CD8$^-$ DCs induce IL-12–independent Th1 differentiation through Delta 4 Notch-like ligand in response to bacterial LPS

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Toll-like receptor (TLR) ligation is believed to skew T cell responses toward T helper (Th)1 differentiation by inducing interleukin (IL)-12 secretion by CD8$^+$ dendritic cells (DCs). However, TLR-dependent Th1 responses occur in the absence of IL-12. To determine how DCs induce Th1 differentiation in the absence of IL-12, we examined the response of IL-12–deficient DCs to bacterial lipopolysaccharide (LPS). We find that LPS activates MyD88-dependent Delta 4 Notch-like ligand expression by CD8$^-$ DCs, and that these cells direct Th1 differentiation by an IL-12–independent and Notch-dependent mechanism in vitro and in vivo. Thus, activation of the two DC subsets by TLR4 leads to Th1 responses by two distinct MyD88-dependent pathways.

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Pathogen eradication requires synergy between the innate and adaptive immune response. DCs are crucial in this respect because they efficiently capture and process antigens and deliver them to T cells, (1, 2). In addition, DCs direct immune responses by cell-to-cell contact and cytokine secretion (3, 4). IL-12 is a heterodimeric cytokine that plays a key role in the induction of cell-mediated immunity to pathogens (4). This cytokine is produced by macrophages and CD8$^+$ DCs upon Toll-like receptor (TLR) ligation (5–7), and in turn, it induces IFN-γ production and Th1 differentiation (8). Thus, IL-12 is an important bridge between the innate and adaptive immune systems.

However, Th1 responses can also take place in the absence of IL-12 (4, 9–11). Consistent with this finding, IL-12 deficiency leads to a much less severe phenotype than IFN-γ deficiency (12, 13). Other pathways that have been implicated in directing Th1 differentiation include direct cell–cell signaling through Notch (14–16). The interactions between Notch receptor and its ligands represent an evolutionary conserved pathway important for cell fate decisions (17). Mammals express four Notch genes (Notch 1–4) and five ligands for Notch from two conserved families, Jagged (Jagged 1 and 2) and Delta (Delta 1, 3, and 4; references 18 and 19). Notch signaling involves regulated proteolysis and nuclear translocation of the cytoplasmic domain of Notch, which functions as a transcription factor (17–19). The role of Notch in regulating lineage decisions in hematopoiesis and in the developing thymus has been well documented (18). However, the role of Notch signaling in mature T cell activation and Th polarization remains controversial (15, 16, 20–22). There is evidence that Notch activation is required to promote Th2 differentiation in vivo (21) and that Delta-like Notch ligands might promote Th1 polarization in vitro and in vivo (14, 16). Furthermore, inhibitors of γ-secretase, an enzyme regulating signaling through all four Notch receptors, block Th1 polarization in vivo and in vitro (15). In contrast, ablation of Notch 1 or RBP-Jk/CSL, which is a mediator of Notch function, had no detectable effect on Th1 polarization in vitro and in vivo (20, 21).

Here, we report that although LPS specifically induces MyD88-dependent expression of IL-12 by CD8$^+$ DCs, it also induces Delta 4 on spleen CD8$^-$ DCs, and that the latter leads to IL-12–independent Th1 differentiation in vivo. Thus, IL-12 and Notch mediate redundant MyD88-dependent pathways to Th1 differentiation in the two major spleen DC subsets, and this redundancy is at least in part responsible for the reported discrepancies in the role of Notch in Th1 development.

The online version of this article contains supplemental material.

RESULTS AND DISCUSSION

DC subsets direct Th1 development in vitro

Among cytokines, IL-4 and IL-12 are factors directing Th2 and Th1 cell development, respectively (4, 23). Antigen presentation by DCs to OVA-specific TCR transgenic $CD4^+$ T cells (OTII) induces Th1 differentiation in vitro, as determined by IFN-γ but not IL-4 secretion, and this is enhanced by DC stimulation with LPS (Fig. 1, A–C, and Figs. S1 and S2 A, which are available at http://www.jem.org/cgi/content/full/jem.20062305/DC1; references 6 and 7). To determine how DC subsets contribute to Th1 T cell differentiation, we performed antigen presentation experiments using $CD8^+$ and $CD8^-$ DCs purified from the spleens of mice injected with LPS and controls. We found that unstimulated $CD8^+$ DCs induce an increase in IFN-γ production, but $CD8^-$ DCs were much less active in this respect (Fig. 1 D). IL-4 and IL-10 were not detected in these same supernatants, suggesting that polarization was primarily to Th1. LPS increased IFN-γ production in T cell cultures containing $CD8^+$ DCs, but to a much greater extent in cultures containing $CD8^-$ DCs, as measured by ELISA and ELISPOT (Fig. 1 D and Fig. S2 B). Thus, steady-state $CD8^+$ DCs promote Th1 differentiation, but TLR ligation enables both DC subsets to skew T cell differentiation in this direction (24).

Splenic DC responses to LPS

IL-12 is produced by activated $CD8^+$ DCs after TLR ligation (5–7), and it skews immune responses by inducing production of IFN-γ and TNF-α by NK cells and by promoting differentiation of Th1 T cells (5, 23, 25). To determine how TLR ligation enhances the ability of DCs to induce Th1 differentiation, we assayed IL-12 production by DCs purified from wild-type and MyD88-deficient mice. Like others, we found that LPS injection or LPS addition to cultures of purified DCs stimulated $CD8^+$ DCs to produce IL-12 in an MyD88-dependent manner but failed to induce production of this cytokine by $CD8^-$ DCs, and neither DC subset produced IL-4 or IL-10 (Fig. 2, A and B, and not depicted; references 6, 7, and 24). Therefore IL-12 secretion cannot account for the Th1 responses induced by LPS-activated $CD8^-$ DCs. IL-12 is not essential for Th1 immune responses (9–11). Other cytokines might direct the residual Th1 response in IL-12–deficient mice, for example IL-18, which promotes IFN-γ production in $CD4^+$ T cells. However, studies with *Toxoplasma gondii* (26) and mycobacteria (27) failed to reveal a function for this cytokine in Th1 development in the absence of endogenous IL-12. IL-23 is another candidate cytokine mediator of Th1 development, but this IL-12–related factor shares the p40 subunit, which is defective in IL-12–mutant mice (9). Therefore, neither IL-18 nor IL-23 can account for the residual Th1 response found in the absence of IL-12.

Delta 1 expression on fibroblasts can promote Th1 differentiation of transgenic CD4 T cells in vitro (14). All four Notch receptors are expressed on mature T cells (18). Less is known about the expression of Notch ligands on DCs. Cultured bone marrow DCs up-regulate Delta 4 upon LPS stimulation, low levels of Delta 1 have been reported on spleen DCs, and both Jagged 1 and 2 are expressed

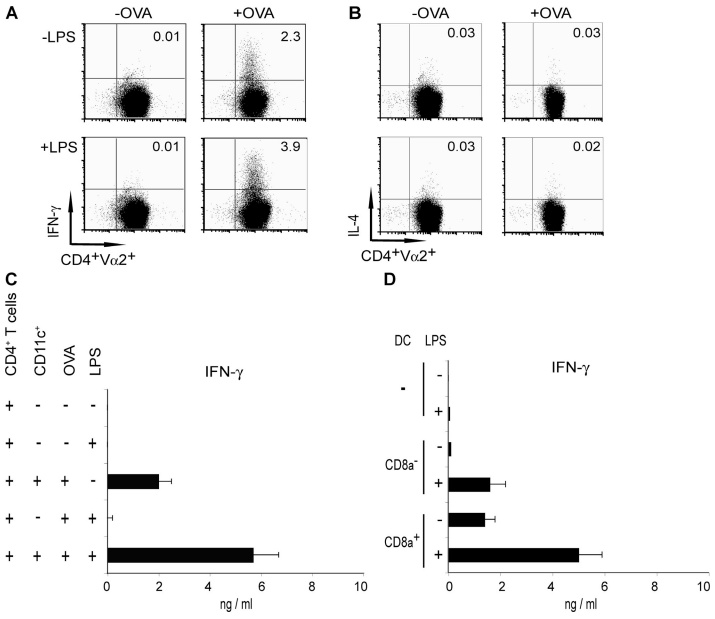

Figure 1. LPS increases the capacity of splenic DC subsets to induce Th1 differentiation. (A and B) FACS plots show intracellular IFN-γ and IL-4 production by $CD4^+$ OTII T cells cultured with antigen and $CD11c^+$ cells (2×10^4/well) purified from C57BL/6 mice injected with 25 μg LPS for 12 h. (C) Bar graph indicates IFN-γ production measured by ELISA in the supernatant of cultures containing $CD4^+$ OTII T cells, antigen, and splenic $CD11c^+$ cells that had been pretreated with 50 ng/ml LPS for 6 h in vitro (D) as in B, except that $CD8^+$ or $CD8^-$ DCs were used as APCs.

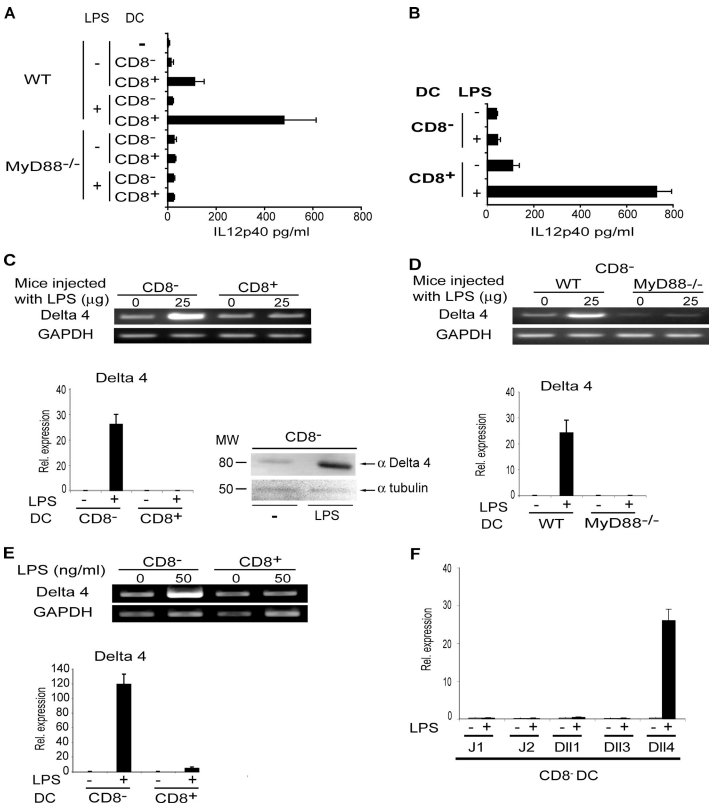

Figure 2. DC subset responses to LPS stimulation. (A) IL-12 secretion by $CD8^+$ and $CD8^-$ DCs purified from LPS-injected $MyD88^{-/-}$ or C57BL/6 mice uninjected controls. Supernatants were harvested after 10 or 48 h (the 48-h time point is shown), and IL-12p40 was measured by ELISA. IL-4 and IL-10 levels were below the limits of detection of the assay (5–10 pg/ml). (B) IL-12 secretion by $CD8^+$ and $CD8^-$ DCs purified from C57BL/6 mice stimulated with 50 ng/ml LPS for 6–10 h in vitro. (C) RT-PCR and quantitative PCR for Delta 4 expression by $CD8^+$ and $CD8^-$ DCs purified from LPS-injected C57BL/6 mice and uninjected controls. (Right) Western blot for Delta 4 using 4×10^5 cells per lane of lysates from $CD8^+$ and $CD8^-$ DCs purified from LPS-injected (12 h) C57BL/6 mice and controls. The blot was reprobed for α-tubulin. (D) RT-PCR and quantitative PCR for Delta 4 expression by $CD8^-$ DCs purified from LPS-injected $MyD88^{-/-}$ or C57BL/6 mice and uninjected controls. (E) RT-PCR and quantitative PCR for Delta 4 expression by $CD8^+$ and $CD8^-$ DCs treated for 6 h with 50 ng/ml LPS and untreated controls. (F) Quantitative PCR for Delta 1 and 3 and Jagged 1 and 2 expression by $CD8^-$ DCs purified from LPS-injected C57BL/6 mice and uninjected controls. GAPDH and β-actin controls were used in RT-PCR and quantitative PCR experiments, respectively.

constitutively on bone marrow and spleen DCs (14, 28). To determine whether spleen DCs up-regulate Notch ligands upon LPS stimulation, we purified $CD8^+$ and $CD8^-$ DCs from mice injected with LPS and measured Delta 4 mRNA and protein expression by conventional and quantitative PCR, as well as by immunoblotting (Fig. 2 C). Although Delta 4 was not expressed by DCs in the steady state, it was rapidly induced on $CD8^-$ but not on $CD8^+$ DCs by LPS, and this effect was MyD88 dependent (Fig. 2 D). IL-12 and Delta 4 synthesis were cell autonomous because isolated $CD8^+$ and $CD8^-$ DCs stimulated with LPS in vitro expressed these molecules, respectively (Fig. 2, B and E). In contrast, Delta 1 and 3 and Jagged 1 and 2 (14) were not up-regulated upon LPS stimulation (Fig. 2 F). Thus, LPS induces MyD88-dependent IL-12 secretion by $CD8^+$ DCs and Delta 4 up-regulation by $CD8^-$ DCs, suggesting that Delta 4 expression by these cells might be the alternative signal for Th1 differentiation.

$CD8^+$ and $CD8^-$ DCs induce Th1 development by different mechanisms

Signaling by direct cell-to-cell contact can also instruct T cell differentiation in the thymus and in the periphery. Notch family members are important mediators of this type of signaling and contribute to essential aspects of thymocyte development and T cell lineage commitment (18, 19). Delta 1 has been implicated in directing Th1 development in vitro and in vivo, and Jagged is believed to induce Th2 differentiation, but the relative roles of IL-12 and the Notch pathway in Th1 differentiation in vivo have not been defined (14, 16). To determine the relative function of IL-12 and Delta 4 in DC-induced Th1 differentiation, we purified DCs from LPS-injected $MyD88^{-/-}$, $IL12p40^{-/-}$, $IL\text{-}12p35^{-/-}$, and wild-type control mice, cultured them with antigen and naive CD4 T cells, and measured IFN-γ production by ELISA and ELISPOT. As expected, induction of IFN-γ secretion by $CD8^+$ DCs was IL-12 dependent (Fig. 3 A and Fig. S3A, which is available

at http://www.jem.org/cgi/content/full/jem.20062305/DC1; references 6, 7, and 25). In contrast, induction of Th1 differentiation by CD8$^-$ DCs was MyD88 dependent but IL-12 independent (Fig. 3 A). To determine whether Delta 4 signaling was required for induction of Th1 differentiation, we blocked it using a soluble Delta 4–mFc fusion protein (sD4-mFc; Fig. 3 B and Figs. S3 B and S4). As Notch receptor signaling requires receptor cross-linking, a mutation was inserted into the Fc portion of the Delta 4–Fc fusion protein, thus disabling its ability to bind to Fc receptors (Delta 4–mFc). The presence of this mutant Delta 4–Fc protein was expected to compete with endogenous Delta 4 for binding to Notch, preventing the normal function of this molecule expressed on cultured cells. We found that the addition of sD4-mFc to cultures of activated CD8$^-$ DCs did not effect T cell proliferation or antigen-dependent T cell activation, as measured by up-regulation of CD69 expression (Fig. S5); however, it inhibited induction of IFN-γ secretion (Fig. 3 B and Fig. S3 B). In contrast, the addition of sD4-mFc to cultures of T cells responding to antigen presented by activated CD8$^+$ DCs did not have any effect on IFN-γ production (Fig. 3 B). We conclude that LPS specifically induces MyD88-dependent Delta 4 expression by CD8$^-$ DCs, and that engagement of this Notch ligand by antigen-responsive T cells directs Th1 T cell differentiation.

To further confirm the role of Delta 4 in Th1 differentiation, we used I-E^k– and Delta 4 ligand–expressing fibroblasts as APCs to differentiate naive AND TCR transgenic CD4 T cells (14). We found that surface Delta 4 expression on APCs (Fig. S6, available at http://www.jem.org/cgi/content/full/jem.20062305/DC1) enhanced Th1 development, as measured by the induction of IFN-γ but not IL-4 production (Fig. 3, C and D). Thus, Delta 4 ligand actively participates in Th1 differentiation.

IL-12–independent Th1 responses by Delta 4 Notch-like ligand in vivo

Several studies have examined the role of Notch signaling in Th polarization, but the results were conflicting (15, 16, 20–22). Although some have found that the Notch pathway can induce IFN-γ production (14–16), others have shown that ablation of Notch 1 or RBP-Jk/CSL, the major mediator of signaling through all four Notch receptors, did not measurably interfere with Th1 responses (14, 20, 21). However, the gene knockout experiments were performed using IL-12–sufficient mice.

To examine the role of Delta 4 in IL-12–independent Th1 T cell differentiation in vivo, we asked whether sD4-mFc could block the differentiation of OVA-specific CD4$^+$ T cells into Th1 cells in IL-12p40$^{-/-}$ mice. OTII T cells were transferred into IL-12p40$^{-/-}$ or control mice that were injected with a mixture of LPS and OVA in the presence or absence of sD4-mFc. CD4 T cell clonal expansion and

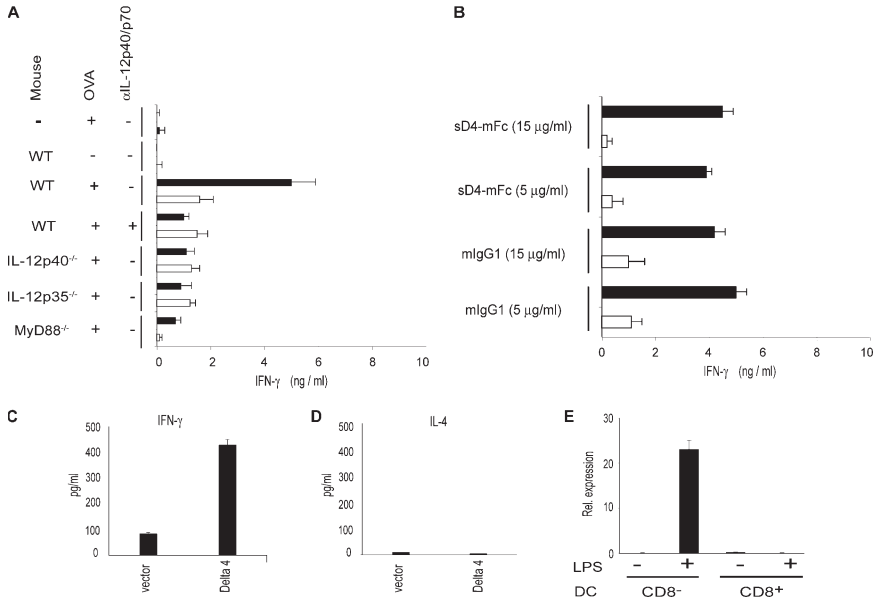

Figure 3. IL-12–independent Th1 differentiation in vitro. (A) IFN-γ secretion was measured by ELISA in cultures containing CD4$^+$ OTII T cells, antigen, and CD8$^+$ DCs (filled bars) or CD8$^-$ DCs (open bars) purified from LPS-injected MyD88$^{-/-}$, IL-12p40$^{-/-}$, IL-p35$^{-/-}$, or C57BL/6 wild-type mice. Anti–IL-12p40/p70 indicates cultures to which anti–IL-12 was added. (B) IFN-γ production measured by ELISA in cultures containing soluble D4-mFc (15 and 5 μg/ml) or mouse IgG (15 and 5 μg/ml) control, CD4$^+$ OTII T cells, antigen, and CD8$^+$ DCs (filled bars) or CD8$^-$ DCs (open bars) purified from LPS-injected C57BL/6 mice. (C and D) Naive CD4 T cells were isolated from AND TCR transgenic mice and cultured in vitro with I-E^{k+} control (vector), Delta 4–expressing APCs (3 × 10^5/well; DCEK hi7) pulsed with 0.1 μg/ml mcc peptide (see Fig. S6). After 5 d, 10^5/well viable T cells were stimulated with plate-bound anti-CD3. Supernatants were taken after 48 h, and cytokine concentrations were determined by ELISA. The results are representative of two independent experiments. (E) Quantitative PCR for Delta 4 expression by splenic DC subsets purified from LPS-injected B10.BR mice and uninjected controls.

IFN-γ production were measured 2 wk after antigen challenge. In agreement with the work of others, we found that LPS and OVA induced antigen-specific T cell clonal expansion and IFN-γ production in wild-type mice, and that this was substantially decreased but not abolished in IL-12$^{-/-}$ recipients (Fig. 4 A; references 7 and 9). Injection of sD4-mFc reduced the IFN-γ production in IL-12$^{-/-}$ mice to nearly baseline levels (Fig. 4). We conclude that Delta 4 Notch-like ligand induces IL-12–independent Th1 differentiation in vivo.

Our experiments show that IL-12 and Notch are redundant and that IL-12 is responsible for up to 85–90% of the Th1 response, whereas the contribution by Notch is only 10–15%. Therefore, blocking Notch in the presence of IL-12 would not be expected to produce major changes in the Th1 response. Furthermore, our data is consistent with the finding that the intracellular domain of Notch binds directly to and activates the T-bet promoter, thereby potentiating Th1 responses (14–16, 22, 29). In BALB/c mice injected with anti-CD40/Poly:IC, CD8$^+$ DCs can also induce CD4$^+$ T cells to produce IFN-γ by an additional IL-12–independent pathway involving CD70 (30).

We have shown that TLR4 ligation by LPS induces MyD88-dependent expression of Delta 4 Notch ligand specifically on the CD8$^-$ DC subset, and that Delta 4 expression by these cells mediates Th1 development, accounting for IL-12–independent Th1 development in vivo. However, this alternative pathway to Th1 differentiation appears to account for only 10–15% of the total Th1 response in wild-type mice. Nevertheless, several IFN-γ–dependent responses can proceed in the absence of IL-12 (4, 9–11). Furthermore, it is important to note that mice and humans deficient in IL-12 are very different from those that are IFN-γ deficient in that the former are only susceptible to a small number of pathogens (12, 13). Therefore, the existence of physiologically important IL-12–independent pathways to stimulate IFN-γ is well documented. Our work identifies one such pathway and the cell that induces it.

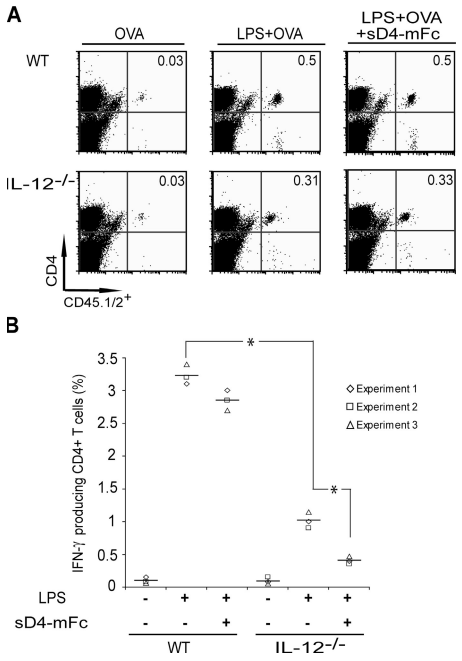

Figure 4. Delta 4 induces IL-12–independent Th1 differentiation in vivo. (A) Dot plots show the number of OTII T cells in the spleens of C57BL/6 SJL (WT) or IL-12$^{-/-}$ mice 2 wk after immunization with soluble OVA (2 mg/mouse) or OVA plus LPS (25 μg/mouse) in the presence or absence of sD4-mFc (100 μg/mouse). Numbers indicate percentages of CD45.1$^+$ or CD45.2$^+$ among CD4$^+$ T cells. (B) Plots show numbers of IFN-γ–producing OTII cells (ICS). Numbers indicate percentages of CD45.1$^+$ or CD45.2$^+$ among CD4$^+$ T cells.

The two DC subsets in the mouse spleen (2, 31) share several features, including a common precursor (32), but they differ in several important respects (2, 31). The two cell types show distinct global gene expression profiles (33). CD8$^+$ DCs have a unique capacity to take up dying cells in vivo and are enriched in components of the MHC I–processing pathway (34), whereas CD8$^-$ DCs are specialized to efficient antigen processing and presentation on MHC II (35). CD8$^+$ DCs are found primarily in the T cell areas, whereas CD8$^-$ DCs reside in bridging channels, the marginal zone, and the red pulp, but they rapidly migrate into the T cell zone upon LPS administration or bacterial infection (5). Finally, CD8$^+$ DCs appear to be more effective in inducing Th1 T cell differentiation (6, 7, 25, 36). Nevertheless, we and others find that both CD8$^+$ and CD8$^-$ DCs support Th1 differentiation (9). After TLR ligation, CD8$^+$ DCs secrete IL-12, whereas the same signaling pathway leads to synthesis of Delta 4 in CD8$^-$ DCs. Why the two types of DCs evolved distinct mechanisms to stimulate Th1 differentiation is not apparent but could be related to their specialization in antigen processing, anatomic location, or simply to an advantage in functional redundancy for regulating the Th balance in immune responses in vivo.

MATERIALS AND METHODS

Animals and procedures. C57BL6/J, C57BL6/SJL, IL-12p40$^{-/-}$, IL12p35$^{-/-}$, and OTII mice were purchased from The Jackson Laboratory. MyD88$^{-/-}$ mice were provided by S. Akira (Osaka University, Osaka, Japan). Mice were used for experiments at the age of 6–8 wk. All mice were housed in specific pathogen-free conditions and were treated in accordance with the institutional Animal Care and Use Committee protocols of The Rockefeller University.

T cell differentiation experiments. CD4$^+$ CD62L^{high} CD25^{low} T cells were purified from the spleens of OTII mice using a CD4$^+$ T cell isolation kit (Miltenyi Biotec), followed by single cell sorting using mAbs against CD62L, CD25, and CD4 (BD Biosciences). IFN-γ production was measured by ELISPOT or by intracellular cytokine staining (ICS). ELISPOT plates (MAHAS; Millipore) were coated with 10 μg of the anti–mouse IFN-γ mAb (clone R4-6A2; BD Biosciences) overnight at room temperature, and plates were blocked by incubation in PBS 1% BSA for 2 h at 37°C. CD4$^+$ T cells (2×10^5/well) were then cultured for 48 h at 37°C in the presence of 3 μM of OVA cognate peptide and CD11c$^+$ DCs or CD11c^{high} CD8$^+$ DEC$^+$ or CD11c^{high} CD8$^-$ DEC$^-$ DCs (2×10^4/well). CD11c$^+$ cells were purified using a CD11c$^+$ isolation kit (Miltenyi Biotec) and where indicated preincubated for 6 h with 50 ng/ml LPS from *Salmonella abortus equi* (Sigma-Aldrich). Splenic CD11c^{high} CD8$^+$ DEC$^+$ and CD11c^{high} CD8$^-$ DEC$^-$ DCs were purified from mice before or 12 h after injection with LPS (25 μg/mouse) by negative selection using CD19,

CD90, and DX5 beads (Miltenyi Biotec) and cell sorting on a FACSVantage (BD Biosciences) using anti–DEC-biotin (Rockefeller University antibody facility) and streptavidin–Pe-Cy7, with anti–CD11c-PE and anti–CD8-FITC (BD Biosciences). The purity of DC subsets was >99%. For Western blot, the following antibodies were used: mouse monoclonal anti-Dll4, anti–α tubulin (R&D Systems and Abcam), anti–rat IgG2a–horseradish peroxidase, and anti–rabbit–horseradish peroxidase (Southern-Biotech and Jackson ImmunoResearch Laboratories). Cells were cultured in RPMI medium (10% FBS, penicillin, streptomycin, sodium pyruvate, and L-glutamine). Plates were developed with anti–IFN-γ, anti–IL-4, or anti–IL-10 biotinylated antibody (BD Biosciences), and spots were visualized with avidin–horseradish peroxidase (Vector Laboratories), followed by DAB as substrate (Invitrogen). Spots were counted in an ELISPOT reader (Autoimmun Diagnostika GmbH).

ICS was performed on a 72-h co-culture of splenic $CD11c^+$ and $CD4^+$ T cells in the presence or absence of 3 μM OTII peptide. Brefeldin A (Sigma-Aldrich) was added in the co-culture for the last 4 h. Cells were harvested, stained for extracellular CD4 and Vα2, and then fixed and stained for IFN-γ or IL-4 (Intracellular Staining kit; BD Biosciences). IFN-γ, IL-4, IL-10, and IL-12 secretion was analyzed using ELISA Set, BD OptEIA (BD Biosciences). Concentrations were determined based on standard curves of recombinant IFN-γ, IL-4, IL-10, and IL-12 provided by the manufacturer.

2 μM CFSE (Invitrogen) -labeled $CD4^+$ OTII T cells (10^6/well) were cultured for 60 h at 37°C with 5% CO_2 in the presence of 3 μM of OVA cognate peptide and $CD11c^{high}$ $CD8^+$ DEC^+ or $CD11c^{high}$ $CD8^-$ DEC^- DCs (10^5/well) purified as described previously. Cells were then stained with APC-conjugate anti-CD4 and PE-conjugate anti-CD69, and T cell proliferation and activation was followed by multicolor flow cytometry (FACSCalibur; Becton Dickinson).

RT-PCR. cDNA was generated from DNase1-treated (DNA free; Ambion) RNA (isolated using RNA-Bee; Tel-Test, Inc.). Fluorogenic probes were obtained from Biosearch Technologies. Quantitative PCR was performed for 40 cycles using an ABI (model 7900HT; Applied Biosystems). Samples were normalized for β-actin contents as described previously (14). Concentrations were determined on the basis of standard curves of plasmid DNA using software provided by the manufacturer.

Plasmid constructions. cDNA for mouse Delta 4 was provided by A. Duarte (Technical University of Lisbon, Lisbon, Portugal) and A. Freitas (Institut Pasteur, Paris, France). To generate soluble Delta 4, the cDNA was truncated at the codon CCC corresponding to proline (amino acid 517). cDNA for Fc (mIgG1) sequence was fused to the 3′ end coding region of mouse Delta 4 cDNA as described previously (37). Soluble D4-mFc was produced by transient transfection of 293T cells and was purified as described previously (38).

Notch ligand binding assay. Delta-Serrate-Lag2 proteins are known to be ligands for Notch 1 and 2 receptors (37, 39). Binding of soluble Delta-Serrate-Lag2 protein to the surface of pro–B cell line 32D was performed as described previously (37).

Delta 4 expression on APCs and in vitro T cell differentiation. DCEK hi7 $I\text{-}E^k$–expressing fibroblasts (provided by D. Amsen, University of Amsterdam, Amsterdam, Netherlands; reference 14) were transduced with a PMXpie retrovirus encoding an IRES GFP-linked mouse Delta 4 cDNA. $I\text{-}E^k$ and CD80 levels were measured by staining with anti–$I\text{-}E^k$ and anti-CD80 antibodies (BD Biosciences; Fig. S6). 3×10^5 DCEK hi7 cells (treated for 1 h at 37°C with 50 μg/ml mitomycin C [Sigma-Aldrich]) were incubated with naive AND CD4 T cells (2.5×10^5 /well; 24-well plates; Falcon) and the 81–103 moth cytochrome C peptide. Viable effector cells were isolated using Ficoll and restimulated at 10^5 cells per well (96-well plate; Falcon) with plate-bound anti-CD3. Cytokine concentration (48-h supernatants) was determined by ELISA (BD Biosciences).

Online supplemental material. Fig. S1 shows purity profiles of naive $CD4^+$ OTII T cells. Fig. S2 shows the role of LPS in the ability of splenic DC subsets to induce Th1 development. Fig. S3 shows the existence of an IL-12–independent Th1 differentiation pathway in vitro. Fig. S4 shows production of soluble Delta 4–mFc fusion protein. Fig. S5 shows the effect of sDelta 4–mFc on T cell activation and proliferation. Fig. S6 shows expression level of Delta 4 in transfected fibroblasts. The online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20062305/DC1.

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