

Jagged1 on Dendritic Cells and Notch on CD4⁺ T Cells Initiate Lung Allergic Responsiveness by Inducing IL-4 Production^{1,2}

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Jagged1, a Notch ligand, and Notch have been implicated in Th2 differentiation, but their role in initiating IL-4 production and Th2 differentiation in vivo and the development of allergic airway responses has not been defined. In this study, we show that Jagged1 is up-regulated on bone marrow-derived dendritic cells (BMDCs) pulsed with allergen and that the transfer of these BMDCs before allergen challenge induces airway hyperresponsiveness (AHR) and eosinophilic airway inflammation. Treatment of CD4⁺ T cells with a γ -secretase inhibitor (GSI), which inhibits Notch signaling, resulted in decreased cytokine production when the cells were cocultured with allergen-pulsed, Jagged1-expressing BMDCs and, after the transfer of allergen-pulsed BMDCs, IL-4-deficient (IL-4^{-/-}) recipients of GSI-treated naive CD4⁺ T cells developed lower levels of AHR, reduced numbers of eosinophils, and lower Th2 cytokine levels when challenged with allergen. In vivo treatment of wild-type mice with Jagged1-Fc enhanced AHR and airway inflammation, whereas the transfer of BMDC transfected with *Jagged1* small interfering RNA (siRNA) cells into WT or IL-4^{-/-} mice before transfer of CD4⁺ T cells resulted in decreased AHR, inflammation, and Th2 cytokines, indicating the critical role for Jagged1 expression on APCs. These data identify the essential role of the interactions between Notch on CD4⁺ T cells and Jagged1 on APCs in the initiation of IL-4 production and Th2 differentiation for the development of AHR and allergic airway inflammation. *The Journal of Immunology*, 2009, 183: 2995–3003.

Asthma is a complex disease characterized by persistent airway inflammation and airway hyperresponsiveness (AHR)⁴ (1) in response to the inhalation of airborne allergens, infectious pathogens, or chemical agents (2). A number of cell types, including Th2 cells, eosinophils, and mast cells are recruited to the lung and activated to release various cytokines and chemokines, enhancing airway inflammation (3). Several clinical and experimental investigations have shown that CD4⁺ T cells, especially Th2-type cells, play a pivotal role in the development of AHR and eosinophilic inflammation (1, 3–10).

Naive Th cells (Th0) have the potential to differentiate into IFN- γ producing Th1 cells, Th2 cells secreting IL-4, IL-5, and IL-13, or IL-17-producing Th17 cells (11). In the process of differentiation, naive T cells first encounter Ags presented by dendritic cells (DCs) in the T cell zones of secondary lymphoid organs; these DCs have intrinsic capacities to drive either Th1 or Th2 responses. When DCs recognize bacterial and viral products via

TLRs, the cells produce IL-12 and induce Th1 polarization (12). Although these TLR-stimulated DCs promote Th1 differentiation, DCs stimulated with agents such as fungal products, parasitic nematodes, or cholera toxin induce Th2 responses (12, 13). Central to initiating Th2 differentiation is IL-4, which synergizes with TCR signals to induce Th2 differentiation through the activation of STAT6, which up-regulates Gata3 (14–16). In animal models of allergic asthma, transfer of Th2-type cells into mice induces airway eosinophilia and AHR following allergen challenge (17, 18). We and others previously demonstrated that IL-4 produced from CD4⁺ T cells played an essential role in the development of AHR and airway inflammation and that exogenous IL-4 administration restored AHR and allergic airway inflammation by driving Th2 differentiation in the sensitization but not the challenge phase (19–21). Although the critical roles for Th2 cytokines in the pathogenesis of allergic asthma have been well established, the initial events resulting in IL-4 release in vivo and its importance as a Th2-initiating event under physiological conditions is not well defined.

Recently, the differential expression of Notch ligands on APCs in concert with Notch receptors on T cells has been shown to promote Th0 differentiation in response to different Th1- or Th2-promoting stimuli (22–25). In vertebrates, there are four Notch receptors (Notch1–4) and five Notch ligands, the Delta-like families (Delta1, Delta3, and Delta4) and the Jagged families (Jagged1 and Jagged2) (26). Notch receptors and their ligands are expressed on the surface of mature lymphocytes and APCs, respectively. Notch proteins are transcriptional activators expressed as transmembrane heterodimeric surface receptors. Ligand binding releases the Notch intracellular domain by proteolytic cleavage; this allows the Notch intracellular domain to enter the nucleus and transactivate genes through its association with the CSL/RBP-J transcription factor and coactivators of the Mastermind-like family (26–28). γ -Secretase inhibitors (GSI) can effectively prevent enzymatic cleavage of the cytoplasmic domain of Notch receptors, thereby inhibiting the downstream signaling events triggered by

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⁴ Abbreviations used in this paper: AHR, airway hyperresponsiveness; BAL, bronchoalveolar lavage; BMDC, bone marrow-derived dendritic cell; DC, dendritic cell; GSI, γ -secretase inhibitor; MCh, methacholine; R_L, lung resistance; siRNA, small interfering RNA; WT, wild type.

activation through these receptors (29). Studies have implicated Notch in the activation (30–33) and differentiation (23, 25, 34) of cells of the peripheral immune system. RBP-J-deficient mice or mice expressing a dominant-negative form of the Mastermind-like protein suppressed both IL-4 production and Th2 responses (23, 35). We recently reported that Notch and Delta1 interactions in vivo inhibit the development of AHR and airway inflammation accompanied by heightened Th1 responses (36).

In this study, we define the important role of Notch-Notch ligand (Jagged1) interactions in vivo in the sensitization phase of the development of AHR and other lung allergic responses. *Jagged1* expression was enhanced on Ag-pulsed bone marrow-derived dendritic cells (BMDCs). Using an approach where Ag-pulsed BMDCs are transferred into naive recipients before allergen challenge, the full spectrum of lung allergic responses can be triggered. Using this model, we demonstrated that inhibition of Notch signaling on CD4⁺ T cells using GSI or the inhibition of Jagged1 expression on BMDCs using small interfering RNA (siRNA) prevented the development of AHR and airway inflammation. In contrast, administration of Jagged1-Fc augmented AHR and airway inflammation. These results indicated that Notch-Notch ligand (Jagged1) interactions in vivo regulated the initiation of allergic airway disease by controlling the induction of IL-4 production, initiating Th2 differentiation.

Materials and Methods

Mice

Wild-type (WT) C57BL/6 and IL-4-deficient (IL-4^{-/-}) mice were purchased from The Jackson Laboratory. The mice were housed under specific pathogen-free conditions and maintained on an OVA-free diet in the Biological Resources Center at National Jewish Health (Denver, CO). Both female and male mice, 8–12 wk of age, were used in these experiments and each experiment was independently performed at least three times with four mice per group ($n = 12$). Controls were matched to the deficient mice with regard to both age and gender in each experimental group. All experimental studies were conducted under a protocol approved by the Institutional Animal Care and Use Committee of National Jewish Health.

BMDC generation

BMDCs were differentiated from bone marrow cells according to the procedure described by Inaba and colleagues (37, 38), with some modifications. Briefly, bone marrow cells were flushed from the femurs and tibias of C57BL/6 mice, washed, and cultured in complete medium (RPMI 1640 containing 10% heat-inactivated FCS, 50 μ M 2-ME, 2 mM L-glutamine, penicillin (100 U/ml) and streptomycin (100 μ g/ml) from Invitrogen and recombinant mouse GM-CSF (10 ng/ml) and recombinant mouse IL-4 (10 ng/ml) from R&D Systems). Nonadherent granulocytes were removed after 48 h of culture and fresh complete medium was added every other day. All cultures were incubated at 37°C in 5% humidified CO₂. After 7 days of culture, > 80% of the cells expressed characteristic DC-specific markers (CD11c⁺) as determined by flow cytometry. For some experiments, BMDCs on day 7 were cultured with OVA (200 μ g/ml; Fisher Scientific) for 24 h. The LPS content in the solution was 1.6 ng/ml. These BMDCs were used in immunoblot analyses or total RNA was extracted from them for real-time PCR. All data were representative of at least three independent experiments.

For siRNA transfection, BMDCs were washed and plated in 24-well plates at a concentration of 2×10^5 cells/well in 400 μ l of serum-free RPMI 1640. After 24 h of transfection, the cells were cultured with or without OVA for 24 h and washed three times with PBS.

BMDC transfection by siRNA

BMDCs were transfected with 21-bp siRNA sequences specific for Jagged1 (5'-CTCGTAATCCTTAATGGTT-3') synthesized and annealed by the manufacturer (Dharmacon). Scrambled siRNA controls were used to establish a baseline response that could be compared with the levels in cells treated with target-specific siRNA. Transfection was conducted as described previously (39, 40). Briefly, 3 μ l of 20 μ M annealed siRNA was incubated with 3 μ l of GenePorter (Gene Therapy Systems) in a volume of 94 μ l of serum-free RPMI 1640 at room temperature for 30 min. This was then added to each well containing BMDCs and incubated for 4 h at 37°C. Three

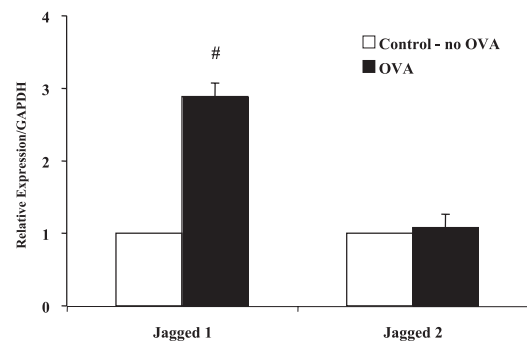


FIGURE 1. Real-time PCR analysis of Notch ligand expression in BMDCs pulsed with OVA. BMDCs from WT mice were incubated with or without OVA for 24 h and mRNA was isolated. The relative expression levels of Notch ligands (Jagged1 or Jagged2) were determined by quantitative real-time PCR. cDNA contents were normalized to levels of GAPDH. Results are from three independent experiments and the results for each group are expressed as mean $\pm$ SEM. #, $p < 0.05$, significant differences comparing BMDCs pulsed with OVA and BMDCs alone.

μ l of GenePorter alone were used as mock controls. After incubation, 500 μ l/well RPMI 1640 supplemented with 20% FCS was added to the cells. Twenty-four hours later, transfected or treated BMDCs were washed and used in subsequent experiments.

CD4⁺ T cell preparation

Purification of CD4⁺ T cells from C57BL/6 mice was conducted as previously described (41). Briefly, spleen cells from naive mice were harvested by mincing the tissues and subsequently passing them through a stainless steel sieve. After washing with PBS, mononuclear cells were isolated by Histopaque gradient centrifugation (Sigma-Aldrich). Purification of CD4⁺ T cells was conducted by negative selection using a mouse CD4⁺ T cell recovery column kit (purity, >95%; Cedarlane Laboratories) in accordance with the manufacturer's instructions. Purity of CD4⁺ T cell populations after purification exceeded 95% as assessed by flow cytometry. For some experiments, isolated CD4⁺ T cells were cultured with GSI (20 μ M) (dibenzazepine; Calbiochem-EMD Biosciences) or DMSO (0.1% final concentration) for 24 h.

Preparation of soluble Jagged1-Fc

The extracellular portion of Jagged1 cDNA (the sequence between nt 1 and 3276) was originally obtained by PCR using C57BL/6 splenocytes as a template. A cDNA for the Fc portion of human IgG1 (IgG1-Fc) was constructed in-frame to the 3' end of the cDNAs encoding the extracellular region of Jagged1 in the expression vector pcDNA3.1 (Invitrogen). Chinese hamster ovary cells were transfected with these pcDNA3.1 plasmid-containing cDNAs for the Jagged1-Fc protein using the FuGENE 6 transfection reagent (Roche Applied Science). After culture of these cells for several days, the supernatants were collected and soluble protein was purified from the concentrated supernatant using HiTrap protein G HP (Amersham Biosciences) according to the manufacturer's instructions.

In vitro coculture of CD4⁺ T cells with BMDCs and Jagged1-Fc

Isolated CD4⁺ T cells from WT mice were pretreated with GSI (GSI/CD4) or DMSO (DMSO/CD4) and IL-2 for 24 h. GSI/CD4 or DMSO/CD4 were cocultured with BMDCs previously pulsed with OVA (200 μ g/ml) at a ratio of 10:1 for 5 days. After culture, viable cells were restimulated with plate-bound anti-CD3 plus anti-CD28 (2 μ g/ml each; R&D Systems) for 3 days. All BMDCs were treated with mitomycin C (50 mg/ml; Sigma-Aldrich) before being cultured with CD4⁺ T cells. Culture supernatants were harvested for cytokine analysis. In some in vitro experiments, isolated CD4⁺ T cells (2×10^6) from WT mice were stimulated with plate-bound anti-CD3 together with plate-bound Jagged1-Fc (5 μ g/ml) or human IgG (5 μ g/ml) for 5 days. Viable CD4⁺ cells were then restimulated with plate-bound anti-CD3 and anti-CD28 (2 μ g/ml, respectively) for 2 days. Supernatants were collected and evaluated by ELISA. All data are representative of at least three independent experiments conducted in triplicate.

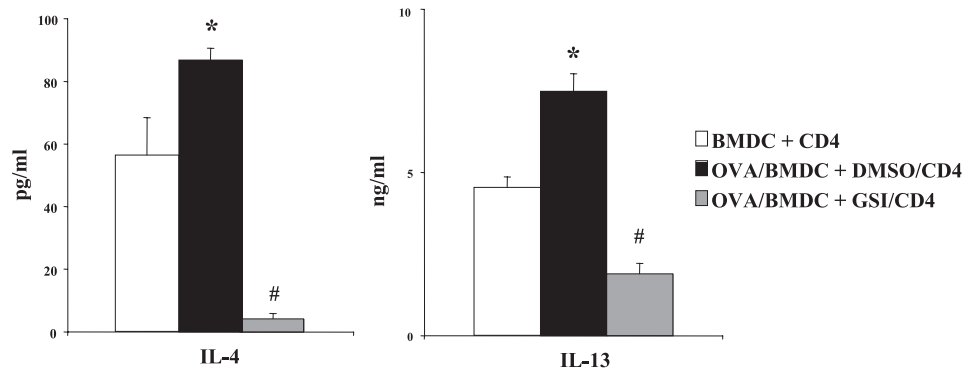


FIGURE 2. Cytokine production from $CD4^+$ T cells pretreated with GSI and cocultured with OVA-pulsed BMDCs. Isolated naive $CD4^+$ T cells from WT mice were incubated with GSI (GSI/CD4) or DMSO (DMSO/CD4) and IL-2 for 24 h. Subsequently, GSI/CD4 or DMSO/CD4 T cells were cocultured with OVA/BMDCs. After 5 days, viable $CD4^+$ T cells were restimulated with plate-bound anti-CD3 and anti-CD28 ($2 \mu\text{g/ml}$, respectively) for 3 days. Supernatants were collected and evaluated by ELISA. The results are representative of three independent experiments conducted in triplicate and are expressed as means $\pm$ SEM. #, $p < 0.05$, significant differences comparing OVA/BMDC plus GSI/CD4, BMDC plus CD4 and OVA/BMDC plus DMSO/CD4; and *, $p < 0.05$, significant differences comparing OVA/BMDC plus DMSO/CD4 and BMDC plus CD4.

Adoptive transfer of BMDCs and $CD4^+$ T cells and administration of Jagged1-Fc

In these transfer protocols, BMDCs were cultured with OVA ($200 \mu\text{g/ml}$) 24 h and instilled intratracheally (2×10^6 cells/recipient). Ten days after the transfer of BMDCs, mice were challenged via the airways with OVA (1% in saline solution) for 20 min on three consecutive days. Forty-eight hours after the last allergen challenge, all assays were conducted. For adoptive transfer of T cells, naive $CD4^+$ T cells (5×10^6) pretreated with GSI (GSI/CD4) or DMSO (DMSO/CD4) were administered i.v. through the tail vein to $IL-4^{-/-}$ recipients, followed by intratracheal administration of OVA-pulsed BMDCs. $IL-4^{-/-}$ mice that received no cells served as controls.

In the Jagged1-Fc protocol, soluble Jagged1-Fc was injected i.p. at a daily dose of $200 \mu\text{g}$ beginning 4 days before through the day following

transfer of OVA-pulsed BMDCs in WT mice before challenge with OVA. As a control, human IgG ($200 \mu\text{g}$) was administered in the same manner.

To assess the effect of Jagged1 knockdown on BMDC activity in vivo, BMDCs transfected with siRNA-Jagged1 were instilled intratracheally into naive C57BL/6 or $IL-4^{-/-}$ mice that received naive $CD4^+$ T cells (5×10^6). The WT or $IL-4^{-/-}$ recipients of untreated BMDCs, BMDCs transfected with reagent alone (mock-treated), or control siRNA (siRNA-scrambled) served as controls.

Assessment of airway function

Airway function was assessed as previously described by measuring changes in lung resistance (R_L) in response to increasing doses of inhaled methacholine (MCh) (42). Data are expressed as percentage change from baseline R_L values obtained after inhalation of saline. The baseline R_L

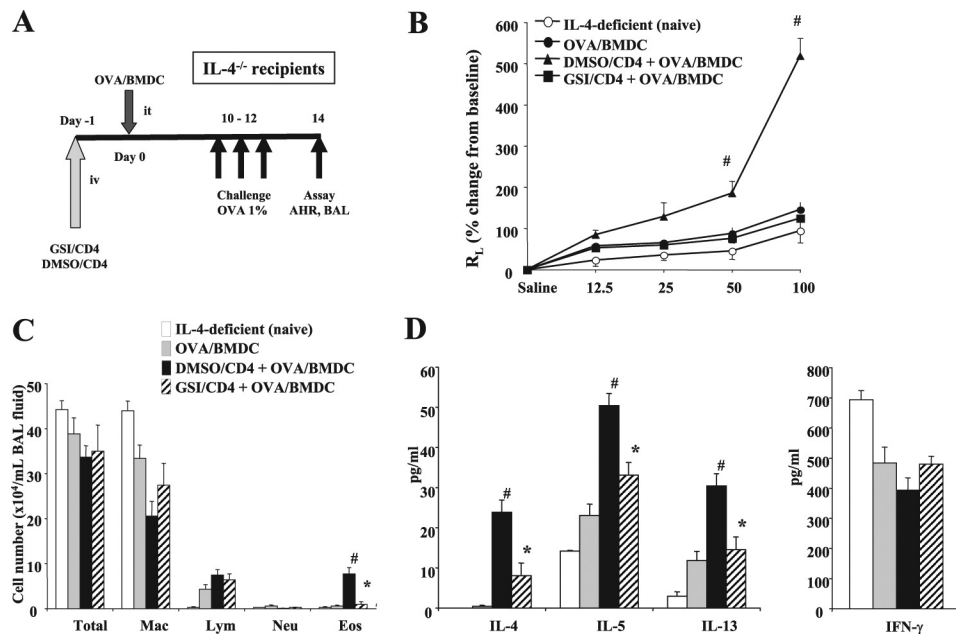


FIGURE 3. Transfer of GSI/CD4 $^+$ T cells and OVA/BMDCs fails to restore lung allergic responses in $IL-4^{-/-}$ mice. **A**, Experimental protocol. Naive WT $CD4^+$ T cells (5×10^6) pretreated with GSI (GSI/CD4) or DMSO (DMSO/CD4) were administered i.v. into $IL-4^{-/-}$ mice, followed by intratracheal administration of OVA/BMDCs (2×10^6). Ten days after the cell transfers, mice received three OVA challenges. $IL-4^{-/-}$ mice that received no $CD4^+$ T cells or OVA/BMDCs alone are also shown. **B**, R_L values were obtained in response to increasing concentrations of inhaled MCh. **C**, Cellular composition of BAL fluid. **D**, Cytokine levels in BAL fluid. Total, Total cells; Mac, macrophages; Lym, lymphocytes; Neu, neutrophils; Eos, eosinophils. Data represent the means $\pm$ SEM ($n = 12$ in each group). #, $p < 0.05$, significant differences comparing DMSO/CD4 plus OVA/BMDC recipients and GSI/CD4 plus OVA/BMDC recipients, OVA/BMDC recipients, or $IL-4^{-/-}$ mice (naive); *, $p < 0.05$, significant differences comparing GSI/CD4 plus OVA/BMDC recipients and DMSO/CD4 plus OVA/BMDC recipients.

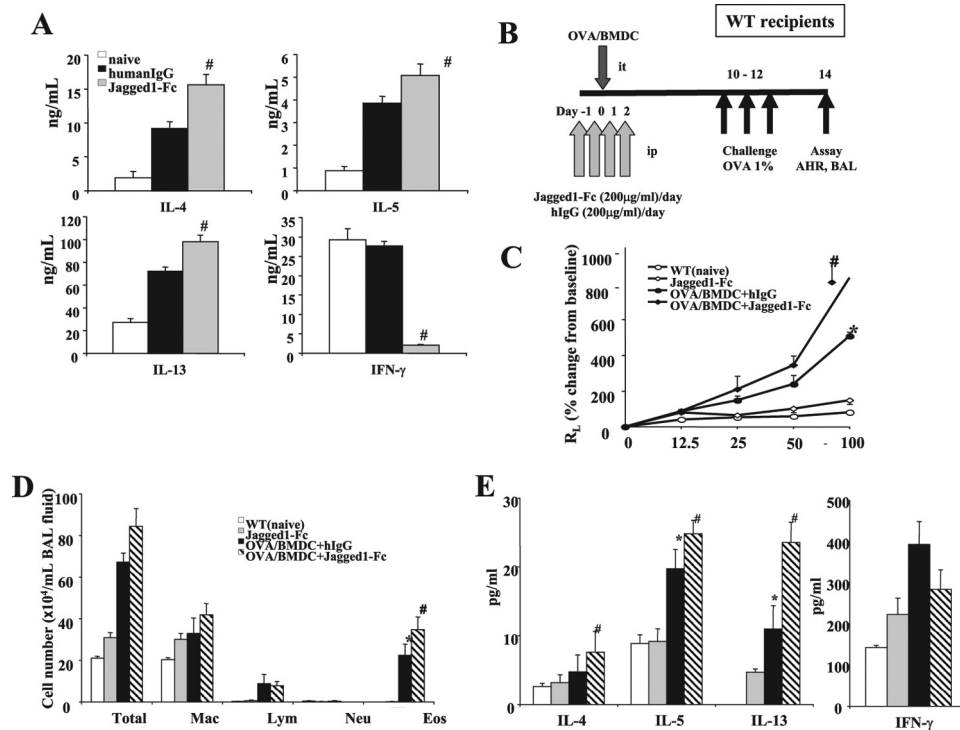


FIGURE 4. Jagged1-Fc stimulated IL-4 production and enhanced allergen-induced AHR and airway inflammation. **A**, Isolated CD4⁺ T cells from naive WT mice were stimulated with platebound anti-CD3/anti-CD28 and Jagged1-Fc or human IgG (5 μ g/ml). Supernatants were collected and cytokine production evaluated. Data represent the mean $\pm$ SEM from three independent experiments conducted in triplicate. #, $p < 0.05$, significant differences between naive CD4⁺ T cells or CD4⁺ T cells stimulated with anti-CD3/anti-CD28 and human IgG and CD4⁺ T cells stimulated with anti-CD3/anti-CD28 and Jagged1-Fc. **B**, Experimental protocol. **C**, AHR. **D**, Cell composition in BAL fluid. **E**, BAL cytokine levels. WT mice were treated with Jagged1-Fc or human IgG following transfer of OVA-pulsed BMDCs and before OVA challenges. Mice received OVA challenges following transfer of Jagged1-Fc alone (Jagged1), OVA-pulsed BMDCs with human IgG (OVA/BMDC+hIgG), and OVA-pulsed BMDCs with Jagged1-Fc (OVA/BMDC+Jagged1). Naive WT mice that received neither Jagged1-Fc nor human IgG were also shown. The results for each group are expressed as the mean $\pm$ SEM ($n = 12$ in each group). #, $p < 0.05$, significant differences between OVA/BMDC plus Jagged1 recipients, OVA/BMDC plus human IgG recipients, Jagged1 recipients, or WT mice (naive); *, $p < 0.05$, significant differences between OVA/BMDC plus human IgG recipients and Jagged1 recipients or WT mice (naive). Total, Total cells; Mac, macrophages; Lym, lymphocytes; Neu, neutrophils; Eos, eosinophils.

responses to saline in the individual groups were not significantly different from each other.

Bronchoalveolar lavage (BAL)

Immediately following measurement of AHR, lungs were lavaged with HBSS (1 $\times$ 1 ml at 37°C) and total leukocyte numbers were analyzed. Differential cell counts were performed under light microscopy by counting at least 200 cells on cytocentrifuged preparations (Shandon Cytospin 2; Thermo Scientific), stained with Leukostat (Fisher Diagnostics), and differentiated by standard hematological procedures in a blinded fashion.

Preparation of RNA and real-time PCR

Total RNA was extracted from BMDCs or siRNA-treated BMDCs using an RNeasy mini kit (Qiagen). Two micrograms of total RNA was used in each reaction primed with oligo-dT to obtain c-DNA. Then, 3 μ l of the synthesized cDNA was used as the template for real-time PCR. Real-time cDNA primers and probes for Jagged1 targeted by siRNA were as follows: forward primer, 5'-CAAAAACCCATCGAGAAACA-3'; reverse primer, 5'-TCCTGATTTTGTACATTTTCGAGTT-3'; probe, 5'-ACGGTCCCCA TTAAGGATTACGAGAA-3'. Jagged2 and GAPDH primers and probes were obtained from Applied Biosystems. The real-time PCRs were performed on an ABI 7700 sequence detection system (Applied Biosystems) with cycling parameters of 50°C for 2 min, 95°C for 10 min, and 40 repeats at 95°C for 15 s and 60°C for 1 min. The $\Delta\Delta$ cycle threshold method was performed for relative quantification of mRNA expression.

Western blot analysis

Proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were treated as recommended by the Ab manufacturer for Jagged1 (Novus Biologicals). For detection of the specific

protein, a sensitive chemiluminescence method was used with an appropriate IgG Ab linked to ab HRP Ab (Pierce).

Measurement of cytokines

Cytokine levels in the BAL fluid and cell culture supernatants were measured by ELISA as previously described (43). IL-4, IL-5, IFN- γ (BD Pharmingen), and IL-13 (R&D Systems) ELISAs were performed according to the manufacturers' directions. The lower limits of detection were 4 pg/ml for IL-4, IL-5, and IL-13 and 10 pg/ml for IFN- γ .

Statistical analysis

Results were expressed as the mean $\pm$ SEM. The t test was used to determine differences between two groups and the Tukey-Kramer test was used for comparisons between multiple groups. Measured values may not be normally distributed because of the small sample sizes. Nonparametric analysis using the Mann-Whitney U test or Kruskal-Wallis test was also used to confirm that the statistical differences remained significant even if the underlying distribution was uncertain. The p values for significance were set to 0.05 for all tests.

Results

Expression of Notch ligand on OVA-pulsed BMDCs

Because expression of the Notch ligands Jagged1 and Jagged2 on APCs has been associated with the development of Th2 responses in vitro (22), we first analyzed the levels of their expressions in BMDCs cultured with/without OVA for 24 h using real-time PCR. The expression of Jagged1 was significantly higher in BMDCs cultured with OVA compared with that in BMDCs cultured in

medium alone (Fig. 1). However, the expression of Jagged2 in BMDCs cultured with OVA showed little increase over that seen with BMDCs alone.

Notch signaling controls cytokine production from CD4⁺ T Cells cocultured with OVA-pulsed BMDCs

To assess whether Notch signaling of CD4⁺ T cells in vitro affected Th1/Th2 polarization, we analyzed cytokine production in cocultures of OVA-pulsed BMDCs (OVA/BMDC) with naive CD4⁺ T cells that were or were not pretreated with GSI to prevent Notch signaling. We previously showed that GSI pretreatment markedly inhibited Notch signaling (36). Naive CD4⁺ T cells isolated from spleens of WT mice were incubated with DMSO (DMSO/CD4) or GSI (GSI/CD4) for 24 h in the presence of IL-2 (20 U/ml). DMSO/CD4 or GSI/CD4 were cocultured with OVA/BMDC, followed by restimulation with plate-bound anti-CD3 and anti-CD28 for 24 h. Supernatants from GSI/CD4 cocultured with OVA/BMDC contained significantly lower levels of IL-4 and IL-13 compared with cultures containing DMSO/CD4 (Fig. 2). These data indicated that pharmacologic inhibition of Notch signaling resulted in markedly reduced cytokine production from CD4⁺ T cells.

Inhibition of Notch signaling on CD4⁺ T cells decreases their ability to promote allergen-induced AHR and airway inflammation in IL-4^{-/-} recipients

To test the functional consequences of GSI treatment of CD4⁺ T cells in vivo, we used a BMDC transfer model in which CD4⁺ T cells were shown to be essential for the development of AHR and airway inflammation (44). To isolate directly the function of transferred CD4⁺ T cells and not that of host CD4⁺ T cells in the initiation of Th2-type allergic airway inflammation, IL-4^{-/-} recipients were used. GSI/CD4 or DMSO/CD4 were transferred into IL-4^{-/-} mice before OVA/BMDC administration, followed by three OVA challenges (Fig. 3A). As shown in Fig. 3B, IL-4^{-/-} mice were incapable of developing AHR or eosinophilic airway inflammation despite receiving OVA/BMDC before challenge. However, IL-4^{-/-} mice that received both OVA/BMDC and DMSO/CD4 developed increased AHR as illustrated by significant increases in R_L in response to increasing doses of inhaled MCh (Fig. 3B). In parallel to the increases in airway responsiveness, the inflammatory cell composition of BAL fluid was altered with significant increases in eosinophil numbers (Fig. 3C). In contrast, IL-4^{-/-} recipients of OVA/BMDC and GSI/CD4 did not develop an increase in airway reactivity above that seen in naive IL-4^{-/-} mice or IL-4^{-/-} recipients of OVA/BMDC alone. Recipients of GSI/CD4 T cells also did not show increases in BAL eosinophil numbers.

The balance between levels of Th1 and Th2 cytokines has been proposed to play an important role in the development of allergic airway inflammation (45). IL-4, IL-5, and IL-13 levels in the BAL of IL-4^{-/-} recipients of DMSO/CD4 T cells were increased, whereas GSI/CD4 T cell recipients showed smaller increases in IL-4, IL-5, and IL-13, but no differences in IFN- γ levels (Fig. 3D).

Effect of Jagged1-Fc on the response of CD4⁺ T cells in WT mice

To define the effects of Jagged1-Fc on cytokine production directly, isolated CD4⁺ T cells (2×10^6) from WT mice were stimulated with plate-bound anti-CD3 alone or together with plate-bound Jagged1-Fc or human IgG (5 μ g/ml) for 5 days and then restimulated with plate-bound anti-CD3 and anti-CD28 (2 μ g/ml, respectively) for an additional 2 days. Supernatants were collected and evaluated by ELISA. As shown in Fig. 4A,

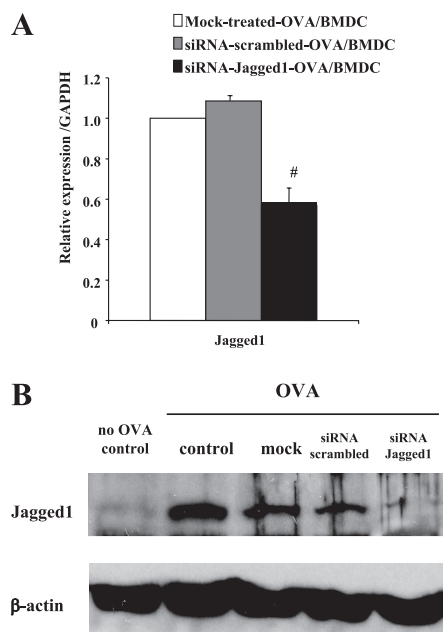


FIGURE 5. Gene silencing in OVA/BMDCs using Jagged1-specific siRNA. BMDCs were transfected with siRNA-Jagged1, siRNA-scrambled, reagent (Gene Porter) alone (mock-treated), or non-transfected cells (control). Transfected BMDCs were incubated with OVA for 24 h and RNA from these transfected BMDCs was collected to assess the expression of Jagged1 by real-time PCR and by Western blotting. *A*, Real-time PCR analysis of Jagged1 levels in transfected BMDCs. Mock-treated results were taken as 1. Results are from three independent experiments. The data for each group are expressed as means $\pm$ SEM. [#], $p < 0.05$, significant differences between siRNA-Jagged1 and mock-treated or siRNA-scrambled BMDCs. *B*, Jagged1 protein levels in transfected BMDCs. BMDCs were unmanipulated (control), transfected with reagent alone (mock), siRNA scrambled, or siRNA-Jagged1. Transfected BMDCs were incubated with OVA for 24 h and cell lysates from these transfected BMDCs were collected to assess the expression of Jagged1 by Western blotting. β -Actin was used as a loading control. Representative of one of three similar experiments.

when T cells were initially cultured with anti-CD3 and human IgG and then restimulated with anti-CD3/anti-CD28, levels of IL-4, IL-5, and IL-13 increased over those seen in the absence of stimulation. Following the addition of Jagged1 in the initial phase, the levels of these cytokines were further increased. In parallel, the levels of IFN- γ were decreased and the decreases were augmented by Jagged1.

To directly determine whether the administration of Jagged1 in vivo regulates AHR and airway inflammation, WT mice were treated with Jagged1-Fc or human IgG as a control following the transfer of OVA-pulsed BMDCs and before OVA challenge (Fig. 4B). The administration of Jagged1-Fc markedly enhanced AHR compared with the administration of (control) human IgG following the transfer of OVA-pulsed BMDCs and OVA challenge (Fig. 4C). In parallel, the administration of Jagged1-Fc to WT mice increased the numbers of eosinophils and the levels of Th2 cytokines in the BAL compared with controls (Fig. 4, *D* and *E*). These data indicated that administration of the Notch ligand Jagged1 can further enhance the development of lung allergic responses, even in WT mice.

Gene silencing in BMDCs treated with Jagged1 siRNA

To further analyze the importance of Notch-Jagged1 interactions, we used the ability of siRNA to reduce *Jagged1*-specific gene

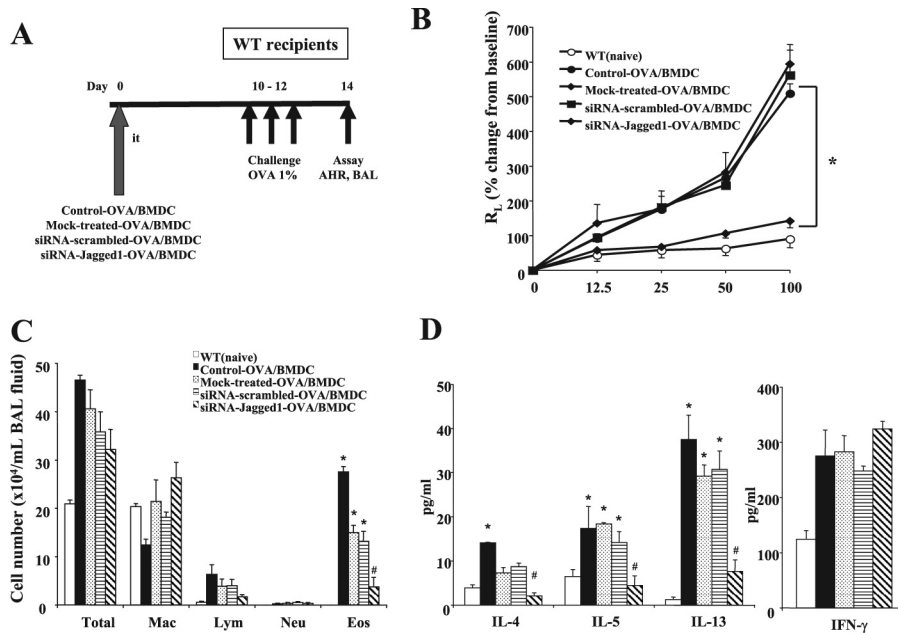


FIGURE 6. Administration of OVA/BMDCs transfected with siRNA-Jagged1 fails to restore lung allergic responses in WT recipients. **A**, Experimental protocol. BMDCs were transfected with siRNA-Jagged1, siRNA scrambled, or mock treated. Subsequently, transfected and untreated BMDCs were incubated with OVA for 24 h before transfer into WT mice. Ten days later, mice received three daily OVA challenges. **B**, AHR. R_L values were obtained in response to increasing concentrations of inhaled MCh. *, $p < 0.05$, significant differences. **C**, Cellular composition of BAL fluid. **D**, Cytokine levels in BAL fluid. Data represent the means $\pm$ SEM ($n = 12$ in each group). #, $p < 0.05$, significant differences between siRNA-Jagged1-OVA/BMDC recipients vs OVA/BMDC, mock-treated-OVA/BMDC, and siRNA-scrambled-OVA/BMDC recipients; *, $p < 0.05$, significant differences between OVA/BMDC, mock-treated-OVA/BMDC, or siRNA-scrambled-OVA/BMDC recipients and WT mice (naive). Total, Total cells; Mac, macrophages; Lym, lymphocytes; Neu, neutrophils; Eos, eosinophils.

expression in BMDCs cultured with OVA. The expression of *Jagged1* mRNA and protein levels in BMDCs were analyzed by real-time PCR using primers flanking the siRNA target sequence and by Western blotting, respectively. The levels of Jagged1 mRNA in BMDCs transfected with siRNA-Jagged1 were decreased by $\sim 50\%$ compared with levels in BMDCs transfected with siRNA-scrambled or mock-treated BMDCs (Fig. 5A). Following pulsing with OVA, Jagged1 protein levels in BMDCs were markedly increased compared with those of nonpulsed BMDCs (Fig. 5B). Following transfection of OVA-pulsed BMDC with siRNA-Jagged1, Jagged1 protein levels were markedly reduced.

Inhibition of Jagged1 in BMDCs decreases their ability to induce allergen-dependent AHR and airway inflammation in WT mice

To determine the functional consequences of *Jagged1* gene silencing in OVA/BMDC, we monitored the effects of the transfer of gene-silenced OVA/BMDC into naive WT mice before allergen challenge (Fig. 6A). This DC-dependent protocol has been shown to be dependent on the Ag pulsing of DCs before transfer and allergen challenge in naive WT mice (44). WT mice received either OVA/BMDC, siRNA-Jagged1-OVA/BMDC, mock-treated-OVA/BMDC, or siRNA-scrambled-OVA/BMDC intratracheally before the three daily OVA challenges. WT recipients of OVA/BMDC, mock-treated OVA/BMDC, or siRNA-scrambled-OVA/BMDC developed significant increases in MCh-induced AHR and airway eosinophilia (Figs. 6, B and C). This was in contrast to the responses following transfer of siRNA-Jagged1-OVA/BMDC, where AHR and airway eosinophilia failed to develop. The levels of Th2 cytokines in the BAL fluid paralleled the findings for AHR and airway eosinophilia with no significant increases in Th2 cytokine levels in the BAL fluid of recipients of the Jagged1-silenced BMDC (Fig. 6D). There were no significant differences among the

recipients of any of the OVA/BMDC groups when levels of IFN- γ were examined (Fig. 6D).

Inhibition of Jagged1 on BMDCs decreases allergen-induced CD4⁺ T Cell/IL-4-dependent AHR and airway inflammation in IL-4^{-/-} recipients

To complement the findings in WT mice and to confirm the direct impact of transferred CD4⁺IL-4⁺ T cells on the initiation of lung allergic responses in vivo, these same populations of OVA/BMDCs were transferred into IL-4^{-/-} recipients before the transfer of naive (WT) CD4⁺ T cells and OVA challenge (Fig. 7A). AHR to inhaled MCh was significantly increased in IL-4^{-/-} recipients of OVA/BMDCs and naive CD4⁺ T cells, but not in those receiving either alone (Fig. 7B). Transfer of mock-treated-OVA/BMDCs or siRNA-scrambled-OVA/BMDCs together with CD4⁺ T cells before OVA challenge resulted in similar increases in MCh-induced AHR, whereas transfer of siRNA-Jagged1-OVA/BMDCs failed to increase AHR. In parallel to the assessment of lung function, the inflammatory cell composition of BAL fluid was also different, with recipients of siRNA-Jagged1-OVA/BMDCs failing to develop significant BAL eosinophilia (Fig. 7C). In addition, Th2 cytokine levels in the BAL fluid of IL-4^{-/-} recipients of siRNA-Jagged1-OVA/BMDCs did not demonstrate the increases seen in the other recipient groups (Fig. 7D); levels of IFN- γ were similar in all groups. The transfer of CD4⁺IL-4⁺ T cells alone was incapable of restoring any of the responses. These data defined the requirement for Jagged1-expressing DCs and CD4⁺IL-4⁺ T cells in the initiation of lung allergic responses in vivo.

Discussion

In allergic asthma, accumulation of CD4⁺ T cells producing Th2 cytokines has been commonly observed in BAL fluid and lung biopsies (4, 45, 46). There is also abundant evidence from animal

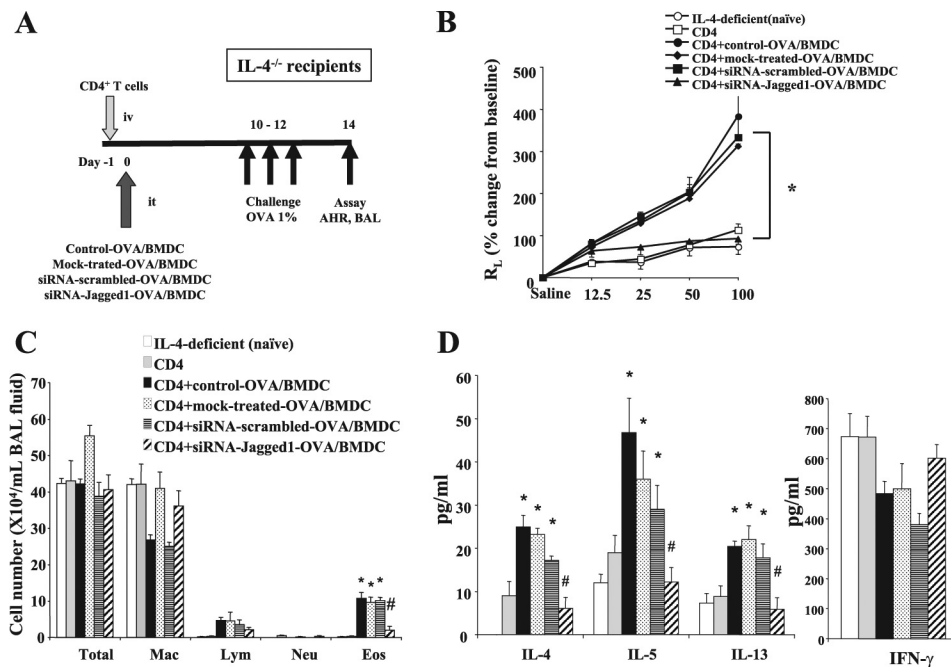


FIGURE 7. Allergen-induced AHR and airway inflammation are not restored in IL-4^{-/-} recipients of siRNA-Jagged1-transfected BMDCs and CD4⁺ T cells. **A**, Experimental protocol. IL-4^{-/-} mice received naive CD4⁺ T cells i.v. and unmanipulated or transfected BMDCs, including siRNA-Jagged1-BMDCs, siRNA-scrambled-BMDCs, or mock-treated-BMDCs, following 24 h incubation with OVA. Ten days later, mice received three daily OVA challenges. Data represent the means $\pm$ SEM ($n = 12$ in each group). **B**, AHR. R_L values were obtained in response to increasing concentrations of inhaled MCh. *, $p < 0.05$, significant differences. **C**, Cellular composition of BAL fluid. #, $p < 0.05$, significant differences comparing CD4 plus siRNA-Jagged1-OVA/BMDC recipients and CD4 plus OVA/BMDC, CD4 plus mock-treated-OVA/BMDC, or CD4 plus siRNA-scrambled-OVA/BMDC recipients. **D**, Cytokine levels in BAL fluid. #, $p < 0.05$, significant differences comparing CD4 plus siRNA-Jagged1-OVA/BMDC recipients and CD4 plus OVA/BMDC, CD4 plus mock-treated-OVA/BMDC, or CD4 plus siRNA-scrambled-OVA/BMDC recipients; *, $p < 0.05$, significant differences between CD4 plus OVA/BMDC, CD4 plus mock-treated-OVA/BMDC, or CD4 plus siRNA-scrambled-OVA/BMDC recipients and IL-4^{-/-} mice (naïve) or CD4 recipients.

studies that IL-4 plays a major role in the development of AHR and the influx of eosinophils as a result of Th2 cell differentiation (10, 18, 37). Thus, IL-4 is an effective and essential initiator of Th2 differentiation, and the development of effective Th2 responses in vivo and in vitro depends on IL-4 (47–50). However, the events that trigger IL-4 production, beginning with the initial encounters of naive T cells with APCs, remain less well understood, especially in vivo and in the development of allergic asthma.

In the present study, Jagged1 but not Jagged2 was found to be up-regulated in BMDCs pulsed with OVA compared with non-pulsed BMDCs, and the elevation was maintained for at least 24 h. The increases in gene expression were paralleled by increases in Jagged1 protein levels. Several reports have suggested that Notch ligand signaling by activated DCs is involved in directing specific Th1 and Th2 polarization on Notch-expressing T cells (22, 23, 25, 51). In vitro, Jagged-expressing APC cell lines were shown to induce Th2 cytokine production preferentially, whereas Delta-expressing cell lines induced IFN- γ production (22). As a rule, the Notch ligands tend to be expressed in a more highly restricted pattern than their receptors. Recent reports have noted the up-regulation of Jagged2 expression on DCs by helminths with induction of Th2 differentiation (52). Cholera toxin treatment also stimulated Jagged2 expression in a c-kit-dependent manner in BMDCs from WT mice that was linked to the development of allergic airway inflammation (53). In experimental autoimmune encephalomyelitis, anti-Jagged1 Ab exacerbated whereas anti-Delta1 Ab reduced the severity of clinical disease (54). Investigation of these Notch pathways has underscored the complex role of APCs in the initiation and regulation of Th1/Th2 differentiation. Notch signaling has been shown to direct Th2 differentiation via GATA3 (55, 56) through IL-4 receptor signaling in a STAT6-dependent fashion

(14, 47, 57). However, little is known about the interaction between Notch on CD4⁺ T cells and Notch ligand on APCs pulsed with OVA in the development of lung allergic responses.

To investigate the role of Notch-Jagged1 interactions on CD4⁺ T cells and APCs in the context of allergen-driven responses, several in vitro and in vivo approaches were followed, including the inhibition of Notch signaling in CD4⁺ T cells using a GSI, the silencing of *Jagged1* expression in APCs, and the use of a BMDC transfer protocol. This BMDC transfer model was shown to induce AHR and airway eosinophilia, but to a less robust extent than in models where mice were systemically sensitized and then challenged via the airways (19). Nonetheless, these responses following BMDC transfer were shown to be dependent on both IL-4-producing CD4⁺ T cells and APCs pulsed with Ag (19). To first determine the consequences of inhibiting Notch signaling in CD4⁺ T cells on the development of lung allergic responses, we used the BMDC transfer model in which the transfer of allergen-pulsed BMDCs intratracheally before allergen challenge was shown to be essential (44). To focus on the role of Notch in inducing IL-4 from CD4⁺ T cells in this model, we used IL-4-deficient recipient mice that have been shown to exhibit a significantly reduced ability to develop AHR and airway eosinophilia, accompanied by decreased BAL IL-13 levels (18, 58). In these IL-4^{-/-} recipients, reconstitution of the full development of lung allergic responses could be achieved by adoptive transfer of naive (WT) CD4⁺ T cells, followed by the transfer of OVA-pulsed BMDCs and allergen challenge. In contrast, the transfer of GSI-treated CD4⁺ T cells failed to restore AHR, eosinophilic inflammation, or Th2 cytokine levels in these IL-4-deficient recipients. These data indicate that in the context of APC interactions with CD4⁺ T cells in vitro or in vivo, Notch signaling is a critical step for the differentiation of naive

CD4⁺ T cells to a Th2 (IL-4 producing) phenotype and the development of lung allergic responses.

Given this role for Notch signaling in the CD4⁺ T cells, we next determined whether the increase in *Jagged1* gene expression and protein levels in OVA-pulsed BMDCs was the critical ligand-mediated event in these responses. A number of approaches were used to confirm the importance of this ligand in triggering Th2 differentiation and the development of lung allergic responses. When Jagged1-Fc was administered in vivo to WT mice together with OVA-BMDCs followed by OVA airway challenges, Jagged1 increased all lung allergic responses, including AHR, airway eosinophilia, and BAL Th2 cytokines levels. In complementary studies, the transfection of BMDCs to silence *Jagged1* gene expression was conducted. Transfection of BMDCs with siRNA was effective in silencing targeted genes and provided a means to examine the capacity of allergen-pulsed and siRNA-modified BMDCs to alter the allergen-specific immune response and Th polarization in recipients (39). In OVA-pulsed BMDCs, we demonstrated that siRNA could be used to target the expression of Jagged1 at the transcription and protein levels, resulting in functional consequences. In WT mice, the transfer of BMDCs silenced by siRNA-Jagged1 failed to trigger AHR or airway (eosinophilic) inflammation. As a result of the silencing of *Jagged1* by siRNA in OVA/BMDC, Th2 cytokine production in BAL fluid was inhibited without affecting IFN- γ production. Together, these data demonstrate the importance of Notch signaling in CD4⁺ T cells and *Jagged1* expression in APCs for the development of these responses in the WT recipients.

Th2 polarization has been reported to occur through both IL-4-dependent and -independent pathways (47–50). To directly examine the impact of Notch-Jagged1 interactions in the context of initiating IL-4 production, we examined the outcomes of administering OVA/BMDCs silenced by siRNA-Jagged1 to IL-4-deficient mice that received naive (WT) CD4⁺ T cells. The transfer of siRNA-Jagged1 OVA/BMDCs together with CD4⁺ T cells failed to restore AHR, airway eosinophilia, or BAL Th2 cytokine levels in these recipients. Thus, it appears that the blockade of Jagged1 on DCs did not influence or default to the expression of other Notch ligands (e.g., Delta4), enhancing IFN- γ production.

These studies demonstrate for the first time that the effects of either preventing Notch signaling by GSI treatment of CD4⁺ T cells or Notch ligand expression through siRNA-Jagged1 silencing of BMDCs resulted in attenuation of the full array of lung allergic responses. Central to this failure was the inability of the CD4⁺ T cells to undergo Th2 differentiation, produce IL-4, and initiate the lung allergic cascade. These data identify the Notch-Jagged1 pathway as a critical initiator and regulator of the development of allergen-induced, Th2-mediated AHR and lung allergic inflammation. Manipulation of this pathway may be particularly effective in the treatment of allergic airway disease.

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Disclosures

The authors have no financial conflict of interest.

References

1. Gavett, S. H., X. Chen, F. Finkelman, and M. Wills-Karp. 1994. Depletion of murine CD4⁺ T lymphocytes prevents antigen-induced airway hyperreactivity and pulmonary eosinophilia. *Am. J. Respir. Cell Mol. Biol.* 10: 587–593.
2. Umetsu, D. T., J. J. McIntire, O. Akbari, C. Macaubas, and R. H. DeKruyff. 2002. Asthma: an epidemic of dysregulated immunity. *Nat. Immunol.* 3: 715–720.
3. Busse, W. W., R. L. Coffman, E. W. Gelfand, A. B. Kay, and L. J. Rosenwasser. 1995. Mechanisms of persistent airway inflammation in asthma: a role for T cells and T-cell products. *Am. J. Respir. Crit. Care Med.* 152: 388–393.
4. Robinson, D. S., Q. Hamid, S. Ying, A. Tsicopoulos, J. Barkans, A. M. Bentley, C. Corrigan, S. R. Durham, and A. B. Kay. 1992. Predominant TH2-like bronchoalveolar T-lymphocyte population in atopic asthma. *New Engl. J. Med.* 326: 298–304.
5. Hogan, S. P., K. I. Mattheai, A. Young, A. Koskinen, I. G. Young, and P. S. Foster. 1998. A novel T cell-regulated mechanism modulating allergen-induced airways hyperreactivity in BALB/c mice independently of IL-4 and IL-5. *J. Immunol.* 161: 1501–1509.
6. Wills-Karp, M., J. Luyimbazi, X. Xu, B. Schofield, T. Y. Neben, C. L. Karp, and D. D. Donaldson. 1998. Interleukin-13: central mediator of allergic asthma. *Science* 282: 2258–2261.
7. De Sanctis, G. T., A. Itoh, F. H. Green, S. Qin, T. Kimura, J. K. Grobholz, T. R. Martin, T. Maki, and J. M. Drazen. 1997. T-lymphocytes regulate genetically determined airway hyperresponsiveness in mice. *Nat. Med.* 3: 460–462.
8. Busse, W. W., and R. F. Lemanske, Jr. 2001. Asthma. *New Engl. J. Med.* 344: 350–362.
9. Cohn, L., J. S. Tepper, and K. Bottomly. 1998. IL-4-independent induction of airway hyperresponsiveness by Th2, but not Th1, cells. *J. Immunol.* 161: 3813–3816.
10. Grunig, G., M. Warnock, A. E. Wakil, R. Venkayya, F. Brombacher, D. M. Rennick, D. Sheppard, M. Mohrs, D. D. Donaldson, R. M. Locksley, and D. B. Corry. 1998. Requirement for IL-13 independently of IL-4 in experimental asthma. *Science* 282: 2261–2263.
11. Weaver, C. T., and K. M. Murphy. 2007. T-cell subsets: the more the merrier. *Curr. Biol.* 17: R61–R63.
12. Kapsenberg, M. L. 2003. Dendritic-cell control of pathogen-driven T-cell polarization. *Nat. Rev. Immunol.* 3: 984–993.
13. Sporri, R., and R. Reis e Sousa. 2005. Inflammatory mediators are insufficient for full dendritic cell activation and promote expansion of CD4⁺ T cell populations lacking helper function. *Nat. Immunol.* 6: 163–170.
14. Murphy, K. M., and S. L. Reiner. 2002. The lineage decisions of helper T cells. *Nat. Rev. Immunol.* 2: 933–944.
15. Finkelman, F. D., S. C. Morris, T. Orekhova, M. Mori, D. D. Donaldson, S. L. Reiner, N. L. Reilly, L. Schopf, and J. F. Urban, Jr. 2000. Stat6 regulation of in vivo IL-4 responses. *J. Immunol.* 164: 2303–2310.
16. Jankovic, D., M. C. Kullberg, N. Noben-Trauth, P. Caspar, W. E. Paul, and A. Sher. 2000. Single cell analysis reveals that IL-4 receptor/Stat6 signaling is not required for the in vivo or in vitro development of CD4⁺ lymphocytes with a Th2 cytokine profile. *J. Immunol.* 164: 3047–3055.
17. Cohn, L., R. J. Homer, A. Marinov, J. Rankin, and K. Bottomly. 1997. Induction of airway mucus production by T helper 2 (Th2) cells: a critical role for interleukin 4 in cell recruitment but not mucus production. *J. Exp. Med.* 186: 1737–1747.
18. Brusselle, G., J. Kips, G. Joos, H. Bluethmann, and R. Pauwels. 1995. Allergen-induced airway inflammation and bronchial responsiveness in wild-type and interleukin-4-deficient mice. *Am. J. Respir. Cell Mol. Biol.* 12: 254–259.
19. Koya, T., N. Miyahara, K. Takeda, S. Matsubara, H. Matsuda, C. Swasey, A. Balhorn, A. Dakhama, and E. W. Gelfand. 2007. CD8⁺ T cell-mediated airway hyperresponsiveness and inflammation is dependent on CD4⁺IL-4⁺ T cells. *J. Immunol.* 179: 2787–2796.
20. Corry, D. B., H. G. Folkesson, M. L. Warnock, D. J. Erle, M. A. Matthay, J. P. Wiener-Kronish, and R. M. Locksley. 1996. Interleukin 4, but not interleukin 5 or eosinophils, is required in a murine model of acute airway hyperreactivity. *J. Exp. Med.* 183: 109–117.
21. Henderson, W. R., Jr., E. Y. Chi, and C. R. Maliszewski. 2000. Soluble IL-4 receptor inhibits airway inflammation following allergen challenge in a mouse model of asthma. *J. Immunol.* 164: 1086–1095.
22. Amsen, D., J. M. Blander, G. R. Lee, K. Tanigaki, T. Honjo, and R. A. Flavell. 2004. Instruction of distinct CD4 T helper cell fates by different notch ligands on antigen-presenting cells. *Cell* 117: 515–526.
23. Tanigaki, K., M. Tsuji, N. Yamamoto, H. Han, J. Tsukada, H. Inoue, M. Kubo, and T. Honjo. 2004. Regulation of $\alpha\beta/\gamma\delta$ T cell lineage commitment and peripheral T cell responses by Notch/RBP-J signaling. *Immunity* 20: 611–622.
24. Minter, L. M., D. M. Turley, P. Das, H. M. Shin, I. Joshi, R. G. Lawlor, O. H. Cho, T. Palaga, S. Gottipati, J. C. Telfer, et al. 2005. Inhibitors of γ -secretase block in vivo and in vitro T helper type 1 polarization by preventing Notch upregulation of Tbx21. *Nat. Immunol.* 6: 680–688.
25. Maekawa, Y., S. Tsukumo, S. Chiba, H. Hirai, Y. Hayashi, H. Okada, K. Kishihara, and Yasutomo. 2003. Delta1-Notch3 interactions bias the functional differentiation of activated CD4⁺ T cells. *Immunity* 19: 549–559.
26. Glimcher, L. H. 2001. Lineage commitment in lymphocytes: controlling the immune response. *J. Clin. Invest.* 108: s25–s30.
27. Artavanis-Tsakonas, S., M. D. Rand, and R. J. Lake. 1999. Notch signaling: cell fate control and signal integration in development. *Science* 284: 770–776.
28. Struhl, G., and A. Adachi. 1998. Nuclear access and action of notch in vivo. *Cell* 93: 649–660.
29. Reizis, B., and P. Leder. 2002. Direct induction of T lymphocyte-specific gene expression by the mammalian Notch signaling pathway. *Genes Dev.* 16: 295–300.
30. Kopan, R., and M. X. Ilagan. 2004. γ -Secretase: Proteasome of the membrane? *Nat. Rev. Mol. Cell Biol.* 5: 499–504.
31. Palaga, T., L. Miele, T. E. Golde, and B. A. Osborne. 2003. TCR-mediated notch signaling regulates proliferation and IFN- γ production in peripheral T cells. *J. Immunol.* 171: 3019–3024.

32. Adler, S. H., E. Chiffolleau, L. Xu, N. M. Dalton, J. M. Burg, A. D. Wells, M. S. Wolfe, L. A. Turka, and W. S. Pear. 2003. Notch signaling augments T cell responsiveness by enhancing CD25 expression. *J. Immunol.* 171: 2896–2903.
33. Eagar, T. N., Q. Tang, M. Wolfe, Y. He, W. S. Pear, and J. A. Bluestone. 2004. Notch 1 signaling regulates peripheral T cell activation. *Immunity* 20: 407–415.
34. Wong, K. K., M. J. Carpenter, L. L. Young, S. J. Walker, G. McKenzie, A. J. Rust, G. Ward, L. Packwood, K. Wahl, L. Delriviere, et al. 2003. Notch ligation by Delta1 inhibits peripheral immune responses to transplantation antigens by a CD8⁺ cell-dependent mechanism. *J. Clin. Invest.* 112: 1741–1750.
35. Tu, L., T. C. Fang, D. Artis, O. Shestova, S. E. Pross, I. Maillard, and W. S. Pear. 2005. Notch signaling is an important regulator of type 2 immunity. *J. Exp. Med.* 202: 1037–1042.
36. Okamoto, M., K. Takeda, A. Joetham, H. Ohnishi, H. Matsuda, C. H. Swasey, B. J. Swanson, K. Yasutomo, A. Dakhama, and E. W. Gelfand. 2008. Essential role of Notch signaling in effector memory CD8⁺ T cell-mediated airway hyperresponsiveness and inflammation. *J. Exp. Med.* 205: 1087–1097.
37. Inaba, K., R. M. Steinman, M. W. Pack, H. Aya, M. Inaba, T. Sudo, S. Wolpe, and G. Schuler. 1992. Identification of proliferating dendritic cell precursors in mouse blood. *J. Exp. Med.* 175: 1157–1167.
38. Inaba, K., M. Inaba, N. Romani, H. Aya, M. Deguchi, S. Ikehara, S. Muramatsu, and R. M. Steinman. 1992. Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J. Exp. Med.* 176: 1693–1702.
39. Hill, J. A., T. E. Ichim, K. P. Kuszniuk, M. Li, X. Huang, X. Yan, R. Zhong, E. Cairns, D. A. Bell, and W. P. Min. 2003. Immune modulation by silencing IL-12 production in dendritic cells using small interfering RNA. *J. Immunol.* 171: 691–696.
40. Shen, L., K. Evel-Kabler, R. Strube, and S. Y. Chen. 2004. Silencing of SOCS1 enhances antigen presentation by dendritic cells and antigen-specific anti-tumor immunity. *Nat. Biotechnol.* 22: 1546–1553.
41. Miyahara, N., K. Takeda, T. Kodama, A. Joetham, C. Taube, J. W. Park, S. Miyahara, A. Balhorn, A. Dakhama, and E. W. Gelfand. 2004. Contribution of antigen-primed CD8⁺ T cells to the development of airway hyperresponsiveness and inflammation is associated with IL-13. *J. Immunol.* 172: 2549–2558.
42. Takeda, K., E. Hamelmann, A. Joetham, L. D. Shultz, G. L. Larsen, C. G. Irvin, and E. W. Gelfand. 1997. Development of eosinophilic airway inflammation and airway hyperresponsiveness in mast cell-deficient mice. *J. Exp. Med.* 186: 449–454.
43. Tomkinson, A., G. Cieslewicz, C. Duez, K. A. Larson, J. J. Lee, and E. W. Gelfand. 2001. Temporal association between airway hyperresponsiveness and airway eosinophilia in ovalbumin-sensitized mice. *Am. J. Respir. Crit. Care Med.* 163: 721–730.
44. Lambrecht, B. N., M. De Veerman, A. J. Coyle, J. C. Gutierrez-Ramos, K. Thielemans, and R. A. Pauwels. 2000. Myeloid dendritic cells induce Th2 responses to inhaled antigen, leading to eosinophilic airway inflammation. *J. Clin. Invest.* 106: 551–559.
45. Wills-Karp, M. 1999. Immunologic basis of antigen-induced airway hyperresponsiveness. *Ann. Rev. Immunol.* 17: 255–281.
46. Mazzearella, G., A. Bianco, E. Catena, R. De Palma, and G. F. Abbate. 2000. Th1/Th2 lymphocyte polarization in asthma. *Allergy* 55: 6–9.
47. Ouyang, W., M. Löhning, Z. Gao, M. Assenmacher, S. Ranganath, A. Radbruch, and K. M. Murphy. 2000. Stat6-independent GATA-3 autoactivation directs IL-4-independent Th2 development and commitment. *Immunity* 12: 27–37.
48. Mohrs, M., C. Holscher, and F. Brombacher. 2000. Interleukin-4 receptor α -deficient BALB/c mice show an unimpaired T helper 2 polarization in response to *Leishmania* major infection. *Infect. Immun.* 68: 1773–1780.
49. Cunningham, A. F., K. Serre, K. M. Toellner, M. Khan, J. Alexander, F. Brombacher, and I. C. MacLennan. 2004. Pinpointing IL-4-independent acquisition and IL-4-influenced maintenance of Th2 activity by CD4 T cells. *Eur. J. Immunol.* 34: 686–694.
50. Kusam, S., L. M. Toney, H. Sato, and A. L. Dent. 2003. Inhibition of Th2 differentiation and GATA-3 expression by BCL-6. *J. Immunol.* 170: 2435–2441.
51. Napolitani, G., A. Rinaldi, F. Berton, F. Sallusto, and A. Lanzavecchia. 2005. Selected Toll-like receptor agonist combinations synergistically trigger a T helper type 1-polarizing program in dendritic cells. *Nat. Immunol.* 6: 769–776.
52. Krawczyk, C. M., J. Sun, and E. J. Pearce. 2008. Th2 differentiation is unaffected by Jagged2 expression on dendritic cells. *J. Immunol.* 180: 7931–7937.
53. Krishnamoorthy, N., T. B. Oriss, M. Paglia, M. Fei, M. Yarlagadda, B. Vanhaesebroeck, A. Ray, and P. Ray. 2008. Activation of c-Kit in dendritic cells regulates T helper cell differentiation and allergic asthma. *Nat. Med.* 14: 565–573.
54. Elyaman, W., E. M. Bradshaw, Y. Wang, M. Oukka, P. Kivisäkk, S. Chiba, H. Yagita, and S. J. Khoury. 2007. Jagged1 and Delta1 differentially regulate the outcome of experimental autoimmune encephalomyelitis. *J. Immunol.* 179: 5990–5998.
55. Amsen, D., A. Antov, D. Jankovic, A. Sher, F. Radtke, A. Souabni, M. Busslinger, B. McCright, T. Gridley, and R. A. Flavell. 2007. Direct regulation of Gata3 expression determines the T helper differentiation potential of Notch. *Immunity* 27: 89–99.
56. Fang, T. C., Y. Yashiro-Ohtani, C. Del Bianco, D. M. Knoblock, S. C. Blacklow, and W. S. Pear. 2007. Notch directly regulates Gata3 expression during T helper 2 cell differentiation. *Immunity* 27: 100–110.
57. Kurata, H., H. J. Lee, A. O'Garra, and N. Arai. 1999. Ectopic expression of activated Stat6 induces the expression of Th2-specific cytokines and transcription factors in developing Th1 cells. *Immunity* 11: 677–688.
58. Herrick, C. A., H. MacLeod, E. Glusac, R. E. Tigelaar, and K. Bottomly. 2000. Th2 responses induced by epicutaneous or inhalational protein exposure are differentially dependent on IL-4. *J. Clin. Invest.* 105: 765–775.