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Notch Signalling Regulates Cytokine Production by CD8+ and CD4+ T Cells

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Abstract

The Notch signalling pathway regulates several aspects of cellular differentiation such as T lineage commitment and effector functions on peripheral T cells; however, there is limited information regarding Notch receptor expression on different T cell subsets and the putative role of the different receptors on T cell effector function. Here, we studied the protein expression of Notch receptors on murine T cells *in vitro* and *in vivo* and analysed the role of the Notch pathway in cytokine production by CD4+ and CD8+ T cells. We found that resting CD4+ and CD8+ T cells do not express Notch receptors, but they upregulate Notch 1 and Notch 2 shortly after *in vitro* and *in vivo* activation. Using a γ-secretase inhibitor, which blocks Notch signalling through all Notch receptors, we demonstrated that the Notch pathway regulates IL-10 production by CD4+ T cells and IFN-γ and IL-17 production by CD8+ T cells. These results suggest that Notch 1 and 2 are expressed by CD4+ and CD8+ T cells and represent the putative Notch receptors that regulate effector functions and cytokine production by these cells.

Introduction

The Notch pathway, initially described in *Drosophila*, regulates different aspects of cellular differentiation in multicellular organisms such as lateral inhibition and border formation. In mammals, there are four Notch receptors (Notch 1, 2, 3 and 4) and five different ligands that are divided in two different families: Jagged (Jagged 1 and 2) and Delta-like (Delta-like 1, 3 and 4) [1–3]. The diverse functions of Notch receptors are mediated through a conserved signalling pathway. Upon binding to its ligands, these receptors undergo two proteolytic cleavages that result in the release of the Notch intracellular domain (ICN). This ICN then translocates to the nucleus where it displaces co-repressors, recruits activators and binds to the transcription factor RBP-j, activating the transcription of several genes, including *hes* transcriptional repressors. The cleavage that releases ICN occurs within the transmembrane domain and is mediated by a multiprotein complex with γ-secretase activity containing presenilin, nicastrin, APH-1 and PEN-2 proteins. Some reports have shown that γ-secretase inhibitors (GSI) and the loss of presenilin or nicastrin function prevent Notch signalling [4, 5].

Although rather controversial, new evidence has assigned a role of the Notch pathway to peripheral T cell responses such as CD4+ T cell activation and polarization [3]. For instance, it has been shown that the inhibition of Notch signalling using a GSI decreases IL-2 production, CD25 expression and the proliferation of activated T cells [6]. On the other hand, the first evidence of Notch involvement in T helper differentiation came from the observation that the Notch ligands, Jagged and Delta, are expressed on dendritic cells following Th1 and Th2 polarizing conditions [7, 8]. However, the exact role of Notch on T cell polarization is still up for debate. For example, it has been shown that mice that lack Notch receptor signalling are unable to mount functional Th2 responses, a fact that reveals a role of the Notch pathway in Th2 differentiation [9, 10]. In contrast, other investigators showed that treatment with either a Notch 1 antisense or a GSI blocks the generation of Th1 cells, therefore suggesting that Notch signalling is involved in the generation of Th1 responses as well [11, 12].

On the other hand, Osborne and collaborators revealed that Notch signalling is also crucial on CD8+ effector functions. Using a GSI to block the Notch pathway, they abrogated effector functions such as cytokine production and cytotoxic activity on CD8+ T cells [13, 14].

In contrast, much less is known about the effects of Notch on the maturation and/or activation of DCs [15]. Both Notch receptors and their ligands are expressed on DC, and it has been shown that bacterial products not

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only upregulate the expression of Notch ligands in DC [7, 8] but also can activate Notch signalling in macrophages and DCs [8, 16]. There is little information about the effects of Notch signalling on DC phenotype or function, although one study suggested that Notch stimulation with Jagged 1 could promote DC to mature in a manner similar to that induced by lipopolysaccharide (LPS) [17].

The information regarding the expression of Notch receptors on cells of the immune system is rather scarce, yet it has been revealed that naive CD4+ T cells express both Notch 1 and Notch 2 mRNAs and that, upon activation, they upregulate the mRNA of all Notch receptors [6, 7, 18]. Using monoclonal antibodies, a recent study also showed that both Notch 1 and Notch 2 are upregulated on polyclonally activated T cells [19]. Therefore, much of the conflicting data regarding the role of Notch receptors on T cell differentiation and effector function could be due to a lack of understanding of the expression patterns of Notch receptors on CD8+ and CD4+ T cells. In fact, most studies that analysed the effect of GSI or which required the forced expression of one particular Notch receptor did not consider the physiological expression of the different receptors on T cells and may have given rise to partially correct information regarding the role of the different Notch receptors on the effector functions of T cells.

Here, we studied the protein expression of different Notch receptors on T cells *in vitro* and *in vivo* and analysed the importance of the Notch pathway in the cytokine production by these cells. We demonstrated that the Notch pathway regulates IL-10 production by CD4+ T cells and IFN-γ and IL-17 production by CD8+ T cells. Moreover, we found that CD8+ and CD4+ T cells do not constitutively express Notch receptors, but upregulate Notch 1 and 2 expression following activation. These results suggest an important role for Notch 1 and 2 receptors on the cytokine production and effector function of CD4+ and CD8+ T cells.

Materials and methods

Mice. The C57BL/6 mice were purchased from the Jackson Laboratories (Bar Harbor, ME, USA), and OT-I Rag−/− and OT-II Rag−/− mice were purchased from Taconic (Hudson, NY, USA). All mice were housed under pathogen-free conditions and were used at 8–16 weeks of age. The animal work was carried out under the institutional regulations of Fundacion Ciencia para la Vida and Facultad de Ciencias, Universidad de Chile, and was locally approved by an ethical review committee.

Antibodies and reagents. The following antibodies were purchased from BD Biosciences (San Jose, CA): anti-CD4 PECy5 (RM 4-5), anti-CD8α FITC (53-6.7), Vα2 TCR PE (B20.1), Vβ5.1, 5.2 TCR FITC (MR9-4), anti-CD25 PE (7D4), anti-CD69 PE (H1.2F3), anti-IAb FITC (25-9-17), anti-IFN-γ PE (XMG1.2), anti-IL-17 PE (TC11-18H10) and anti-IL-10 APC (JES5-16E3). The following antibodies were bought from eBioscience (San Diego, CA, USA): anti-CD40 APC (1C10), anti-CD80 FITC (16-10A1), anti CD83 APC (Michel-17), anti-CD86 PE (GL-1), anti-IFN-γ Alexa Fluor 647 (XMG1.2) and anti-CD16/32 (93). Anti-Notch 1 PE (HMN1-12), anti-Notch 2 PE (HMN2-35), anti-Notch 3 PE (HMN3-133), anti-Notch 4 PE (HMN4-14) and their isotype control, Armenian hamster IgG PE (HTK888), were bought from BioLegend (San Diego, CA, USA). The GSI, N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT) (γ-secretase inhibitor IX 565770), was bought from Calbiochem (San Diego, CA, USA). Lipopolysaccharide from *Salmonella typhosa* was bought from Sigma-Aldrich (St. Louis, MO, USA).

Isolation and activation of splenic dendritic cells. Splenic dendritic cells from C57BL/6 mice were isolated by mechanical disaggregation of the spleen and tissue digestion with collagenase D (1 mg/ml) (Roche, Mannheim, Germany) in the presence of DNAse I (20 μg/ml) (Roche) for 45 min at 37 °C. The cells were then filtered through a 90-μm mesh filter, and the erythrocytes were lysed using a hypotonic lysis buffer. Finally, the DCs were positively selected using CD11c microbeads from Miltenyi Biotec (Bergisch Gladbach, Germany).

To evaluate the effect of the GSI on DC function, in some experiments, splenic DC was activated with LPS (100 ng/ml) for 24 h in the presence of 5 μM DAPT or the vehicle (DMSO). The cells were then analysed by flow cytometry to asses the expression of co-stimulatory molecules, and the supernatants were collected and analysed using the mouse inflammatory cytometric bead array (CBA) (BD Biosciences), following the manufacturer's instructions.

Isolation of CD4+ and CD8+ T cells and co-culture with splenic DCs. Splenic CD4+ T cells from OT-II mice and CD8+ T cells from OT-I mice were isolated by positive selection using anti-CD4 and anti-CD8 microbeads, respectively (Miltenyi Biotec). Splenic DCs and OT-I CD8+ T cells were co-cultured at a 1:5 ratio in the presence of 2 nM of the ovalbumin (OVA) peptide $_{257–264}$ (New England Peptide, Gardner, MA, USA) for 3 days. Splenic DCs and OT-II CD4+ T cells were co-cultured at a 1:5 ratio in the presence of 5 μM of the OVA peptide $_{323–339}$ (New England Peptide) for 4 days. In some of the experiments, prior to the co-culture with DCs, the T cells were labelled with 5 μM carboxyfluorescein succinimidyl ester (CFSE) (Molecular Probes, Eugene, OR) to analyse proliferation. To test the effect of Notch signalling inhibition during T cell activation, some co-culture experiments were carried out in the presence of 5 or 10 μM DAPT (Calbiochem).

Flow cytometry and intracellular cytokine staining. To analyse the expression of different Notch receptors and

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other cell-surface markers, the cells were incubated for 20 min at 4 °C with anti-CD16/32, washed with PBS + 2% FBS and then incubated for another 20 min with a different combination of antibodies. The cells were washed again with PBS + 2% FBS and then analysed on a FACSCanto II flow cytometer (BD Biosciences).

To determine intracellular cytokine production on T cells activated with splenic dendritic cells in the presence of DAPT (Calbiochem), following co-culture, the T cells were reactivated with PMA (0.25 μM) (Sigma-Aldrich) and ionomycin (1 μg/ml) (Sigma-Aldrich) in the presence of 1 μl of GolgiPlug (BD Biosciences) for 4 h. The cells were permeabilized and fixed with the Cytofix/Cytoperm Kit (BD Biosciences) and stained with anti-IL-10, anti-IL-17 and anti-IFN-γ antibodies.

Adoptive transfer experiments. Splenic CD4+ T cells from OT-II mice and CD8+ T cells from OT-I mice were isolated by positive selection using anti-CD4 and anti-CD8 microbeads, respectively (Miltenyi Biotec). The cells were labelled with 10 μM CFSE (Molecular Probes) and adoptively transferred to C57BL/6 recipient mice. Then, 24 h later, the mice were immunized with 1 mg/mouse of OVA protein (Sigma-Aldrich) in complete Freund's adjuvant (Sigma-Aldrich), and 3 days later, the peripheral lymph nodes (PLN) were harvested. The cells were isolated by mechanical disaggregation, and the cellular suspension was filtered through a 90-μm mesh filter. Erythrocytes were lysed using a hypotonic lysis buffer. The cells were then stained following the protocol for cytometric labelling, as shown earlier.

Cytokine secretion analysis. To determine cytokine secretion by T cells activated with splenic dendritic cells in the presence of DAPT, T cells obtained from the co-culture were reactivated with PMA (0.25 μM) (Sigma-Aldrich) and ionomycin (1 μg/ml) (Sigma-Aldrich) for 4 h. Following the reactivation, the supernatants were recovered and stored at −80 °C until further analysis. To analyse cytokine secretion during the co-culture of OT-I cells with dendritic cells, supernatants of the co-culture were obtained at 24, 48 and 72 h and stored at −80 °C until further analysis. The supernatants were analysed using the mouse Th1/Th2/Th17 CBA (BD Biosciences), following the manufacturer's instructions.

Statistical analysis. The data are presented as the mean ± SEM and were analysed using the Student's t-test or one-way ANOVA with a Bonferroni post-test. The statistical analyses were performed using Prism 4 (GraphPad Software Inc., La Jolla, CA, USA).

Results

Notch signalling regulates cytokine production by CD4+ and CD8+ T cells

Owing to the existence of conflicting data regarding the role of the Notch pathway on peripheral T cell responses, we first studied the role of Notch signalling on IFN-γ and IL-10 production by CD4+ and CD8+ T cells, two cytokines that have been previously studied as possible Notch targets on CD4+ T cells [14, 20]. For this, we cultured splenic dendritic cells (DCs) with OT-I or OT-II cells for 3 or 4 days in the presence of the OVA peptide and the γ-secretase inhibitor DAPT. After the co-culture, the T cells were re-stimulated with PMA plus ionomycin in the presence of GolgiPlug (brefeldin A) to study intracellular cytokine production. As shown in Fig. 1A, the presence of DAPT during the co-cultures slightly reduced IFN-γ production but significantly reduced IL-10 production by CD4+ T cells. Although we could not detect IL-10 production by CD8+ T cells (data not shown), we found a significant reduction in the number of CD8+ T cells that produced IFN-γ when these cells were co-cultured in the presence of DAPT (Fig. 1A).

Next, we analysed the secretion of other cytokines by CD8+ T cells co-cultured with DCs in the presence of DAPT for 3 days and then re-stimulated with PMA plus ionomycin. As shown in Fig. 1B, TNF and IL-2 production was not affected by DAPT treatment; however, in agreement with our previous finding, we observed a significant reduction in IFN-γ secretion by CD8+ T cells. Surprisingly, we detected considerable amounts of IL-17 in the supernatant of control CD8+ T cells (most likely due to the presence of Tc17 cells, which consist of IL-17-secreting CD8+ T cells), and the production of this cytokine was reduced when these cells had been previously co-cultured with DCs in the presence of DAPT (Fig. 1B). This novel finding suggests that Notch signalling not only participates in IFN-γ production by CD8+ T cells but is also important for the production of other cytokines such as IL-17.

To discard a possible contamination of Th17 CD4+ T cells, which may have accounted for the presence of IL-17 in the supernatant, we performed intracellular cytokine staining for IL-17 production on CD8+ T cells re-activated with PMA plus ionomycin after the co-culture with DCs. As shown in Fig. 1C, only approximately 1% of the CD8+ T cells produced IL-17, which probably represents a population of Tc17 cells that were present during our co-cultures. An important number of these CD8+ IL-17+ cells also produced IFN-γ. Notably, the production of both cytokines, IL-17 and IFN-γ, was reduced when CD8+ T cells were co-cultured with DCs in the presence of DAPT (Fig. 1C). Furthermore, we could not detect CD4+ T cells in these co-cultures (data not shown), confirming the absence of contaminating Th17 cells in the co-culture.

To understand whether or not the reduction in IL-17 production observed in the GSI-treated CD8+ T cells was because of a block on the differentiation of Tc17 cells or because of an inhibition of IL-17 production by Tc17 cells, we analysed early IL-6 production during the

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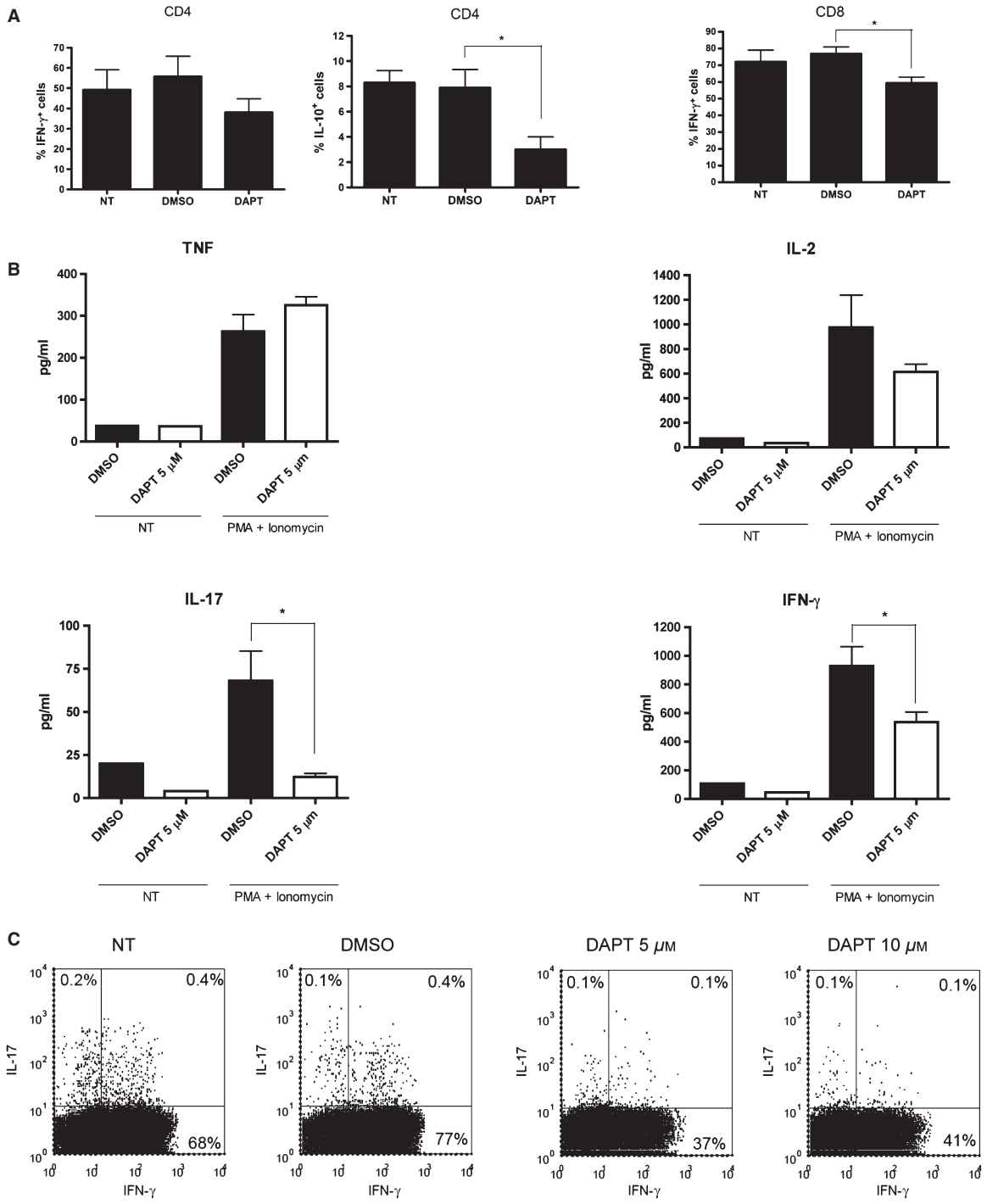


Figure 1 Notch signalling is necessary for IFN-γ and IL-17 production by CD8+ T cells and IL-10 production by CD4+ T cells. OT-I and OT-II cells were cultured with splenic dendritic cells and the ovalbumin (OVA) peptide in the presence of 5 μM DAPT or DMSO as the control vehicle. After 3 or 4 days of culture, the OT-I and OT-II cells were recovered and re-stimulated with PMA, ionomycin and brefeldin A to study intracellular cytokine production by CD4+ and CD8+ T cells (A). OT-I cells were cultured with splenic dendritic cells and the OVA peptide in the presence of 5 μM DAPT or DMSO as the control vehicle. After 4 days of culture, OT-I cells were recovered and re-stimulated with PMA and ionomycin to determine cytokine secretion by cytometric bead array (B). OT-I cells were co-cultured with splenic dendritic cells and the OVA peptide in the presence of 5 μM DAPT, 10 μM DAPT or DMSO as the control vehicle for 72 h. After co-culture, the cells were recovered and re-stimulated with PMA and ionomycin in the presence of brefeldin A to analyse IL-17 and IFN-γ production on CD8+ T cells through intracellular cytokine staining. The dot plots present IL-17 and IFN-γ production in a gate of CD8+ T cells (C). The data in (A) and (B) represent the mean ± SEM of three independent experiments; $*P < 0.05$.

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co-culture of CD8+ T cells with DCs. As shown in Fig. 2, DAPT only slightly reduced IL-6 production during the co-culture, but blocked IL-17 production, suggesting that Notch signalling is not directly affecting Tc17 differentiation, but it might be critical for the production of IL-17 by CD8+ T cells.

In agreement with our previous findings, we observed no difference in IL-2 or TNF production in the presence of the GSI (Fig. 2). The differences in IL-17 production were not because of a variation in the number of cells during the co-culture, because the number of cells that were recovered after 3 days of culture in the presence of DAPT was very similar to the number in the presence of the control vehicle DMSO (data not shown).

Next, we analysed the effect of the GSI on other parameters such as proliferation and activation markers on CD4+ and CD8+ T cells. As shown in Fig. 3A, the addition of DAPT to the co-cultures of T cells and DCs did not affect activation markers such as CD25 or CD69 on T cells, and only slightly delayed the proliferation of CD4+ and CD8+ T cells, as assessed by CFSE dilution (Fig. 3B–C). We also evaluated whether or not the Notch pathway is involved in the cytotoxic function of CD8+ T cells, and we observed that the addition of DAPT to the co-cultures did not affect granzyme B or perforin production by CD8+ T cells (data not shown). All together, these results show that Notch signalling regulates cytokine production by CD4+ and CD8+ T cells without affecting the activation status of these cells.

To asses whether the effect we observed on cytokine production was because of an effect of the GSI on DC function during the co-cultures, we activated splenic DC with LPS in the presence of DAPT and evaluated cytokine production and co-stimulatory molecule expression on this population. We observed no differences in TNF, MCP-1, IL-10 and IL-6 production or CD80, CD86, CD83, IAb and CD40 expression on DCs treated with DAPT compared with DMSO (Figure S1).

CD4+ and CD8+ T cells express Notch 1 and Notch 2 upon activation

To understand which Notch receptor is responsible for the cytokine production by CD8+ and CD4+ T cells, we analysed Notch receptor expression on different T cell populations. For this, we analysed the expression of Notch 1, 2, 3 and 4 on OT-II and OT-I T cells activated *in vitro* with DCs in the presence of the OVA peptide at different time points after the co-culture. Our results indicate that resting CD4+ and CD8+ T cells do not express detectable levels in any of these receptors; however, when the OT-II and OT-I T cells were activated with splenic DCs, they both strongly upregulated Notch 1 expression following 48 h of co-culture (Fig. 4A and Figure S2). Notch 2 expression was also upregulated on CD4+ and CD8+ T cells, but not as dramatically as Notch 1 (Fig. 4A and Figure S2). The other Notch receptors, Notch 3 and Notch 4, were not upregulated at

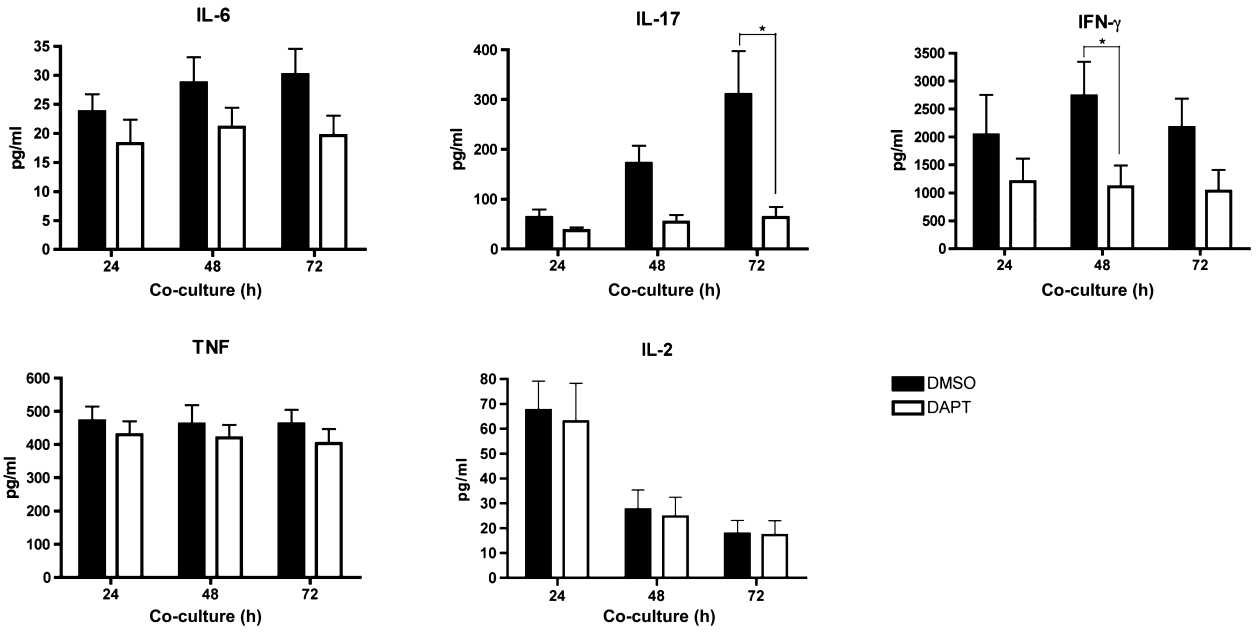


Figure 2 Notch signalling is necessary for IL-17 production by Tc17 cells. OT-I cells were co-cultured with splenic dendritic cells and the ovalbumin peptide in the presence of 5 μM DAPT or DMSO as the control vehicle, and the supernatant of the co-culture was analysed at 24, 48 and 72 h for cytokine production by cytometric bead array. These data represent the mean ± SEM of at least three independent experiments; *$P < 0.05$.

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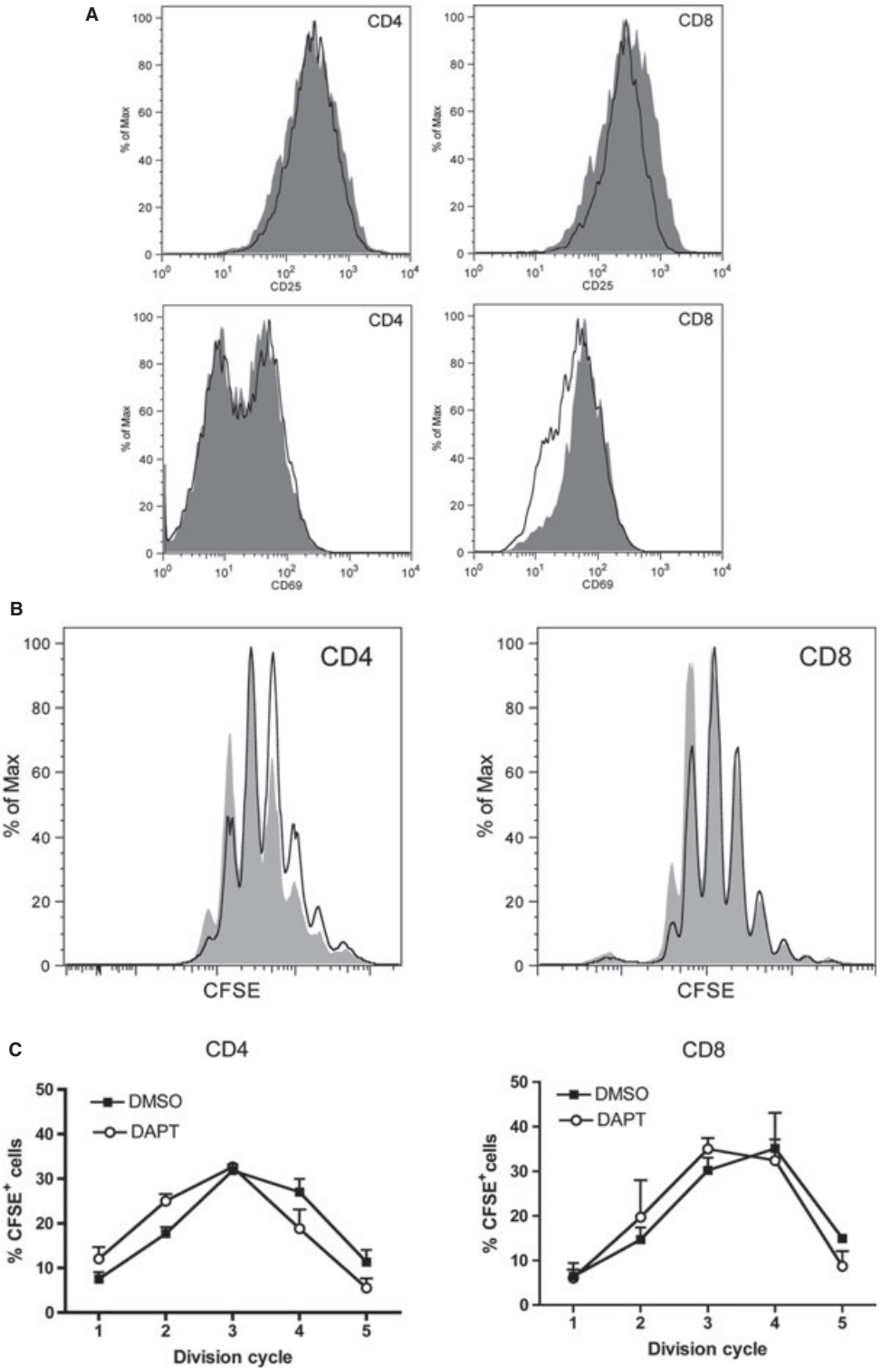


Figure 3 Notch signalling is not necessary for activation marker expression or the proliferation of CD4+ and CD8+ T cells *in vitro*. CFSE-labelled OT-I and OT-II cells were co-cultured with splenic dendritic cells and the ovalbumin peptide in the presence of 5 μM DAPT (empty histogram) or DMSO (filled histogram) as the control vehicle. Four days later, CD4+ (upper panel) and CD8+ T cells (lower panel) were analysed for the expression of activation markers (A) or were evaluated for their capacity to proliferate through CFSE dilution (B). The percentage of cells in each division cycle was calculated based on CFSE dilution (C). These data are representative of three independent experiments.

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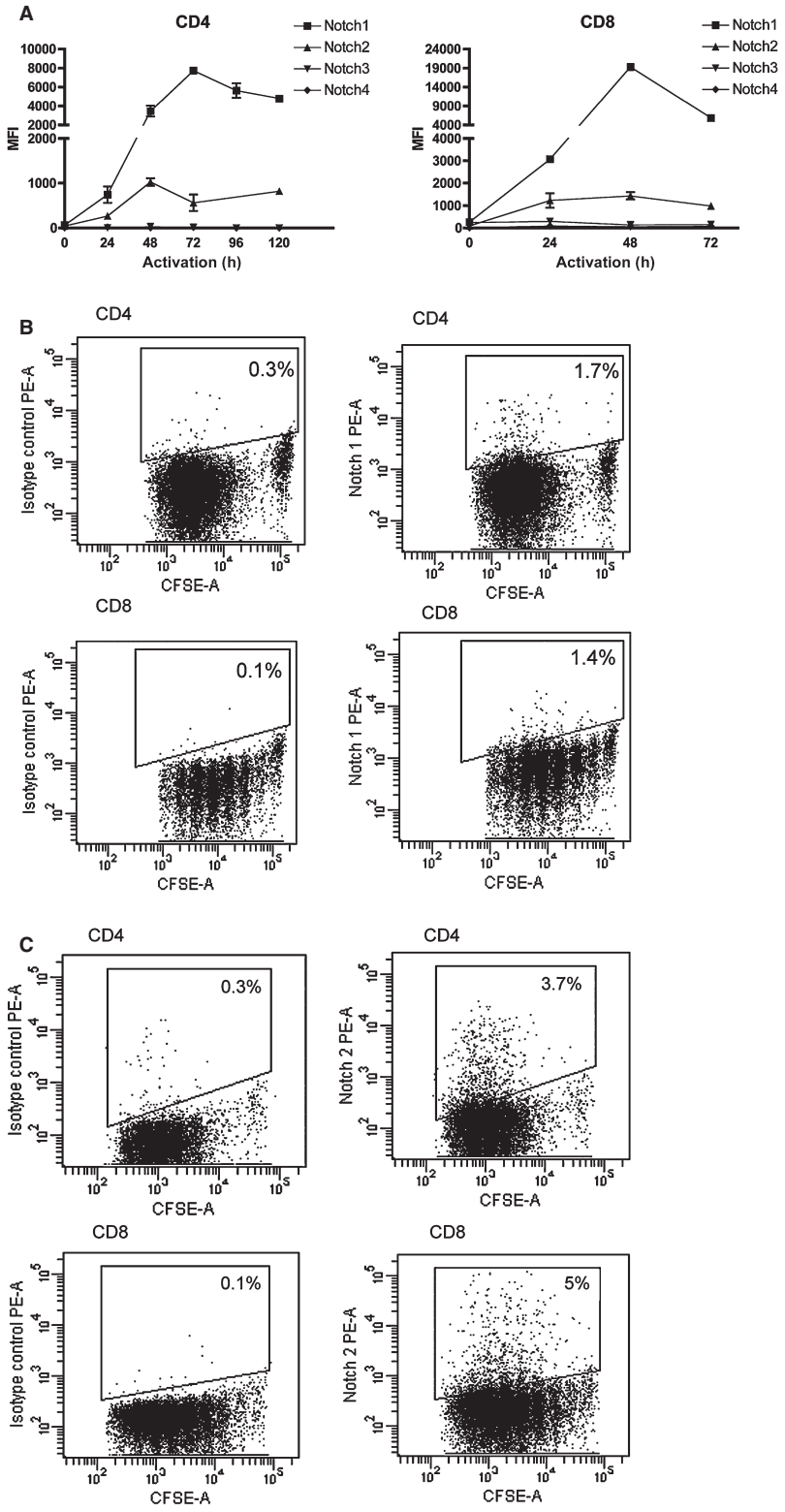


Figure 4 Notch 1 and Notch 2 are expressed on activated CD4+ and CD8+ T cells. OT-II and OT-I T cells were co-cultured with splenic dendritic cells in the presence of the ovalbumin (OVA) peptide, and Notch receptor expression on T cells [mean fluorescence intensity (MFI)] was analysed at different time points on CD4+ and CD8+ cells by flow cytometry (A). OT-II and OT-I cells were labelled with CFSE and adoptively transferred to C57BL/6 mice. The mice were then immunized with OVA protein (1 mg/mouse), and 72 h later, Notch 1 (B) and Notch 2 (C) expression on the transferred cells was analysed by flow cytometry. The data in (A) represent the mean ± SEM of three independent experiments. The data in (B) and (C) are representative of three independent experiments.

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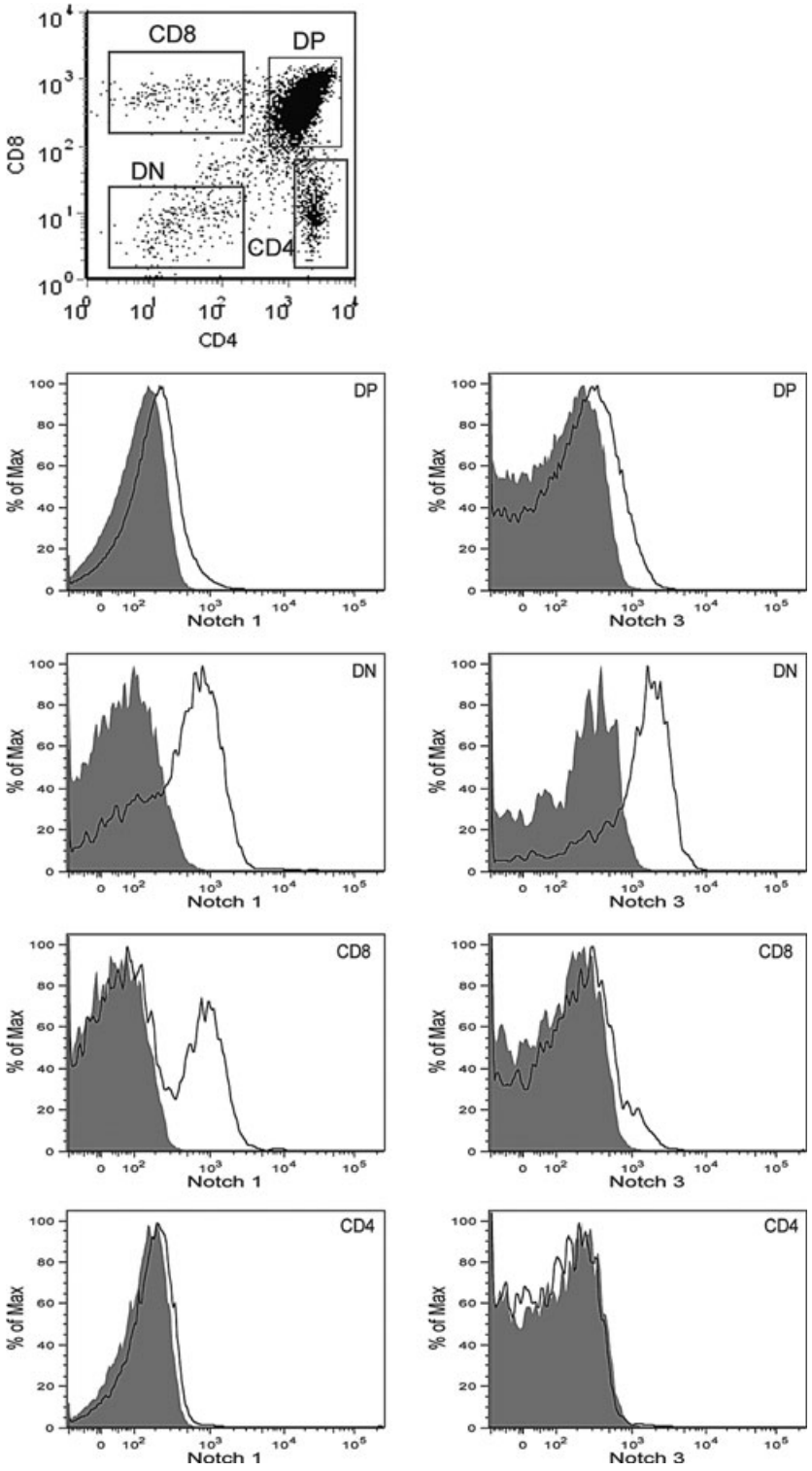


Figure 5 Notch 1 and 3 are expressed by different thymic subpopulations. Different subpopulations of thymocytes from C57BL/6 mice were analysed for the expression of Notch receptors by flow cytometry. DP, CD4/CD8 double-positive thymocytes; DN, CD4/CD8 double-negative thymocytes. The shaded histogram represents the staining with each isotype control. This figure is representative of three independent experiments.

all during the co-culture (Fig. 4A). This finding suggests that Notch 1 and possibly Notch 2 may be important for CD4+ and CD8+ T cell function.

Next, we tested whether or not Notch 1 and Notch 2 are also upregulated *in vivo* on T cells. For this, CD4+ T cells from OT-II mice and CD8+ T cells from OT-I

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mice were isolated, labelled with CFSE and adoptively transferred to C57BL/6 mice. The mice were then immunized with the OVA protein; 24 h later, the peripheral lymph nodes were removed, and Notch expression was analysed on adoptively transferred cells. As shown in Fig. 4B–C, Notch 1 was expressed by a small population (around 1%) of CD4+ and CD8+ T cells, while Notch 2 was expressed by 4–5% of CD4+ and CD8+ T cells. We also observed a shift in the mean fluorescence intensity (MFI) on the bulk population of CD4+ and CD8+ T cells stained with Notch 1 and Notch 2 antibodies versus their isotype control (402 ± 40 versus 216 ± 15 for Notch 1 on CD4+ T cells; 127 ± 23 versus 7 ± 4 for Notch 2 on CD4+ T cells; 569 ± 238 versus 237 ± 85 for Notch 1 on CD8+ T cells and 317 ± 68 versus 101 ± 60 for Notch 2 on CD8+ T cells). These data confirm the results obtained for the activation of CD4+ T and CD8+ T cells *in vitro* and propose a role of Notch 1 and Notch 2 receptors on the effector function of T cells.

Notch 1 and Notch 3 are expressed on different subpopulations in the thymus

Finally, we analysed Notch receptor expression in the different cellular populations in the thymus of C57BL/6 mice. We found that Notch 2 and Notch 4 were not expressed in the thymus; however, we detected Notch 1 expression on double-negative (DN) and single-positive CD8 T cells (Fig. 5), which is in accordance with previous findings by the group of Fiorini [19]. We also detected Notch 3 expression on the CD4/CD8 double-negative population (Fig. 5). This population presented high levels of CD25 expression, intermediate expression of CD44 and no CD3 expression and therefore probably corresponded to the DN2 or DN3 subpopulation (data not shown).

Discussion

Notch proteins influence cell-fate decisions in many developmental systems; however, new evidence suggests that the Notch pathway is gaining recognition as a key regulator of peripheral T cell responses such as T cell polarization and T cell effector functions. In this regard, previous data suggest a role of the Notch signalling pathway on the generation of Th1 and Th2 responses. For instance, it has been shown that mice that lack signalling through all Notch receptors are unable to mount functional Th2 responses, which demonstrates a role of the Notch pathway on Th2 differentiation [9, 10]. On the other hand, other investigators have demonstrated that treatment with either a Notch 1 antisense or a γ-secretase inhibitor blocks the generation of Th1 responses [11, 12]. Much of the conflicting data regarding the role of Notch receptors on T cell differentiation and effector function could be due to a lack of understanding of the expression patterns of Notch receptors on different T cell subsets.

Our data reveal that Notch receptors present a distinctive expression pattern on diverse subpopulations of T cells *in vitro* and *in vivo.* Here, we report that resting CD4+ and CD8+ T cells do not express detectable levels of Notch receptors but strongly upregulate Notch 1 and Notch 2 upon *in vitro* and *in vivo* activation. Although our *in vitro* studies of T cell activation revealed that all activated CD4+ and CD8+ T cells express Notch 1 and Notch 2 receptors, our *in vivo* experiments showed that only a small population of CD4+ and CD8+ T cells upregulate Notch expression. However, in our *in vivo* studies, we observed a shift in the MFI using Notch 1 and Notch 2 antibodies in the bulk population of CD4+ and CD8+ T cells compared with the isotype control. These low levels of Notch 1 and Notch 2 expression observed in our *in vivo* assays could be functionally relevant for T cell effector responses. Further studies should be designed to gain an understanding of the phenotype and properties of this subpopulation of T cells that upregulate Notch receptor expression upon activation. Although we could not detect Notch 3 or Notch 4 receptor expression on peripheral T cells, this fact does not discard the possibility that, although expressed at low levels on the cell surface (below our detection threshold), they may still be relevant on peripheral T cell function. However, as the Notch 1 and Notch 2 receptors are the only Notch receptors highly expressed during T cell activation, we propose that Notch signalling on CD4+ and CD8+ T cells is probably mainly mediated by these receptors.

The data presented herein raise the possibility that both Notch 1 and Notch 2 receptors may have non-redundant functions on CD4+ T cells. This idea is supported by published results showing a major effect on T cell function in a setting of the combined genetic ablation of both Notch 1 and Notch 2 (but not each one alone) in CD4+ T cells [21, 22]. Additionally, the fact that Notch 2 may be important for CD8+ T cell function is supported by previous results, which revealed that a Notch 2 deficiency altered effector function on CD8+ T cells [23].

When analysing the role of the Notch pathway on cytokine production by CD8+ T cells, and in agreement with previous findings [14], we found that this pathway regulates IFN-γ production by these cells. We also noticed that other cytokines such as TNF and IL-2 were not affected by treatment with the GSI. This leads us to propose that the pharmacological inhibition of the Notch pathway does not affect the production of all cytokines but that it selectively blocks the production of certain cytokines by T cells.

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Surprisingly, in our system, after the co-culture of naive CD8+ T cells with DCs, we also detected a small Tc17 population that produced measurable amounts of IL-17. Using the GSI, we observed a reduction in IL-17 production in the co-culture, which strongly suggests that Notch signalling is important for IL-17 production by CD8+ T cells *in vitro*. Previous evidence for a role of the Notch pathway on IL-17 production came from studies on CD4+ T cells, where the groups of Lukacs and Osborne demonstrated that *Rorc* and *IL17* gene promoters are direct transcriptional Notch targets [24, 25]. As some reports have described Notch signalling in DCs [17, 26, 27], we analysed cytokine production by LPS-activated DCs in the presence of DAPT. Although we could not detect any differences on IL-6 production by LPS-activated DCs, we cannot discard an effect of the GSI on IL-6 production by DCs during their interaction with T cells. Further studies should be conducted to demonstrate whether or not Notch signalling is necessary for IL-6 production by T cells, dendritic cells or both populations during DC–T cell co-cultures.

We also showed that the treatment with GSI reduced IFN-γ production by CD4+ T cells. This observation is in agreement with the results of previous studies [12] and indicates that signalling through the Notch 2 receptor may be necessary to generate Th1 responses. In spite of this, to demonstrate a role of Notch receptors on Th1 responses, it would be crucial in our system to determine whether or not other cytokines related to Th2 responses, such as IL-4, are also reduced in the presence of the GSI.

Using the GSI, we demonstrated that Notch signalling is involved in IL-10 production by CD4+ T cells. One previous study addressed the role of Notch signalling in IL-10 production by Th1 polarized cells and showed that the transfection of T cells with constitutively active forms of all Notch receptors increased the percentage of IL-10-producing Th1 cells [20]. It has been suggested that IL-10 production by Th1 cells may be critical in the protection of Th1-induced pathology. Therefore, all of these data support the idea that Notch signalling may be involved in the induction of Th1 responses but also in the counteracting mechanisms developed to avoid uncontrolled Th1 responses, such as the production of anti-inflammatory cytokines like IL-10.

In contrast to a previous report [6], we did not observe a significant delay in T cell proliferation or a reduction in activation markers on T cells activated in the presence of the GSI. These differences may be explained by the use of different GSIs (we used DAPT versus compound E used by Pear and collaborators). Moreover, Pear *et al.* reported that the effect of compound E on T cell proliferation and the activation markers was absolutely dependent on the concentration of the OVA peptide used, where a major effect was achieved at low concentrations of the peptide and, therefore, low levels of stimulation. In our report, we used high concentrations of the OVA peptide and obtained high levels of activation, and for this reason, a reduction in activation markers or T cell proliferation may not necessarily occur. This high level of T cell stimulation may also preclude us from observing an effect of the GSI on granzyme B and perforin expression on CD8+ T cells, as described by Osborne and collaborators [13].

When analysing Notch receptor expression on thymocytes, we discovered that a subpopulation of double-negative cells in the thymus expressed Notch 3. These observations contrast with data obtained by MacDonald and collaborators [19], where they produced a monoclonal antibody against Notch 2 to analyse different thymic subpopulations and found that Notch 2 was expressed at intermediate levels on common lymphoid precursors and CD117+ early intrathymic subsets, but completely disappeared at subsequent stages of T cell development. Although they tested this antibody for cross-reaction with Notch 1 and Notch 2 proteins, they did not test a cross-reaction with other members of the Notch family such as Notch 3. On the other hand, recent reports using mRNA and protein expression on different thymic subpopulations demonstrated that Notch 1 and Notch 3 are expressed on double-negative thymocytes, while Notch 2 and Notch 4 expression could not be detected at any stage during the lymphopoietic process [28]. Further studies of Notch receptor expression on double-negative thymic subpopulations using more specific markers such as CD117 are needed to solve these discrepancies. However, the fact that DN2-DN3 thymocytes express the Notch 3 receptor supports the idea that Notch receptors may have a role on TCR selection, because TCR recombination occurs during the DN3 stage [29].

Although the present study suggests a role for Notch in T cell cytokine production, all of our loss-of-function experiments were based on the pharmacological inhibition of the Notch pathway using a γ-secretase inhibitor. The γ-secretase mediates signalling from and the degradation of type-I transmembrane proteins such as CD44, N-cadherin, and Notch ligands Jagged and Delta [30]; thus by using a GSI, we could be inhibiting the proteolitic cleavage of several targets not related to Notch signalling. Therefore, although our results suggest that Notch signalling may be regulating cytokine production by T cells, we cannot rule out that this is because of the inhibition of the proteolytic cleavage of other gamma secretase targets not related to Notch signalling. Further studies should clarify the relevance of these GSI off-targets on cytokine production by T cells. Furthermore, the use of GSI in DC–T cell co-cultures could lead to non-cell autonomous effects because DCs also express Notch receptors. For this reason, we tested the effect of DAPT on DC effector functions such as cytokine production and co-stimulatory molecule expression. Our results show that none of the effector functions tested on DCs were

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affected by our GSI, suggesting that the GSI is mainly targeting Notch signalling on T cells in our system.

In summary, the data presented herein lead us to propose that both Notch 1 and Notch 2 receptors, expressed by activated CD4+ and CD8+ T cells, may regulate important effector functions such as cytokine production on T cells.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Figure S1 DAPT does not affect effector functions of dendritic cells. Splenic dendritic cells were activated with LPS for 24 h in the presence of 5 μM DAPT or the vehicle DMSO. Supernatants were collected and analysed by cytometric bead array (A), and dendritic cells were analysed by flow cytometry to determine co-stimulatory molecule expression (B). Grey shaded histogram: not treated DCs, black empty histogram: activated DCs plus DMSO, grey empty histogram: activated DCs plus DAPT. The data in (A) represent the mean ± SEM of three independent experiments. The data in (B) is representative of three independent experiments.

Figure S2 Notch 1 and Notch 2 receptors are upregulated on CD4+ and CD8+ T cells *in vitro*. OT-II (A) and

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OT-I (B) T cells were co-cultured with splenic dendritic cells in the presence of the OVA peptide, and Notch receptor expression on T cells was analysed at different time points on CD4+ and CD8+ cells by flow cytometry. The data in (A) and (B) are representative of three independent experiments.

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