Constitutive Notch signalling promotes CD4–CD8– thymocyte differentiation in the absence of the pre-TCR complex, by mimicking pre-TCR signals

Alison M. Michie1, Angela C. Chan2, Maria Ciofani3, Michael Carleton4, Juliette M. Lefebvre5, Yiping He6, David M. Allman7, David L. Wiest8, Juan Carlos Zúñiga-Pflücker3 and David J. Izon2,9

1Division of Cancer Sciences and Molecular Pathology, Section of Experimental Haematology, Royal Infirmary, University of Glasgow, Glasgow G31 2ER, UK
2Telethon Institute for Child Health Research, Cancer Biology Division, 100 Roberts Road, Subiaco, Western Australia 6008, Australia
3Department of Immunology, Sunnybrook Research Institute, University of Toronto, 2075 Bayview Avenue, Toronto, Ontario M4N 3M5, Canada
4Rosetta Inpharmatics LLC, 401 Terry Avenue North, Seattle, WA 98109, USA
5Hopital Necker Enfants maîlades, INSERM U 768, Développement normal et pathologique du système immunitaire, Batiment KIRMISSON, 149 Rue de Sévres, 75743 Paris cedex 15, France
6Ludwig Center for Cancer Genetics and Therapeutics, The Johns Hopkins Kimmel Cancer Center, 1650 Orleans Street, CRB 520, Baltimore, MD 21231, USA
7Department of Pathology and Laboratory Medicine, University of Pennsylvania School of Medicine, 230 John Morgan Building, 36th and Hamilton Walk, Philadelphia, PA 19104-6082, USA
8Division of Basic Science, Fox Chase Cancer Center, 333 Cottman Avenue, Philadelphia, PA 19111, USA
9Haematology and Leukaemia, St Vincent’s Institute, 9 Princes Street, Fitzroy, Victoria 3065, Australia

Keywords: Notch, pre-TCR signalling, T-cell development, thymus

Abstract

Notch1 signalling is essential for the commitment of multipotent lymphocyte precursors towards the αβ T-cell lineage and plays an important role in regulating β-selection in CD4+CD8– double-negative (DN) thymocytes. However, the role played by Notch in promoting the development of CD4+CD8+ double-positive (DP) thymocytes is poorly characterized. Here, we demonstrate that the introduction of a constitutively active Notch1 (ICN1) construct into RAG2/2 lymphocyte precursors resulted in the generation of DP thymocytes in vitro T-cell culture systems. Notably, developmental rescue was dependent not only on the presence of an intact Notch1 RAM domain but also on Delta-like signals, as ICN1-induced DP development in RAG2/2 thymocytes occurred within an intact thymus or in OP9-DL1 co-cultures, but not in OP9-control co-cultures. Interestingly, ICN1 expression in SLP-76−/− precursors resulted in only a minimal developmental rescue to the immature CD8+ single-positive stage, suggesting that Notch is utilizing the same signalling pathway as the pre-TCR complex. In support of this, ICN1 introduction resulted in the activation of the ERK–MAPK-signalling cascade in RAG7−/− thymocytes. Taken together, these studies demonstrate that constitutive Notch signalling can bypass β-selection during early T-cell development by inducing pre-TCR-like signals within a T-cell-promoting environment.

Introduction

T-cell differentiation is initiated when a foetal liver (FL)- or bone marrow (BM)-derived T-lymphoid progenitor enters the thymus. These cells are broadly designated double-negative (DN) cells as they lack expression of CD4 and CD8. DN thymocytes undergo an ordered development that can be subdivided into four stages based on the surface expression of CD44 and CD25. DN1 cells (CD25−CD44+) are multipotent and can reconstitute the T, B, DC and NK lineages (1). DN2 cells (CD25+CD44+) have the ability to give rise to T, NK and dendritic cell (DC) lineage cells, but lack the ability to give rise to B lineage cells (2). TCRβ rearrangement (or the β-selection checkpoint) is completed at the DN3 stage...
Notch promotes pre-TCR-like signals

(24, 25). Moreover, other Notch receptors may function during or affect survival of cells undergoing recombination, suggesting that Notch may either be involved in VDJ recombination or play a unique role during early T-cell development. Indeed, Notch genes encode a family of transmembrane receptors that regulate cell fate decisions, cell number, and cell position (5). Four mammalian Notch homologues (Notch1-4) have been identified in the immune system (6). Notch signalling is initiated when a Notch receptor binds ligand, from either the Delta or Jagged family (6). Delta family members have been identified in the immune system (6). Notch signalling has the capacity to mimic pre-TCR-derived signals. This is supported by the finding that ICN1-expressing RAG-γ/δ mice retrovirally expressed ICN1 only induced ectopic development to the DN3 stage, as observed in RAG-γ/δ, CD3ζ-/- or SLP-76-/- mice models (4). DN4 cells (CD25+CD44+) are rapidly dividing blasts destined to become CD4+CD8+ double-positive (DP) thymocytes through a transient immature CD8+ single-positive (ISP) stage (4). TCRα rearrangement is initiated and fully completed at the DP stage (4). DP thymocytes are then subjected to a series of selection criteria that result in their maturation towards CD4+CD8- or CD4-CD8+ single-positive (SP) T cells that express αβ TCRs with a moderate affinity for self-MHC (4).

Notch genes encode a family of transmembrane receptors that regulate cell fate decisions, cell number, and cell position (5). Four mammalian Notch homologues (Notch1-4) have been identified in the immune system (6). Notch signalling is initiated when a Notch receptor binds ligand, from either the Delta or Jagged family (6). Delta family members are sufficient to induce thymic-independent T-cell development in vitro (7, 8). Ligand binding by Notch leads to proteolytic cleavages that result in nuclear translocation of the Notch intracellular (ICN) domain (9, 10) where it interacts with CBF-1, Suppressor of Hairless and Lag-1 (CSL), a constitutive transcriptional repressor (11, 12). After Notch binding, CSL becomes a transcriptional activator and, in conjunction with co-factors that include Mastermind-like (MAML) proteins, induces transcription of downstream targets (13). Direct targets of activated CSL include several Hairy/Enhancer of Split genes, pTxα, and Notch1 itself (14–18).

Of the four Notch receptors, Notch1 is likely to play several unique roles during early T-cell development. Indeed, mice expressing a conditional Notch1-targeted deletion displayed a block at the earliest stage of T-cell development and an excess of thymically derived B cells (19). Similar findings were observed in conditional CSL-deficient mice (20). In contrast, constitutive expression of full-length intracellular Notch1 (ICN1) in wild-type haemopoietic stem cells (HSCs) induced ectopic T-cell development in the BM, concurrent with a block in B-cell maturation at the earliest developmental stage (21). Similar results were obtained by stimulating lymphoid progenitors with Delta-like 1 (Dll1) expressing stromal cells in culture (7, 8). Appropriate control of Notch signalling during T-cell development is essential, as maintenance of high levels of Notch signalling beyond the DN4 stage of development leads to a block in further thymocyte differentiation and the development of leukaemia (22, 23). Interestingly, a distinct phenotype was observed in mice carrying a conditional deletion of Notch1 under the control of the Lck-Cre-expressing transgene, which resulted in the survival of DN4 cells that have abnormal VDJ recombination, suggesting that Notch may either be involved in VDJ recombination or affect survival of cells undergoing recombination (24, 25). Moreover, other Notch receptors may function during early thymocyte development. Indeed, Notch3 over-expression leads to phenotypes similar to Notch1 (26). Nevertheless, mice carrying inactivated Notch2 or Notch3 genes fail to display defects in early T-cell development (27, 28), suggesting that Notch1 is of central importance at this stage.

Several reports have indicated that constitutive Notch signalling cannot rescue T-cell development beyond the β-selection checkpoint in mice lacking intact pre-TCR signalling. In support of this, RAG-γ/δ mice retrovirally expressed ICN1 only induced ectopic development to the DN3 stage in the BM, however, development to the DP stage, accompanied by massive cell expansion, was observed upon introduction of a TCRβ transgene (29). Likewise in the OP9-DL1 system, RAG-γ/δ thymocytes were blocked at the DN3 stage, even in the presence of continuous exposure to Dll1 ligand; however, addition of pre-TCR signalling resulted in DP development (25, 30). Similar to constitutive Notch1 expression, transgenic intracellular Notch3-IC expression was also unable to rescue development of pre-TCR-deficient thymocytes to the DP stage (26).

To further investigate potential functions of Notch signalling at the β-selection checkpoint, we utilized foetal thymic organ culture (FTOC) and OP9 stromal co-cultures to assess the effect of constitutively active Notch signalling in lymphoid progenitors that were deficient in specific components required for pre-TCR-mediated differentiation. In contrast to previous results where ICN-transduced RAG-γ/δ BM precursors were not found to progress to the DP stage, we now find that ICN1-expressing RAG-γ/δ lymphoid precursors developed to the DP stage in both FTOC and OP9-DL1 stromal cell cultures. Indeed, we demonstrate that developmental progression was dependent on the presence of an intact RAM domain within ICN1, as well as additional signals initiated by Dll1-mediated ligation of other Notch family members, the latter of which is normally provided by the thymic microenvironment. Subsequent experiments using precursors deficient in specific pre-TCR-signalling molecules demonstrated that Notch1-mediated thymocyte differentiation required SLP-76, but not CD3ζ, suggesting that constitutive Notch signalling has the capacity to mimic pre-TCR-derived signals. This is supported by the finding that ICN1 expression in developing DN thymocytes resulted in the activation of ERK–MAPK. Taken together, these results demonstrate that ICN1 can bypass the requirement for RAG activity at the β-selection checkpoint in the thymic microenvironment.

Methods

Mice and reagents

CD3ζ-/- mice (31) were the generous gift of Paul Love, National Institutes of Health, Bethesda, MD, USA. SLP-76-/- (32) and RAG-2-/- (33) (Taconic Farms, Germantown, NY, USA) mice were maintained in the Abramson Cancer Research Center. RAG-1-/- mice (34) (Animal Resources Centre, Perth, Western Australia, Australia) were maintained in the Research Facility at The Telethon Institute for Child Health Research. The plasmids, Mig ICN1, comprising the entire human Notch1 intracellular domain (amino acids

Downloaded from https://academic.oup.com/intimm/article-abstract/19/12/1421/778731 by guest on 08 December 2018
Results

Notch1 rescues the DN to DP block in T-cell development in RAG\(^{-/-}\) mice

It has previously been demonstrated that retroviral-mediated ICN1 expression in BM-derived lymphoid precursors in TCR\(\beta\)-sufficient mice leads to ectopic T-cell development in the BM that is blocked at the CD4+CD8\(^{+}\) stage in vivo (23). As expression of ICN1 in RAG\(^{-/-}\) (TCR\(\beta\) deficient) HSCs induced ectopic T-cell development in the BM that was blocked at the DN3 stage of development, it was suggested that ectopic T-cell development beyond the \(\beta\)-selection checkpoint required additional signals (e.g. from the pre-TCR complex).

To determine whether the thymic microenvironment can influence ICN1 activity to promote development of pre-TCR-deficient thymocytes to the DP stage, RAG\(^{-/-}\) FL was transduced with ICN1 and used to reconstitute FTOCs. Surprisingly, RAG\(^{-/-}\) ICN1 cells developed a significant DP population in FTOC compared with RAG\(^{-/-}\) cells transduced by the MigR1 (control) retroviral vector (Fig. 1A and B). CD4 and CD8 SP cells are also present but as RAG\(^{-/-}\) mice are unable to rearrange TCR genes, these cells are likely to be ISPs. Analysis of CD25-surface expression among DN thymocytes revealed that ICN1-transduced RAG\(^{-/-}\) FL failed to down-regulate CD25 and almost all DN were held at the DN3 stage (Fig. 1C and D). However, as ICN1 can induce CD25 up-regulation on DN, DP and mature T cells, analysis of CD25 expression may not be a valid marker to use in defining the DN subset to which an ICN-transduced thymocyte belongs (38, 39). Consequently, and in contrast to previously published data in the BM (29), we demonstrate that constitutive Notch activity induced RAG\(^{-/-}\)-derived thymocytes to traverse the \(\beta\)-selection checkpoint in the thymic microenvironment.

Notch1 rescue of DP development in RAG\(^{-/-}\) BM cells in FTOC is dependent on the presence of an intact RAM domain

Notch1 has clearly defined structural domains (Fig. 2A). The extracellular portion of Notch1 consists of 36 epidermal growth factor repeats that bind homologous structures on the Notch ligands, Delta and Jagged (40). The intracellular region of human Notch1 is comprised of a RAM domain that contains a high-affinity binding site for CSL (41) and a functional nuclear localization signal sequence (42, 43); seven iterated ankyrin (ANK) repeats, which are essential for Notch function (44); a second functional nuclear localization sequence (42, 45); a transcriptional activation domain (TAD) bounded at its C-terminus by an OPA sequence (16) and a C-terminal PEST domain, likely important for protein stability.

To identify which structural domains of ICN1 are necessary to rescue DP development, several ICN1 deletion mutants were individually transduced into RAG\(^{-/-}\) BM cells and subsequently assayed by FTOC for their ability to promote the generation of DP thymocytes. The \(\Delta W\) ICN1 mutant, lacking the 10 N-terminal amino acids from the RAM domain, failed to rescue DP development of RAG\(^{-/-}\) BM in FTOC (Fig. 2B), suggesting that the complete RAM domain of Notch1 is critical for DP thymocyte generation from RAG\(^{-/-}\) precursors. This result was unexpected as the \(\Delta W\)
ICN1 construct induced ectopic development to the DP stage in the BM when expressed in wild-type HSCs (35) and suggests that the pre-TCR complex can cooperate with a sub-optimal Notch signal to generate DP thymocytes. As expected the ΔANK and ΔTAD mutants did not rescue DP development in RAG−/−-derived cells in FTOC, similar to their failure to induce ectopic DP development in HSCs from wild-type mice (data not shown).

Fig. 1. ICN1 rescues CD4+CD8+ development in RAG−/− precursors in a thymic microenvironment. ICN1-expressing RAG-1−/− FL used to reconstitute RAG−/− FTOC was analysed by FACS after 14 days and found to develop a significant population of DP thymocytes. MigR1 (A) or ICN1-infected FL (B) was cultured in FTOC for 14 days prior to FACS analysis for CD4 and CD8 (mean ± SEM: MigR1 versus ICN1 DP % = 0.13 ± 0.1 versus 41.48 ± 9.6). CD25 and CD44 expression gated on lineage negative cells was analysed on MigR1 (C) or ICN1-infected FL (D) FTOC after 14 days culture (mean ± SEM: MigR1 versus ICN1 DN3 % = 55.61 ± 11.2 versus 78.06 ± 6.2). The results are representative of four independent experiments.

Fig. 2. Notch1 rescue of DP development in RAG−/− BM cells in FTOC is dependent on the presence of an intact RAM domain. (A) Schematic of Notch1 and mutant constructs. EGF repeats = epidermal growth factor repeats, RAM = RAM domain, N1 = nuclear localization signal sequence 1, ANK = ankyrin repeats, N2 = nuclear localization signal sequence 2, TAD = transactivation domain, P = PEST sequence (full-length Notch1); ICN1 = the cytoplasmic region of Notch1, ΔW = ICN1 lacking the first 10 amino acids. (B) The ICN1 mutant, ΔW, cannot rescue DP development in RAG-1−/− precursors (mean ± SEM: ICN1 versus ΔW DP % = 33.7 ± 3.3 versus 0.6 ± 0.1). These results are representative of two independent experiments with each condition carried out in at least duplicate in individual experiments.
RAG\(^{−/−}\)-derived FL expressing ICN1 develops into DP thymocytes when cultured on OP9-DL1 stromal cells

To better understand the requirements for ICN1 to drive RAG\(^{−/−}\)-derived lymphocyte progenitors towards the DP stage of development, we cultured ICN1-transduced FL cells on either OP9 stromal cells or OP9 cells engineered to express the Notch ligand, Dll1 (OP9-DL1) (8). Similar to the FTOC results, ICN1-expressing FL cells from RAG\(^{−/−}\) mice differentiated into DP T cells when cultured on OP9-DL1 cells (Fig. 3). Rescue to the DP stage was only observed in cells expressing moderate levels of ICN1. Moreover, DP development required Dll1 signalling as ICN1-expressing FL cells from RAG\(^{−/−}\) mice did not develop to the DP stage when cultured on parental OP9 cells. These results suggest that Dll1-initiated signals synergize with ICN1 signals to traverse the β-selection checkpoint in RAG\(^{−/−}\) cells and reveal the possibility that additional Notch family members are required to promote the generation of DPs in ICN1-transduced RAG\(^{−/−}\) thymocytes. To test this, we co-cultured RAG\(^{−/−}\) FL with OP9-DL1 in the presence or absence of a Notch-signalling inhibitor, γ-secretase inhibitor, and analysed for the presence of DPs after 12 days (Fig. 4). Specifically, GSI treatment should not alter DP development if ICN1 is the sole source of Notch signalling, as the ICN1 constitutively expressed in our transduced cells cannot be cleaved by γ-secretase (46). As predicted, this analysis revealed a significant reduction in the percentage of ISP and DP thymocytes in the GSI-treated ICN1-RAG\(^{−/−}\) FL:OP9-DL1 co-cultures compared with vehicle control (dimethyl sulfoxide), confirming that signalling through additional Notch family members was

Fig. 3. ICN1 expressing FL cells from RAG\(^{−/−}\) mice develop into DP cells when cultured on OP9-DL1 stromal cells. Sorted DN3 RAG-2\(^{−/−}\) cells from a 7-day co-culture on OP9-DL1 cells were infected with MigR1 or ICN1 and cultured for 6 days on control OP9 or OP9-DL1 cells in the presence of IL-7 or Flt3L. Only GFP\(^{low}\) ICN1 RAG-2\(^{−/−}\) cells cultured on OP9-DL1 cells and not on OP9-control cells developed to the DP stage. These results are representative of two independent experiments.
required to promote optimal progression of ICN1-transduced DN thymocytes towards the DP stage of development in the absence of the pre-TCR complex.

**The generation of CD4^+CD8^+ DP thymocytes is dependent on the presence of SLP-76**

The above results suggest that ICN1 mimicked pre-TCR signals to induce DP cell development in RAG^-/- FL. RAG^-/- FL cells were infected with ICN1 and then co-cultured for 12 days on OP9-DL1 cells in the presence or absence of 1 μM GSI for the last 6 days prior to FACS analysis for CD4 and CD8. Addition of GSI reduced the percentage of DP thymocytes generated from RAG^-/- FL cells in the presence of ICN1 [mean ± SEM: dimethyl sulfoxide (DMSO) versus GSI DP % = 23.7 ± 5.0 versus 5.4 ± 2.4, \( P < 0.05 \)]. These results are representative of four independent experiments.

**Fig. 4.** Additional signals derived from Notch family proteins aid in the promotion of DP thymocyte development from ICN1-transduced RAG^-/- FL. RAG^-/- FL cells were infected with ICN1 and then co-cultured for 12 days on OP9-DL1 cells in the presence or absence of 1 μM GSI for the last 6 days prior to FACS analysis for CD4 and CD8. Addition of GSI reduced the percentage of DP thymocytes generated from RAG^-/- FL cells in the presence of ICN1 [mean ± SEM: dimethyl sulfoxide (DMSO) versus GSI DP % = 23.7 ± 5.0 versus 5.4 ± 2.4, \( P < 0.05 \)]. These results are representative of four independent experiments.

required to promote optimal progression of ICN1-transduced DN thymocytes towards the DP stage of development in the absence of the pre-TCR complex.

**Fig. 5.** The generation of DP thymocytes by ICN1 is dependent on the presence of SLP-76. (A) ICN-1 expressing SLP-76^-/- BM used to reconstitute FTOC was analysed by FACS after 14 days and found to develop a small population of ISP CD8 but no DP (mean ± SEM: MigR1 versus ICN1 DP % = 0.65 ± 0.1 versus 0.74 ± 0.3). The results are representative of three independent experiments with each condition carried out in at least duplicate in individual experiments. (B) ICN1 expressing CD3e^-/- BM-reconstituted FTOC was analysed by FACS after 14 days and found to develop DP thymocytes (mean ± SEM: MigR1 versus ICN1 DP % = 4.13 ± 3.0 versus 40.2 ± 0.1). The results are representative of two independent experiments with each condition carried out in at least duplicate in individual experiments.
To better delineate the point at which Notch1 affects pre-TCR signalling, CD3e−/− BM was transduced with control and ICN1-expressing retroviruses and placed into FTOC. ICN1-transduced CD3e−/− lymphoid precursors were found to generate DP thymocytes (Fig. 5B). Again, ICN1-transduced CD3e−/− DN cells did not down-regulate CD25 (data not shown). Collectively, these results reveal that within the thymic microenvironment, Notch1 signalling may mimic proximal pre-TCR signalling as evidenced by DP generation in pre-TCR complex-deficient backgrounds; however, constitutive Notch signals are unable to efficiently replace downstream effectors, such as SLP-76, that are required to transduce these ‘pre-TCR-like’ signals.

**ICN1 expression in DN thymocytes in FTOC leads to an up-regulation of ERK–MAPK activity**

To address the mechanism used by Notch1 to induce differentiation of RAG−/−-derived DN thymocytes, we hypothesized that introduction of ICN1 into developing thymocytes would activate the ERK–MAPK-signalling pathway. This was based on previously published reports showing that ERK–MAPK was activated downstream of the pre-TCR complex in developing thymocytes upon formation of the pre-TCR complex and that constitutive Ras/Raf-mediated signalling can induce the development of DP thymocytes in RAG−/− mice (47).

Therefore, we took advantage of a gene gun technique that allows for the introduction of ERK–MAPK activity reporter plasmids in vivo (48). Introduction of ICN1 together with the Elk1 reporter plasmids into RAG−/− FTOCs induced a 5-fold increase in luciferase activity relative to the reporter plasmids alone (Fig. 6). As previously shown (48), introduction of the plasmid encoding a functionally rearranged TCRβ chain also elicited a similar elevation in luciferase activity (Fig. 6). A substantial increase in luciferase activity was also observed in FTOCs transfected with a constitutively active Ras plasmid (RasV12). Together, these findings indicate that ICN1 has the capacity to activate the ERK–MAPK-signalling cascade, thus, providing a mechanism for its ability to drive the maturation of DN thymocytes towards the DP stage of development.

**Discussion**

Our previous results established that constitutive Notch signalling accelerates thymocyte development towards the DP stage (22). Ectopic production of DPs in the BM required an intact pre-TCR-signalling pathway as constitutive Notch signalling failed to drive development past the DN3 stage of development. Indeed, introduction of a TCRβ transgene allowed Notch to promote thymocyte development towards the DP stage. Analogous results were observed in Notch3-IC transgenic mice bred onto a pTα-deficient background (49). Notch signalling induced by Dll1 ligand was unable to induce DP development in RAG−/− cells cultured on OP9-DL1 stromal cells in absence of pre-TCR signals (25). Similarly, transgenic Notch1-IC expression on a RAG−/− background was only able to rescue DP development when the mice were given a single dose of anti-CD3 antibody.

In light of these results, what accounts for our ability to observe DP development from RAG−/− progenitors in FTOC? Clearly, the thymic microenvironment is important as this organ and not the BM provided the proper milieu for Notch-induced DP development from RAG−/− lymphocyte precursors. Our results from the OP9-DL1 cultures, in which DP development from RAG−/− progenitors required both ICN1 expression and DI1 signals, demonstrate that Delta-initiated signalling is the critical difference between the BM and thymic microenvironments. As Notch receptors are the targets of Delta-like ligands and addition of GSIs reduced DP development in ICN1-transduced RAG−/− thymocytes, our results establish that RAG−/− precursors require signals from Notch receptors in addition to ICN1 to promote DP development. Consistent with this, Notch2 and Notch3 are expressed during early thymocyte development. Indeed, it is interesting to note that DP thymocytes generated from the OP9-DL1 cultures expressed moderate levels of green fluorescent protein (GFP) [corresponding to ICN1 expression (22)], which suggests that there is selection for specific levels of ICN1 that drive development beyond the DN3 stage in RAG−/− lymphocyte progenitor cells. Notch2 and/or Notch3 may be responsible for modulating the effects of ICN1 signalling, thus shaping subsequent downstream events. Interestingly, Delta 1 can bind to Notch2 and Notch3 (50, 51). Therefore, OP9-DL1-mediated activation of Notch2 and Notch3 could lead to enhanced total intracellular Notch signalling, promoting RAG−/− precursors to navigate the β-selection checkpoint. This potential mechanism would explain why ICN1-transduced RAG−/− DN does not become DP when cultured on OP9 cells. It therefore follows that high intracellular Notch levels may potentiate recruitment of signalling modules analogous to a high copy CD3 transgene and block T-cell development (52). However, it cannot be ruled out that the
Delta-initiated signalling may provide CSL-independent signals that synergize with the CSL-dependent signalling provided by ICN1.

Relevant to this, phosphorylated AKT substituted for Notch in RAG−/− thymocytes expressing a TCRβ transgene (53). Thus, optimal levels of ICN and Delta-induced Notch signalling may be sufficient to achieve a signalling threshold required for DP differentiation in the absence of pre-TCR signals. Our results are complementary to a recent report demonstrating that genetic inactivation of Notch1 in DN thymocytes, by introducing a dominant-negative MAML construct, results in an arrest in T-cell development at the DN stage (54). In addition, our data are applicable to oncogenic Notch signalling as it is now known that >50% of acute T-cell leukaemias are associated with Notch1 mutations (55). As a subset of these tumours is associated with pre-β-selection phenotypes, it is possible that ICN1 provides important pre-TCR signals that contribute to transformation. Interestingly, a recent study has demonstrated that ICN1 can induce ectopic DP development in RAG−/− host mice and that these animals eventually succumbed to a DP-derived leukaemia with a longer latency compared with wild-type animals (56).

Of note, the ability of ICN1 to promote the events associated with β-selection in RAG−/− precursors was incomplete, as expression of ICN1 only rescued differentiation to the DP stage, not proliferation (data not shown). This suggests that ICN1 can substitute for some but not all pre-TCR signals.

In addition to the thymic microenvironment and Delta-like initiated signals, DP development from RAG−/− precursors requires specific structural motifs within the intracellular domain. These domains include the RAM, ANK and TAD domains of Notch1. An intact PEST domain is likely required, as transgenic Notch-IC mice lacking the PEST domain did not rescue DP development in RAG−/− mice (30). However, an additional reason for the lack of rescue in these mice may be because the transgene-encoding Notch1-IC expression was under the control of the Lck proximal promoter, which may result in lower levels of Notch signalling. Although the RAM domain is not required for ectopic DP development from wild-type lymphocyte precursors (35), our studies suggest that it is required in the absence of a pre-TCR. The RAM domain likely provides optimal ICN1:CSL interactions, contributing to maximally efficient signalling. Consistent with this possibility, leukaemia induction in ΔW ICN1 mutants was delayed relative to ICN1 (35). In addition, the RAM domain may also provide survival functions as ΔW ICN1 lacks the high-affinity tryptophan residue required to rescue thymocytes from steroid-induced apoptosis (57). Although survival-mediated functions may be required to overcome the RAG-dependent developmental block, it is not sufficient as Bcl-2 and Bcl-XL in RAG−/−HSCs did not promote DP development in reconstituted FTOC ([53] and data not shown).

An important question is the mechanism by which ICN1 expression leads to DP development in RAG−/− lymphocyte precursors. Two pathways affected by Notch signalling are E2A and nuclear factor-κB (NF-κB). Multiple studies show that Notch inhibits E2A signalling, and loss of E2A is sufficient to induce DP development in RAG−/− thymocytes (21, 58, 59). The relationship between Notch and NF-κB is less clear; however, the latter pathway can also influence pre-TCR signalling (60). The effects of both E2A and NF-κB are likely distal to SLP-76 suggesting that these molecules are not the primary effectors of Notch signalling in RAG−/− thymocytes. Nevertheless, changes in their expression together with changes at the pre-TCR itself may lower the net signalling threshold. We hypothesize that constitutive Notch signalling provides the optimal intracellular milieu so that sub-threshold signals provided by the thymic microenvironment result in signal transduction and development beyond the DN3 stage. The rate-limiting signals provided by Notch are largely proximal as enforced Notch1 signalling only provides a partial rescue of the DN3 developmental block in SLP-76−/− mice. In addition, anti-CD3 treatment does not rescue the SLP-76 defect (61), which is consistent with our hypothesis that Notch1 is mimicking the pre-TCR signal. Further evidence that ICN1 expression restored pre-TCR signalling was derived from the use of reporter assays in FTOCs. It has previously been established that the ERK–MAPK-signalling cascade is essential for the transition of DN thymocytes to the DP stage (62, 63). Moreover, we have shown previously that ERK–MAPK-signalling cascade is activated upon formation of the pre-TCR complex in developing thymocytes in vivo (48). Here, we demonstrate that Notch1 significantly up-regulated Elk-dependent luciferase activity indicating that Notch1 was inducing ERK–MAPK signalling, thus providing further evidence that ICN1 can mimic pre-TCR signals.

The current results are seemingly at odds with our previous findings demonstrating that ICN1 inhibited ERK–MAPK activity in retrovirally transduced Jurkat cells. A potential explanation is that Jurkat cells are human leukaemia cells that are phenotypically at a later stage of differentiation (CD4 SP) than the thymic progenitors utilized in the current experiments. In addition, immature and mature thymocytes respond differently to stimulation (64, 65) and possess different transcription factor signatures (66).

Pre-TCR signalling is wholly dependent on transduction through the CD3 complex (31, 67). Our finding that Notch1 drives development of CD3+:−/− precursors to the DP stage confirms that Notch1 can mimic pre-TCR signalling, but does not require expression of the intact pre-TCR complex to do so. Notch1 expression in DN 1–4 precursors strongly suggests that Notch and the pre-TCR are intimately linked (68). It has been implied that pTα is a downstream target of Notch1 (29, 39) and experiments using a pTα reporter in conjunction with CSL constructs provide strong evidence that this is the case (18). It is therefore probable that Notch1 both inhibits E2A and induces premature pTα expression in pre-TCR−/− precursors (49, 59). Therefore, the role of Notch1 may be to prime early DN cells to respond to TCRβ rearrangement which occurs at the DN3 stage (3). Interestingly, it has been demonstrated that Notch1 is not required beyond the late DN3 stage for normal T-cell development to proceed (69). Therefore, a short pulse of Notch1 signal may be required at the DN1/2 stages as its removal from BM and T-cell precursors completely abrogates β T-cell development (24, 25). Consistent with this, Notch−/− DN3 cells exhibit impaired VDJ rearrangement (24).

In summary, our data reveal that ICN1 has the potential to mimic pre-TCR signals by inducing ERK–MAPK activity and subsequently generate DP from RAG−/− and CD3+−/−.
precursors. The generation of DP thymocytes requires the expression of a construct containing all the domains of intracellular Notch1 (ICN1) and MigR1-ICN1 and MigR1 4W retroviral plasmids and incisive comments on the manuscript. The authors also wish to thank Paul Love for his generous gift of the CD3ε- mice, Gary Kortekty for the use of SLP-76-/- mice and Ada Krusbeek and Jennifer Punt for stimulating discussions. J.C.Z.-P. is a Canada Research Chair in Developmental Immunology.

Funding
National Institutes of Health to D.A., D.W.; Leukemia and Lymphoma Society Scholar Awards to D.A.; Australian National Health and Medical Research Council project (#384144) to D.I.

Acknowledgements
The authors wish to thank Warren Pear for the MigR1, MigR1-ICN1 and MigR1 4W retroviral plasmids and incisive comments on the manuscript. The authors also wish to thank Paul Love for his generous gift of the CD3ε- mice, Gary Kortekty for the use of SLP-76-/- mice and Ada Krusbeek and Jennifer Punt for stimulating discussions. J.C.Z.-P. is a Canada Research Chair in Developmental Immunology.

Abbreviations
ANK ankyrin
APC allophycocyanin
BM bone marrow
CL CBF-1, Suppressor of Hairless and Lag-1
dendritic cell
D/LR DNA Loading Ratio
DN CD4+ CD8- double negative
DP CD4+CD8+ double positive
FL foetal liver
FTOC foetal thymic organ culture
GFP green fluorescent protein
GSI γ-secretase inhibitor
HSC haemopoietic stem cell
ICN1 constitutively active full-length intracellular Notch 1
ISP immature CD8+ single positive
MAML Mastermind like
NF-κB nuclear factor-κB
SP CD4+CD8- or CD4+CD8+ single positive
TAD transcriptional activation domain

References
Notch promotes pre-TCR-like signals


