RESEARCH ARTICLE

A new role for Notch in the control of polarity and asymmetric cell division of developing T cells

Mirren Charnley1,2,3,¶, Mandy Ludford-Menting3,*, Kim Pham1,3,4,5,¶ and Sarah M. Russell1,3,4,¶

ABSTRACT

A fundamental question in biology is how single cells can reliably produce progeny of different cell types. Notch signalling frequently facilitates fate determination. Asymmetric cell division (ACD) often controls segregation of Notch signalling by imposing unequal inheritance of regulators of Notch. Here, we assessed the functional relationship between Notch and ACD in mouse T cell development. To attain immunological specificity, developing T cells must pass through a pivotal stage termed β-selection, which involves Notch signalling and ACD. We assessed functional interactions between Notch1 and ACD during β-selection through direct presentation of Notch ligands, DL1 and DL4, and pharmacological inhibition of Notch signalling. Contrary to prevailing models, we demonstrate that Notch signalling controls the distribution of Notch1 itself and cell fate determinants, α-adaptin and Numb. Furthermore, Notch and CXCR4 signalling cooperated to drive polarity during division. Thus, Notch signalling directly orchestrates ACD, and Notch1 is differentially inherited by sibling cells.

This article has an associated First Person interview with the first author of the paper.

KEY WORDS: Asymmetric cell division, β-selection, CXCR4, DN3, Developing T cell, Notch

INTRODUCTION

Developing multicellular organisms must produce multiple cell types from one progenitor cell. This is achieved by sequential bifurcations in the fate of daughter cells following cell division. Fate bifurcation is driven by various means, including random differences in the daughters, different positional cues, or controlled segregation of fate determinants differentially into the two daughter cells, a process termed asymmetric cell division (ACD) (Dewey et al., 2015; Schweisguth, 2015; Venkei and Yamashita, 2018). A common mediator of fate bifurcation, which uses all of these processes, is the Notch signalling pathway (Artavanis-Tsakonas et al., 1999; Fortini, 2009; Liu et al., 2017; Sjöqvist and Andersson, 2017). Notch proteins are transmembrane receptors that respond to ligands belonging to the Delta and Jagged family, which are present on adjacent cells (Bray, 2016). Notch signalling directly regulates transcription to influence cell fate decisions, such as proliferation, death, multi-potency, differentiation and self-renewal (Fortini, 2009). Understanding how Notch signals differently in different tissue contexts is a subject of intense investigation (Bray, 2016). Differential Notch signalling in two daughter cells often occurs indirectly through the differential inheritance of regulators of Notch (Bray, 2016). Indeed, some of the most frequently observed fate determinants controlled by ACD are the Notch regulators, Numb and α-adaptin (Daeden and Gonzalez-Gaitan, 2018; Dewey et al., 2015; Rhyu et al., 1994; Schweisguth, 2015). Consequently, the prevailing view is that, although an important executor of cell fate, Notch does not directly determine the direction of bifurcation in fate (Schweisguth, 2015; Venkei and Yamashita, 2018).

We considered the possibility that Notch proteins might play a more active role in dictating the direction of fate bifurcation. One observation suggestive of a controlling role for Notch showed that Notch signalling was required for asymmetric distribution of Numb during ACD of Drosophila neural progenitors (Bhat, 2014; Pinto-Texeira and Desplan, 2014). We have previously observed that Notch was polarised during interphase in developing T cells (Pham et al., 2015), leading to two non-mutually exclusive hypotheses: (1) that Notch might remain asymmetric during division, and thus be asymmetrically partitioned in the daughters, and (2) that Notch might control partitioning of cell fate determinants during division. We tested those two hypotheses in this study using an in vitro model of T cell development.

T cell development is a highly tractable model for the study of cell fate, with well-established in vitro models and an extensive knowledge of the molecular pathways involved (Janas et al., 2010; Mohtashami et al., 2010; Schmitt and Zuniga-Pflucker, 2002; Van de Walle et al., 2013; Yui and Rothenberg, 2014). Many aspects of T cell biology share mechanisms of fate control with other developmental systems, and both Notch signalling and ACD operate in developing and mature T cells (Chang et al., 2007; Oliaro et al., 2010; Tajbakhsh et al., 2009). ACD occurs specifically during the β-selection phase of T cell development (Pham et al., 2015). In this critical phase, the developing T cells termed double-negative (DN) 3 cells must balance self-renewal, genomic DNA recombination to produce a functional T cell receptor chain, and differentiation. Notch signalling is required for progression through β-selection and subsequent fate choices (Ciofani et al., 2004; Ciofani and Zuniga-Pflucker, 2005).

We exploited two model systems to explore the role of Notch1 during division of developing T cells; the OP9-DL1 co-culture, and surfaces functionalised with Delta-like 1 (DL1, also known as DLL1) (Janas et al., 2010; Mohtashami et al., 2010; Schmitt and Zuniga-Pflucker, 2002; Van de Walle et al., 2013; Shukla et al., 2017). We show that the interaction of Notch1 with its ligands, DL1 and DL4 (also known...
signalling on the polarisation of developing T cells in interphase. To discern whether Notch1 is a passive participant or active driver of polarity in developing T cells, we first assessed the role of Notch in dictating cell fate in developing T cells. Together, these data show that Notch signalling directly acts as a polarity cue, and that Notch1 is differentially inherited by the progeny of an ACD. This indicates that Notch signalling plays a key role in dictating cell fate in dividing T cells.

RESULTS

Notch signalling drives polarisation during interphase and cell division

To discern whether Notch1 is a passive participant or active driver of polarity in developing T cells, we first assessed the role of Notch signalling on the polarisation of developing T cells in interphase.

Using established methods (Janas et al., 2010; Mohtashami et al., 2010; Schmitt and Zuniga-Pflucker, 2002; Van de Walle et al., 2013), we drove the development of mouse T cells from hematopoietic stem cells using the OP9-DL1 stromal cell line that expresses the Notch ligand, DL1. To ensure that the cells were poised to, but had not yet entered the β-selection checkpoint, we sorted for the DN3a stage by flow cytometry and reseeded onto OP9-DL1 cells. Notch signalling was disrupted using the γ-secretase inhibitor (dibenzazepine, DBZ) (De Kloe and De Strooper, 2014), which recapitulates the effect of Notch1 deletion in developing T cells (Hadland et al., 2001). DBZ has the potential to also inhibit γ-secretase-mediated cleavage of substrates, such as the β-amyloid precursor protein (APP) (De Kloe and De Strooper, 2014), but APP is not expressed in developing T cells (Mingueneau et al., 2013), and the effect of DBZ mimics that of removing Notch1 or Notch ligands in T cell development (Ciofani et al., 2004). As previously published (Ciofani et al., 2004), Notch inhibition in DN3 cells reduced differentiation to the DN4 and double-positive (DP) stages, reduced cell proliferation, and increased cell death (Fig. S1).

To determine the role of Notch signalling in polarity, we performed immunofluorescence microscopy to assess the localisation of α-tubulin and markers of cell polarity in fixed DN3a cells in interphase. We chose an early time point (15 h) where inhibiting Notch signalling had little or no impact on the number of attached DN3a cells (Fig. 1A), but slightly reduced microtubule-organising centre (MTOC) polarisation to the interface between the stromal cell and the DN3a cell. (C) As previously seen (Pham et al., 2015), Notch1 was polarised to the interface (Fig. 1C). Remarking, the protein of interest was assigned as diffuse or polarised, where polarised was defined as a clear enrichment of fluorescence at the interface. (D) Notch1 and (E) CXCR4 were recruited to the cell–cell interface in over half the cells, and this was severely disrupted in the presence of DBZ. (F) CD25 was not polarised and unperturbed by inhibiting Notch signalling. The cell fate regulators (G) α-adaptin and (H) Numb were also polarised and this was reliant on intact Notch signalling. Representative projected z-stack images are shown on the left, where ‘no treat’ shows a polarised example and DBZ shows a non-polarised example for each test protein. Quantification is shown on the right. Total number of cells analysed: MTOC=809 and 589, Notch1=130 and 110, CXCR4=158 and 113, CD25=68, 67, α-adaptin=131 and 105 and Numb=158 and 110, for no treat and DBZ, respectively. Scale bars: 10 µm. The asterisks in the images indicate the interface between the DN3a and OP9-DL1 stromal cells. n=3 independent experiments. All data are represented as means±s.e.m.*P<0.05, **P<0.01, ***P<0.001 (unpaired t-test).

as DLL4), drives the polarisation of Notch1 itself, and the polarisation of cell fate determinants, α-adaptin and Numb. Together, these data show that Notch signalling directly acts as a polarity cue, and that Notch1 is differentially inherited by the daughters of an ACD. This indicates that Notch signalling plays a key role in dictating cell fate in developing T cells.
Notch1, CXCR4, α-adaptin and Numb from above 70% to between 24 and 42%, similar to the level of polarisation observed with the CD25 control (Fig. 1F). These data suggest that Notch signalling was required for the polarisation of DN3a cells.

We next asked whether Notch1 was polarised during cell division, and whether Notch signalling was required. Dividing cells were scored as undergoing symmetric cell division (SCD) if the protein of interest was symmetrically distributed between the two daughter cells, and as undergoing ACD if the protein of interest was more concentrated in one of the daughter cells (Fig. 2A). As previously seen (Pham et al., 2015), Numb and α-adaptin were asymmetric in 55 to 66% of dividing cells, and we further show that Notch1 and CXCR4 were also asymmetric in 55 and 51% of dividing cells, while CD25 was asymmetric in only 29% (Fig. 2B–F). Inhibiting Notch signalling reduced the asymmetry of the markers in dividing cells to the same level as the CD25 control. CXCR4 acts as a polarity cue for ACD (Pham et al., 2015), so the effect of Notch signalling on CXCR4 polarisation indicates that these cues could cooperate to trigger ACD. These data together indicate that Notch1 was polarised during division of DN3a cells, and suggest that Notch signalling is required for the polarisation of Notch1, CXCR4, α-adaptin and Numb.

Surfaces functionalised with the Notch ligand, DL1, were sufficient to induce polarisation during cell division

The pharmacological inhibition experiments above showed that γ-secretase was necessary for polarisation of DN3a cells during interphase and division. To confirm that these effects were through Notch, and to assess whether Notch signalling was sufficient for polarity, we used surfaces functionalised with the Notch ligand, DL1. DL1 was coupled to Protein A (PA)-coated surfaces via an Fc linker to control the orientation of DL1 relative to the cell (Makaraviciute and Ramanaviciene, 2013; Song et al., 2012; Toda et al., 2011). Growth factors essential to DN3a survival and differentiation were included in the cultures (Janas et al., 2010), but these were dispersed throughout the culture medium and only the Notch ligand was presented to the cells with a defined orientation. As others have shown (Janas et al., 2010), surfaces functionalised with Fc-DL1 supported DN3 differentiation, albeit not as efficiently as OP9-DL1 cells (Fig. S2). The frequency and duration of divisions on the Fc-DL1 surfaces was comparable to that on the OP9-DL1 cells (Fig. S3). After 2 h of culture the number of attached cells was unaffected by the surface coating (Fig. 3A). The MTOC, identified by α-tubulin staining, was assigned as polarised if the MTOC was recruited to the cell–substrate interface and non-polarised if the MTOC was absent from the interface (Fig. 3B). MTOC polarisation was increased on the Fc-DL1 surfaces, in comparison to PA alone (Fig. 3C). The recruitment of α-adaptin and Numb to the interface was also increased on Fc-DL1 surfaces (Fig. 3D–F) and this was maintained at 15 h (Fig. S4). These data demonstrate that the immobilised DL1 ligand alone was sufficient to trigger the recruitment of α-adaptin and Numb to the functionalised surface.

We then asked whether the surfaces coated with Fc-DL1 were capable of triggering asymmetry during division. First, DN3a cells were cultured on surfaces functionalised with Fc-DL1 or PA for 15 h, fixed and labelled with α-tubulin and test proteins. (A) To determine polarisation in dividing DN3a cells the dividing cells were identified from α-tubulin staining and scored as undergoing SCD if the protein of interest was symmetrically distributed between the two daughter cells or ACD if the protein of interest was more concentrated in one of the daughter cells. Dividing DN3a cells were analysed for polarisation of: (B) Notch1, (C) CXCR4, (D) CD25, (E) α-adaptin or (F) Numb. Representative projected z-stack images are shown on the left, where ‘no treat’ shows a polarised example and DBZ a non-polarised example for each test protein. Quantification is shown on the right. Inhibiting Notch signalling significantly reduced the percentage of cells undergoing ACD. Total number of cells analysed: Notch1=57 and 44, CXCR4=43 and 41, CD25=30 and 30, α-adaptin=46 and 56 and Numb=40 and 45, for no treat and DBZ, respectively. Scale bars: 10 µm. n=3–5 independent experiments. All data are represented as mean±s.e.m. *P<0.05, ***P<0.001 (unpaired t-test).
or CD25, imaged and blind scored using fluorescence microscopy. α-adaptin and Numb had equal or greater polarity on Fc-DL1 functionalised surfaces, as compared to OP9-DL1 cells, and this response was specific to surfaces functionalised with DL1 (Fig. 4A–C). Similar to what was seen for DN3a cells cultured on OP9-DL1 cells, Notch1 was asymmetric in 65% of dividing cells when cultured on Fc-DL1 functionalised surfaces and CXCR4 was asymmetric in 56% of cells (Fig. 4D). Given that both Notch1 and its regulators, α-adaptin and Numb, were polarised, we assessed whether they were on the same or opposing sides of the dividing cell. For dividing cells in which both Notch1 and Numb were polarised, 84% showed recruitment of both proteins into the same daughter cell (Fig. 4E). Similarly, Notch1 was co-recruited into the same daughter cell as α-adaptin (86% of polarised divisions) and CXCR4 (79% of polarised divisions). This indicates that Notch ligation by DL1 drove polarisation during division, leading to the preferential recruitment of Notch1, CXCR4, Numb and α-adaptin into one daughter cell.

To complement the analysis of the fixed cells and quantify the extent of asymmetry, DN3a cells were transduced with fluorescently tagged α-adaptin and Numb, cultured in cell paddocks (Day et al., 2009) onto which OP9-DL1 stromal cells had been allowed to attach overnight or were coated with PA or PA and Fc-DL1 and imaged using time-lapse microscopy. The fluorescence intensity was quantified in dividing cells and expressed as polarisation ratios (PR; calculated as the difference in fluorescence between the two halves divided by the sum of the fluorescence). A low PR value indicates that the fluorescence is symmetric in the dividing cells, a high PR value indicates that it is asymmetric. A diffuse marker was also transduced into the DN3a cells (either GFP or mCherry) to determine the background level of asymmetry and remove any artefacts (e.g. out-of-focus cells) (Charnley and Russell, 2017; Pham et al., 2015; Shimoni et al., 2014). The overexpression of the protein of interest had little or no effect on proliferation, differentiation or the level of polarity (Fig. S5A–D) (Pham et al., 2015, 2013). To ensure against possible artefacts related to the nature of the tag (Couturier et al., 2014; Short, 2014), we compared the polarity of α-adaptin and Numb and fluorescent protein asymmetry triggered by Notch ligation was coordinated with the polarity of mitotic spindle orientation during ACD. Notch ligation by DL1 drove polarisation during division, leading to the preferential recruitment of Notch1, CXCR4, Numb and α-adaptin into one daughter cell.
using the same strategy as the flat surfaces. The proportion of polarised divisions observed for the PA and Fc-DL1 functionalised beads was similar to the proportion observed for the flat surfaces (Fig. 6A). The angle of the mitotic spindle relative to the polarity cue was measured to determine the division angle (Fig. 6B). When the dividing cell was in contact with OP9-DL1 cells or Fc-DL1 functionalised beads, higher PRs correlated with greater division angles (Fig. 6C,D). Binning the data to separate cells based on division angle revealed that divisions perpendicular to the cue (division angles greater than 45°) showed higher PR values, indicating polarised divisions, compared with divisions that were parallel to the cue (Fig. 6E,F). This demonstrates that the protein asymmetry observed was coordinated with the orientation of the mitotic spindle, indicative of ACD. Thus, Notch ligands were necessary and sufficient to coordinate polarity and the spindle to orchestrate ACD.

**Notch functionally interacts with other cues to orchestrate ACD**

The only directional cue presented to DN3a cells that promoted polarisation in the above experiments was DL1. However, the CXCR4 ligand, CXCL12, is required for T cell differentiation (Janas et al., 2010; Tussiwand et al., 2011) and was included in our cultures. We have previously found that ACD induced by OP9-DL1 stromal cells depends upon CXCR4 signalling in DN3a cells (Pham et al., 2015). This, combined with our observations above that CXCR4 polarisation depends on Notch signalling and that surfaces functionalised with the Notch ligand DL1 induce asymmetry of α-adaptin and Numb in dividing DN3a cells, raises the question of how these two receptors cooperate. We therefore assessed whether cells cultured on Fc-DL1 functionalised surfaces could still polarise after pharmacological inhibition of one or both of the CXCR4 and Notch signalling pathways, using AMD3100 or DBZ, respectively (Fig. 7A; Fig. S7A–C).

![Fig. 5. Dividing DN3a cells polarise cell fate determinants, α-adaptin and Numb, on functionalised surfaces. DN3a cells expressing a diffuse protein (GFP or mCherry) and α-adaptin were cultured onto surfaces pre-coated with PA, PA+Fc-DL1 (DL1) or OP9-DL1 stromal cells, and imaged using time lapse microscopy for 20 h. Representative projected z-stack images are shown on the left, and quantification on the right. (A) Culturing DN3a cells on OP9-DL1 stromal cells and Fc-DL1 increased the proportion of dividing cells with high PRs. Number of divisions analysed: OP9-DL1=208, PA=102 and Fc-DL1=109. (B) A similar trend was observed for DN3a cells transduced with Numb. Number of divisions analysed: OP9-DL1=148, PA=48 and Fc-DL1=45. Scale bars: 10 µm. n=3–8 independent experiments; magenta line on violin plots indicates the median value. ***P<0.001 (KS test). See Fig. S6 for PR scatter plots.](https://doi.org/10.1242/jcs.235358)
and CXCR4 signalling were required for optimal polarisation of α-adaptin in dividing DN3a cells, with the least polarisation occurring when both inhibitors were present. The effect of disrupting CXCR4 signalling was intriguing given that CXCL12 is diffuse in the cultures, so we looked specifically at the localisation of the two receptors. As expected, Notch1 was polarised in response to surface-bound DL1 in dividing DN3a cells (Fig. 7B). Notch1 polarisation was strongly inhibited by the Notch inhibitor, but only minimally affected by inhibition of CXCR4 signalling. CXCR4 was also polarised in response to presentation of the Notch ligand, and this polarisation was dependent on signalling through both Notch1 and CXCR4 (Fig. 7C). These data indicate that Notch ligation can drive the asymmetric polarisation of CXCR4 and that CXCR4 and Notch cooperate as non-redundant cues for ACD, albeit with Notch playing a more dominant role.

Given the complexity of interactions in the thymus (Petrie and Zúñiga-Pflücker, 2007), we evaluated two other ligands for the regulation of ACD in DN3a cells. DL4 is an alternative ligand for Notch1 (Hozumi et al., 2004, 2008; Koch et al., 2008), and we assessed whether it behaved similarly to DL1. Indeed, both ligands induced equivalent polarisation of α-adaptin in dividing DN3a cells (Fig. 7D; Fig. S7D). VCAM-1 contributes to stromal cell–DN3 interactions and its presence is associated with improved in vitro generation of T cells (Shukla et al., 2017; Abe et al., 2010). We therefore assessed the impact of Fc-VCAM-1 on DL4-mediated polarisation during division by functionalising the surfaces with both ligands. Interestingly, the inclusion of Fc-VCAM-1 slightly reduced the level of polarisation (Fig. 7D,E; Fig. S7E). One possible explanation for this decrease is that the presence of VCAM-1 altered the interaction of the DL1 and CXCL12 ligands with their receptors. Indeed, on the Fc-DL4+Fc-VCAM-1 surfaces the polarisation of both Notch1 and CXCR4 was also slightly reduced (Fig. 7F,G). This effect correlated with a decrease in polarisation at interphase of the MTOC and α-adaptin (Fig. S8A–C), but was not associated with an effect on differentiation (Fig. S8D–F). These data show that the slight reduction in ACD observed when VCAM-1 was included in the functionalised surfaces was associated with reduced polarisation of Notch1 and CXCR4. Collectively, these data show that multiple cues can interact to enhance or reduce the extent of ACD.

**DISCUSSION**

It is well established that Notch controls diversification in cell fate in a number of developmental systems. However, most of the proposed mechanisms assume that Notch executes decisions that have been made stochastically or are imposed by unequal expression of regulators of Notch (Dewey et al., 2015; Schweisguth, 2015; Venkei and Yamashita, 2018). Here, we demonstrate that Notch1 can directly trigger asymmetry in daughter cells, by steering ACD and by partitioning itself asymmetrically in the two daughter cells (Fig. 8).

Two model systems were used to explore the role of Notch1 during division of developing T cells; the OP9-DL1 co-culture, and surfaces functionalised with Fc-DL1. The OP9-DL1 co-culture system has been extensively used to study T cell development in vitro, and mimics physiological early T cell development with high fidelity (Janas et al., 2010; Mohtashami et al., 2010; Schmitt and Zúñiga-Pflücker, 2002; Van de Walle et al., 2013). Surfaces functionalised with Notch ligands have also provided insights into the mechanisms controlling T cell development (Janas et al., 2010; Tussiwand et al., 2011; Dallas et al., 2005). Furthermore, there is a growing interest in using surfaces functionalised with Notch ligands for the in vitro generation of T cells for therapeutic treatments, such as cancer immunology (Gehre et al., 2015; Shukla et al., 2017; Suzuki et al., 2006 and as reviewed in Singh and Zuniga-Pflucker, 2018). Recently, it was demonstrated that it is possible to direct human hematopoietic stem cells along the T cell lineage utilising functionalised surfaces in serum-free conditions (Shukla et al., 2017), which would circumvent issues associated with xenogenic culture and enable the generation of clinical-grade pro-T cells.

Functionalised surfaces for the controlled presentation of proteins have been widely used to study fundamental biology, including adhesion and migration (Charnley et al., 2012; Gavard et al., 2004; Kovacs et al., 2002; Silvestre et al., 2009), division (den Elzen et al.,
immune responses (Grakoui et al., 1999; Manz and Groves, 2010; Mossman et al., 2005; Charnley et al., 2009) and T cell development (Janas et al., 2010; Tussiwand et al., 2011; Dallas et al., 2005). Here, we demonstrated that these surfaces successfully mimicked another aspect of T cell development; developing T cells cultured on the surfaces underwent ACD and this process was coordinated by external cues and restricted to the DN3a stage of development. This work also highlights that functionalised surfaces can be used to manipulate the microenvironment of cells and systemically test the influence of extrinsic factors on ACD.

The interaction of Notch1 with the DL1 ligand drove the polarisation of fate determinants that are asymmetrically distributed in several models of ACD, namely α-adaptin and Numb. Notch signalling also triggered the polarisation of CXCR4, which is a polarity cue for ACD of DN3a cells (Pham et al., 2015), and the polarisation of Notch1 itself. Thus, the orientated presentation of the Notch1 ligand was sufficient to trigger polarity during division in developing T cells. The role of Notch in controlling localisation of Numb is analogous to that observed in Drosophila ganglion mother cells (Bhat, 2014), indicating that its role as a polarity cue could be more broadly conserved.

In addition to its role in controlling polarity, we demonstrate that, in dividing DN3a cells, Notch1 was asymmetrically distributed between the two daughter cells. Asymmetric partitioning of Notch1 itself during an ACD provides direct mechanism to establish variation in Notch signalling levels. Interestingly, Notch1 localisation is also controlled at cytokinesis in dividing sensory organ precursor cells (Coumailleau et al., 2009; Couturier et al., 2012; Trylinski et al., 2017). However, in these cells Notch was localised at the interface between two daughter cells during division, rather than polarised to one side of the mother cell. Using developing T cells as a model system of ACD, we demonstrated that Notch1 is asymmetrically inherited, which could provide a direct and novel mechanism to establish differential Notch signalling in the two daughters (Fig. 8).

Fig. 7. Different polarity cues cooperate to regulate ACD. DN3a cells expressing a diffuse protein (GFP or mCherry) and α-adaptin were cultured onto surfaces pre-coated with PA, PA+Fc-DL1 (DL1), PA+Fc-DL4 (DL4) or PA+Fc-DL4+Fc-VCAM-1 (D+V) and imaged using time lapse microscopy for 20 h. The addition of (A) either DBZ or AMD3100 (AMD) or a combination of both inhibitors caused a reduction in the level of ACD. Number of divisions: DBZ=68, AMD3100=65, DBZ+AMD3100=51. (B) The polarisation of Notch1 in dividing cells was reduced by DBZ, but only slightly affected by AMD3100. (C) Conversely, CXCR4 polarisation was reduced by the inhibition of either Notch or CXCR4 signalling. Number of divisions analysed=55, 48 and 42 for no treat, DBZ and AMD3100, respectively. (D) Fc-DL4-coated surfaces triggered ACD and the inclusion of Fc-VCAM-1 led to a reduction in the asymmetric segregation of α-adaptin during division. Number of divisions analysed: DL4=67; DL4+VCAM=58. The presence of VCAM-1 in the functionalised surfaces also resulted in reduced polarisation of (E) α-adaptin, (F) Notch1 and (G) CXCR4 in dividing cells. Number of divisions analysed: DL4=56; DL4+VCAM=44 and DL4=64 (α-adaptin); DL4=67; DL4+VCAM=71 (Notch1 and CXCR4). n=3–6 independent experiments; scatter plots represent mean±s.e.m., magenta line on violin plots indicates median value; *P<0.05, **P<0.01, ***P<0.001 (KS test for violin plots and unpaired t-test for scatter plots). See Fig. S7 for PR scatter plots.

Fig. 8. A model of the role of Notch in controlling cell polarity and ACD. Notch ligation of DL1, presented either by the OP9-DL1 stromal cell or DL1 functionalised surfaces, orchestrates polarisation during the division of DN3a cells. CXCR4 signalling cooperates with Notch to drive this asymmetry during division. This results in the differential inheritance of the cell fate determinants, α-adaptin and Numb, CXCR4 and Notch1 itself, which were preferentially recruited into the same daughter cell.
More work is needed to clarify how this impacts on cell fate determination during β-selection. Notch1 signalling is required for T cell development past the β-selection stage, but peaks at the DN3a stage (Mingueu et al., 2013) and is dramatically downregulated following β-selection (Yashiro-Ohtani et al., 2009). The role of Notch1 in enhancing ACD, and our finding that ACD is restricted to DN3a cells (current study and Pham et al., 2015) suggests that Notch could act in part to determine the developmental stage at which ACD occurs. Additionally, the segregation of Notch signalling by ACD might enable one daughter cell to progress past the β-selection stage, and the other to self-renew. A molecular basis for such a scenario is suggested by the recent finding that Notch regulates Cdkn1b-mediated proliferation during β-selection (Zhao et al., 2019). The co-inheritance of Notch antagonists with the Notch receptor suggests that this daughter cell is primed, but not necessarily active, for Notch signalling. In contrast, the daughter cell that receives less Notch1 presumably has less need to express the Notch antagonists. Interestingly, a similar asymmetry of both Notch and Numb was recently suggested in hematopoietic stem cells, where the daughter cell that inherits less Numb and Notch is more likely to proliferate and differentiate (Loeffler et al., 2019). In developing T cells, the endurance of this balance of inheritance of positive and negative regulators of Notch signalling, and its effect on cell fate, will require analysis of the dynamics of Notch signalling over several generations.

Notch1, pre-T cell receptor (TCR) and CXCR4 signalling cooperate to drive the developing T cell through β-selection, and the presence of both CXCR4 and Notch1 signalling is required for survival on the functionalised surfaces (Ciofani et al., 2004; Ciofani and Zuniga-Pflucker, 2005; Janas et al., 2010; Trampont et al., 2010). Our results indicate that they also cooperate to dictate polarity and ACD in developing T cells. A functional interaction between Notch1 and CXCR4 has been demonstrated in a number of cell systems (Chairamonte et al., 2015; Cong et al., 2017; Ho et al., 2017; Wang et al., 2009; Xiao et al., 2017), but the molecular basis for this interaction is not clear. The co-recruitment of Notch1 and CXCR4 could indicate that they physically interact, either via a direct interaction of the receptors or their ligands. The striking loss of CXCR4 polarisation when Notch signalling is inhibited suggests that Notch is upstream of the chemokine receptor. Taken together, these findings indicate that Notch1 and CXCR4 have complementary and non-redundant roles in dictating polarity to control ACD.

These experiments reveal a new paradigm by which Notch controls cell fate. In developing T cells, Notch plays a key role in dictating its own asymmetry during cell division and influencing asymmetry of other fate determinants. This work focused on developing T cells, but given the influence of Notch on Numb asymmetry in neural precursor cells, this might be a more general paradigm.

**Materials and Methods**

**Primary developing T cell co-culture**

Mouse hematopoietic stem cells [isolated from C57BL/6 fetal liver at embryonic day (E)14.5] were seeded onto OP9-DL1 stromal cells (received from Juan-Carlos Zúñiga-Pflucker, University of Toronto, Sunnybrook Health Sciences Centre, Toronto, Ontario, Canada) at a 1:1 ratio in six-well plates (2×10^5) in minimal essential medium α modification supplemented with fetal calf serum (10% v/v), glucose (1 mM), β-mercaptoethanol (50 μM), sodium pyruvate (1 mM), HEPES (10 mM), penicillin-streptomycin (100 ng/ml), mouse interleukin 7 (IL-7, 1 ng/ml) and mouse FMS-like tyrosine kinase 3 (Flt-3, 5 ng/ml). Hematopoietic cells were harvested via forceful pipetting and co-cultured on fresh OP9-DL1 stromal cells every 3–8 days. All mice were maintained in a specific pathogen-free environment with food and water freely available. All experiments on mice were performed with the approval of the Animal Experimentation Ethics Committee of the Peter MacCallum Cancer Centre.

**Retroviral transduction**

Phoenix E cells (provided by Garry Nolan, Stanford University, Stanford, CA) were maintained at 37°C and at 10% CO2 in Dulbecco’s minimal essential medium supplemented with fetal calf serum (10% v/v), L-glutamine (1 mM) and penicillin/streptomycin (100 ng/ml). Calcium phosphate transfection was performed on Phoenix E cells with 10–20 μg of the following pMSCV retroviral constructs: mCherry (Addgene #52114), GFP (Addgene #20672), GFP–Numb, mCherry–Numb, mCherry–α-adaptin, GFP–α-adaptin and GFP–tubulin (Ting et al., 2012; Pham et al., 2013) in 10 cm dishes (Corning). Viral supernatant was harvested 48 h after transfection and added to six-well plates that had been pre-coated with 15 μg/ml RetroNectin (Takara Bio Inc.) and blocked with 2% BSA. After addition of the viral supernatant, plates were spun at 2000 g for 1 h and incubated for 1 h at 37°C. 5×10^5 hematopoietic cells (day 4 co-culture) were added, and plates were spun for 1 h at 1200 g.

**Flow cytometry**

Developing T cell subsets were purified by staining for the cell surface markers CD44 and CD25 to discriminate between DN1–DN4, CD28 to discriminate between DN3a and DN3b, and CD4 and CD8 to discriminate between the DN and DP populations. Hematopoietic cells were harvested from OP9-DL1 co-cultures by forceful pipetting and stained on ice for 1 h with the following antibodies: biotin-conjugated anti-CD4 (1:2000; BD Pharmingen, 553045), biotin-conjugated anti-CD8a (1:2000; BD Pharmingen, 553029), PerCP/Cy5.5-conjugated anti-CD44 (1:300; Biolegend 103032), APC-eFluor 780-conjugated anti-CD25 (1:300; eBioscience, 47-0251-82) and Pe/Cy7-conjugated anti-CD28 (1:200; Biologend, 122014). Subsequently, the DN3a cells were rinsed and stained with BV421 Streptavidin (1:600; BD Biosciences, 563259) for 1 h on ice prior to sorting. DN3a cells were isolated on a FACs (FACS Aria III; BD Biosciences) based on surface expression (CD28^+^, CD25^+^, CD44^+^, CD4^+^ and CD8^+^). Retrovirally transduced cells were sorted 72 h after transduction and also sorted on the basis of GFP and mCherry fluorescence. For the analysis of T cell differentiation, cells were stained with Pacific Blue-conjugated anti-CD4 (1:600; BD Pharmingen, 558107), APC-eFluor 780-conjugated anti-CD8a (1:300; eBioscience, 47-0081-82), PerCP/Cy5.5-conjugated anti-CD44 (1:300; Biolegend 103032), APC-conjugated anti-CD25 (1:300; eBioscience, 17-0251-82), Pe/Cy7-conjugated anti-CD28 (1:200; Biologend, 122014) and Sytox Green viability stain (30 nM; Molecular Probes, S34860).

**Substrate functionalisation**

Functionalised surfaces were used to individually present ligands to the developing T cells. Protein A (PA) was used to couple the Fc-linked ligands to the surface to ensure optimum orientation of the ligand (Makaroviciute and Ramanaviciene, 2013; Song et al., 2012; Toda et al., 2011). Glass coverslips, silicon dioxide beads (5 μm), PDMS cell paddocks or 24-well plates were coated with PA (235 nM, 1 h), rinsed with PBS and further functionalised, if required, with Fc-DL1 (86 nM; R&D Systems, 5026-DL), Fc-DL4 (86 nM; BioVision, P1163), Fc-VCAM-1 (19 nM; R&D Systems, 643V) or Fc-DL4 and Fc-VCAM-1 (86 nM+19 nM) for 30 min. The substrates were then washed in PBS and medium before cell deposition. For live-cell imaging, PDMS cell paddocks (120 μm × 120 μm) (Day et al., 2009) were rendered hydrophilic by exposure to air plasma at 1.5×10^-2^ mbar for 10 min and placed into an eight-well chamber slide (Ibidi) prior to protein functionalisation.

**Proliferation and development of DN3 cells on the functionalised surfaces**

For the analysis of the ability of functionalised surfaces to support developing T cell proliferation and differentiation, 24-well plates were coated with PA, PA plus Fc-linked proteins or OP9-DL1 stromal cells. 10^5
DNA3a cells were added to an eight-well chamber slide (Thermo Fisher Scientific) precoated with PA, PA plus Fc-linked proteins or OP9-DL1 stromal cells and cultured for 2 or 15 h. For stromal cell-free culture, cells were cultured in the presence of Flt-3 (5 ng/ml), CXCL12 (10 nM) and IL-7 (10 ng/ml) (Janas et al., 2010). Cells were then fixed, permeabilised, and labelled with primary antibodies for α-tubulin (1:800; Rocklands, 600-401-880 or 200-301-880) and test proteins, Notch1 (1:200; Abcam, ab27526), α-adaptin (1:200; Abcam, Ab2730), Numb (1:200; Abcam, Ab4147), CXCR4 (1:200; BD Pharmingen, 551968), CD25 (1:1000; BD Pharmingen, 553070), and the appropriate secondary antibodies and then mounted with Prolong gold antifade (Molecular Probes). The slides were examined using an FV1000 microscope (Olympus) and 40× objective lens (0.95 NA Oil Plan Apochromat) or FV3000 microscope (Olympus) and 60× objective lens (1.35 NA Oil Plan Apochromat). 3D images of the cells were acquired with a z distance of 0.5 µm and maximum intensity projections of z sections created using ImageJ. For quantifications, the investigator was blind to the sample allocation at the moment of scoring. For the analysis of polarisation during interphase, α-tubulin staining was used to identify cells with the MTOC at the cell–cell/cell–substrate interface and the cell was scored depending on whether the protein of interest was diffuse or enriched at the MTOC (Fig. 1C). To determine polarisation during division, mitotic cells were identified by the presence of a mitotic spindle and the cell division was assigned as asymmetric if the fluorescence of the test protein was evenly distributed between the two daughter cells and asymmetric if the fluorescence was greater in one of the daughter cells than the other daughter cell (Fig. 2A). The co-inheritance of Notch with other fate determinants (specifically Numb, α-adaptin and CXCR4) was assessed by selecting dividing cells that were asymmetric for both proteins for analysis and scoring it as co-inherited if both proteins were concentrated within the same nascent daughter cell.

**Live-cell image acquisition and analysis**

For live-cell imaging, 2×10^5 DNA3a cells were added to eight-well chamber slides (Ibidi) with the PDMS cell paddocks precoated with PA, PA plus Fc-linked proteins or OP9-DL1 stromal cells. For stromal cell-free culture, cells were cultured in the presence of Flt-3 (5 ng/ml), CXCL12 (10 nM) and IL-7 (10 ng/ml) (Janas et al., 2010). Images were captured on a spinning disc confocal system fitted with an inverted microscope and temperature-controlled chamber maintained at 37°C and 5% CO₂. Images are acquired using a 4× air objective (0.95 NA) and multiple stage positions are recorded every 3 min for 20 h, with 8-slice z-stacks of 1 µm thickness. As described in Charnley and Russell (2017), Olario et al. (2010), Pham et al. (2015), Shimoni et al. (2014), the polarisation of fluorescent markers in the nascent daughter cells was quantified by measuring the total fluorescence intensity of each daughter cell and applying a ‘Polarisation Ratio’ (PR) equation [(PR=(ΣH1−ΣH2)/(ΣH1+ΣH2))) where the difference in total intensity between Daughter 1 (ΣH1) and Daughter 2 (ΣH2) is divided by the sum of intensities in both Daughter 1 (H1) and Daughter 2 (H2). A PR of 0.17 was used as a cutoff to designate cells as undergoing ACD (PR>0.17) or SCD (PR<0.17). This cutoff was determined from the spread of the diffuse protein to result in a minimal number of the diffuse protein divisions being assigned as asymmetric (Shimoni et al., 2014). On the functionalised surfaces, only cells that had not interacted with another cell for at least 30 min prior to division were selected for analysis. This enabled the isolation of the effect of the immobilised protein on ACD. To compare the distribution of PR values between the different cell culture platforms, outliers (which were identified by their high PR diffuse values and consisted of less than 10% of dividing cells) were removed (Charnley and Russell, 2017) and the data was re-plotted as violin plots using the kernel density function.

To assess the role of the orientation of the polarity cue, 5 µm silicon dioxide beads were coated with PA or PA plus Fc-DL1 using the same protocol outlined for the 2D substrates. The orientation of division relative to the polarity cue was determined for dividing DNA3a cells in contact with functionalised beads and OP9-DL1 stromal cells (Fig. 6B). For DNA3a cells in contact with OP9-DL1 stromal cells, the division angle was determined by measuring the angle between the interface between the DNA3a cell and OP9-DL1 stromal cell and the long axis of the dividing cell. For the functionalised beads, a line was drawn between the centre of mass of the bead and the centre of mass of the dividing cell. The division angle was defined as the angle orthogonal to the angle between this line and the long axis of the dividing cell.

**Notch and CXCR4 inhibition protocol**

To disrupt Notch signalling, we used the γ-secretase inhibitor dibenzazepine (DBZ, 0.05 µM) (van Es et al., 2005). DBZ prevents the cleavage of Notch by γ-secretase, and so prevents the translocation of the cytoplasmic portion of Notch to the nucleus and therefore inhibits Notch signalling. To analyse the effect of Notch signalling on proliferation and differentiation, purified DNA3 cells were co-cultured on OP9-DL1 stromal cells in the presence or absence of DBZ (0.05 µM, Calbiochem) and assessed by flow cytometry. To determine the effect of Notch and CXCR4 inhibition on ACD, purified DNA3a cells were cultured on OP9-DL1 stromal cells or Fc-DL1 functionalised surfaces in the presence of AMD3100 (2 µg/ml, Sigma-Aldrich) or DBZ (0.05 µM, Calbiochem) for ~30 mins prior to the start of the time lapse imaging or for 15 h prior to fixing for immunofluorescence analysis.

**Statistical analysis**

All data were collected from two to eight independent experiments. For scatter and bar plots, data is shown as mean±s.e.m., the median value is indicated on the violin plots. The following tests were used for the statistical analysis. Student’s unpaired two-way t-test with unequal variance was used to determine the difference between mean values for the scatter plots and calculated using Microsoft Office Excel. For the PR scatter plots from the time lapse microscopy experiments, paired t-tests were performed in Graphpad Prism between control (diffuse protein) and test protein PR values. The violin plots were analysed for differences in the distribution of the data using two-sample Kolmogorov–Smirnov (KS) test (Young, 1977) in Graphpad Prism software. The level of statistical significance is indicated as *P<0.05, **P<0.01, ***P<0.001.

**Acknowledgements**

We thank Juan Carlos Zúñiga-Pflücker (University of Toronto, Sunnybrook Health Sciences Centre, Toronto, Canada), for OP9 stromal cell lines. This work was performed in part at the Biointerface Engineering Hub at Swinburne, part of the Victorian node of the Australian National Fabrication Facility (ANFF), a company established under the National Collaborative Research Infrastructure Strategy to provide nano- and microfabrication facilities for Australia’s researchers. We thank Louise Cheng (Peter MacCallum Cancer Centre) and Helena Richardson (La Trobe University) for comments on the manuscript.

**Competing interests**

The authors declare no competing or financial interests.

**Author contributions**

Conceptualization: M.C., S.M.R.; Investigation: M.C., M.L.-M., K.P.; Writing - original draft: M.C., S.M.R.; Writing - review & editing: M.C., M.L.-M., K.P., S.M.R.; Funding acquisition: M.C., S.M.R.

**Funding**

We acknowledge support from the Schweizerischer Nationalfonds zur Förderung der Wissenschaftlichen Forschung (Swiss National Science Foundation, SNSF) (grants PA00P3_142120 and P300P3_154664 to M.C.), the Australian Research Council (FT0990405 to S.M.R.) and the National Health and Medical Research Council (APP1099140 to S.M.R.).

**Supplementary information**

Supplementary information available online at http://jcs.biologists.org/lookup/doi/10.1242/jcs.235358#supplemental

**References**

High expression of C-X-C chemokine receptor 4 and Notch1 is predictive of chemokine system.


Directional Delta and Notch trafficking in Sara endosomes during asymmetric cell fate control and signal integration in development.


