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Differential Regulation of Notch Ligands in Dendritic Cells upon Interaction with T Helper Cells

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Abstract

The Notch signalling pathway has recently been linked to T helper 1 (Th1)/T helper 2 (Th2) cell polarization via a mechanism involving differential expression of Notch ligands, Delta-like and Jagged, in antigen-presenting cells. However, whether stimuli other than pathogen-derived factors are involved in the regulation of Notch ligand expression in dendritic cells (DCs) remains unknown. Here, we address the effect of T helper cells (Th1 and Th2) on Delta-like 4 and Jagged 2 expression in bone marrow–derived DCs. We demonstrate that both Th1 and Th2 cells induce Delta-like 4 mRNA expression in DCs, in a process that is, in part, mediated by CD40 signalling. In contrast, only Th2 cells induce a significant increase in Jagged 2 mRNA levels in DCs. Additionally, we show that IL-4, a hallmark Th2 cytokine, plays a role in Jagged 2 expression, as evidenced by the fact that cholera toxin, a Th2-promoting stimulus, induces Jagged 2 mRNA expression in DCs only in the presence of IL-4. Finally, we demonstrate that DCs also express Notch 1 and that this expression is downregulated by IL-4. These data suggest that Notch ligands are differentially regulated in DCs: Delta-like 4 is regulated by T helper cells and by pathogen-derived Th1 stimuli, whereas Jagged 2 is regulated by Th2 cells and pathogen-derived Th2-promoting stimuli. Based on our results, we propose that the positive feedback loop that Th2 cells exert on T cell polarization may involve the induction of Jagged 2 expression in DCs.

Introduction

Notch signalling is an evolutionarily conserved signal transduction pathway, which controls diverse aspects of development and tissue homoeostasis. In mammals, there are four different Notch receptors (Notch 1–4) and five Notch ligands (Jagged 1 and 2, Delta-like 1, 3 and 4). Receptor–ligand interactions initiate two successive cleavages in Notch, resulting in the release of its intracellular domain (ICN). Following this cleavage, the ICN domain translocates to the nucleus, where it binds to the transcription factor CSL/RBP-j and MAML proteins. In the absence of ICN, CSL/RBP-j binds co-repressors, inhibiting the transcription of several genes. ICN displaces these co-repressors, recruits co-activators and activates the transcription of many target genes, including *hes-1* transcriptional repressors. Importantly, ICN has also been shown to regulate the expression of proteins involved in T cell activation and function, such as NF-κB, T-bet, IFN-γ, IL-10 and GATA3 [1–4].

The most well-characterized Notch function in the immune system is its role in lymphopoiesis, where Notch is required for T cell commitment from a multipotent progenitor [5, 6]. In support of this, Notch 1-deficient mice present a blockage of T cell development and B lymphoid precursor differentiation [7], and CSL/RBP-j-deficient mice present an elevated number of B cells in the thymus [8].

In addition to this involvement in lymphopoiesis, Notch also plays important roles in determining several aspects of peripheral T cell responses [9–13]. It has been demonstrated that after activation, T cells increase the expression of all four Notch receptors [10, 14]. Furthermore, inhibition of Notch signalling reduces T cell proliferation as well as cytokine production and CD25 expression [4, 9, 15].

Many studies implicate Notch signalling in T helper 1 and T helper 2 (Th1 and Th2) differentiation [16]. T cells derived from CSL/RBP-j-deficient mice (which cannot signal through any Notch receptor) produce increased IFN-γ and decreased IL-4 levels compared with wild-type mice [10, 17], suggesting that Notch may be involved in Th2 responses. In support of this involvement, mice expressing a dominant negative form of MAML, which

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results in the blockage of signalling through all Notch receptors, have reduced GATA3 expression and are not capable of mounting functional Th2 responses [2, 13].

Other studies suggest a role of Notch receptors in Th1 responses. Transgenic expression of antisense Notch 1 constructs or treatment of T cells with a γ-secretase inhibitor, which inhibits Notch signalling, results in reduced ability to generate Th1 responses. Moreover, γ-secretase inhibitor treatment also reduces Tbx21 expression, a transcription factor involved in the generation of Th1 responses [11, 12]. Finally, using Notch 1-deficient mice, Notch receptor was shown to have no effect on polarization of the immune response [18]. Discrepancies between the reports described earlier may be attributable to functional redundancy between different Notch receptors.

Substantial evidence supports the hypothesis that dendritic cells (DCs) shape the development of polarized immune responses in a pathogen-dependent manner [19]. Thus, several studies have focused on the expression of Notch ligands in these cells and how they are regulated. Pathogen-derived Th1-promoting stimuli have been shown to induce Delta-like 4 expression, whereas Th2-promoting stimuli induce Jagged 2 expression in DCs, strongly suggesting that differential expression of these ligands in DCs may affect the generation of Th1 and Th2 responses [10].

In addition to decoding pathogen-derived signals, DCs have recently been proposed to orchestrate the development of the immune response, by integrating signals derived from other immune system cells, such as T, B, NK, NKT and mast cells [20–22]. T cells have been shown to be necessary to temper the early innate response, by reducing cytokine production by NK cells and DCs [23], while memory and polarized T cells have been proposed to influence the outcome of subsequent T effector responses, by delivering information through DCs. Thus, DCs may act as messengers between memory or polarized T cells and naïve $CD4^+$ T cells [24, 25].

Here we show that, in addition to the known stimulatory effects of pathogen-derived signals on the induction of Delta-like 4 and Jagged 2 in DCs, the interaction of these cells with either Th1 or Th2 cells induces a dramatic increase in Delta-like 4 mRNA expression in DCs. Our results show that this upregulation is because, at least in part, of CD40 signalling during DC/T cell contact. Importantly, we demonstrate that Jagged 2 mRNA expression is induced in DCs, both following interaction with Th2 cells and when DCs are activated with Th2-type stimuli in the presence of IL-4. Interestingly, IL-4 not only induced Jagged 2 expression but also blocked Notch signalling in DCs. Combined, these data support the hypothesis that the positive feedback loop in Th2 responses may be mediated by induction of Jagged 2 expression in DCs, while Delta-like 4 may act as a co-stimulatory molecule, as it is upregulated both by pathogen-derived factors and during T helper/DC interactions.

Materials and methods

Mice. C57BL/6 and OT-II mice were housed under pathogen-free conditions and were used at 8–16 weeks of age. All animal work was conducted according to institutional regulations of Fundacion Ciencia para la Vida and Facultad de Ciencias, Universidad de Chile, and was approved by the local ethical review committee.

Reagents and antibodies. The following antibodies and reagents were obtained from BD Biosciences (San Jose, CA, USA): anti-CD11c PE (HL3), anti-CD80 FITC (16-10A1), anti-CD86 FITC (GL1), anti-IAb FITC (25-9-17), anti-CD40 (3/23), anti-CD3 (145-2C11), anti-CD28 (37.51), anti-IL-4 (11B11), anti-IFN-γ (XMG1.2), rmIL-4 and rm-GM-CSF. The anti-CD154 APC antibody (MR1) was obtained from e-Bioscience (San Diego, CA, USA). Recombinant mouse IL-12 was obtained from R&D Systems (Minneapolis, MN, USA). LPS from *Salmonella typhosa*, cholera toxin (CT) from *Vibrio cholera*, prostaglandin E2 (PGE2), PMA and ionomycin were all obtained from Sigma-Aldrich (St Louis, MO, USA). The CpG oligonucleotide (ODN1826) was obtained from InvivoGen (San Diego, CA, USA).

Differentiation of bone marrow–derived dendritic cells (BMDCs). Bone marrow was obtained from the tibias and femurs of C57BL/6 mice (up to 12 weeks old). Erythrocytes were eliminated using a hypotonic lysis buffer, and cells were cultured at 1×10^6 cells/ml in RPMI + 10% FCS in the presence of rmGM-CSF (10 ng/ml). For some experiments, cells were differentiated in the presence of IL-4 (1 ng/ml). On days 2 and 4, 75% of the culture medium was replaced with 1 ml of fresh medium containing rmGM-CSF ± IL-4. On day 6, cells were recovered by pipetting and $CD11c^+$ cells were purified using anti-CD11c microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). Purity of the preparation was checked by fluorescence-activated cell sorting based on CD11c expression.

Activation of BMDCs. Purified BMDCs were activated with anti-CD40 (10 μg/ml) or the following Th1-promoting stimuli: LPS (100 ng/ml) and CpG (10 μg/ml), or Th2-promoting stimuli: CT (1 μg/ml) and PGE2 (1 μM). For some experiments, IL-4 (1 ng/ml) was added during the activation of the DCs.

Generation of Th1 and Th2 cells and co-culture with BMDCs. Th1 and Th2 cells were generated as previously described [12, 26], with minor modifications. Briefly, splenic $CD4^+$ T cells from OT-II mice were isolated by positive selection using anti-CD4 microbeads (Miltenyi Biotec). The cells were incubated at 1×10^6 cells/ml in Iscove's modified Dulbeccos's medium (IMDM) + 10% FBS, with plate bound anti-CD3

(1 μg/ml) and soluble anti-CD28 (1 μg/ml). For generation of Th1 cells, $CD4^+$ T cells were incubated with IL-12 (10 ng/ml) and anti-IL-4 antibodies (10 μg/ml) in IMDM + 10% FBS. For generation of Th2 cells, $CD4^+$ T cells were incubated with IL-4 (20 ng/ml) and anti-IFN-γ antibodies (10 μg/ml). After 3 days of culture, T cell activation was stopped by transferring the T cells to new, uncoated wells. Cells were further incubated in IMDM + 10% FBS for 2 days and then analysed by intracellular staining for IL-4 and IFN-γ production.

BMDC and T helper cells were co-cultured at a ratio of 1:1 in the presence of 5 μM OVA peptide (323–339) for 24 h. BMDCs were then positively selected using CD11c microbeads (Miltenyi Biotec). Jagged 2 and Delta-like 4 mRNA expression in BMDCs was analysed by real-time PCR.

Real-time PCR. RNA was extracted using an RNeasy mini kit from QIAgen (Hilden, Germany), and cDNA was prepared using the Superscript first-strand system for RT-PCR from Invitrogen (Carlsbad, CA, USA). Real-time PCR was performed using a Stratagene MX3000p instrument and the PCR Master Mix, RT2 Real-time SYBR Green/Rox from SuperArray (Frederick, MD, USA). The following primer sequences were used: Delta-like 4 (sense 5′-GCA CCA ACT CCT TCG TCG TC-3′, antisense 5′-GTT TCC TGG CGA AGT CTC TG-3′); Notch 1 (sense 5′-CAG CTT GCA CAA CCA GAC AGA C-3′, antisense 5′-ACG GAG TAC GGC CCA TGT T-3′); Hes 1 (sense 5′-CCG GCA TTC CAA GCT AGA G-3′, antisense 5′-TCA CCT CGT TCA TGC ACT CG-3′); and HPRT (sense 5′-CTC CTC AGA CCG CTT TTT GC-3′, antisense 5′-TAA CCT GGT TCA TCA TCG CTA ATC-3′). Jagged 2 primers were obtained from SuperArray. For an endogenous control, we used HPRT, and relative mRNA expression was calculated using the ΔΔCt method.

Intracellular cytokine staining. T helper cells differentiated in the presence of cytokines, or after co-culture with preconditioned BMDCs, were activated with PMA (0.25 μM) and ionomycin (1 μg/ml) in the presence of 1 μl GolgiPlug (BD Biosciences) for 4 h. Cells were permeabilized and fixed using Cytofix/Cytoperm (BD Biosciences) and stained with anti-IL-4 and anti-IFN-γ antibodies.

Statistical analysis. Data are presented as the mean ± SEM and were analysed using Student's *t*-test or a one-way ANOVA test (coupled with a Bonferroni post-test).

Results

Delta-like 4 mRNA expression is induced in DCs after interaction with T helper cells

Previous studies have established that Th1 cells favour the generation of Th1 responses, while Th2 cells favour the generation of Th2 responses [24, 25]. This observation implies the existence of a positive feedback loop involving DCs as messengers between T cell populations. Based on previous reports implicating Notch signalling in T cell polarization, we decided to investigate whether T helper cells could by themselves modulate Notch ligand expression in DCs. To address this issue, we generated BMDCs from C57BL/6 mice and co-cultured them with Th1 and Th2 cells generated *in vitro* from OT-II mice. Th1 and Th2 cells were routinely subjected to intracellular cytokine staining to analyse IFN-γ and IL-4 production (Fig. 1A). After a short, 24-h co-culture in the presence of OVA peptide, DCs were isolated using CD11c microbeads and analysed for Delta-like 4 and Jagged 2 mRNA expression by real-time PCR. Notably, DCs were found to upregulate Delta-like 4 after co-culture with either Th1 or Th2 cells, but only co-culture with Th2 cells significantly upregulated Jagged 2 mRNA expression in DCs (Fig. 1B). Although some reports state that T cells express Notch ligands, after co-culture with DCs, Th1 and Th2 cells expressed lower levels of Delta-like 4 and Jagged 2 mRNA than non-treated DCs (data not shown). Therefore, the Jagged 2 and Delta-like 4 mRNA induction observed in DCs following co-culture with T helper cells is unlikely to be because of contamination with mRNA from T cells.

Because differences in Delta-like 4 and Jagged 2 mRNA levels in DCs may be attributed to intrinsic differences in the ability of Th1 and Th2 cells to activate DCs, we tested whether Th1 and Th2 cells activate DCs to the same extent. DCs were co-cultured with Th1 or Th2 cells for 24 h in the presence of OVA peptide. As shown in Fig. 1C, following a short, 24-h co-culture with T helper cells, DCs significantly upregulated MHC-II and CD86 expression. Notably, there were no differences in co-stimulatory molecule or MHC-II expression between DCs activated with either Th1 or Th2 cells. Moreover, this upregulation was similar to the induction of MHC-II and CD86 expression observed in DCs activated with an anti-CD40 agonist antibody (Fig. 1D). These results demonstrate that both Th1 and Th2 cells are able to fully activate DCs and that differences observed in Notch ligand expression in DCs following interaction with T helper cells are not because of differences in the capacity of Th1 and Th2 cells to activate DCs.

Next, we tested whether Th1 and Th2 cells in our system are able to bias T cell polarization through a direct effect on DCs. After a short 24-h co-culture with Th1 or Th2 cells, DCs were purified using CD11c microbeads and subjected to a second co-culture with naïve $CD4^+$ T cells for an additional 5 days to analyse T cell polarization. In agreement with previous data, DCs co-cultured for 24 h with Th1 cells induced Th1 responses, whereas DCs co-cultured with Th2 cells induced Th2 responses (Fig. 1E, F).

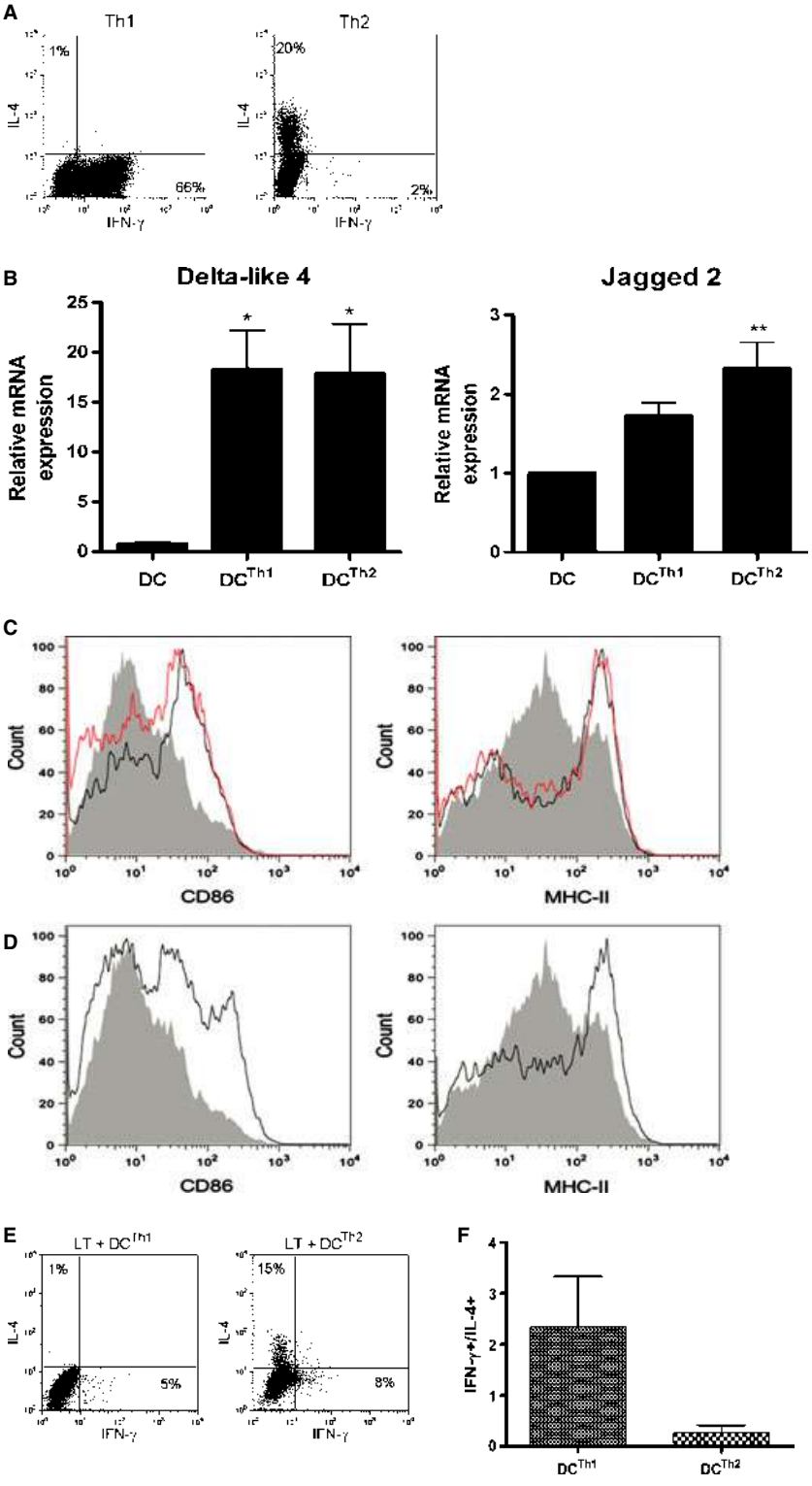

Figure 1 Th1 and Th2 cells induce Delta-like 4 and Jagged 2 expression in dendritic cells (DCs). (A) OT-II Th1 and Th2 cells were generated as described in the Materials and Methods section and subjected to intracellular cytokine staining for IFN-γ and IL-4 production before co-culture with DCs. (B) Bone marrow–derived DCs (BMDCs) from C57BL/6 mice were co-cultured for 24 h with Th1 (DC^{Th1}) or Th2 (DC^{Th2}) cells in the presence of 5 μM of OVA peptide$_{323–339}$. DCs were isolated and analysed by real-time PCR to assess Delta-like 4 and Jagged 2 mRNA expression. (C) MHC-II and CD86 expression was analysed in DCs after 24-h co-culture with Th1 (open black histogram) or Th2 cells (open red histogram) and compared with non-treated DCs (filled grey histogram). (D) MHC-II and CD86 expression was analysed in DCs activated for 24 h with the anti-CD40 antibody (10 μg/ml) (open black histogram) and compared with non-treated DCs (filled grey histogram). (E) BMDCs from C57BL/6 mice were co-cultured for 24 h with Th1 or Th2 cells in the presence of 5 μM of OVA peptide$_{323–339}$. DCs were then purified from the co-cultures and further incubated with naïve $CD4^+$ T cells for 5 days in the presence of OVA peptide$_{323–339}$. The dot plots show IL-4 and IFN-γ production by T cells after co-culture with DCs pre-incubated with Th1 cells (DC^{Th1}) or Th2 cells (DC^{Th2}). (F) The ratio of IFN-γ^+ T cells to IL-4^+ T cells obtained after co-culture with DCs pre-incubated with Th1 cells (DC^{Th1}) or Th2 cells (DC^{Th2}) is shown. Data shown represent the average of three independent experiments. *$P < 0.05$, **$P < 0.01$. Th1, T helper 1; Th2, T helper 2.

Delta-like 4 expression in DCs is induced by CD40 signalling

During DC/T cell contact, interaction between CD40 and CD154 is essential for the induction of maturation, co-stimulatory molecule expression and cytokine production by DCs [27]. Given the fact that Delta-like 4 mRNA in DCs is highly induced following interaction with T helper cells, we asked whether this upregulation is mediated by CD40 signalling. First, we tested whether Th1 and Th2 cells are able to express CD154 (CD40L) to determine whether these cells are able to activate CD40 signalling in DCs. For this purpose, we analysed

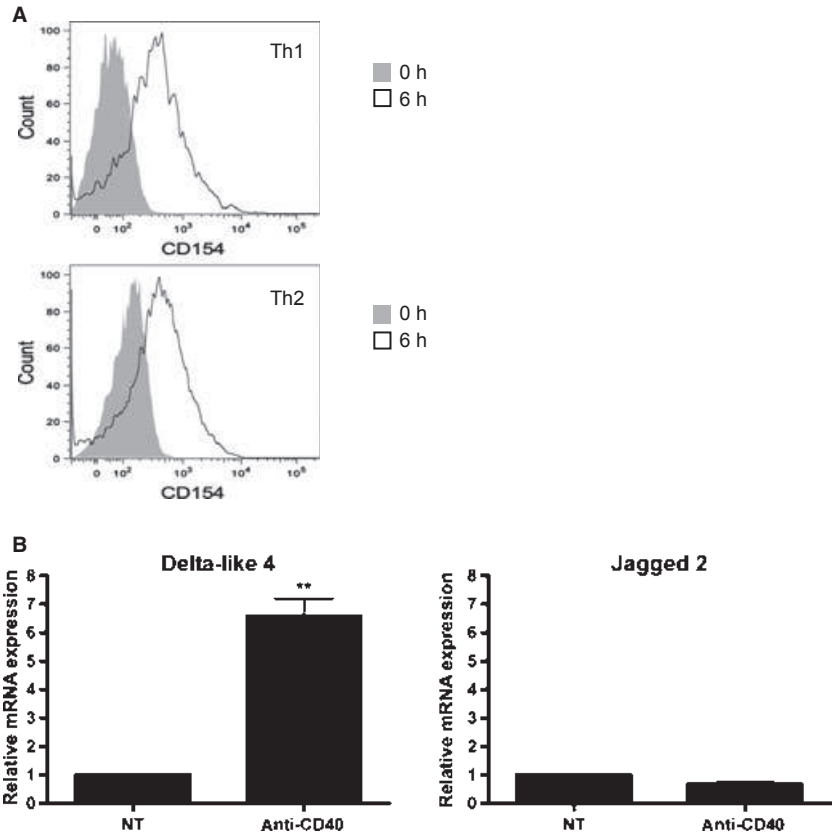

Figure 2 CD40 signalling induces Delta-like 4 expression in dendritic cells (DCs). (A) Th1 and Th2 cells from OT-II mice were co-cultured for 0 (filled grey histogram) and 6 h (open histogram) with bone marrow–derived DCs (BMDCs) from C57BL/6 in the presence of 5 μM OVA peptide$_{323–339}$. Cells were then analysed for CD154 expression by flow cytometry. (B) BMDCs from C57BL/6 mice were activated with the anti-CD40 antibody (10 μg/ml) for 6 h. DCs were isolated using CD11c microbeads, and Delta-like 4 and Jagged 2 mRNA expression in DCs was analysed by real-time PCR. Data shown represent the average of at least three independent experiments. $^{**}P < 0.01$. Th1, T helper 1; Th2, T helper 2.

CD154 expression in Th1 and Th2 cells before and after co-culture with DCs and observed that CD154 is absent in Th1 and Th2 cells prior to co-culture but is upregulated 6 h after interaction with DCs (Fig. 2A). Importantly, the level of CD154 expression in Th1 and Th2 cells, as measured by mean fluorescence intensity, was very similar 6 h after activation (668 for Th1 cells and 706 for Th2 cells).

Next, we used an anti-CD40 agonist antibody to activate DCs in the absence of other stimuli and analysed Delta-like 4 mRNA expression at different time points. Delta-like 4 was rapidly induced, with transient expression peaking 6 h after DC activation with the anti-CD40 antibody (data not shown). Following 6 h of DC activation with the anti-CD40 antibody, we observed a sixfold upregulation of Delta-like 4 mRNA expression compared with non-treated controls (Fig. 2B). Moreover, our results show that CD40 signalling in DCs induces Delta-like 4 expression while at the same time slightly downregulates Jagged 2 mRNA expression (Fig. 2B). Notably, the level of Delta-like 4 induction following activation with T helper cells (Fig. 1B) was always higher than following DC activation with the anti-CD40 agonist antibody (Fig. 2B). Moreover, blocking the CD40/CD154 interaction with antibodies against CD154 did not affect Delta-like 4 mRNA expression in DCs following contact with T helper cells (not shown). All these data reinforce the idea that although CD40 signalling may contribute to the induction of Delta-like 4 mRNA in DCs, other factors are necessary for optimal induction of Delta-like 4 mRNA in DCs during interaction with T helper cells.

IL-4 induces Jagged 2 expression in pathogen-activated BMDCs

After demonstrating that Th2 cells induce Jagged 2 mRNA expression in DCs, we sought to determine whether IL-4, a hallmark Th2 cytokine, is involved in this process. To test this, we analysed Jagged 2 mRNA expression in DCs treated with Th1- (LPS, CpG) or Th2-promoting stimuli (CT, PGE2) in the presence or absence of IL-4. As shown in Fig. 3A, addition of Th2-promoting stimuli alone was unable to induce Jagged 2 or Delta-like 4 mRNA expression in DCs. In contrast, Th1-promoting stimuli upregulated Delta-like 4 mRNA expression ($P < 0.05$ for LPS treatment and $P < 0.01$ for CpG treatment compared with untreated cells) but had no effect on Jagged 2 mRNA expression (Fig. 3A). Furthermore, addition of exogenous IL-4 during DC differentiation and activation strongly induced Jagged 2 expression in DCs treated with CT ($P < 0.01$ compared with no IL-4) but did not change Delta-like 4 mRNA

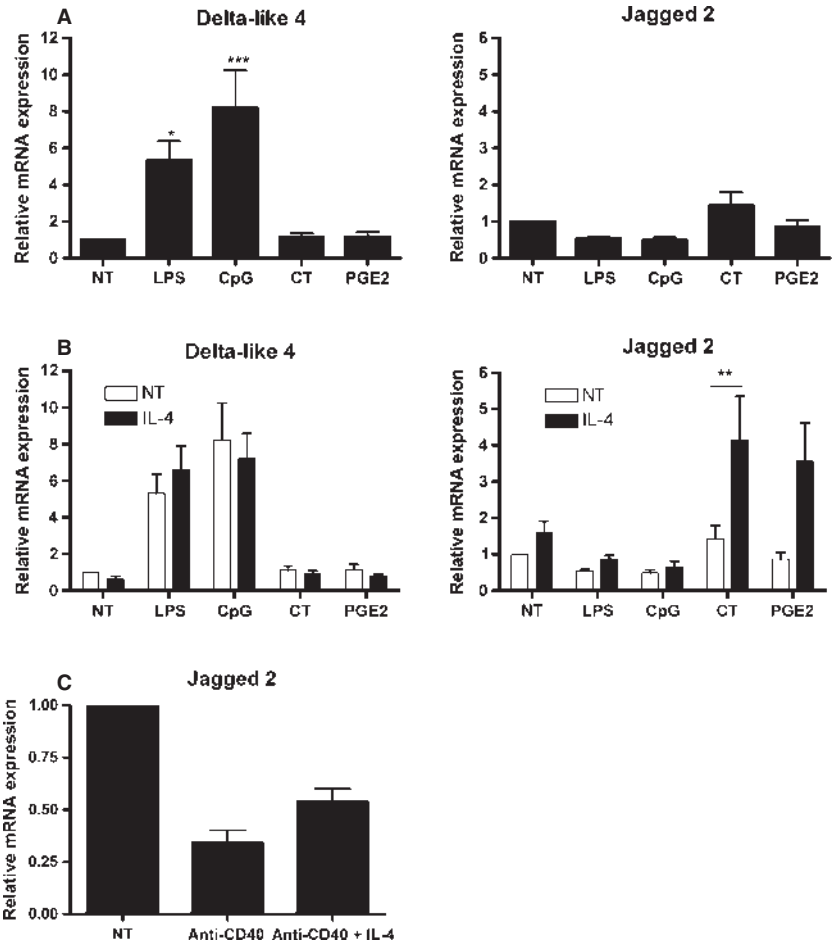

Figure 3 IL-4 induces Jagged 2 expression in dendritic cells (DCs) under Th2 stimulation. (A) Bone marrow–derived DCs (BMDCs) from C57BL/6 mice were activated for 6 h with different Th1- or Th2-promoting stimuli. Delta-like 4 and Jagged 2 mRNA expression in DCs was analysed by real-time PCR. (B) BMDCs from C57BL/6 mice were generated in the presence or absence of IL-4 (1 ng/ml) and activated for 6 h either with different Th1- or Th2-promoting stimuli (C) or with the anti-CD40 antibody (10 μg/ml). Delta-like 4 and Jagged 2 mRNA expression in DCs was analysed by real-time PCR. Data shown represent the average of at least three independent experiments. Th1, T helper 1; Th2, T helper 2.

expression in DCs activated with any stimulus (Fig. 3B). To determine whether IL-4 alone is responsible for Jagged 2 induction in DCs following interaction with Th2 cells, we activated DCs with the anti-CD40 agonist antibody in the presence of IL-4. As shown in Fig. 3C, IL-4 was not able to restore Jagged 2 expression following CD40 signalling–mediated inhibition, which suggests that other unknown factors produced by or present on Th2 cells may be responsible for Jagged 2 mRNA upregulation in DCs during DC/Th2 interaction.

IL-4 turns off Notch signalling in DCs

During analysis of Notch ligand expression in BMDCs, we determined that, in addition to the expression of Delta-like 4 and Jagged 2, BMDCs also express Notch 1 mRNA. Moreover, in addition to upregulating Jagged 2 expression (Fig. 3B), differentiation and activation of BMDCs from C57BL/6 mice in the presence of IL-4 resulted in reduced Notch 1 mRNA expression (Fig. 4). Accordingly, expression of Hes-1, a known target and

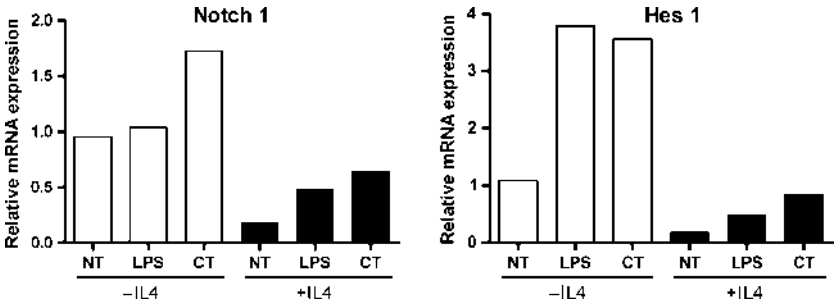

Figure 4 IL-4 turns off Notch signalling in dendritic cells (DCs). Bone marrow–derived DCs from C57BL/6 mice were activated for 6 h with LPS (100 ng/ml) or cholera toxin (1 ug/ml), in the presence or absence of IL-4 (1 ng/ml). Notch 1 and Hes-1 relative mRNA expression was analysed by real-time PCR.

reporter of Notch signalling, was also reduced in DCs following IL-4 treatment (Fig. 4).

These data demonstrate that, in addition to its inductive effect on Jagged 2 mRNA expression, IL-4 plays a role in downregulating Notch signalling in DCs.

Discussion

In addition to the well-characterized role of Notch signalling in lymphopoiesis, this pathway is important in determining peripheral $CD4^+$ T cell responses, such as lymphocyte activation and differentiation into Th1 and Th2 lineages [9–13]. Moreover, data presented here demonstrate that Delta-like 4 and Jagged 2 expression in DCs is induced by Th1- and Th2-promoting stimuli, respectively, in agreement with results from Flavell *et al.* [10]. In addition, recent evidence has shown that Jagged 2-deficient DCs are severely impaired in their ability to generate Th2 responses *in vitro* [28]. These observations strongly suggest that differential expression of these Notch ligands in DCs affects the generation of Th1 and Th2 responses.

DCs coordinate the development of the immune response, by integrating signals derived from the pathogen, damaged tissues and other cells of the immune system [20, 21]. A number of investigations have demonstrated that the well-characterized positive feedback loop associated with Th1 and Th2 responses is mediated by the effect of these T cells on DCs, which act as messengers between naïve and effector T cells [24, 25]. Matzinger's group demonstrated that, in addition to secreted cytokines, direct cell-to-cell contact between DCs and effector T cells is necessary to instruct DCs to favour the polarization of T cells towards Th1 or Th2 responses [24]. These observations led us to hypothesize that Delta-like 4 and Jagged 2 may be involved in positive feedback loops regulating Th1 and Th2 responses, respectively.

To test this hypothesis, we determined whether Delta-like 4 and Jagged 2 expression was induced by T helper cells or their cytokines, in addition to pathogen-derived factors. The cytokine IL-4 has been shown to play an essential role in the generation of Th2 responses, by inducing the expression of GATA3, which is responsible for the production of Th2-associated cytokines by T cells [29]; however, its effect on DCs has remained controversial. Interestingly, we found that CT, a Th2-promoting stimulus, was able to induce Jagged 2 mRNA expression only in DCs treated with IL-4. Thus, in contrast with Delta-like 4, Jagged 2 expression depends on the presence of IL-4 during DC differentiation and/or activation. These data favour the idea that Jagged 2 is involved in the positive feedback loop regulating Th2-polarized responses, because it can be induced in pathogen-activated DCs during ongoing Th2 responses, where IL-4 may be available.

By analysing the effect of T helper cells on Notch ligand expression in BMDCs, we demonstrated that Delta-like 4 mRNA is highly upregulated in DCs following interaction with either Th1 or Th2 cells. In fact, Delta-like 4 expression in DCs activated with T helper cells is 15-fold higher than in non-treated cells. Although we demonstrated that DC activation with the anti-CD40 agonist antibody induces the expression of Delta-like 4 in DCs, the level of Delta-like 4 induction is only sixfold higher than in non-treated controls. Moreover, blocking the CD40/CD154 interaction during DC/T cell contact, using an antibody against CD154, had no affect on Delta-like 4 expression in DCs. All these data support the idea that CD40 signalling is only one of the factors contributing to Delta-like 4 expression in DCs following interaction with T helper cells and that other factors, such as cytokines released during DC/T cell contact, may potentiate Delta-like 4 expression in DCs.

Because Delta-like 4 expression is induced in DCs by CD40 signalling, it is very unlikely that Delta-like 4 is involved in the positive feedback loop regulating Th1-polarized responses and suggests that Delta-like 4 may be a co-stimulatory molecule. In support of this notion, Delta-like 4 expression is induced under conditions (such as LPS activation or following DC interaction with T helper cells) that normally upregulate other co-stimulatory molecules. Moreover, Sheffold's group demonstrated that Notch ligation on T cells by the different Notch ligands results in differential effects on T cell activation [30]. Using *in vitro* stimulation assays with immobilized ligands, they demonstrate that Delta-like 4 is the only Notch ligand that induces the activation and proliferation of T cells. This observation is in accordance with the hypothesis that Delta-like 4 expressed on DCs may serve as a co-stimulatory molecule.

Although we observed a twofold upregulation of Jagged 2 mRNA expression in DCs following interaction with Th2 cells, the anti-CD40 agonist antibody alone was unable to induce Jagged 2 expression in DCs, suggesting that CD40 signalling is necessary but not sufficient for Jagged 2 upregulation. Taken together, these data suggest that upregulation of Jagged 2 mRNA expression in DCs following interaction with Th2 cells requires other unknown factors.

In addition to Notch ligands, DCs also express Notch 1 on their surface. The Vyas' group has shown that both T cells and DCs express Notch 1 and their ligands, enabling bidirectional communication via the Notch signal transduction pathway after formation of immunological synapses [31], although the function of the population of DCs that express Notch 1 and the possible role that this pathway plays in DC biology remains to be determined. In agreement with these findings, we demonstrated that BMDCs also express Notch 1 mRNA. Others have observed that $CD8^+$ $CD205^+$ splenic DCs

are specialized for the generation of FoxP3$^+$ regulatory T cells [32]. However, recent data have demonstrated that regulatory T cells express Jagged 1 and that their function is inhibited when the interaction between Notch 1 and Jagged 1 is blocked [33]. Combined, this evidence raises the possibility that Notch 1 expression in DCs may be involved in the maintenance of tolerance, through the generation of regulatory T cells or by regulating the function of these cells. Interestingly, we observed that IL-4 blocks Notch 1 and simultaneously induces Jagged 2 expression in DCs, suggesting the existence of a negative feedback loop between Notch and its ligands in the same cell. In fact, the existence of this negative feedback loop has already been described in other models, such as *Caenorhabditis elegans* [34, 35].

In summary, although these results should be corroborated with protein expression studies on Notch ligands in DCs, our data demonstrate that Delta-like 4 and Jagged 2 mRNA levels are upregulated in DCs, not only in response to pathogens, but also after interaction with T helper cells. While Delta-like 4 mRNA is upregulated in DCs upon interaction with either Th1 or Th2 cells, Jagged 2 is only induced following DC interaction with Th2 cells. Although these results suggest that a role of Delta-like 4 in positive feedback loop regulation of Th1 responses is unlikely, they do raise the possibility that positive feedback loop regulation of Th2 responses may be mediated by differential expression of Jagged 2 in DCs.

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Conflicts of interest

The authors declare no financial or commercial conflicts of interest.

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