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Jagged1 on Dendritic Cells and Notch on CD4⁺ T Cells Initiate Lung Allergic Responsiveness by Inducing IL-4 Production¹⁻²

Masakazu Okamoto,* Hiroyuki Matsuda,* Anthony Joetham,* Joseph J. Lucas,* Joanne Domenico,* Koji Yasutomo,* Katsuyuki Takeda,* and Erwin W. Gelfand³*¹

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)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 Toll-like receptors, 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...
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 μM 2-ME, 2 mM l-glutamine, penicillin (100 U/ml) and streptomycin (100 μg/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, BMDCs 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 μg/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 × 105 cells/well in 400 μl 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-nt siRNA sequences specific for Jagged1 (5'-CTCGTAAATCTTTAACAGT-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 μl of 20 μM annealed siRNA was incubated with 3 μl of GenePorter (Gene Therapy Systems) in a volume of 94 μl 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 μl of GenePorter alone were used as mock controls. After incubation, 500 μl/well RPMI 1640 supplemented with 20% FCS was added to the cells. Twenty-four hours later, transfected or untreated 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 har- vested by mincing the tissues and subsequently passing them through a stainless steel sieve. After washing with PBS, mononuclear cells were iso- lated 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 (purification > 95%; Cedarlane Laboratories) in ac- cordance with the manufacturer’s instructions. Purity of CD4+ T cell popu- lations after purification exceeded 95% as assessed by flow cytometry. For some experiments, isolated CD4+ T cells were cultured with GSI (20 μM) (dibenzazepine; Calbiochem-EMD Biociences) 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 C57BL6 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 extra- cellular 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 μg/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 μg/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 × 105) from WT mice were stimulated with plate-bound anti-CD3 together with plate-bound Jagged1-Fc (5 μg/ml) or human IgG1 (5 μg/ml) for 3 days. Viable CD4+ cells were then restimulated with plate-bound anti-CD3 and anti-CD28 (2 μg/ml, respectively) for 2 days. Supernatants were collected and evaluated by ELISA. All data are repre- sentative of at least three independent experiments conducted in triplicate.
Adoptive transfer of BMDCs and CD4+ T cells and administration of Jagged1-Fc

In these transfer protocols, BMDCs were cultured with OVA (200 µg/ml) for 24 h and instilled intratracheally (2 x 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 x 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.

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 x 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 values obtained after inhalation of saline.

Transfer of OVA-pulsed BMDCs in WT mice before challenge with OVA. As a control, human IgG (200 µg) was administered in the same manner.

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 µg/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 ± 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.

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 x 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. 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 ± 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.
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 × 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 cytospin 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 μl 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'-TCCTGATTTTTGACATTTTCGAGTT-3'; reverse primer, 5'-ACGGTCCCCAACTCATCCAGAGAAACA-3'; probe, 5'-CAAAAACCCCATCGAGAAACAT-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 ΔΔCt 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 ± 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/C4D) or GSI (GSI/C4D) for 24 h in the presence of IL-2 (20 U/ml). DMSO/C4D or GSI/C4D were cocultured with OVA/BMDC, followed by restimulation with plate-bound anti-CD3 and anti-CD28 for 24 h. Supernatants from GSI/C4D cocultured with OVA/BMDC contained significantly lower levels of IL-4 and IL-13 compared with cultures containing DMSO/C4D (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/C4D or DMSO/C4D 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/C4D developed increased AHR as illustrated by significant increases in Rf, 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/C4D 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/C4D 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/C4D T cells were increased, whereas GSI/C4D 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 × 10⁶) from WT mice were stimulated with plate-bound anti-CD3 alone or together with plate-bound Jagged1-Fc or human IgG (5 μg/ml) for 5 days and then restimulated with plate-bound anti-CD3 and anti-CD28 (2 μg/ml, respectively) for an additional 2 days. Supernatants were collected and evaluated by ELISA. As shown in Fig. 4A, 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
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 ~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
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 induction 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 vivo or in vitro, 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 cytokine 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 allergenspecific 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 illustrate 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 key effect 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
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