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Low-Dose Lipopolysaccharide Affects Lung Allergic Responses by Regulating Jagged1 Expression on Antigen-Pulsed Dendritic Cells

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Key Words
Asthma · Dendritic cells · Endotoxin · Notch ligands

Abstract
Background: Notch signaling pathways govern immune function and the regulation of Th1 and Th2 differentiation. We previously demonstrated essential interactions between Notch on CD4+ T cells and Jagged1 on antigen-presenting cells in Th2 differentiation for the full development of allergen-induced airway hyperresponsiveness (AHR) and allergic airway inflammation. ***Methods:*** Bone marrow-derived dendritic cells (BMDCs) were differentiated and incubated with different preparations of ovalbumin (OVA), including lipopolysaccharide (LPS)-depleted and LPS-spiked preparations. In some experiments recipient mice also received soluble Jagged1-Fc in addition to allergen-pulsed BMDCs. Ten days following transfer of BMDCs, mice were exposed to three airway challenges with OVA, and airway responsiveness to inhaled methacholine, airway inflammation and cytokine production were monitored 48 h later. Notch ligand expression was assessed by real-time PCR. ***Results:*** Induction of Jagged1 expression on antigen-pulsed BMDCs was dependent on low-dose endotoxin. In vivo, transfer of endotoxin-free, antigen-pulsed BMDCs failed to induce AHR or airway eosinophilia on allergen challenge. However, administration of exogenous Jagged1-Fc together with endotoxin-free, allergen-pulsed BMDCs fully restored the responses to allergen challenge. ***Conclusions:*** These data demonstrate that LPS regulates the expression of Jagged1 on BMDCs, which is essential for the full development of lung allergic responses.

Introduction

The pathogenesis of allergic asthma is the result of both genetic and environmental factors [1]. Exposure to airborne endotoxin or lipopolysaccharide (LPS), a component of Gram-negative bacteria, in early childhood may be responsible for shifting the CD4+ T cell balance from a Th2-dominant response in newborns to a Th1 response later in life [2]. Several studies have suggested that the reduction in microbial exposure in children in more industrialized societies is responsible for the increased prevalence of allergy [3, 4]. As an extension of this notion, microorganism exposure is proposed to be necessary for the protective differentiation of the immune system and may play an important role in preventing pulmonary allergic responses [5, 6].

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Recent studies demonstrated that Notch ligand-Notch receptor interactions govern cell fate decisions in T cells such as the polarization of Th1 and Th2 cells [7–10]. In vertebrates, there are four Notch receptors (Notch1–4) and five Notch ligands, the Delta-like families (Delta1, Delta3, Delta4) and Jagged families (Jagged1, Jagged2) [11, 12]. γ-Secretase inhibitors, which effectively prevent the enzymatic cleavage of the cytoplasmic domain of Notch receptors, inhibit the downstream signaling events triggered through these receptors [13]. We recently reported that interactions of Notch and Delta1, a Notch ligand, inhibited development of airway hyperresponsiveness (AHR) as well as airway inflammation and were accompanied by heightened Th1 responses in the challenge phase of a mouse model of asthma [14]. Based on these findings, we suggested that the differential expression of Notch ligands on bone marrow-derived dendritic cells (BMDCs) in concert with Notch receptors on T cells promotes Th1 or Th2 differentiation and may be involved in regulating development of allergen-induced AHR and airway inflammation [14, 15]. Both preventing Notch signaling by γ-secretase inhibitor treatment of CD4+ T cells or reducing Jagged1 expression through si-RNA-Jagged1 silencing of dendritic cells (DCs) resulted in attenuation of the full array of lung allergic responses [15]. Here, we demonstrate the critical role of endotoxin in the upregulation of Jagged1 on DCs in the development of these lung allergic responses.

Animals and Methods

Mice

C57BL/6 mice were purchased from Harlan Laboratories (Indianapolis, Ind., USA). The mice were housed under specific pathogen-free conditions and maintained on an ovalbumin (OVA)-free diet in the Biological Resources Center at National Jewish Health. Both female and male mice, 8–12 weeks of age, were used in these experiments, and each experiment was independently performed at least 3 times with 4 mice/group (n = 12). Controls were matched to the 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.

LPS Depletion

Prior to depletion, the LPS content of the commercial OVA (cOVA) preparation (Fisher Scientific, Pittsburgh, Pa., USA) was 8–10 ng/ml. Endotoxin Detoxi-Gel™ (Thermo Scientific, Rockford, Ill., USA) was used according to the manufacturer's instructions to remove >99% of the contaminating LPS in the OVA solution, resulting in <0.001 μg/ml LPS (purified OVA, pOVA), as measured by limulus amebocyte assay (BioWhittaker Inc., Walkersville, Md., USA).

BMDC Generation and Priming

BMDCs were differentiated from bone marrow cells according to the procedure described by Inaba and colleagues [16, 17], with some modification [15]. After 7 days, more than 90% of the cells expressed characteristic DC-specific markers (CD11c+) as determined by flow cytometry. In some experiments, BMDCs on day 7 were cultured with or without pOVA, pOVA plus low-dose LPS (loLPS; 10 ng/ml), cOVA (200 μg/ml) or high-dose LPS (hiLPS; 100 ng/ml) for 24 h.

Preparation of RNA and Real-Time PCR

Total RNA was extracted from BMDCs using an RNeasy mini kit (Qiagen Inc., Valencia, Calif., USA). Two micrograms of total RNA was used in each reaction primed with oligo-dT to obtain cDNA and 3 μl of the synthesized cDNA was used as the template for real-time PCR. Real-time cDNA primers and probes for Jagged1, Delta4 and GAPDH primers and probes were obtained from Applied Biosystems (Foster City, Calif., USA). 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 ΔΔ cycle threshold method was performed for relative quantification of mRNA expression.

Administration of Jagged1-Fc to Recipients of OVA-Pulsed BMDC and Allergen Challenge

BMDCs cultured with pOVA (200 μg/ml), cOVA (200 μg/ml) or LPS (100 ng/ml) for 24 h were instilled intratracheally (2×10^6 cells/recipient) into recipient mice. Soluble Jagged1-Fc [15] was injected intraperitoneally at a daily dose of 200 μg beginning 4 days before through the day following transfer of pOVA-pulsed BMDCs in wild-type (WT) mice. Ten days after the transfer of BMDCs, mice were challenged via the airways with OVA (1% in saline) for 20 min on 3 consecutive days. Forty-eight hours after the last allergen challenge, all assays were performed. As a control, human IgG (200 μg) was administered in the same manner.

Assessment of Airway Function

Airway function was assessed as previously described by measuring changes in lung resistance (RL) in response to increasing doses of inhaled methacholine [18]. Data are expressed as percent change from baseline RL values obtained after inhalation of saline. The baseline RL responses to saline in the individual groups were not significantly different from each other.

Bronchoalveolar Lavage

Immediately following measurement of AHR, lungs were lavaged with HBSS (1 × 1 ml 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 (Cytospin 3; Thermo Fisher Scientific, Waltham, Mass., USA), stained with Leukostat (Fisher Diagnostics, Pittsburgh, Pa., USA), and differentiated by standard hematological procedures in a blinded fashion.

Measurement of Cytokines

Cytokine levels in the bronchoalveolar lavage (BAL) fluid and cell culture supernatants were measured by ELISA as previously described [19]. IL-4, IL-5, IFN-γ (BD Pharmingen, San Diego, Calif., USA) and IL-13 (R&D Systems, Minneapolis, Minn., USA)

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 means ± SEM. The t test was used to determine differences between two groups and the Tukey-Kramer test was used for comparisons between multiple groups. As measured values may not be normally distributed due to the small sample sizes, nonparametric analysis using the 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

LPS Regulates Expression of Notch Ligand, Jagged1 and Delta4 in OVA-Pulsed BMDCs

The relative expression ratios of Notch ligands regulate T cell polarization [8]. The expression of the Notch ligand, Jagged1, on antigen-presenting cells has been associated with the development of Th2 responses, whereas Delta4 expression has been associated with Th1 responses [8]. To first determine whether different doses of LPS result in differential expression levels of these Notch ligands, we analyzed levels of Jagged1 and Delta4 expression in BMDCs from donor mice following culture with LPS-free OVA (pOVA), pOVA together with loLPS, cOVA or hiLPS alone for 24 h using quantitative real-time PCR. The expression of Jagged1 was significantly higher when BMDCs were cultured for 24 h with pOVA in the presence of loLPS or with cOVA than in BMDCs cultured with purified OVA alone or BMDCs alone; similar levels of Jagged1 were expressed on BMDCs cultured with hiLPS (fig. 1a). These data demonstrated that loLPS together with OVA were essential to increasing the expression of Jagged1 on BMDC. The levels of Delta4 expression increased in an LPS dose-dependent manner, with the highest levels detected in the presence of hiLPS (fig. 1b).

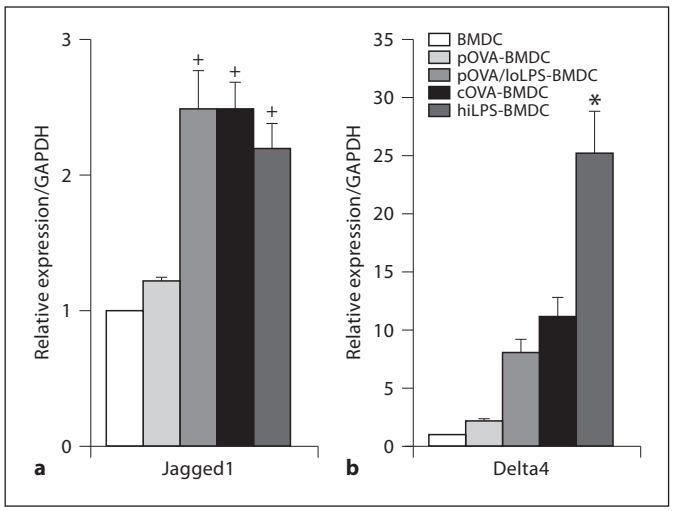

Fig. 1. Notch ligand expression in BMDCs exposed to LPS with or without pOVA. BMDCs from WT mice were incubated with or without pOVA, pOVA plus loLPS (10 ng/ml), cOVA or hiLPS (100 ng/ml) for 24 h, and mRNA was isolated. The relative expression levels of Jagged1 and Delta4 were determined by quantitative real-time PCR. cDNA contents were normalized to levels of GAPDH. Results are from 3 independent experiments and the results for each group are expressed as means ± SEM. $^{+}$ $p < 0.05$ between BMDCs cultured alone or cultured with pOVA and BMDCs cultured with pOVA plus loLPS, cOVA, or hiLPS. * $p < 0.05$ between BMDCs cultured with hiLPS and other groups. Representative of 1 of 3 similar experiments.

Effects of Different Doses of LPS with Antigen in BMDC Transfer WT Recipients

To test whether the transfer of BMDCs cultured with antigen and different doses of LPS induced allergic lung responses in WT recipients, we used a model in which transferred OVA-pulsed BMDCs were essential to the development of AHR and airway inflammation [20]. To focus on the role of LPS in the initiation of Th2-type allergic airway inflammation, BMDCs pulsed either with cOVA (cOVA-BMDC), pOVA (pOVA-BMDC), pOVA together with loLPS (pOVA/loLPS-BMDC) or hiLPS (hiLPS-BMDC) were transferred into WT recipients followed by 3 OVA challenges 10 days later (fig. 2a). As shown in figure 2b and c, recipients of cOVA-pulsed or pOVA/loLPS-BMDCs developed AHR and eosinophilic airway inflammation. In contrast, recipients of pOVA-BMDC or hiLPS-BMDC failed to develop significant alterations in airway function or eosinophilic inflammation.

The relative levels of Th1 and Th2 cytokines have been proposed to play an important role in the development of allergic airway inflammation and AHR [21]. IL-4, IL-5 and IL-13 levels in the BAL of WT recipients of cOVA-BMDCs or pOVA/loLPS-BMDCs were increased compared to the levels seen in recipients of pOVA-BMDCs or hiLPS-BMDCs; no differences in IFN-γ levels were observed among the 3 groups (fig. 2d). These data demonstrate that the combination of antigen-pulsed BMDCs and loLPS or cOVA-BMDCs resulted in the development of lung allergic responses on subsequent allergen challenge. Moreover, under conditions where the levels of

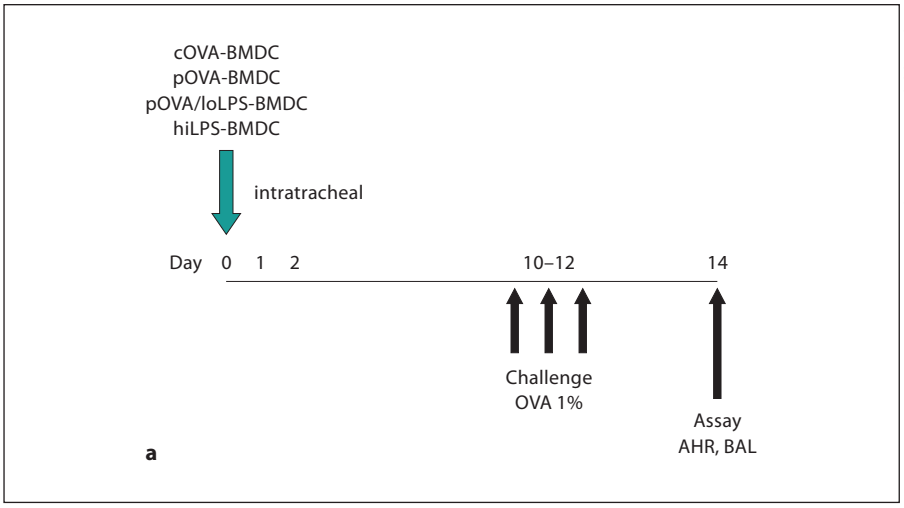

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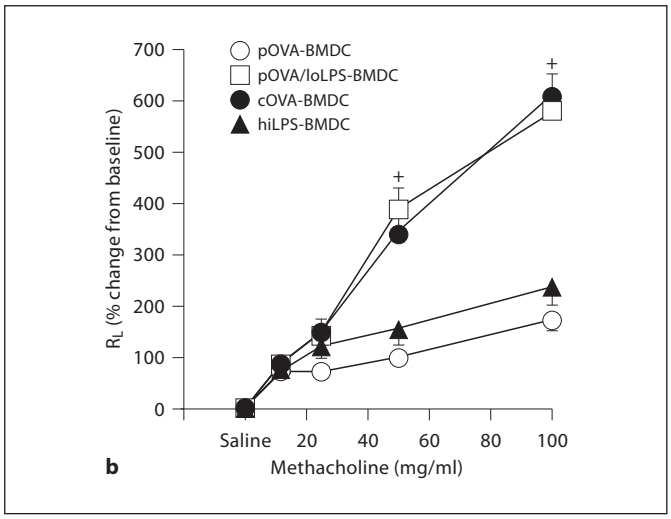
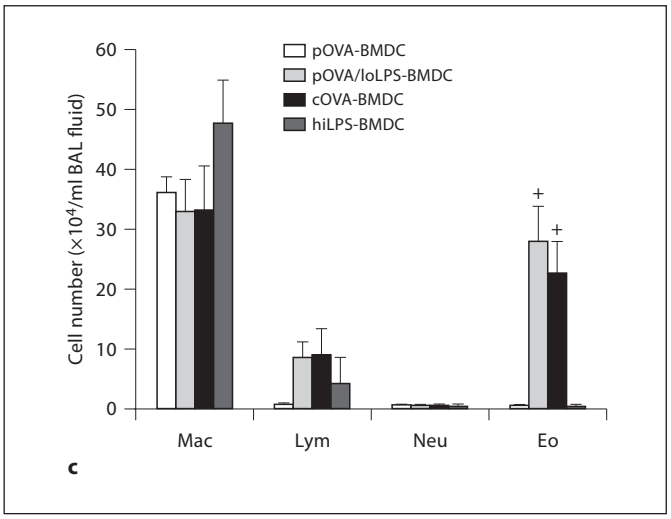
Fig. 2. Transfer of BMDCs cultured with loLPS plus antigen develop AHR and airway inflammation. Experimental protocol illustrating the time frame for transfer of BMDCs cultured with pOVA, pOVA with loLPS, cOVA or hiLPS and allergen challenge (**a**), AHR (**b**), cell composition in BAL fluid (**c**) and BAL cytokine levels (**d**). Prior to pOVA challenge, mice received pOVA-BMDCs, pOVA/loLPS-BMDCs, cOVA-BMDCs or hiLPS-BMDCs. Mac = Macrophages; Lym = lymphocytes; Neu = neutrophils; Eo = eosinophils. Data represent means ± SEM (n = 12 in each group). $^{+}p < 0.05$ between groups of cOVA-BMDC or pOVA/loLPS-BMDC and groups of pOVA-BMDC or hiLPS-BMDC.





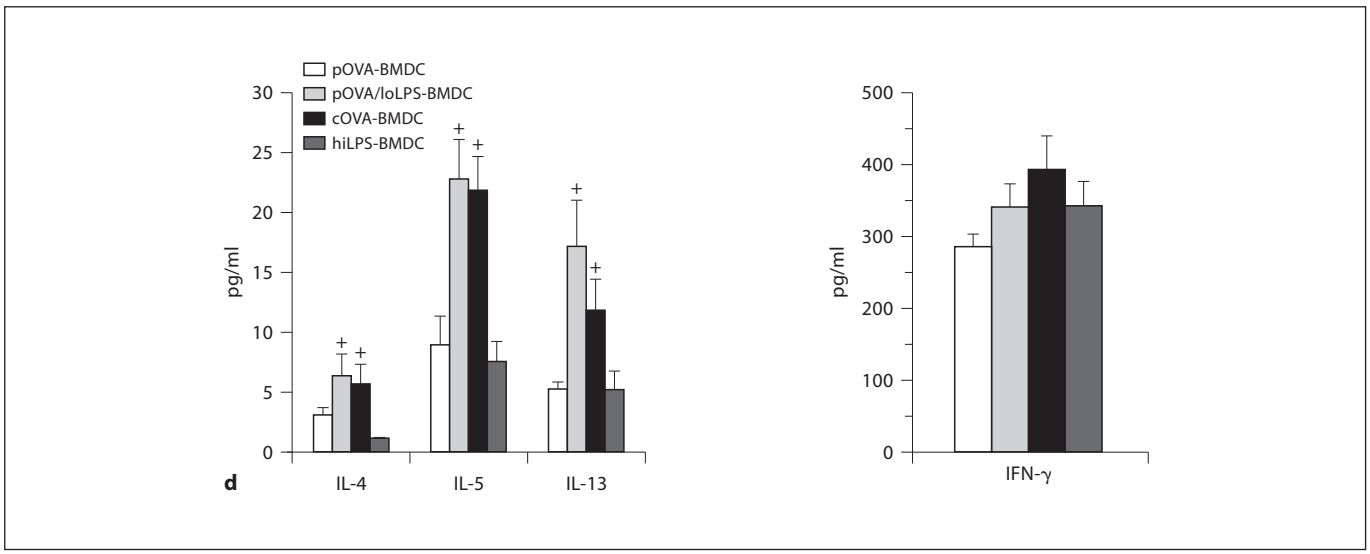
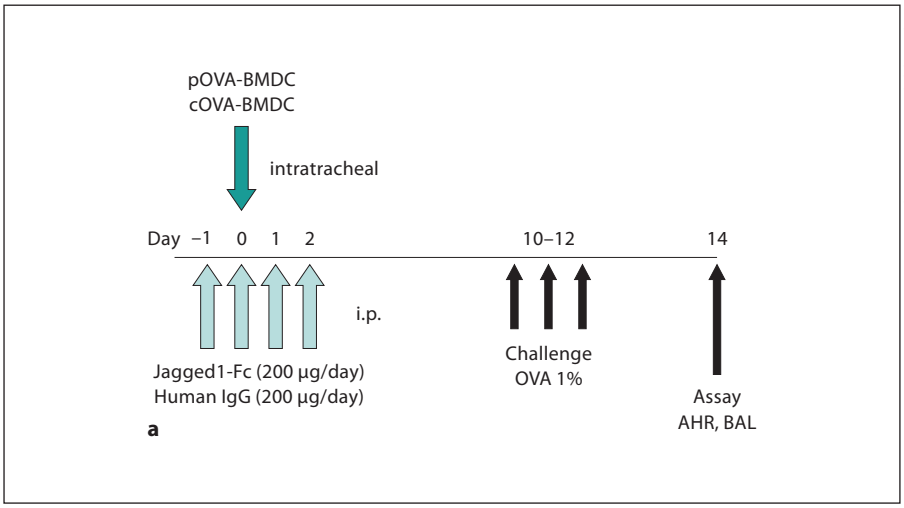


Fig. 3. Allergen-induced AHR and airway inflammation are restored in recipients of pOVA-BMDCs following administration of Jagged1-Fc. Experimental protocol illustrating the time frame for challenge with pOVA and administration of Jagged1-Fc (**a**), AHR (**b**), cell composition in BAL fluid (**c**) and BAL cytokine levels (**d**). Prior to pOVA challenge, mice received Jagged1-Fc, pOVA-BMDCs, pOVA-BMDCs/human IgG, pOVA-BMDCs/Jagged1-Fc or cOVA-BMDCs. Mac = Macrophages; Lym = lymphocytes; Neu = neutrophils; Eo = eosinophils. Data represent means ± SEM (n = 12 in each group). $^{+}$ $p < 0.05$ between groups of pOVA-BMDCs/Jagged1-Fc or cOVA-BMDCs and all other groups.



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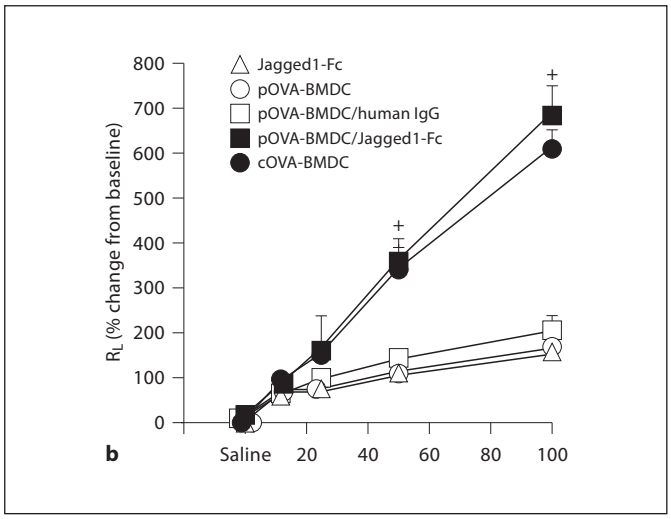

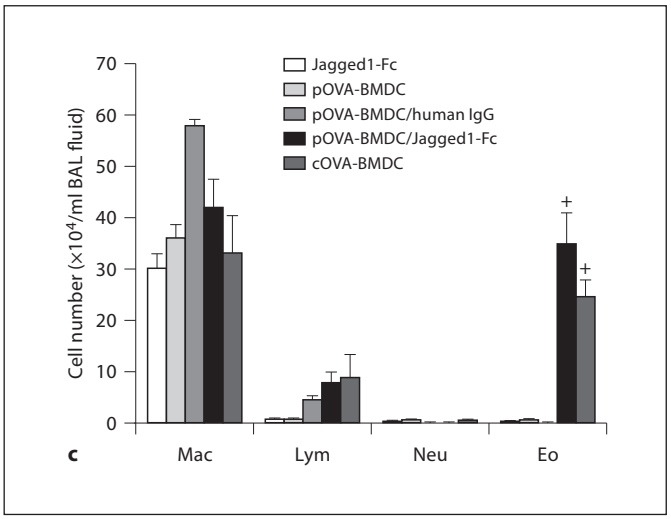

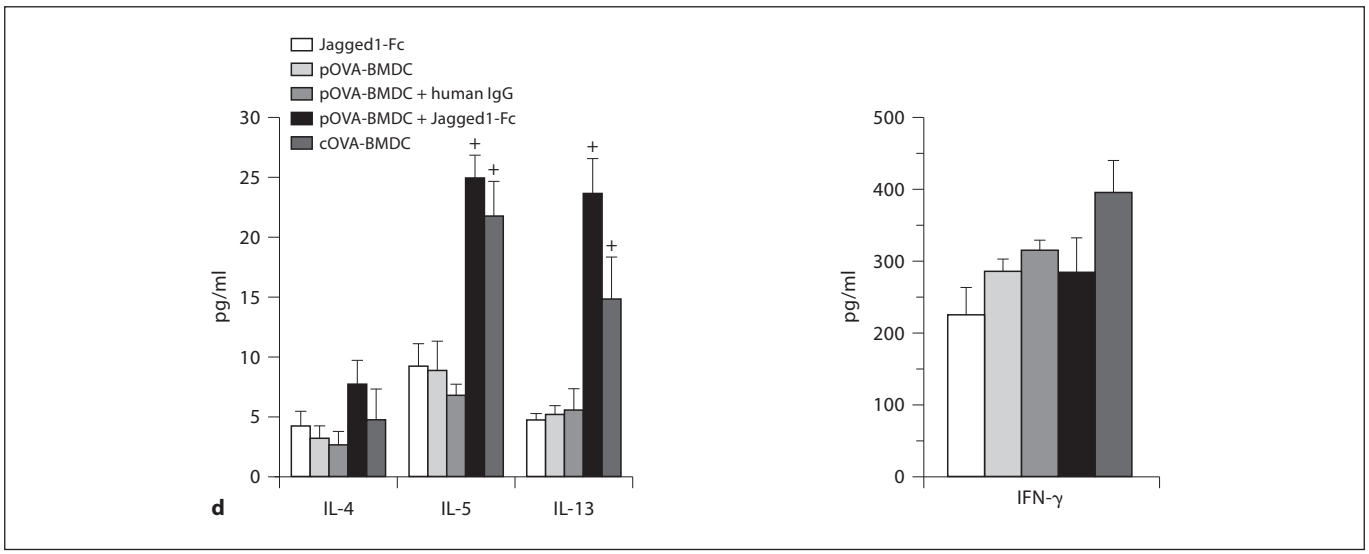

Jagged1 were not increased (pOVA) or where Jagged1 was increased but in association with high levels of Delta4 expression (hiLPS-BMDC), lung allergic responses failed to develop following BMDC transfer.

Effects of Jagged1-Fc in WT Recipients of pOVA-Pulsed BMDCs

To further assess the functional consequences of Jagged1 in vivo, Jagged1-Fc was administered beginning 4 days before through the day following transfer of OVA-pulsed BMDCs (fig. 3a). Mice which received pOVA-BMDCs together with Jagged1-Fc or cOVA-BMDCs developed increased AHR, illustrated by significant increases in RL in response to increasing doses of inhaled methacholine (fig. 3b) and a marked BAL eosinophilia (fig. 3c). In contrast, recipient mice of Jagged1-Fc alone, pOVA-BMDC plus human IgG or pOVA-BMDCs alone did not develop any increases in airway responsiveness or BAL eosinophil numbers. Examination of cytokine levels in the BAL fluid of these mice revealed that levels of IL-4, IL-5 and IL-13 were elevated in recipients of pOVA-BMDCs together with Jagged1-Fc or cOVA-BMDCs, whereas Th2 cytokine levels in the BAL fluid of recipients of Jagged1-Fc alone, pOVA- BMDCs and human IgG or pOVA-BMDCs alone were significantly lower (fig. 3d). IFN-γ levels were little affected. These data identify an essential role for Jagged1 in the full development of lung allergic responses. In the absence of LPS, where BMDCs do not upregulate expression of Jagged1, administration of exogenous Jagged1 can restore responsiveness in recipients of pOVA-pulsed BMDCs.

Discussion

Notch receptors and their ligands are expressed on the surface of mature lymphocytes and antigen-presenting cells, respectively. Notch proteins are transcriptional activators expressed first 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 CSL/RBP-J transcription factor and coactivators of the Mastermind-like family [12, 22]. DCs recognize and take up antigens in the peripheral tissues and then present processed peptides to the surface-bound MHC molecules, which are recognized by T cells, initiating T cell priming [23, 24]. Notch receptors in CD4+ T cells regulate initial IL-4 production as demonstrated using an RBP-J-deficient mouse [25, 26]. Moreover, Fang et al. [27] reported that inhibition of Notch using the dominant-negative Mastermind-like family mouse abolished IL-4 production and Th2 responses via GATA-3 from CD4+ T cells stimulated by OVA-pulsed BMDCs. In Th1-promoting DCs expressing high levels of Delta4, there was a reciprocal decrease in the expression levels of Jagged1 [28].

In this regard, microorganisms have been shown to be capable of decreasing the levels of Jagged1 and upregulating expression of Delta4, leading to an increased level of Th1-promoting and a reduced level of Th2-promoting Notch ligands on the cell surface of DCs [29]. Thus, distinct Notch ligands and the relative levels of these ligands appear capable of triggering differential effects through Notch receptor signaling, thus determining the fate of T helper cell differentiation. The LPS content in OVA solutions has been noted to play an important role in several responses including endothelial cell activation [30] and T cell activation [31], and we reasoned that such effects could be mediated through Notch ligand-Notch receptor interactions. However, given the plurality of Notch ligands and Notch receptors as well as their interplay dictating outcomes, it remains unclear how T cell fate is ultimately determined. One possibility is that the relative levels or ratio of one Notch ligand to another is important and that quantitative differences in signaling underlie determination of transcriptional specificity and induction of distinct effector cell types.

We determined that the concentration of LPS in the cOVA used here was 8–10 ng/ml before depletion of LPS by immobilized polymyxin B, and pOVA was prepared for comparison. Using these two preparations of OVA we compared their ability to induce Jagged1 mRNA by real-time PCR in BMDCs. pOVA-BMDCs failed to increase levels of Jagged1 compared to the cOVA-BMDCs, and this lack of effectiveness could be overcome by adding small amounts of LPS (10 ng/ml) to the cultures. hiLPS (100 ng/ml) in the absence of antigen pulsing also increased the expression of Jagged1 on BMDCs, but was more remarkable for inducing the highest levels of Delta4 expression. These results demonstrated that the relative expression levels of Jagged1 and Delta4 on BMDCs could be altered by different concentrations of LPS.

We then tested the functional consequences of these manipulations in a BMDC transfer model. We previously showed that Jagged1 interactions with the Notch receptor on CD4+ T cells was essential for initiating lung allergic responses in allergen-challenged mice [15]. Transfer of LPS-free, antigen-pulsed BMDCs failed to induce AHR, airway eosinophilia or Th2 cytokine production

when compared to responses in recipients of cOVA-BMDCs or pOVA/loLPS-BMDCs. The failure of recipients of pOVA-BMDCs to respond to allergen challenge was fully overcome when Jagged1-Fc was also administered prior to allergen challenge. Jagged1 alone was ineffective without antigen-pulsed BMDCs. Taken together, these data identify Jagged1 as a major regulator of the development of lung allergic responses following allergen-pulsed BMDC transfer.

There have been several reports of a role for LPS in the initiation of allergen-induced lung responses in asthmatics [32–36]. Timing of the exposure appears critical as high levels of endotoxin may be protective at certain stages of asthma [37]. In corticosteroid-resistant asthmatics, LPS levels in BAL fluid were higher than in corticosteroid-sensitive patients and the prolonged exposure to LPS induced a functional steroid resistance in monocytes and increased IL-6 release [38]. A role for LPS in initiating lung allergic responses in mice has been demonstrated when exposure to allergen was carried out in the absence of systemic sensitization together with adjuvant [31, 39]. In the model used here, a similar set of conditions may have prevailed; in the absence of systemic sensitization a critical role for LPS in priming BMDCs could be demonstrated. In humans, exposure to endotoxin at an early age, such as in a farm environment with livestock, has been shown to attenuate the development of Th2-related disease, including atopic asthma. These findings, among others, formed the basis of the hygiene hypothesis [40]. The results in the present study support this notion, as demonstrated by the findings that small amounts of endotoxin enhanced the development of lung allergic responses through increased expression of the Notch ligand Jagged1, an essential contributor to Th2 differentiation. In contrast, exposure to large amounts of endotoxin inhibited the development of lung allergic responses through increased expression of the Notch ligand Delta4, despite higher levels of expression of Jagged1.

These data demonstrate that in the development of lung allergic responses Notch ligand-Notch receptor interactions represent important regulators of the differentiation of T lymphocytes, in part dictated by the balance between Jagged1 and Delta4 expression, which is in turn regulated by levels of LPS in the airways. Given the ubiquitous nature of endotoxin, both Notch receptors and Notch ligands represent important targets for intervention in allergic asthma.

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