

# Human immature myeloid dendritic cells trigger a T<sub>H</sub>2-polarizing program via Jagged-1/Notch interaction

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**Background:** The mechanisms by which human dendritic cells (DCs) activate a T<sub>H</sub>1-polarizing or T<sub>H</sub>2-polarizing program are still partially unclear.

**Objective:** Study of the mechanisms responsible for the T<sub>H</sub>1/T<sub>H</sub>2-polarizing activity of human circulating myeloid DCs before and after ligation of their Toll-like receptors (TLRs).

**Methods:** IL-4 and IFN- $\gamma$  production by CD4<sup>+</sup> T cells was assessed in cocultures with myeloid DCs before or after TLR triggering. Expression of Jagged-1 and Delta-4 Notch ligands and of GATA-3 and T-box expressed in T cells transcription factors was evaluated by real-time quantitative PCR. Signal transducer and activator of transcription 4 and 6 phosphorylation was assessed by flow cytometry. Knockdown of Jagged-1 or Delta-4 was performed by transfection of DCs with appropriate silencing mRNAs.

**Results:** Myeloid immature DCs constitutively expressed Jagged-1, which induces in CD4<sup>+</sup> T cells a T<sub>H</sub>2 polarization, as shown by Jagged-1 gene silencing. The T<sub>H</sub>2 polarization associated with high GATA-3/T-box expressed in T cells ratio and was at least partially dependent on the early induction of IL-4. Maturation of DCs by TLR ligation resulted in the reduction of Jagged-1 and upregulation of Delta-4, which was at least in part responsible for the polarization of CD4<sup>+</sup> T cells to the T<sub>H</sub>1 phenotype.

**Conclusion:** CD4<sup>+</sup> T-cell responses are usually characterized by a prevalent T<sub>H</sub>2 phenotype unless TLRs are triggered on DCs by microbial components. (J Allergy Clin Immunol

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**Key words:** Dendritic cells, T<sub>H</sub>1, T<sub>H</sub>2, Toll-like receptors, Notch, Jagged-1, Delta-4, STAT-4, STAT-6, IL-4, IL-12

The adaptive effector CD4<sup>+</sup> T<sub>H</sub>-mediated immune response is highly heterogeneous, on the basis of the development of distinct subsets characterized by different cytokine production profiles. Initially, 2 polarized forms of CD4<sup>+</sup> T effector cells were identified and named as T<sub>H</sub>1 or T<sub>H</sub>2 cells.<sup>1,2</sup> T<sub>H</sub>1 cells produce IFN- $\gamma$  and are mainly devoted to the protection against intracellular microbes, whereas T<sub>H</sub>2 cells produce IL-4, IL-5, IL-9, and IL-13 and are involved in the protection against gastrointestinal nematodes, but are also responsible for allergic disorders.<sup>3</sup> An intermediate phenotype, able to produce both T<sub>H</sub>1-type and T<sub>H</sub>2-type cytokines and known as T<sub>H</sub>0, also exists,<sup>3</sup> but its real function and its relationship with T<sub>H</sub>1 and T<sub>H</sub>2 cells have not been clearly defined. Recently, a novel subset distinct from both T<sub>H</sub>1 and T<sub>H</sub>2 cells and named T<sub>H</sub>17 has been described.<sup>4</sup> T<sub>H</sub>17 cells originate under different polarizing conditions than T<sub>H</sub>1 or T<sub>H</sub>2 cells and play a major role in the protection against extracellular bacteria but are also pathogenic, at least in some murine autoimmune disorders.<sup>5</sup>

T<sub>H</sub>1 polarization in both mice and human beings appears to be partly dependent on the activity of cytokines produced by dendritic cells (DCs) and natural killer cells. Both the early production of IL-12 and IFN- $\gamma$  is critical for the T<sub>H</sub>1 polarization,<sup>6</sup> with IL-12 able to activate a cascade of transcription factors, such as the signal transducer and activator of transcription (STAT)-4 and T-box expressed in T cells (T-bet), which results in the activation of the IFN- $\gamma$  gene promoter.<sup>7</sup> By contrast, the mechanisms responsible for T<sub>H</sub>2 polarization have been unclear for many years, despite the demonstration that early production of IL-4 in the absence of IL-12 may be a critical event.<sup>7</sup> Indeed, after interaction with its receptor, IL-4 activates STAT-6,<sup>8</sup> which in turn induces the activation of GATA-3 and c-maf, which act as binding factors for IL-5 and IL-13, or IL-4 genes, respectively.<sup>9</sup> However, the cell responsible for early IL-4 production and the mechanisms of its stimulation remain unclear. Recently, lung epithelial cells have been found to produce IL-25, a cytokine that at least in mice seems to initiate proallergic responses by inducing early IL-4 production and GATA-3 expression in activated T cells.<sup>10</sup> However, the T<sub>H</sub>2 polarization has also been reported to occur through an IL-4-independent pathway.<sup>11</sup> Another mechanism of T<sub>H</sub>2 polarization in mice has also been found to be the interaction between Notch receptors on T cells and their ligands present on DCs.<sup>12</sup> Indeed, the inhibition of Notch signaling by either the deletion of RBP-J or the

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Supported by the Italian Ministry of Education (40%), the Italian Ministry of Health grant by Ente Cassa di Risparmio, Florence, Italy (AIDS Project), EU Projects SENS-IT-IV (FP6-LSBH-CT-2006-018861), INNOCHEM (FP6-LSHB-CT-2005-518167), Agenzia Spaziale Italiana MoMa (WP ref: 1B1241-X5), and the German Federal Ministry of Education and Research (BMBF): 0313066F, 0313068C01 and 01 GS 0413 (NGFN-2: SIPAGE).

Disclosure of potential conflict of interest: The authors have declared that they have no conflict of interest.

Received for publication July 10, 2007; revised December 24, 2007; accepted for publication January 3, 2008.

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0091-6749/\$34.00

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doi:10.1016/j.jaci.2008.01.004

**Abbreviations used**

BDCA: Blood dendritic cell antigen  
 DAPT: N(N-[3,5-difluorophenacetyl-L-alanyl]-S-phenylglycine t-butyl ester  
 DC: Dendritic cell  
 iDC: Immature dendritic cell  
 STAT: Signal transducer and activator of transcription  
 T-bet: T-box expressed in T cells  
 TCR: T-cell receptor  
 TLR: Toll-like receptor

expression of a dominant-negative form of MAML identifies defects in IL-4 production and  $T_H2$  responses.<sup>12-14</sup> This impairment in RBP-J-deficient T cells is completely overcome by the addition of IL-4 during the induction phase. According to this model, Jagged-1 expressed on DCs promotes a  $T_H2$  polarization probably via both an IL-4-dependent and an IL-4-independent pathway.<sup>12</sup> By contrast, the interaction of Notch with its Delta ligand on the surface of murine DCs results in  $T_H1$  polarization.<sup>12</sup> More recently, the understanding of Notch-RBP-J-mediated  $T_H2$  differentiation mechanism was further clarified by the demonstration that Notch-mediated binding of RBP-J to the regulatory region of exon 1a in the Gata3 locus regulates GATA-3 expression.<sup>15</sup>

On the basis of all these findings, in this study we hypothesized that the interaction between Notch receptors on  $CD4^+$  T cells and their ligands expressed by DCs may also be involved in human  $T_H1/T_H2$  polarization. To this end, the expression of the Notch ligands Jagged-1 and Delta-4 was evaluated on human myeloid DCs in either their immature state or after maturation induced by triggering of their Toll-like receptors (TLRs). The results showed that immature DCs (iDCs) promoted a  $T_H2$  polarization in both naive and memory  $CD4^+$  T cells because of the interaction between Notch and its Jagged-1 ligand, which resulted in the maintenance of high GATA-3/T-bet ratios and STAT-6 phosphorylation. However, stimulation of TLR on the same DCs induced strong Delta-4 upregulation, which allowed these cells to activate in  $CD4^+$  T cells a  $T_H1$ -polarized program.

**METHODS****Donors**

Human PBMCs used in this study were obtained in accordance with the ethical standards of the responsible regional committee on human experimentation.

For reagents and antibodies, flow cytometry analysis, isolation of  $CD4^+$ ,  $CD14^+$ ,  $CD45RA^+$ ,  $CD45RO^+$  and BDCA-1<sup>+</sup> cell, generation of DCs and of short term T-cell cultures, RNA isolation, cDNA synthesis and real-time quantitative RT-PCR, evaluation of TCR V $\beta$  repertoire by spectratyping, Western blot analysis, siRNA delivery, and gene silencing, refer to Methods in the Online Repository at [www.jacionline.org](http://www.jacionline.org).

**Statistical analysis**

Statistical analysis was performed by using the Student *t* test.

**RESULTS****Culturing  $CD4^+$  T cells with myeloid iDCs results in the development of  $T_H2$ -polarized cells**

To understand the role of myeloid DCs on the process of  $T_H1/T_H2$  polarization, we compared the cytokine production profile of  $CD4^+$  T-cells present in streptokinase-stimulated or *Dermatophagoides*

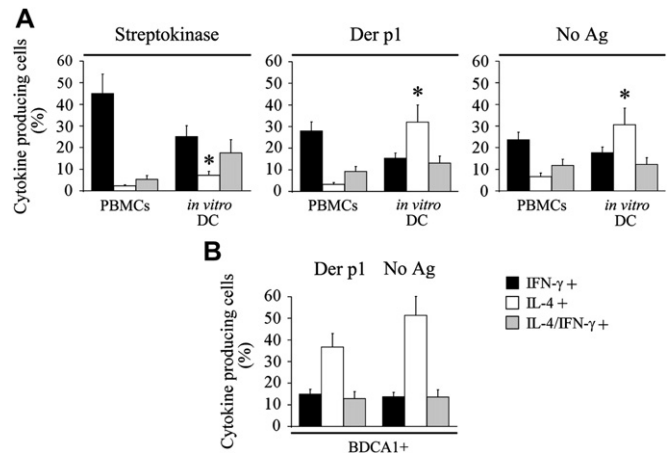


**FIG 1.** Circulating myeloid iDCs activate a  $T_H2$ -polarizing program in  $CD4^+$  T cells. **A**, PBMCs or *in vitro*-derived iDCs plus autologous  $CD4^+$  T cells were cultured for 16 days with or without antigen. Columns represent mean values  $\pm$  SEs ( $n = 5$ ) of the proportions of cytokine-producing  $CD4^+$  T cells. \* $P < .05$ . **B**,  $T_H2$  polarization induced on  $CD4^+$  lymphocytes by BDCA-1<sup>+</sup> cells. For explanations, see A. Ag, Antigen.

*pteronyssinus* group 1 (Der p 1)-stimulated PBMCs of healthy non-atopic donors with that of  $CD4^+$  T cells stimulated with the same antigens in presence of autologous DCs obtained *in vitro* by culturing  $CD14^+$  cells with IL-4 plus GM-CSF. As shown in previous studies,<sup>16</sup> after stimulation of whole PBMCs with each of the 2 antigens, or even in their absence,  $CD4^+$  T cells exhibited a prevalently  $T_H1$ -oriented phenotype (Fig 1, A; see this article's Fig E1, A, in the Online Repository at [www.jacionline.org](http://www.jacionline.org)). By contrast,  $CD4^+$  T cells stimulated with streptokinase or Der p 1 in presence of autologous  $CD14^+$ -derived DCs, and even those cultured under these conditions in absence of any exogenous antigen, exhibited a prevalent production of IL-4 versus IFN- $\gamma$ , thus showing an opposite, prevalently  $T_H2$ -oriented, phenotype (Fig 1, A; Fig E1, A). The possibility that the  $T_H2$ -polarizing effect of iDCs could reflect some artifact induced by the *in vitro* technique used for their differentiation was excluded by the observation that myeloid iDCs, freshly purified from PBMCs by immunomagnetic cell sorting with an anti-blood dendritic cell antigen (BDCA)-1 mAb, also activated a  $T_H2$ -polarized program in  $CD4^+$  T cells, irrespective of whether they were cultured in the presence or absence of an exogenous antigen (Fig 1, B; Fig E1, B). Finally, to evaluate whether the  $T_H2$  polarization triggered by myeloid iDCs could be related to a specific involvement of naive or memory  $CD4^+$  T cells or both, total  $CD4^+$ , as well as  $CD4^+CD45RA^+$  or  $CD4^+CD45RO^+$ , T cells were cocultured with BDCA-1<sup>+</sup> cells under the conditions discussed. As shown in this article's Fig E2, A (see the Online Repository at [www.jacionline.org](http://www.jacionline.org)), the  $T_H2$ -polarizing effect of DCs involved both naive ( $CD4^+CD45RA^+$ ) and memory ( $CD4^+CD45RO^+$ ) T cells. As shown by carboxyfluorescein diacetate succinimidyl ester staining, this effect was selectively exerted on the proliferating subset of the  $CD4^+$  T-cell populations. By contrast, nonproliferating naive  $CD4^+$  T cells did not show any ability to produce cytokines, whereas nonproliferating memory  $CD4^+$  T cells rather showed the ability to produce IFN- $\gamma$  (Fig E2, B).

 **$T_H2$  polarization triggered by immature myeloid DCs is a result of the Jagged-1/Notch interaction**

To investigate the mechanisms responsible for the  $T_H2$ -polarizing activity by myeloid iDCs on human  $CD4^+$  T cells, we took

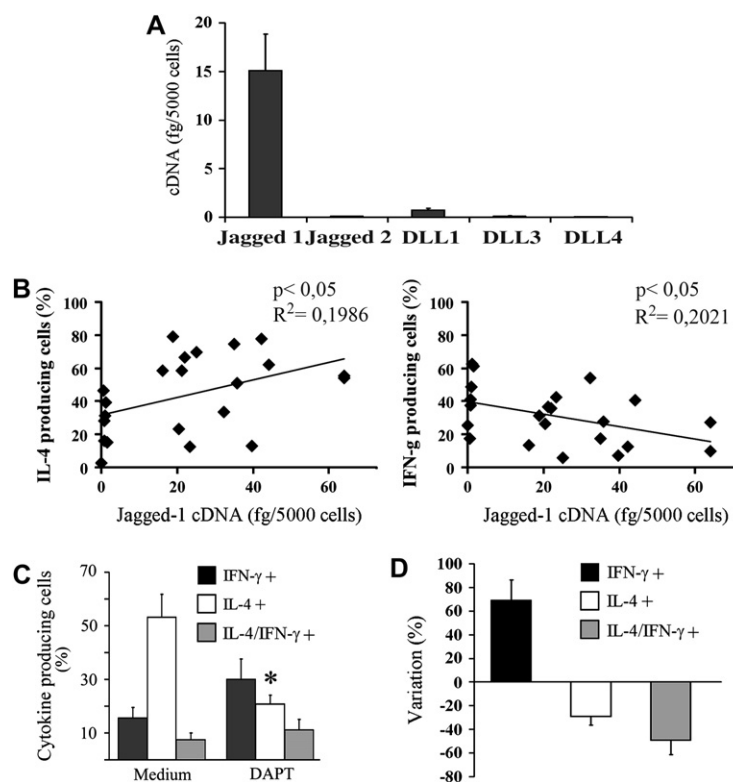


**FIG 2.** The  $T_H2$ -polarizing program triggered by immature BDCA-1<sup>+</sup> cells is mediated by the Jagged-1/Notch interaction. **A**, Notch ligands mRNAs expression in iDCs (mean values  $\pm$  SEs;  $n = 11$ ). **B**, Correlation between Jagged-1 mRNAs in freshly isolated BDCA-1<sup>+</sup> cells and proportions of IL-4-producing or IFN- $\gamma$ -producing CD4<sup>+</sup> cells ( $n = 22$ ). **C**, CD4<sup>+</sup> lymphocytes cocultured with BDCA-1<sup>+</sup> cells without or with DAPT (mean values  $\pm$  SEs;  $n = 6$ ). \* $P < .05$ . **D**, Columns represent cytokines production variation between CD4<sup>+</sup> T cells cocultured with BDCA-1<sup>+</sup> cells transfected with human Jagged-1-siRNA or with GFP-siRNA as control (mean values  $\pm$  SEs;  $n = 3$ ). GFP, Green fluorescent protein; fg, femtograms; DLL, delta-like ligand.

advantage of the observation that murine DCs use the Notch pathway to instruct T-cell differentiation toward the  $T_H2$  or  $T_H1$  lineage, inasmuch as of the 2 Notch ligand families, Jagged induced  $T_H2$ , whereas Delta induced the alternate  $T_H1$  fate.<sup>14,15,17</sup> On the basis of this finding, we investigated whether the Notch/Notch ligand interaction could provide an explanation for the phenomena described.

To this end, we first measured the expression of Notch ligands on human iDCs by using quantitative RT-PCR. Both CD14<sup>+</sup>-derived DCs and freshly purified BDCA-1<sup>+</sup> cells expressed significantly higher amounts of Jagged-1 than of all other Notch ligands (Fig 2, A). The expression of Jagged-1 was also confirmed at protein level by both the Faser technique, applied to flow-cytometry analysis, and Western blot (see this article's Fig E3, A and B, in the Online Repository at [www.jacionline.org](http://www.jacionline.org)). Of note, the Jagged-1 mRNA expression by freshly isolated myeloid iDCs positively correlated with the proportion of IL-4-producing and negatively correlated with the proportion of IFN- $\gamma$ -producing CD4<sup>+</sup> T cells obtained after coculture (Fig 2, B). In agreement with the possible implication of the Jagged-1/Notch interaction in the  $T_H2$  polarization, the presence in culture of N-[3, 5-difluorophenacetyl-L-alanyl]-S-phenylglycine t-butyl ester (DAPT), a Notch signaling inhibitor,<sup>18</sup> reduced the  $T_H2$  polarizing activity of myeloid iDCs (Fig 2, C). More importantly, the selective silencing of Jagged-1 by means of specific siRNA (see this article's Fig E4, A and B, in the Online Repository at [www.jacionline.org](http://www.jacionline.org)) reduced the ability of iDCs to polarize the

CD4<sup>+</sup> T cells toward the  $T_H2$  phenotype (Fig 2, D).

### TLR ligation on DCs shifts their polarizing activity from $T_H2$ to $T_H1$ profile

In previous studies,<sup>12</sup> it was demonstrated that TLR triggering can modulate the expression of the Notch ligand repertoire by iDCs and shifts the  $T_H2$  polarizing program to  $T_H1$ . We therefore investigated the effect of ligation of TLRs both on the ability of DCs to polarize CD4<sup>+</sup> T cells toward the  $T_H2$  or  $T_H1$  phenotype and on the expression of their Notch ligands.

As shown in Fig 3, A and B, ligation of TLR4 with LPS or TLR7/TLR8 with R-848 resulted in a strong reduction of IL-4 production and a strong increase of IFN- $\gamma$  production by autologous CD4<sup>+</sup> T cells. We therefore asked whether the  $T_H1$  cells expanded in the presence of myeloid DCs after their TLR triggering belonged to the same subset of CD4<sup>+</sup> T cells that polarized to  $T_H2$  cells in the presence of myeloid iDCs. As shown in Fig 3, C,  $T_H1$  polarization induced by TLR7/TLR8 ligation on DCs involved the same CD4<sup>+</sup> T cells that polarized to the  $T_H2$  profile in the presence of iDCs, as revealed by the expression of the same T-cell receptor (TCR) V $\beta$  patterns. These data clearly indicate that the *in vitro* polarization toward the  $T_H2$  or  $T_H1$  phenotype by immature or mature DCs, respectively, is a result of the immunomodulation of the same CD4<sup>+</sup> T cells.

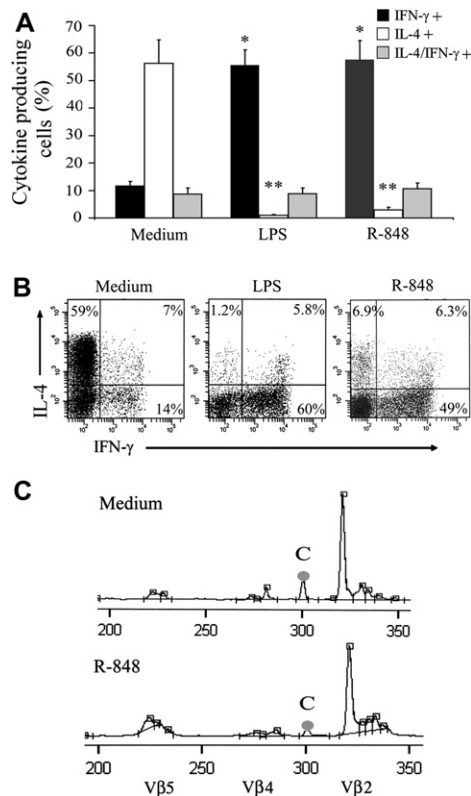


**FIG 3.** Ligation of TLR4 by LPS or TLR7/8 by R-848 on freshly derived BDCA-1<sup>+</sup> cells induces a shifting of their polarizing program from T<sub>H</sub>2 to T<sub>H</sub>1. **A**, CD4<sup>+</sup> T lymphocytes were cocultured with BDCA-1<sup>+</sup> cells with or without LPS or R-848 and assessed by flow cytometry for cytokine production (mean values  $\pm$  SEs; n = 7). \* $P$  < .001; \*\* $P$  < .0001. **B**, One representative flow-cytometric analysis. **C**, TCR V $\beta$  expression on CD4<sup>+</sup> T lymphocytes cocultured with BDCA-1<sup>+</sup> cells with or without LPS. One representative experiment is shown.

The loss of the ability of DCs to induce a T<sub>H</sub>2 polarization after their R-848-induced maturation appeared to be associated with the upregulation of Delta-4 and the downregulation of Jagged-1, without any significant effect on all other Notch ligands (Fig 4, A; see this article's Fig E5 in the Online Repository at [www.jacionline.org](http://www.jacionline.org)). We therefore asked whether Delta-4 was directly involved in the T<sub>H</sub>1 polarization. As shown in Fig 4, B, the selective silencing of Delta-4 on R-848-matured DCs by means of specific siRNA (see this article's Fig E6 in the Online Repository at [www.jacionline.org](http://www.jacionline.org)) significantly, even if not completely, reduced their ability to polarize CD4<sup>+</sup> T cells toward the T<sub>H</sub>1 phenotype. In agreement with already published results,<sup>16,19</sup> ligation either of TLR4 by LPS or of TLR7/TLR8 by R-848 associated with the production by DCs of T<sub>H</sub>1-polarizing cytokines and chemokines, such as IL-12 and CXCL10 (data not shown), thus suggesting that different mechanisms may be involved in the T<sub>H</sub>1 polarization.

### T<sub>H</sub>2 polarization induced by Jagged-1/Notch interaction is related to high GATA-3/T-bet ratio and is partially dependent on the production of IL-4

We asked whether the T<sub>H</sub>2-polarizing program triggered by human myeloid iDCs through Jagged-1/Notch interaction correlated with the high GATA-3/T-bet ratios. To this end, the mRNA levels of both the T<sub>H</sub>2-associated GATA-3 and the T<sub>H</sub>1-associated T-bet transcription factors were measured at different periods in CD4<sup>+</sup>

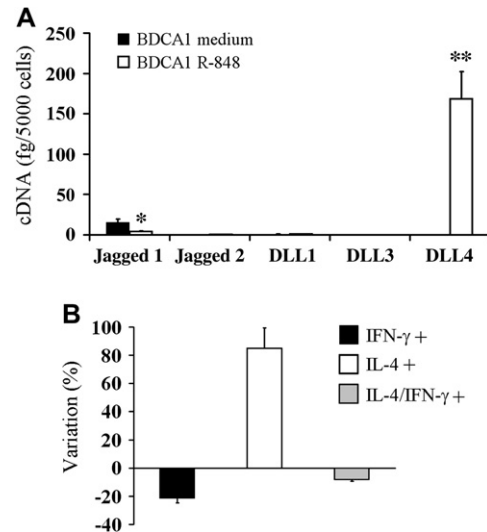


**FIG 4.** The T<sub>H</sub>1-polarizing program triggered by mature BDCA-1<sup>+</sup> cells is partially mediated by the Delta-4/Notch interaction. **A**, Expression of Notch ligands mRNAs in BDCA-1<sup>+</sup> cells cultured with or without R-848 (mean values  $\pm$  SEs; n = 9). \* $P$  < .05; \*\* $P$  < .01. **B**, Columns represent the variation in cytokines production between CD4<sup>+</sup> T cells cocultured with R848-matured BDCA-1<sup>+</sup> cells transfected with hDelta-4-siRNA or GFP-siRNA as control (mean values  $\pm$  SEs; n = 3). *fg*, Fentograms; *DLL*, delta-like ligand.

T-cell suspensions cocultured with autologous myeloid iDCs or with the same DCs stimulated with R-848. In the presence of myeloid iDCs, CD4<sup>+</sup> T-cell suspensions maintained virtually unchanged their GATA-3 mRNA levels until day 4 of culture, whereas CD4<sup>+</sup> T cells cocultured with R-848-stimulated myeloid DCs strongly upregulated T-bet starting from 12 hours of culture, resulting in a significant reduction of the GATA-3/T-bet ratio (Fig 5, A). Moreover, on day 16, in cultures containing myeloid iDCs, the GATA-3/T-bet ratio appeared to be significantly higher than the ratio measured at the beginning under the same culture conditions, confirming the T<sub>H</sub>2 polarization of CD4<sup>+</sup> T cells stimulated by iDCs (Fig 5, A). Of note, the addition of DAPT to the cocultures of BDCA-1<sup>+</sup> cells and autologous CD4<sup>+</sup> T cells also significantly reduced the GATA-3/T-bet ratio (Fig 5, B).

Finally, we asked whether the cascade of transcriptional events leading to T<sub>H</sub>2 polarization was a result of the production by the T<sub>H</sub> cell of even small concentrations of IL-4 which, after its interaction with the IL-4 receptor on the surface of the same cell, was able to activate STAT-6. To ascertain this point, phosphorylation of both STAT-6 and STAT-4 in CD4<sup>+</sup> T cells cultured with BDCA-1<sup>+</sup> cells in the absence or presence of R-848 or IL-4 (added as positive control) was assessed. Myeloid iDCs induced detectable STAT-6 phosphorylation starting on day 2 (Fig 5, C, upper panel). By contrast, ligation of TLR7/TLR8 by R-848 on the same BDCA-1<sup>+</sup> cells, because of the ability of this compound to induce IL-12 production,<sup>19</sup> was able to induce a rapid STAT-4 phosphorylation (Fig 5, C, lower panel). Moreover, the addition in culture of an anti-IL-4 Ab significantly reduced the T<sub>H</sub>2-polarizing effect exerted by myeloid iDCs on autologous CD4<sup>+</sup> T cells (Fig 5, D).

## DISCUSSION

Dendritic cells are a complex and heterogeneous family of cells that play an important role in the innate as well as the subsequent

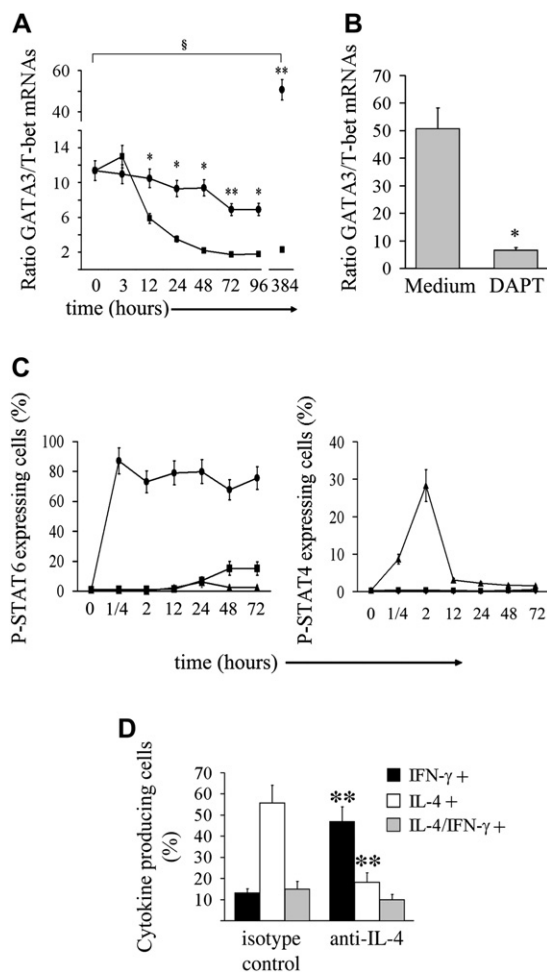


**FIG 5.** The  $T_H2$ -polarizing program induced by iDCs associates with high GATA-3/T-bet mRNA ratios and depends on the production of IL-4. **A**, GATA-3/T-bet mRNA ratios in  $CD4^+$  lymphocytes cocultured with BDCA-1 $^+$  cells without (circles) or with (squares) R-848 (mean values  $\pm$  SEs;  $n = 4$ ). **B**, GATA-3/T-bet mRNA ratio in  $CD4^+$  lymphocytes cultured for 16 days with BDCA-1 $^+$  cells with or without DAPT. **C**, STAT-6 or STAT-4 phosphorylation in  $CD4^+$  lymphocytes cultured with IL-4 (circles), or BDCA-1 $^+$  cells without (squares) or with R-848 (triangles; mean values  $\pm$  SEs;  $n = 5$ ). **D**, Inhibition of iDC-induced  $T_H2$  polarization by neutralization of IL-4 (mean values  $\pm$  SEs;  $n = 6$ ). \* $P < .05$ ; \*\* $P < .01$ ; § $P < .01$ . P-STAT, Phosphorylated signal transducer and activator of transcription.

adaptive immune responses because they are capable of antigen uptake, processing, and presentation to T cells.<sup>20</sup> At least 2 distinct pathways of DC development have been identified in mice, myeloid and lymphoid,<sup>21,22</sup> which differ in phenotype, localization, and function.<sup>21</sup> In human beings, DCs represent 1% to 2% of PBMCs and are made up of at least 2 major subsets distinguished by their reactivity with a panel of mAbs, as well as by their function.<sup>23</sup> The myeloid DCs subset is BDCA-1 $^+$  and CD11c $^+$ , whereas lymphoid DCs are BDCA-2 $^+$ , BDCA-4 $^+$ CD11c $^-$ CD123 $^{\text{bright}}$  cells. In addition, it is well known that CD14 $^+$  monocytes can give rise to myeloid iDCs after their culturing with GM-CSF plus IL-4.<sup>23</sup> It has been reported that human CD14-derived CD11c $^+$  DCs predominantly polarize T cells toward a  $T_H1$  profile, whereas the CD11c $^-$  DC subset induces T cells to prevalent production of  $T_H2$  cytokines, and the 2 subsets have therefore been named as DC1 and DC2, respectively.<sup>24</sup>

The results of this study demonstrated that human myeloid iDCs trigger a  $T_H2$ -polarizing program in circulating  $CD4^+$  T cells, but after their TLR ligation, the same DCs shifted their  $T_H$ -polarizing program toward a prevalent  $T_H1$  phenotype. Because CD11c $^+$  myeloid DCs showing a  $T_H1$ -polarizing effect, and therefore named DC1,<sup>24</sup> underwent CD40L or LPS activation, they likely correspond to our TLR-activated mature myeloid DCs.

Interestingly, the  $T_H2$ -polarizing effect of myeloid iDCs was independent of the addition in culture of an exogenous antigen. This does not exclude a critical role for TCR stimulation in the  $T_H2$ -polarizing effect, because in the case of DCs cocultured with  $CD4^+$  T cells in the absence of any exogenous antigen, presentation of self-peptides is probably involved. Accordingly, the analysis of the TCR V $\beta$  expression by spectratyping revealed that the subset of proliferating  $T_H2$ -polarized  $CD4^+$  T cells consisted of an oligoclonal population, whereas the nonproliferating subset appeared to contain mainly polyclonal T cells (data not shown).

The most important finding emerging from this study was the demonstration that the  $T_H2$ -polarizing program triggered by myeloid iDCs on  $CD4^+$  T cells reflected the interaction of Notch receptor on T cells with Jagged-1 on myeloid iDCs, whereas up-regulation of Delta-4 associated with  $T_H1$  polarization. First, Jagged-1 was highly expressed on human myeloid iDCs, and its expression positively or negatively correlated with the ability of these cells to promote the development of IL-4-producing or IFN- $\gamma$ -producing T cells, respectively. Second, after TLR ligation on iDCs, Jagged-1 mRNA levels significantly decreased, whereas Delta-4 levels were strongly upregulated. Finally, the impairment of Notch signaling by specifically silencing Jagged-1 mRNA inhibited the ability of myeloid iDCs to trigger a  $T_H2$ -polarizing program. By contrast, silencing of Delta-4 mRNA on mature DCs only partially interfered with the ability of these cells to trigger a  $T_H1$ -polarizing program. However, the interaction with mature (R-848-stimulated) DCs promoted in  $CD4^+$  T cells the phosphorylation of STAT-4, which follows the interaction between IL-12 and the IL-12 receptor, thus supporting the concept that both an IL-12-independent<sup>17</sup> and an IL-12-dependent<sup>19</sup> pathway are involved in the  $T_H1$  differentiation induced by mature DCs.

Murine  $T_H2$  polarization induced by Jagged/Notch interaction has also been found to occur via either an IL-4-dependent or an IL-4-independent pathway. The results of this study demonstrate that the interaction between human iDCs and  $CD4^+$  T cells resulted in STAT-6 phosphorylation, which usually follows the interaction of IL-4 with its receptor on the  $T_H$  cell. Moreover, the  $T_H2$ -polarizing program triggered by myeloid iDCs was significantly inhibited by the addition in culture of an anti-IL-4 Ab, supporting the concept that it was at least partially dependent on the production of IL-4 by the  $T_H$  cell itself.

The results of these study may have important implications for the understanding of both protective and pathogenic mechanisms operating in autoimmune and allergic disorders. The finding that myeloid iDCs presenting a self-peptide in the absence of their TLR ligation by microbial products trigger a  $T_H2$ -polarizing program in  $CD4^+$  T cells may be expression of a protective mechanism devoted to avoid more dangerous  $T_H1$ -mediated or  $T_H17$ -mediated inflammatory processes in response to autoantigens. Likewise, the fact that after their TLR ligation by microbial

components the same cells become able to trigger an opposite,  $T_H1$ -polarized, program in  $CD4^+$  T cells is certainly important for a better protection against infectious agents, but it can also account for the role of infections in the development of dangerous  $T_H1$ -mediated (and perhaps  $T_H17$ -mediated) effector responses under conditions of tolerance breakdown. With regard to allergy, the results of this study may provide a convincing explanation for the hygiene hypothesis.<sup>25</sup> This hypothesis suggests that the increased prevalence of allergy in Western countries reflects the reduced microbial burden during early childhood and/or even during pregnancy.<sup>26,27</sup> However, the immunologic mechanisms by which the reduced microbial burden during childhood can result in the increased prevalence of allergy are still controversial, with some authors suggesting the role of a missing immune deviation from  $T_H2$  to  $T_H1$ <sup>28,29</sup> and others claiming the importance of a reduced immune suppression.<sup>30</sup> Our findings are fully in agreement with the results of a recent study showing that 2 bacterial species identified in cowsheds of farms, *Acinetobacter lwoffii* F78 and *Lactococcus lactis*, were able to reduce allergic reactions in mice by inducing a  $T_H1$ -polarizing program in DCs<sup>31</sup> via the induction of high IL-12 production and upregulation of Delta-4 expression.<sup>31</sup> Taken together, these data clearly favor the importance of a missing immune deviation as a main explanatory mechanism for the hygiene hypothesis.<sup>32</sup>

Our findings also have obvious implications for both prevention and therapy of allergic disorders. Indeed, treatment of children or even newborns at high risk for allergy with innocuous adjuvants promoting stimulation of TLRs on DCs may reduce the risk of developing  $T_H2$  responses against environmental allergens. Moreover, specific immunotherapeutic strategies based on the use of microbial or synthetic adjuvants that induce TLR ligation on DCs<sup>19,33</sup> may be effective and also safe in subjects with allergy. Of note, indeed, in the study by Debarry et al,<sup>31</sup> only negligible signs of  $T_H1$ -driven inflammation were observed after repeated intranasal treatment of mice with rather high doses of the bacterial preparations. The safety of allergen-specific  $T_H1$  responses is also proved by the observation that virtually all healthy individuals without allergy possess circulating Der p 1-specific  $T_H1$ -polarized cells.<sup>29</sup> Similarly, children without allergy exhibit peanut-specific  $T_H1$  responses, and children who have outgrown their peanut allergy also show a  $T_H1$  skewing to peanut antigens,<sup>34</sup> without exhibiting allergic inflammation or other types of inflammatory symptoms.

**Clinical implications:** These findings may have important implications for the explanation of the hygiene hypothesis and the development of both preventive and immunotherapeutic strategies in allergic disorders.

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## METHODS

### Reagents and antibodies

The cell culture medium used was RPMI 1640 (Seromed, Berlin, Germany), supplemented with 2 mmol/L L-glutamine, 1% nonessential amino acids, 1% pyruvate, and  $2 \times 10^{-5}$  mol/L 2-mercaptoethanol (ME) (Gibco Laboratories, Grand Island, NY). Fluorescein isothiocyanate (FITC)-conjugated, phycoerythrin-conjugated, allophycocyanin-conjugated, or peridinin chlorophyll protein-conjugated anti-CD1a, anti-CD3, anti-CD4, anti-CD8, anti-CD11c, anti-CD14, anti-CD16, anti-CD20, anti-CD40, anti-CD45RA, anti-CD45RO, anti-CD80, anti-CD83, anti-CD86, anti-CD123, anti-HLA-DR, anti-IFN- $\gamma$ , anti-IL-4, anti-pho-STAT6, anti-pho-STAT4, and isotype-matched control mAbs were purchased from Becton Dickinson Biosciences (Mountain View, Calif). The neutralizing anti-IL-4 Ab was from R&D Systems (Minneapolis, Minn). LPS and brefeldin A were purchased from Sigma Chemical Co (St Louis, Mo). R-848 (Resiquimod, S28463) was purchased by 3M Pharmaceutical (St Paul, Minn). IL-2 was kindly provided by Eurocetus (Milan, Italy). DAPT was purchased from Calbiochem (Darmstadt, Germany). The 5,6-carboxyfluorescein diacetate, succinimidyl ester (CFDA-SE) was from Molecular Probes (Eugene, Ore).

### Isolation of CD4<sup>+</sup>, CD14<sup>+</sup>, CD45RA<sup>+</sup>, CD45RO<sup>+</sup> and BDCA-1<sup>+</sup> cells

CD14<sup>+</sup> cells were positively selected from PBMCs by immunomagnetic cell sorting (Miltenyi Biotec, Bisley, Germany), as described elsewhere.<sup>E1</sup> CD3<sup>+</sup>CD4<sup>+</sup> cells were isolated from the same PBMCs by using the CD4 isolation kit II according to the manufacturer's instructions (Miltenyi Biotec). BDCA-1<sup>+</sup> myeloid DCs were isolated from the same PBMCs by using the BDCA-1 isolation kit (Miltenyi Biotec). CD45RA<sup>+</sup> and CD45RO<sup>+</sup> T cells were isolated from CD4<sup>+</sup> purified T cells by immunomagnetic depletion of CD45RO<sup>+</sup> or CD45RA<sup>+</sup>, respectively.

### Generation of DCs and of short-term T-cell cultures

Dendritic cells were generated by culturing CD14<sup>+</sup> cells in medium supplemented with 5% FCS (Hyclone, South Logan, Utah), 50 ng/mL GM-CSF, and 20 ng/mL IL-4 (R&D Systems). On day 7, cells were analyzed by flow cytometry for CD1a, CD14, CD40, CD80, CD83, CD86, and HLA-DR expression.

CD4<sup>+</sup> cells were stimulated with autologous DCs (5:1 ratio) in the absence or presence of Der p 1 (10  $\mu$ g/mL) or streptokinase (1000 U/mL) in the presence of recombinant IL-2 (100 U/mL; Eurocetus, Milan, Italy) in medium supplemented with 5% AB serum (Cambrex, Walkersville, Md). Short-term T-cell cultures were contemporary obtained from the PBMCs of the same subjects under the conditions described. In additional experiments, CD4<sup>+</sup> cells were stimulated with autologous DCs in the presence or in the absence of DAPT (5  $\mu$ mol/L), LPS (100 ng/mL), and R-848 (2  $\mu$ g/mL). In 3 separate experiments, total CD4<sup>+</sup>, CD4<sup>+</sup>CD45RA<sup>+</sup>, and CD4<sup>+</sup>CD45RO<sup>+</sup> cells were labeled with CFDA-SE (1  $\mu$ mol/L) and then stimulated with autologous purified BDCA-1<sup>+</sup> cells. After 16 days of *in vitro* culture, cells were recovered and analyzed by flow cytometry for cytokines production, on PMA (10 ng/mL) and ionomycin stimulation (1  $\mu$ mol/L), and in some cases for T-cell proliferation. Furthermore, CFDA-SE<sup>+</sup> and CFDA-SE<sup>-</sup> cell fractions were isolated by FACS sorting (FACSARIA; Becton Dickinson Biosciences) for spectratyping analyses. The relative proliferating index was calculated as follows: relative proliferating index = Geometric Mean Fluorescence of nonproliferating cells – Geometric Mean Fluorescence of total cells.

### RNA isolation, cDNA synthesis, and real-time quantitative RT-PCR

Total RNA was extracted by using the RNeasy Micro Kit (Qiagen, Hilden, Germany) and treated with DNase I to eliminate possible genomic DNA contamination. Taq-Man RT-PCR was performed as described elsewhere.<sup>E2</sup> Primers and probes used were purchased from Applied Biosystems (Warrington, United Kingdom).

### Evaluation of V $\beta$ repertoire by spectratyping

Total RNA was obtained by Trizol extraction protocol. All the RNA preparation was used to synthesize the first strand of the cDNA. This procedure was performed by using Sensiscript Reverse Transcriptase Kit (Qiagen S.p.a, Milan, Italy) according to the manufacturer's instructions. cDNA was checked for quality and quantity by PCR-specific reaction that amplifies a region between exon 1 and exon 2 of both TCR constant region genes (TCR C $\beta$ 1/C $\beta$ 2). TCR V $\beta$  gene family expression was analyzed by using a Multiplex-Hot Start PCR. By this method, 46 functional rearrangements of TCR V $\beta$  gene families in 5 reactions were studied. The amplified PCR products were size-fractionated on 2.2% miniagarose gel, and an aliquot of each labeled PCR product was loaded on 6% denaturing polyacrylamide sequencing gel and analysed on an ALF DNA-sequencing machine (Amersham-Pharmacia Biotech, Uppsala, Sweden). The data were analysed by the Fragment Manager software program (Amersham-Pharmacia Biotech).

### Western blot analysis

Whole cell extracts were prepared by lysing cells in RIPA buffer (Pierce Biotechnology, Rockford, Ill) containing a mixture of protease inhibitors, phosphatase inhibitors, and EDTA for 1 hour at 4°C. Cell extracts were centrifuged to pellet cell debris, and supernatants were analyzed for total protein content by using the Bradford Assay (Bio-Rad Laboratories, Hercules, Calif). Lysates were boiled at 95°C for 5 minutes for denaturation, and serial dilutions of proteins were prepared by adding an equal volume of Laemmli sample buffer.

Immunoblotting of cellular extracts was performed after SDS-PAGE on 12% TRIS-HCl Ready gels (Bio-Rad Laboratories), and proteins were then transferred to a nitrocellulose membrane by electroblotting. Equal loading across lanes and equal transfer were verified by staining all membranes with Ponceau-S and all gels with Brilliant Blue. Nonspecific binding was blocked for 4 hours at room temperature with 5% skim milk powder in TRIS-buffered saline containing 0.1% Tween. Membranes were then incubated for 1 hour with a goat anti-Jagged-1 primary antibody (C-20; 1:200 in 5% skim milk powder in TRIS-buffered saline containing 0.1% Tween; Santa Cruz Biotechnology, Santa Cruz, Calif), washed extensively, and incubated for an additional hour with a horseradish peroxidase-conjugated rabbit anti-goat IgG secondary antibody (1:1000; R&D Systems). The specific bands were visualized by the use of an enhanced chemiluminescence detection kit (Amersham Biosciences, Piscataway, NJ) according to the manufacturer's instructions.

Even loading of proteins was confirmed by stripping and reprobing membranes for the housekeeping protein  $\beta$ -actin, using a specific mouse monoclonal horseradish peroxidase-conjugated antibody (1:50,000; Sigma Chemical Co).

### siRNA delivery and gene silencing

A total of  $0.5 \times 10^6$  BDCA-1<sup>+</sup> cells and  $0.5 \times 10^6$  CD14<sup>+</sup>-derived DCs were transiently transfected respectively with 500 pmol hJagged1-siRNA (Iba, Gottingen, Germany) or hDLL4-siRNA (Ambion, Austin, Tex) by electroporation using the Amaxa nucleofection system with the human dendritic cell nucleofector kit (Amaxa GmbH, Cologne, Germany). As control, GFP-siRNA (Iba) was used. After the electroporation, cells were plated at a density of  $0.5 \times 10^6$  cells/mL, rested for 2 hours, and then stimulated with R-848 (2  $\mu$ g/mL) or with medium alone. To determine the extent of mRNA and protein knockdown, cells were harvested at 12 hours for RT-PCR and after 48 hours for Western blot analyses. Cells, after 12 hours, were also cocultured with CD4<sup>+</sup> T cells under the conditions described.

### Flow cytometry analysis

Flow cytometry analysis of cell suspensions for surface molecules and intracellular cytokines was performed as detailed elsewhere.<sup>E2</sup> Cells were analyzed on a BDLSRII cytofluorimeter using the Diva software (Becton Dickinson Biosciences), and  $10^4$  cells were analyzed for each experiment. The FASER technique was performed as indicated by the manufacturer (Faser kit-FITC, Miltenyi Biotec). Briefly, cells were labeled with a FITC-conjugated anti-Jagged-1 mAb (R&D Systems) and then incubated with FcR blocking reagent and FITC-Activator reagent for 10 minutes in the dark at 4 to 8°C.

After incubation, cells were washed and then incubated with FcR blocking reagent and FITC-Enhancer reagent for 10 minutes in the dark at 4 to 8°C. After extensive washing, the amplification of the FITC fluorescence was performed for an additional 2 rounds. Cells were then analyzed on a BDLSR II cytometer using the Diva software (Becton Dickinson). The area of positivity was determined by using an isotype-matched mAb. A total of  $10^5$  events for each sample were acquired.

### RNA isolation, cDNA synthesis, and real-time quantitative RT-PCR

Total RNA was extracted by using the RNeasy Micro Kit (Qiagen, Hilden, Germany) and treated with DNase I to eliminate possible genomic DNA

contamination. Taq-Man RT-PCR was performed as described elsewhere.<sup>E2</sup> Primers and probes used were purchased from Applied Biosystems (Warrington, United Kingdom).

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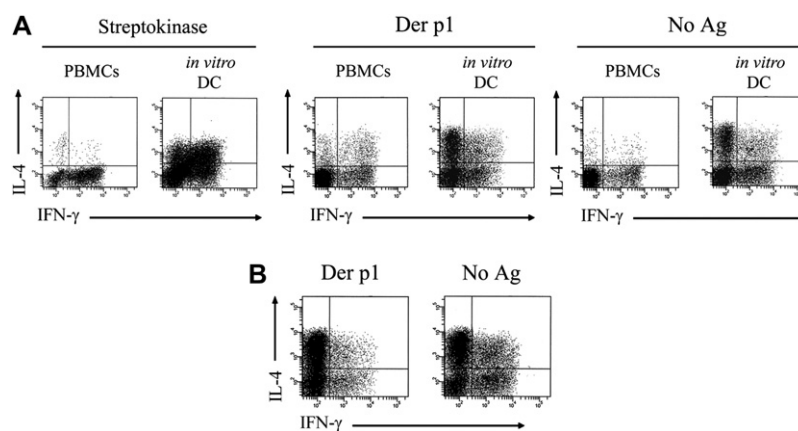


**FIG E1.** Circulating myeloid iDCs activate a T<sub>H</sub>2-polarizing program in CD4<sup>+</sup> T cells. **A**, Unfractionated PBMC or *in vitro*-derived iDCs plus autologous CD4<sup>+</sup> T cells were cultured for 16 days with or without streptokinase or Der p 1. One representative flow-cytometric analysis is shown. **B**, T<sub>H</sub>2 polarization induced on CD4<sup>+</sup> lymphocytes by freshly sorted BDCA-1<sup>+</sup> cells. For explanations, see A. Ag, Antigen.

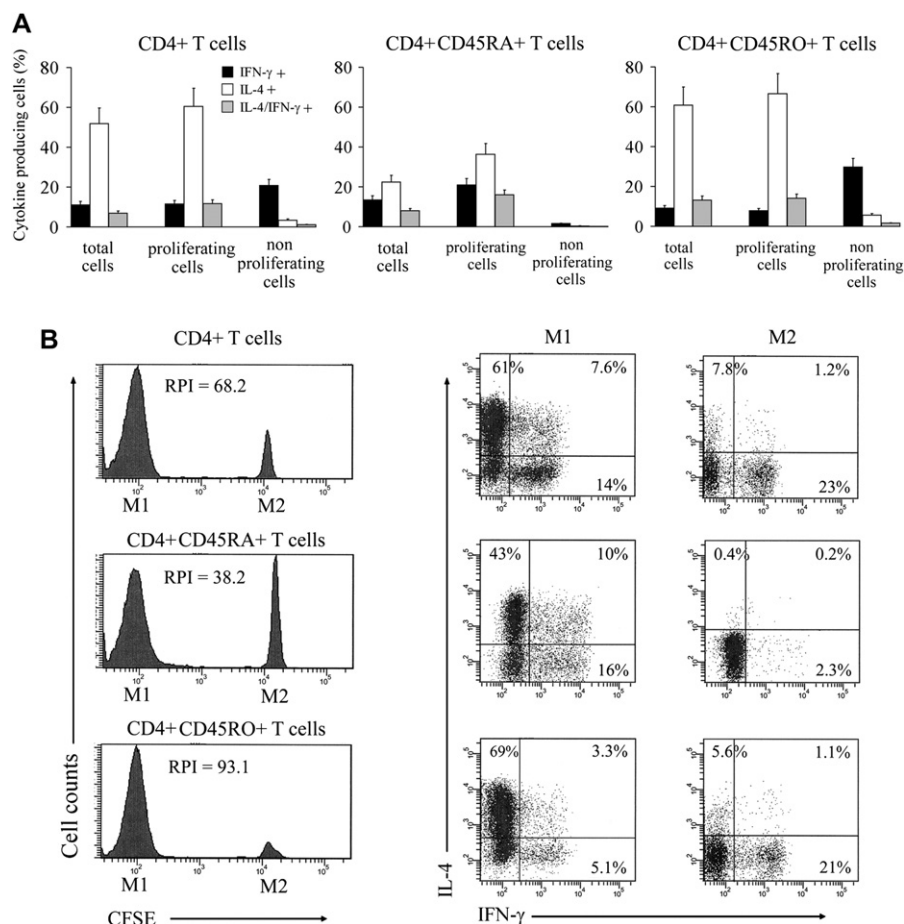


**FIG E2.** BDCA-1<sup>+</sup> cells selectively trigger a T<sub>H</sub>2-polarizing program in a proliferating subset of both naive and memory CD4<sup>+</sup> T cells. **A**, Total CD4<sup>+</sup> or separated CD4<sup>+</sup>CD45RA<sup>+</sup> or CD4<sup>+</sup>CD45RO<sup>+</sup> T cells were cocultured with autologous BDCA-1<sup>+</sup> cells and assessed by flow cytometry for cytokine production and proliferative activity. Columns represent mean values  $\pm$  SEs ( $n = 5$ ) of cytokine-producing CD4<sup>+</sup> T lymphocytes. **B**, One representative experiment is shown. *M1*, Proliferating cells; *M2*, nonproliferating cells; *RPI*, relative proliferating index.

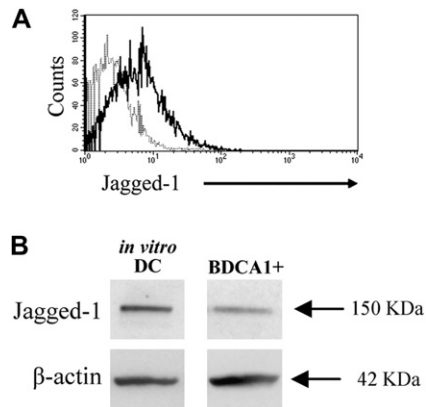


**FIG E3.** Jagged-1 expression by *in vitro*-derived iDCs and freshly isolated BDCA-1<sup>+</sup> cells. **A**, Jagged-1 expression by *in vitro*-derived iDCs was assessed by using the FASER technique associated with flow cytometry analysis. One representative experiment is shown. Dotted line represents the staining obtained with an isotype-matched mAb. **B**, Jagged-1 expression by *in vitro*-derived iDCs and freshly isolated BDCA-1<sup>+</sup> cells was assessed by Western blot technique. One representative experiment is shown.  $\beta$ -Actin expression is also reported as control of appropriate protein extraction and gel loading.

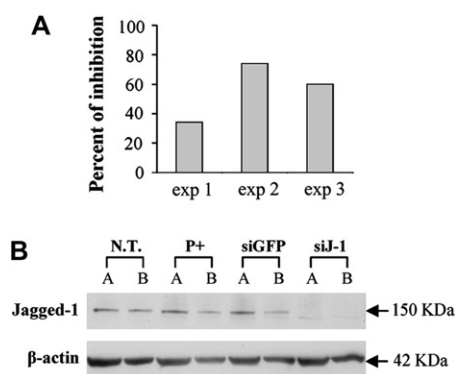


**FIG E4.** Jagged-1 expression by BDCA-1<sup>+</sup> cells on transfection with hJagged-1-siRNA. **A**, Jagged-1 mRNA expression was assessed by real-time RT-PCR 12 hours after transfection of BDCA-1<sup>+</sup> with hJagged-1-siRNA or GFP-siRNA as control. *Columns* represent the percentage of inhibition of Jagged-1 mRNA expression obtained in 3 different experiments. **B**, Jagged-1 protein expression was assessed by Western blot technique on BDCA-1<sup>+</sup> cells 48 hours after transfection. *NT*, Not transfected; *P*<sup>+</sup>, electroporated only; *siGFP*, GFP-siRNA; *siJ-1*, hJagged-1-siRNA; *A*, 28 μg proteins; *B*, 14 μg proteins. β-Actin expression is also reported as control of appropriate protein extraction and gel loading. *exp*, Experiment.

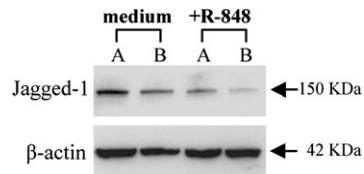


**FIG E5.** Ligation of TLR7/8 by R-848 on freshly derived BDCA-1<sup>+</sup> cells inhibits Jagged-1 expression. Jagged-1 protein expression was assessed by Western blot technique on BDCA-1<sup>+</sup> cells after 48 hours of culture in the presence or absence of R-848. A, 28  $\mu$ g proteins; B, 14  $\mu$ g proteins.  $\beta$ -Actin expression is also reported as control of appropriate protein extraction and gel loading.

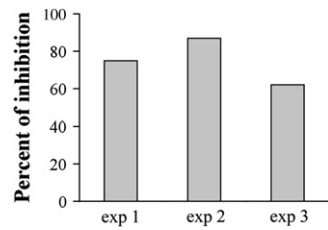


**FIG E6.** Delta-4 expression by BDCA-1<sup>+</sup> cells on transfection with hDelta-4-siRNA. Delta-4 mRNA expression was assessed by real-time RT-PCR 12 hours after transfection of BDCA-1<sup>+</sup> with hDelta-4-siRNA or GFP-siRNA as control. *Columns* represent the percentage of inhibition of Delta-4 mRNA expression obtained in 3 different experiments. *exp*, Experiment.