 in *cis*. This property of Dll3 was also observed when C2C12-N1 cells were replaced with Notch1-transfected NIH3T3 cells in the co-culture assay (Figure 7d). These results confirm the previous findings showing that Dll3 cannot activate Notch signalling when expressed in *trans* with the receptor⁸ but they also extend our

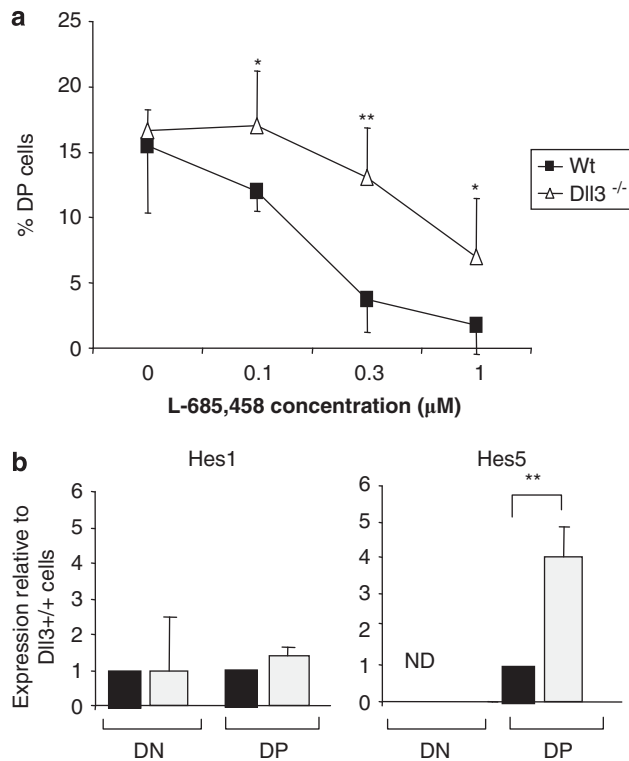


Figure 6 T-cell production by *Dll3*^{-/-} precursors is dependent on Notch cleavage by γ -secretases. **(a)** Lineage negative DN cells isolated from the thymus of either *Dll3*^{+/+} or *Dll3*^{-/-} mice were cultured on OP9-Dll1 cells in the presence or absence of the γ -secretase inhibitor L-685,458 (Calbiochem, gsi-X) at the indicated concentrations. FACS analysis of CD4 versus CD8 expression was made 8 days after commencement of the culture. Graph shows the frequency of DP cells at the end of the culture period *Dll3*^{+/+} (filled squares), *Dll3*^{-/-} (open triangles). There was a statistically significant difference in the frequency of DP cells between *Dll3*^{+/+} and *Dll3*^{-/-} cells at $P=0.018$ ($0.1 \mu\text{g ml}^{-1}$), $P=0.0006$ ($1 \mu\text{g ml}^{-1}$) and $P=0.0289$ ($10 \mu\text{g ml}^{-1}$). **(b)** Expression of Notch target genes *Hes-1*, *Hes-5* and *Dtx1* in *Dll3*^{+/+} (black bars) and *Dll3*^{-/-} (grey bars) DN and DP thymocytes using real time PCR. **Statistically significant difference in *Hes5* expression is shown $P=0.005$. Data show the average gene expression (s.d. obtained from analysis of FACS-purified DN and DP cells generated from three independent samples from *Dll3*^{-/-} and *Dll3*^{+/+} mice. ND, not detected.

understanding of Dll3, in that unlike Dll1, Dll3 does not completely *cis*-inhibit Notch signalling.

DISCUSSION

Until now the investigation of the role of Notch ligands in the thymus have focused on their role in thymic stromal cells, which express Dll1, Dll4 and Jagged 1 ligands.^{4-6,11} Here we have identified a novel cell autonomous role for Dll3 in thymocytes during the differentiation of $\alpha\beta$ -T cells. The Dll3 ligand cannot activate Notch signalling when expressed in *trans*, but can inhibit Notch signalling although not as efficiently as other DSL ligands when expressed in *cis*. A deficiency in Dll3 leads to increased positive selection of SP thymocytes whereas negative selection of autoreactive T cells remains intact. In DP cells the loss of Dll3 leads to increased expression of the Notch target gene *Hes5* as well as TCR signalling. Therefore the Dll3 ligand appears to have evolved a novel regulatory role in the modulation of Notch signalling and this property is utilised by the immune system during T-cell differentiation.

Notch signalling is crucial to normal T-cell development and dysregulation of this signalling pathway through inherited or spontaneously acquired somatic mutations can lead to T-cell leukaemia.²⁷ Most focus on the role of Notch ligands in T-cell development has been on stromal cells. Cell autonomous inhibition and intracellular association with Notch is a property of the DSL ligands when they are overexpressed in *cis*.²⁸⁻³¹ Dll3, unlike Dll1 and Jagged1, is unable to activate Notch when expressed in *trans*. Through evolution the Dll3 protein has undergone several changes with respect to the other Delta-like ligands. The DSL domain that is crucial in ligand binding to Notch receptors³² and the epidermal growth factor-like repeats 1 and 2 of Dll3 are highly divergent compared with the other related Notch ligands.^{14,33} Also the Dll3 protein lacks key lysine residues in its C-terminal domain, which are important for ubiquitination and association with PDZ domain proteins.³⁴⁻³⁷ Our findings are similar to those of Ladi *et al.*⁸ who also found that Dll3 was unable to activate Notch in *trans* but can function in *cis*-inhibition of Notch signalling. In support of an inhibitory role for Dll3 in thymocyte development we found that the loss of Dll3 leads to an increase in *Hes5* expression in DP cells, a direct target gene of Notch signalling.²⁴ Dll3 deficient thymocytes were also less susceptible to the inhibitory effects of a γ secretase inhibitor that blocks ligand-dependent Notch cleavage and this lead to a significant increase in the frequency of DP cells in OP9-Dll1 stromal cultures with Dll3 deficient thymocytes. This result would be expected if Dll3 deficient thymocytes had a constitutively higher basal level of Notch signalling compared with wild-type cells that would promote the differentiation of DP cells.

The changes in Notch signalling in *Dll3*^{-/-} DP cells were not associated with any significant change to cell surface expression of differentiation markers including CD3, CD5 or CD69 but the mutant DP cells did show an increase in TCR signalling with enhanced dephosphorylation of the tyrosine kinase p56lck that associates with the TCR and increased phosphorylated-Erk staining. The changes in TCR signalling were also observed in DP cells in both intact *Dll3*^{-/-} mice as well as in *Dll3*^{-/-}-derived DP cells in mixed irradiation chimeras. There is evidence that Notch1 and Numb can associate with the TCR at the surface of T cells,³⁸ which raises speculation that perhaps Dll3 may act in DP cells to sequester Notch away from the TCR and in turn this could help to modulate TCR signalling and in turn limit the potential for positive selection. In the absence of Dll3, Notch signalling becomes more active and this could synergize with TCR signals to promote T-cell differentiation. The increase in TCR signalling in DP cells resulted in enhanced positive selection of SP thymocytes that was most evident in mixed irradiation chimeras showing that Dll3 acts cell autonomously in thymocytes. Importantly, the loss of Dll3 did not affect the fidelity of negative selection of autoreactive thymocytes. The ubiquitin E3 ligase Itch has recently been shown to regulate JunB in Th cells and is important in the regulation of Th2 cell differentiation.³⁹ Itch can ubiquitinate Notch receptors and target it for proteolytic degradation by the proteasome. Notch signalling has been shown to be important in Th2 cell differentiation that is induced by Notch ligands expressed by professional APCs.^{3,40-42} *Itchy* mice develop a late onset of an autoimmune like disease characterized by lymphoproliferation in the spleen, lymph nodes and thymic medulla.^{43,44} Also when combined with an activated Notch1 allele the *Itch* mutation could synergize with Notch to cause autoimmune disease in mice.⁴⁵ Although the loss of Dll3 could lead to an increase in positive selection of SP cells, it did not affect clonal deletion of autoreactive T cells. The 3A9 *TCR:insHel* model is a validated model to study organ-specific autoimmune diseases such as type 1 diabetes and heterozygous mutations in autoimmune susceptibility genes such

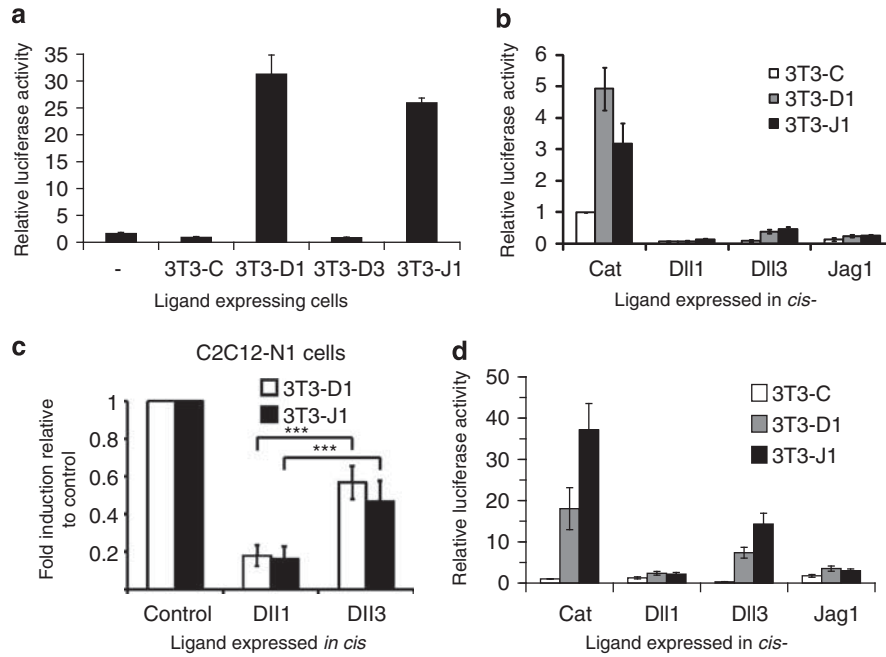


Figure 7 Dll3 mediates *cis*-inhibition of Notch. (a) The CBF1/Su(H)/Lag1 responsive promoter TP-1 coupled to a luciferase reporter (TP1-luc) was introduced into C2C12 cells stably expressing a full-length Notch1 receptor (C2C12-N1), the C2C12-N1 cells were co-cultured with NIH3T3 cells stably expressing either Dll1 (3T3-D1), Jagged1 (3T3-J1) or Dll3 (3T3-D3). Error bars represent the s.d. of the mean. A representative experiment is shown. (b) *Cis*-inhibition of Notch signalling by Dll3 in C2C12-N1 cells. Notch reporter activity in C2C12-N1 cells transfected with either Dll1, Jagged1 or Dll3 ligands and the TP1-luc reporter following co-culture with either control (3T3-C; white bars), 3T3 cells expressing Dll1 (3T3-D1; grey bars) or Jagged1 (3T3-J1; black bars). Data are expressed relative to Cat control co-cultured with 3T3-C cells. Representative experiments are shown. Error bars represent the s.d. of the quotient. (c) *Cis*-inhibition of Notch signalling by Dll3 in C2C12-N1 cells. Notch reporter activity in C2C12-N1 cells transfected with either Dll1 or Dll3 ligands and the TP1-luc reporter following co-culture with 3T3 cells expressing Dll1 (3T3-D1; white bars) or Jagged1 (3T3-J1; black bars). Data from four independent experiments are represented as fold induction (ligand over control cells) relative to control. One-way analysis of variance was performed and significance determined using the Tukey's multiple comparison test. *** $P < 0.001$. Error bars represent the s.d. from the mean. Notch signalling was induced at least fourfold in all experiments. (d) *Cis*-inhibition of Notch signalling by Dll3 in NIH3T3 cells. Notch reporter activity in NIH3T3 cells transfected with Notch1, TP1-luc reporter and either Dll1, Jagged1 or Dll3-HA ligands and then co-cultured with the same 3T3-C (white bars), 3T3-D1 (grey bars) or 3T3-J1 cells (black bars) as described above. Data are expressed relative to Cat control co-cultured with 3T3-C cells. A representative experiment is shown. Error bars represent the s.d. of the quotient.

as *Aire*, *Il2* and *Roquin* can all lead to a breakdown in self tolerance and rapid onset of type 1 diabetes.^{21,46,47} However the *Dll3*^{-/-} mutation in autoreactive thymocytes did not affect clonal deletion and did not lead to the aberrant survival of autoreactive T cells in the periphery. In addition, we have not observed any significant change in Th1, Th2 or Th17 cell differentiation *in vivo*.

These studies are the first to show that Dll3 has an important and non-redundant role in the immune system to regulate T-cell development. Further studies are required to understand exactly how Dll3 functions to regulate Notch signalling in T cells but this may be of wider importance given the important role that Dll3 has in the development of the nervous system and mutations in Dll3 have been shown to be responsible for human disease.^{14,17,48}

METHODS

Mice

Dll3^{-/-} mice were bred on a (C57BL/6×129) F1 background at the Victor Chang Cardiac Research Institute, Darlinghurst, Sydney, Australia. The irradiation chimeras used E14 fetal liver cells from *Dll3*^{-/-} or *Dll3*^{+/+} mice on a C57BL/6J background (backcrossed > 10 generations) that were syngeneic with the C57BL/6 CD45.1 congenic mice. CD45.1 congenic mice were purchased from the Animal Resources Centre, Murdoch, Western Australia, Australia. The 3A9 TCR transgenic mice on a B10.Br background were bred with *Dll3*^{-/-} mice and backcrossed for six generations before use in the fetal liver chimera assay.

B10.BR, B10.Br *insHel* transgenic mice and B10.BR 3A9 TCR CD45.1 congenic mice were all bred at the Australian Phenomics Facility at the Australian National University (ANU). All experiments were performed in accordance with animal ethics regulations.

Cell lines

The OP9-GFP and OP9-Dll1 stromal cells were obtained from Dr J-C Zuniga-Pflucker (University of Toronto, Canada).⁶ The cells were maintained in α -minimum essential medium supplemented with penicillin/streptomycin and 20% fetal calf serum. C2C12, C2C12-N1²⁵ and NIH3T3 were obtained from the ATCC and maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum.

Generation of Dll3-specific antibody

Guinea pig antisera were raised against the peptide SADWNHPEDGDSRS mapping to residues 558–571 near the C-terminus of mouse Dll3 and affinity-purified according to the manufacturer's instructions (PSL GmbH, Heidelberg, Germany). Western blotting and was performed as previously described.²⁵

Notch signalling reporter assays

Mouse complementary DNAs of Dll1, Jagged1 and Dll3 were cloned, using the Gateway system (Invitrogen, Victoria, Australia) into the expression vector pCMX. Dll3 was Gateway-cloned into pCAG-IRES-HA-Puro to create pCAG-Dll3HA-iPuro. NIH3T3 cells overexpressing mDll3 were generated by stably transfecting pCAG-Dll3HA-iPuro followed by selection in 1.5 $\mu\text{g ml}^{-1}$ puromycin for 10 days. Isolated cell clones were picked, expanded and analysed for

expression of Dll3 by immunofluorescence and western blotting with an anti-haemagglutinin antibody.

Transfections were performed using LipofectAMINE and Plus reagent (Invitrogen) according to the manufacturer's instructions. Luciferase assay transfections in 12-well trays contained 14 ng of CMV-renilla plasmid, 350 ng of p6xTP1-Luc and 350 ng of each expression plasmid or pCAT-CMX control plasmid. Co-cultures were established by addition of 2×10^5 NIH3T3 cells stably transfected with vector (3T3-C), NIH3T3 cells expressing mDll1 (3T3-D1) or mJagged1 (3T3-J1).⁴⁹ Co-cultures were harvested 24 h after transfection in 200 μ l of passive lysis buffer (Promega, Sydney, Australia). Transient transfection of NIH3T3 cells and co-culture with 3T3-C, 3T3-D1 and 3T3-J1 cells was performed as in Ladi *E et al.*⁸ Firefly and renilla luciferase activities were assayed using the dual-luciferase reporter system (Promega) and measured on a FLUOstar Optima Luminometer (BMG LabTech, Victoria, Australia). Firefly luciferase counts were normalised against renilla luciferase counts to account for differences in transfection efficiency.

Flow cytometry

Single cell suspensions from thymus, spleen, lymph node and bone marrow were prepared from mice at 5 and 10 weeks of age and stained for three or four colour FACS analysis using standard procedures. Results were analysed with Cell Quest software (BD Biosciences, San Jose, CA, USA) on a FACS Calibur flow cytometer. The antibodies used in these experiments included: anti-B220-APC or Prcp (clone RA3-6B2, Pharmingen, Sydney, Australia), anti-CD4-APC or PE (clone GK1.5), anti-CD8-Prpc or FITC (clone 53-5.8; Pharmingen), anti-Thy1-PE, anti-TCR β -FITC (clone H57-597; Pharmingen), anti-TCR $\gamma\delta$ (clone GL3), anti-CD3-FITC or PE (clone 2C11), anti-CD44 APC, anti-CD69-PE, anti-CD25-PE or APC (clone PC-61) and anti-CD5-biotin.

For intracellular staining, cells were incubated with primary antibodies, washed and fixed and permeabilised with cytofix/cytoperm buffer (BD Pharmingen, Sydney, Australia) for 20 min at 4 °C, the cells were washed with perm/wash buffer (BD Pharmingen) and incubated with the anti-Dll3 antibody or a guinea pig isotype control overnight at 4 °C. Cells were washed and stained with anti-guinea pig-PE+FC block antibody for 3 h at 4 °C and the cells were washed and analysed by FACS. To examine intracellular signalling in thymocytes, cells were stained with primary antibodies to cell surface markers and then fixed and permeabilised. Antibodies, pLckY505-FITC, pErk-FITC, Bcl-2-FITC or isotype controls-FITC (all from BD Pharmingen) were added for 3 h at 4 °C and the cells were washed and analysed by FACS. Anti-phospho-Gsk-3 β was purchased from Cell Signalling Technology (Danvers, MA, USA).

Real time PCR

Complementary DNA was generated from DNaseI-treated (DNA free, Ambion, Melbourne, Australia) RNA (isolated using Trizol reagent, Invitrogen, Life Technologies, Carlsbad, CA USA). Primers and fluorogenic probes for *Hes1* and *Hes5* were purchased from Applied Biosystems (Melbourne, Australia). Quantitative PCR was performed for 40 cycles using an iCycler iQ (Bio-Rad, Hercules, CA, USA). The cycle threshold values for experimental samples were converted to relative complementary DNA levels based on the standard curve for each primer set. Expression levels for individual samples were normalised to the house keeping gene ubiquitin conjugating enzyme (Ubc) calculated similarly from the standard curve for those primers. These normalized values are presented in units and represent the change in cycle threshold values subtracted from *Dll3*^{-/-} versus *Dll3*^{+/+} cells. These data were collected from FACS-purified DN and DP samples from three individual mice.

Irradiation chimeras

Fetal liver cells from *Dll3*^{+/+} and *Dll3*^{-/-} animals (on the C57BL/6 background) were collected from E14 post coital embryos, cell suspensions were injected at different cell ratios with fetal liver cells from wild-type CD45.1 embryos and were injected into sublethally irradiated CD45.1 mice receiving two doses of 5 Gy γ -irradiation (Cs source, CSIRO, Canberra, Australia) given 3 h apart. Animals were given water-containing antibiotics for the first 3 weeks and mice were analysed between 8–12 weeks post injection. Reciprocal chimeras were also set up whereby wild-type bone marrow cells from CD45.1+ donor animals, which were injected into either irradiated *Dll3*^{+/+}

or *Dll3*^{-/-} mice. The recipients were analysed 10–12 weeks after the transfer. Fetal liver cells from *Dll3*^{+/+} and *Dll3*^{-/-} animals (on the 3A9 TCR transgenic B10.Br background) were mixed with fetal liver cells from 3A9 TCR-CD45.1 congenic mice and these were injected intravenously at a 50:50 ratio to irradiated B10.Br or B10.Br-*insHel* transgenic recipient mice.

In vitro T-cell differentiation assays

Lineage negative HSCs were prepared by magnetic antibody cell separation (MACs) of fetal liver or bone marrow cell suspensions made from either *Dll3*^{+/+} or *Dll3*^{-/-} animals. Briefly, bone marrow cells were cultured with a cocktail of biotinylated antibodies including anti-CD4, anti-CD8, anti-TCR β , anti-TCR $\gamma\delta$ and anti-B220 (all from Pharmingen). Cells were washed and incubated with streptavidin magnetic microbeads (Miltenyi Biotech, Sydney, Australia) and the cells were passed over a magnetic field through an LS column. Non-bound cells were collected, washed and set up in culture at 5×10^5 cells per well in a 24-well plate. Cells were cultured in medium containing α -minimum essential medium+20% fetal calf serum+penicillin-streptomycin and glutamine, 5 ng ml⁻¹ interleukin-7 and 5 ng ml⁻¹ Flt3L on either control OP9-GFP or OP9-Dll1 cells at 37 °C. After 21 days, cells were collected and analysed by FACS. To study the effect of the L-685,458 (gsi-X, Calbiochem) or JLK6 (gsi-XI, Calbiochem, San Diego, CA, USA) treatment on T-cell development, bone marrow-derived cells were cultured on OP9-Dll1 cells in the presence or absence of various concentrations of the two inhibitors, and T-cell development was assessed by flow cytometry.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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