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Gerard F. Hoyne

*University of Notre Dame Australia, gerard.hoyne@nd.edu.au*

Gavin Chapman

Yovina Sontani

Sharon E. Pursglove

Sally L. Dunwoodie

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A cell autonomous role for the Notch ligand Delta-like 3 in αβ T-cell development

Gerard F Hoyne1,2, Gavin Chapman3,4, Yovina Sontani1,5, Sharon E Pursglove3 and Sally L Dunwoodie3,4

Notch signalling is critical to help direct T-cell lineage commitment in early T-cell progenitors and in the development of αβ 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 αβ 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 radiation 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 thatDll1 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.3 In mammals, Notch signalling is mediated by the Delta-like (Dll1, Dll4) and Serrate-like (Jagged1, Jagged2) DSL ligands.4–6 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.10–13 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 of Notch and is 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 found 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 in vitro. Using RNA prepared from fluorescence-activated cell sorting (FACS)
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).\(^{15}\) 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-\(\alpha\beta\)(14%) and CD8\(^{+}\) TCR-\(\beta\) (10%) cells and was also detected in TCR-\(\alpha\)lo DP and DN thymocytes cells (Figure 1c). 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\(^{+/+}\) mice, which serves as a negative control. The data are representative of one of four experiments performed with a total of 12 mice. \(\alpha\)-Dll3 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 Dll1HA transfected (D1) nor from untransfected cells (ND). Dll3 expression showed (grey line) and the Jagged2 (stippled bars) in thymocytes at different stages of T-cell development. Data represent the average expression in three independent experiments performed with a total of 12 mice. (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 Dll1HA transfected (D1) nor from untransfected cells (ND). Dll3 expression showed (grey bars) and Jagged2 (stippled bars) in thymocytes at different stages of T-cell development. Data represent the average expression in three independent experiments performed with a total of 12 mice. (c) Thymocytes were stained initially with anti-CD4, CD8, TCR-\(\alpha\) on the C-terminus of the Dll3 protein that is highly divergent to other DSL ligands.

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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 Dll1HA transfected (D1) nor from untransfected cells (ND). Dll3 expression was demonstrated on the same blot using an antibody against mouse Dll1. (c) Thymocytes were stained initially with anti-CD4, CD8, TCR-\(\beta\) 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-\(\beta\) 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).
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 2β-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 TCRβ development, we made use of the OP9-Dll1 stromal line, which can support T-cell development in vitro along both TCRβ+ and TCRγδ+ cell lineages. 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 cells into irradiated CD45.1 recipients. Thymus derived progenitors were able to compete effectively with 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.
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 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-Ak.2

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, Air2.22 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
Effect of loss of Dll3 on both positive and negative selection of 3A9 T 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 allotypic expression of CD45 at the cell surface. In the Dll3+/CD45.1 chimaera 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 chimaeras 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 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 pGSK3β. (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. (e) Overlay histograms comparing expression of Bcl2 expression in spleen CD4+ and CD8+ T cells. The isotype control antibody is shown as a black line. (f) Graph shows the pLCKY505 geomean expression of CD45.2+ DP cells from individual chimaeric mice of Dll3+/+ mice (open triangles). The difference in mean is significant at P=0.001. Each symbol represents data from an individual mouse. (g) Representative histograms showing overlay plots of Dll3+/− (grey shade) and Dll3−/− (black fill) CD45.2+ DP cells stained with antibodies to CD3, CD5 and CD69, respectively.
Figure 5 Enhanced positive selection of HSR-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 CD121 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+/+ and 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 2.10,11 L-685,458 potently inhibited cell proliferation in these assays consistent with previous observations.10,13 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 μM) 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 Dll1–/– 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+/+ or Dll3–/– 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 potent 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 receptor but they also extend our
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.34–35 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 synergise 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 proteosome. Notch signalling has been shown to be important in Th2 cell differentiation that is induced by Notch ligands expressed by professional APCs.40–42 Itch 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 synergise 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 TCRinsHel model is a validated model to study organ-specific autoimmune diseases such as type 1 diabetes and heterozygous mutations in autoimmune susceptibility genes such as...
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 autoimmune 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/6 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 congenic background were bred with *Dll3\(^{+/+}\ )* mice and backcrossed for six generations before use in the fetal liver chimera assay.

**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-N1\(^{25}\) 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-Dll3-HA-iPuro. NIH3T3 cells expressing mDll3 were generated by stably transfecting pCAG-Dll3-HA-iPuro followed by selection in 1.5 μg ml\(^{-1}\) puro-mycin for 10 days. Isolated cell clones were picked, expanded and analysed for

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**Figure 7** Dll3 mediates *cis*-inhibition of Notch. (a) The CBF1/Sul/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.

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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 x 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 μl 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.5 Firefly and renilla luciferase activities were assessed 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 RA3-682, Pharmingen, Sydney, Australia), anti-CD4-APC or PE (clone GK1.5), anti-CD8-APC or Prcp or FITC (clone 53-5.8; Pharmingen), anti-Thy-1-PE, anti-Tcrβ-FITC, anti-c-KIT-FITC (clone H9F1, Pharmingen), anti–CD45R0 (clone GL3), anti-CD3-FITC or PE (clone 145-2C11), 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 cytometric/permeabilisation buffer (BD Pharmingen, Sydney, Australia) for 20 min at 4°C, the cells were washed with permeabilisation 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-3β 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 the housekeeping gene ubiquitin conjugating enzyme (Ubc) calculated for differences in transfection efficiency.

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Conflict of Interest
The authors declare no conflict of interest.


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Supplementary Information accompanies the paper on Immunology and Cell Biology website (http://www.nature.com/icb)