Notch signaling in T cells is essential for allergic airway inflammation, but expression of the Notch ligands Jagged 1 and Jagged 2 on dendritic cells is dispensable

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Background: Allergic asthma is characterized by a T_H2 response induced by dendritic cells (DCs) that present inhaled allergen. Although the mechanisms by which they instruct T_H2 differentiation are still poorly understood, expression of the Notch ligand Jagged on DCs has been implicated in this process.
Objective: We sought to establish whether Notch signaling induced by DCs is critical for house dust mite (HDM)–driven allergic airway inflammation (AAI) *in vivo*.
Methods: The induction of Notch ligand expression on DC subsets by HDM was quantified by using quantitative real-time PCR. We used an HDM-driven asthma mouse model to compare the capacity of Jagged 1 and Jagged 2 single- and double-deficient DCs to induce AAI. In addition, we studied AAI in mice with a T cell–specific deletion of recombination signal–binding protein for immunoglobulin Jκ region (RBPJκ), a downstream effector of Notch signaling.
Results: HDM exposure promoted expression of Jagged 1, but not Jagged 2, on DCs. In agreement with published findings, *in vitro*–differentiated and HDM-pulsed Jagged 1 and Jagged 2 double-deficient DCs lacked the capacity to induce AAI. However, after *in vivo* intranasal sensitization and challenge with HDM, DC-specific Jagged 1 or Jagged 2 single- or double-deficient mice had eosinophilic airway inflammation and a T_H2 cell activation phenotype that was not different from that in control littermates. In contrast, RBPJκ-deficient mice did not experience AAI and airway hyperreactivity.
Conclusion: Our results show that the Notch signaling pathway in T cells is crucial for the induction of T_H2-mediated AAI in an HDM-driven asthma model but that expression of Jagged 1 or Jagged 2 on DCs is not required. (J Allergy Clin Immunol 2017;■■■:■■■-■■■.)

Key words: *Allergic asthma, Notch signaling, Gata-3, T_H2 immunity, T_H1 immunity, house dust mite, Jagged, recombination signal–binding protein for immunoglobulin Jκ region, cytokines*

Allergic asthma is a T_H2 cell–mediated disease characterized by chronic airway inflammation, airway hyperreactivity, and episodes of bronchoconstriction. Inflammatory dendritic cells (DCs) are necessary for induction of T_H2 immunity to inhaled house dust mite (HDM) allergen in mice, as was shown in CD11c–diphtheria toxin receptor mice in which DCs were specifically depleted by diphtheria toxin exposure.[1] Lung-resident DCs continuously sample the airway lumen for the presence of allergens, such as HDM, and once activated, these cells mature and migrate to the draining lymph nodes to activate naive T cells.[2] On antigenic stimulation by DCs, T_H2 cell differentiation is initiated whereby the polarizing cytokine IL-4, which induces phosphorylation and activation of signal transducer and activator of transcription 6, enhances expression of the key T_H2 transcriptional regulator Gata-3.[3] T_H2 cells are potent producers of cytokines that induce IgE synthesis (IL-4), recruit eosinophils (IL-5), and cause smooth muscle hyperreactivity and goblet cell hyperplasia (IL-13).

Therefore initiation of T_H2 cell differentiation through the IL-4/signal transducer and activator of transcription 6 axis is suggestive of an autocrine loop that leads to expansion of IL-4–producing T cells. However, the primary origin of IL-4, which induces the T_H2 response, remains unclear.

One of the pathways that has been implicated in the initiation of T_H2 cell differentiation is the Notch signaling pathway. It has been demonstrated that Notch signaling has the capacity to induce T_H2 cell differentiation by (1) directly activating the upstream *Gata3* gene promoter and (2) regulating *Il4* gene transcription through activation of a 3′ enhancer.[4-6] Both of these are dependent on a nuclear complex that includes recombination signal–binding protein for the immunoglobulin Jκ (RBPJκ) region and the coactivator mastermind-like 1 (MAML1). Notch signaling in $CD4^+$ T cells is required for physiologic T_H2 responses to parasite antigens, as was shown in mice deficient for the RBPJκ region or the Notch 1 and Notch 2 receptors[4] and in mice expressing dominant negative MAML.[7] Moreover, pharmacologic inhibition of γ-secretase, the enzyme that liberates the intracellular Notch domain from the plasma membrane, allowing it to function as a transcription factor in the nucleus, led to decreased T_H2 cytokine production after immunization with ovalbumin (OVA) in an asthma model.[8]

Several lines of research support that the Notch ligands Delta-like ligand (DLL) and Jagged instruct T_H1 and T_H2 cell differentiation, respectively.[9] Surface DLL expression was shown to promote T_H1 cell generation and to reduce T_H2 responses,

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Abbreviations used
AAI: Allergic airway inflammation
BAL: Bronchoalveolar lavage
BMDC: Bone marrow–derived dendritic cell
cDC: Conventional dendritic cell
DC: Dendritic cell
DLL: Delta-like ligand
EYFP: Enhanced yellow fluorescent protein
HDM: House dust mite
MedLN: Mediastinal lymph node
MoDC: Monocyte-derived dendritic cell
OVA: Ovalbumin
qRT-PCR: Quantitative real-time PCR
RBPJκ: Recombination signal–binding protein for immunoglobulin Jκ region
RSV: Respiratory syncytial virus
WT: Wild-type

whereas Jagged-expressing antigen-presenting cells stimulated T_H2 effector generation.[6] Jagged 1 can be upregulated on DCs by stimuli that promote T_H2 cell responses, such as through thymic stromal lymphopoietin, which is produced by diesel exhaust particle–treated human bronchial epithelial cells,[10] and on stimulation with *Trypanosoma brucei*–derived antigens, as well as TNF, *Dermatophagoides pteronyssinus* group 7 allergen (Der p 7), and low-dose LPS.[11-13] Jagged 1 was shown to be crucial in the induction of a T_H2 response in a model of airway hyperresponsiveness using OVA-pulsed, *in vitro*–cultured, GM-CSF bone marrow–derived dendritic cells (BMDCs).[14] Although evidence was provided that Jagged 2 is dispensable for the induction of T_H2 cells *in vivo*,[15,16] Jagged 2 was shown to have the capacity to induce T_H2 cell differentiation *in vitro*.[15] Correspondingly, DLL1 and DLL4 ligands are induced on DCs by stimuli that elicit T_H1 responses and have the capacity to induce T_H1 differentiation *in vitro*.[17,18]

In contrast to this model, it has been hypothesized that Notch signaling acts as a general amplifier of helper T-cell responses rather than an instructive director of specific cell fates. This could be through either enhancing proliferation, cytokine production, and antiapoptotic signals[19-21] or boosting antigen sensitivity through promotion of costimulatory signals in T cells.[22,23]

Therefore in this report we aimed to determine whether Notch signaling is critical for HDM-driven allergic airway inflammation (AAI) *in vivo*. In particular, we questioned whether Jagged 1 and Jagged 2 on DCs are required for the induction of polarization of naive T cells into T_H2 cells. We found that expression of Jagged 1 or Jagged 2 on DCs is not required, whereas T cells do need Notch signals, specifically to differentiate into T_H2 cells.

METHODS

For detailed methods, including mice used, experimental protocols and statistical analysis, see the Methods section in this article's Online Repository at www.jacionline.org.

RESULTS

Jagged 1 is upregulated on *in vitro* GM-CSF BMDCs on exposure to HDM

Because several research groups have shown a role for Jagged in the orchestration of T-cell responses by using GM-CSF BMDCs,[6,11,14-16] we first investigated the expression of Notch ligands on BMDCs on stimulation with the pro-T_H2 stimulus HDM and the pro-T_H1 stimulus LPS. GM-CSF BMDCs were cultured from wild-type (WT) mice and sorted at day 9 into $CD11c^+$MHC class $II^{int}F4/80^-CD115^+$ GM–monocyte-derived dendritic cells (MoDCs), $CD11c^+MHCII^{high}F4/80^-CD115^-$ GM-DCs, and $CD11c^+MHCII^{int}F4/80^+CD115^+$ GM-macrophages (Fig 1, *A*) based on the study by Helft et al.[24] On HDM stimulation, *Jag1* mRNA was upregulated on GM-MoDCs and GM-DCs, whereas LPS stimulation induced upregulation of DLL4 mRNA on GM-MoDCs and GM-macrophages. Expression of *Jag2* and DLL1 was not altered on GM-CSF BMDCs on stimulation (Fig 1, *A*). Thus *Jag1* mRNA is substantially upregulated on *in vitro* GM-CSF BMDCs after HDM stimulation.

Jagged is crucial during the sensitization phase in a model that uses GM-CSF BMDCs to induce AAI

To delete *Jag1* and *Jag2* specifically in DCs, we used $Jag1^{fl/fl}$ and $Jag2^{fl/fl}$ mice, in which the *Jag* loci contain loxP sites, as well as CD11c-Cre transgenic mice, expressing Cre recombinase under the control of the DC-specific CD11c promoter. Efficiency of CD11c-Cre–mediated deletion was confirmed in CD11c-Cre transgenic $ROSA^{EYFP}$ mice with Cre-mediated excision of a loxP-flanked transcriptional STOP sequence. GM-CSF BMDCs were cultured from CD11c-Cre×$ROSA^{EYFP}$ and WT×$ROSA^{EYFP}$ mice with GM-CSF. Analysis of enhanced yellow fluorescent protein (EYFP) expression by means of flow cytometry indicated that GM-CSF BMDC subsets manifested Cre-mediated deletion in 70% to 74% of the cells (see Fig E1, *A*, in this article's Online Repository at www.jacionline.org).

Next, we analyzed Jagged mRNA expression in DCs from CD11c-Cre transgenic $Jag1^{fl/fl}Jag2^{fl/fl}$ mice ($Jg1Jg2^{\Delta CD11c/\Delta CD11c}$) and $Jg1Jg2^{+/+}$ control mice (Fig 1, *B*). We found reduced expression of *Jag1* and *Jag2* compared with that seen in WT DCs in all GM-CSF BMDC subsets. Finally, recombination of *Jag1* and *Jag2* was confirmed on genomic DNA of GM-CSF BMDCs from $Jg1Jg2^{\Delta CD11c/\Delta CD11c}$ mice compared with $Jg1Jg2^{+/+}$ mice (see Fig E1, *B*).

To confirm that Jagged expression on DCs is essential for AAI induction by means of intratracheal transfer of allergen-pulsed GM-CSF BMDCs, we sensitized WT mice with HDM-pulsed total GM-CSF BMDCs from $Jg1Jg2^{\Delta CD11c/\Delta CD11c}$ or $Jg1Jg2^{+/+}$ mice and challenged the mice with HDM (Fig 2, *A*). HDM-stimulated $Jg1Jg2^{+/+}$ GM-CSF BMDCs induced AAI, as evidenced by a significant increase in numbers of eosinophils, macrophages, neutrophils, B cells, T cells, and DCs in bronchoalveolar lavage (BAL) fluid compared with numbers in mice sensitized with PBS-treated GM-CSF BMDCs (Fig 2, *B*). In contrast, GM-CSF BMDCs from $Jg1Jg2^{\Delta CD11c/\Delta CD11c}$ mice lacked the capacity to induce AAI (Fig 2, *B*). Accordingly, numbers of IL-4^+, IL-5^+, IL-13^+, IFN-γ^+, and IL-$17A^+$ T cells in BAL fluid (Fig 2, *C*) or Gata-3^+ T_H2 cells in mediastinal lymph nodes (MedLNs; Fig 2, *D* and *E*) were reduced when mice were sensitized with $Jg1Jg2^{\Delta CD11c/\Delta CD11c}$ GM-CSF BMDCs compared with control DCs. Numbers of Rorγt^+ T_H17 cells or $Foxp3^+$ regulatory T cells in MedLNs were not different between the 2 groups of mice, and T box–containing protein expression was not detected (data not shown).

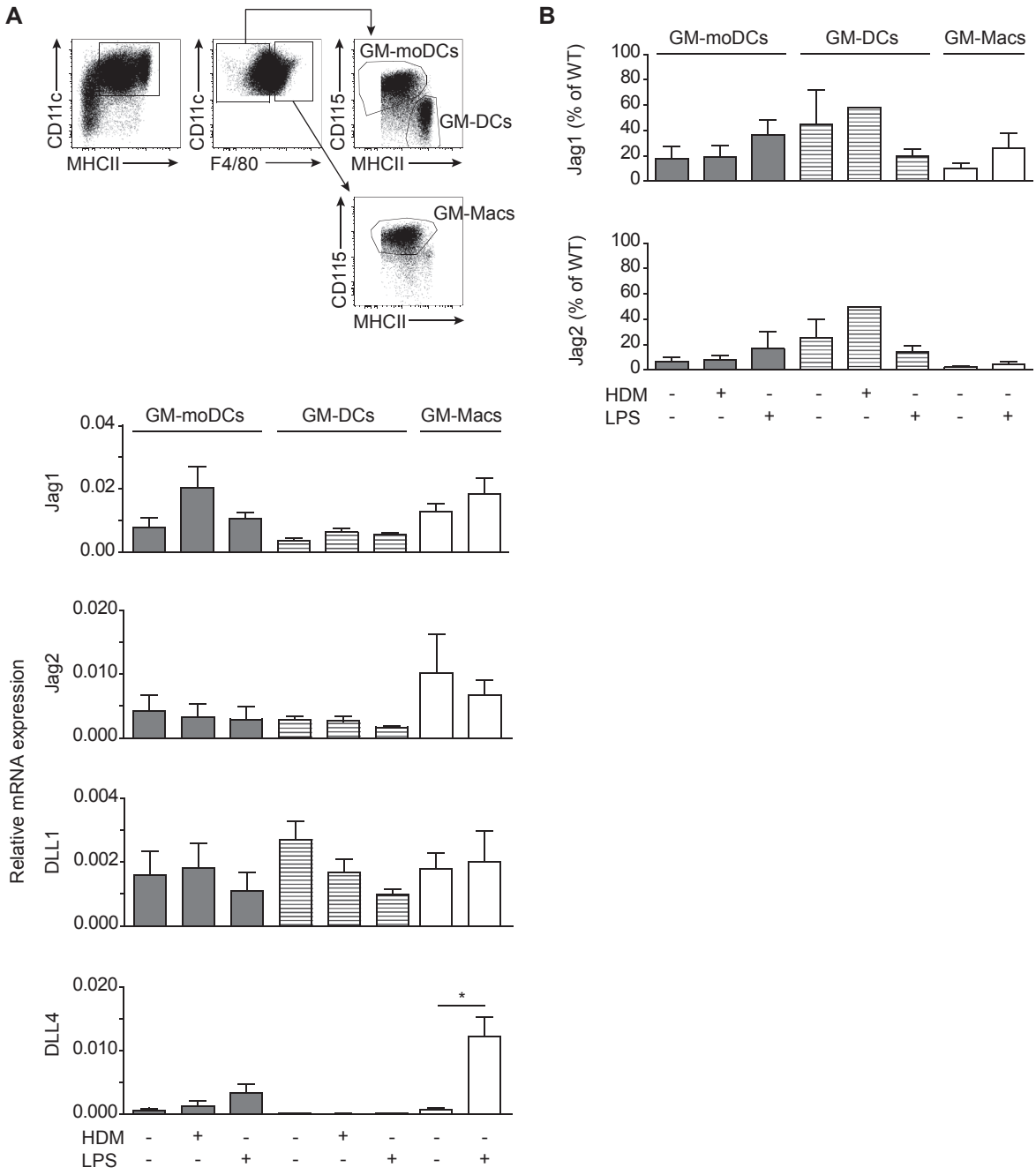


FIG 1. Jagged 1 is upregulated on BMDCs on HDM exposure, and CD11c-Cre is effective in GM-CSF BMDCs. **A,** Flow cytometric gating strategy for BMDC subsets from C57BL/6 mice. Live cells were analyzed for CD11c and MHC class II and gated as indicated *(top)*. mRNA expression of the indicated Notch ligands quantified by using quantitative real-time PCR (qRT-PCR) in GM-MoDCs, GM-DCs, and GM-macrophages and stimulated overnight in the presence or absence of HDM or LPS, as indicated *(bottom)*. **B,** Quantification of relative Jagged 1 and Jagged 2 expression by using qRT-PCR in BMDC subsets that were fluorescence-activated cell sorted from $Jg1Jg2^{\Delta CD11c/\Delta CD11c}$ mice compared with WT C57BL/6 mice, which were set to 100%. Data are shown as means + SEMs of 4 mice per group in 1 experiment, except for HDM-stimulated GM-DCs (n = 2 in Fig 1, *A*, and n = 1 in Fig 1, *B*). $^*P < .05$, Mann-Whitney *U* test.

The defective capacity of $Jg1Jg2^{\Delta CD11c/\Delta CD11c}$ GM-CSF BMDCs (see Fig E2, *A*, in this article's Online Repository at www.jacionline.org) to induce T_H2 polarization *in vivo* was likely not due to cell-intrinsic defects because these DCs expressed similar levels of costimulatory molecules (see Fig E2, *B*), DLL1, and DLL4 (see Fig E2, *C*) and produced similar amounts of proinflammatory cytokines (see Fig E2, *D*), as did control DCs on *in vitro* activation with a variety of stimuli.

Finally, to investigate whether expression of Jagged 1 and Jagged 2 is perhaps also required during the challenge phase of AAI induction, $Jg1Jg2^{\Delta CD11c/\Delta CD11c}$ or $Jg1Jg2^{+/+}$ mice were sensitized with WT GM-CSF BMDCs and challenged with HDM. We found

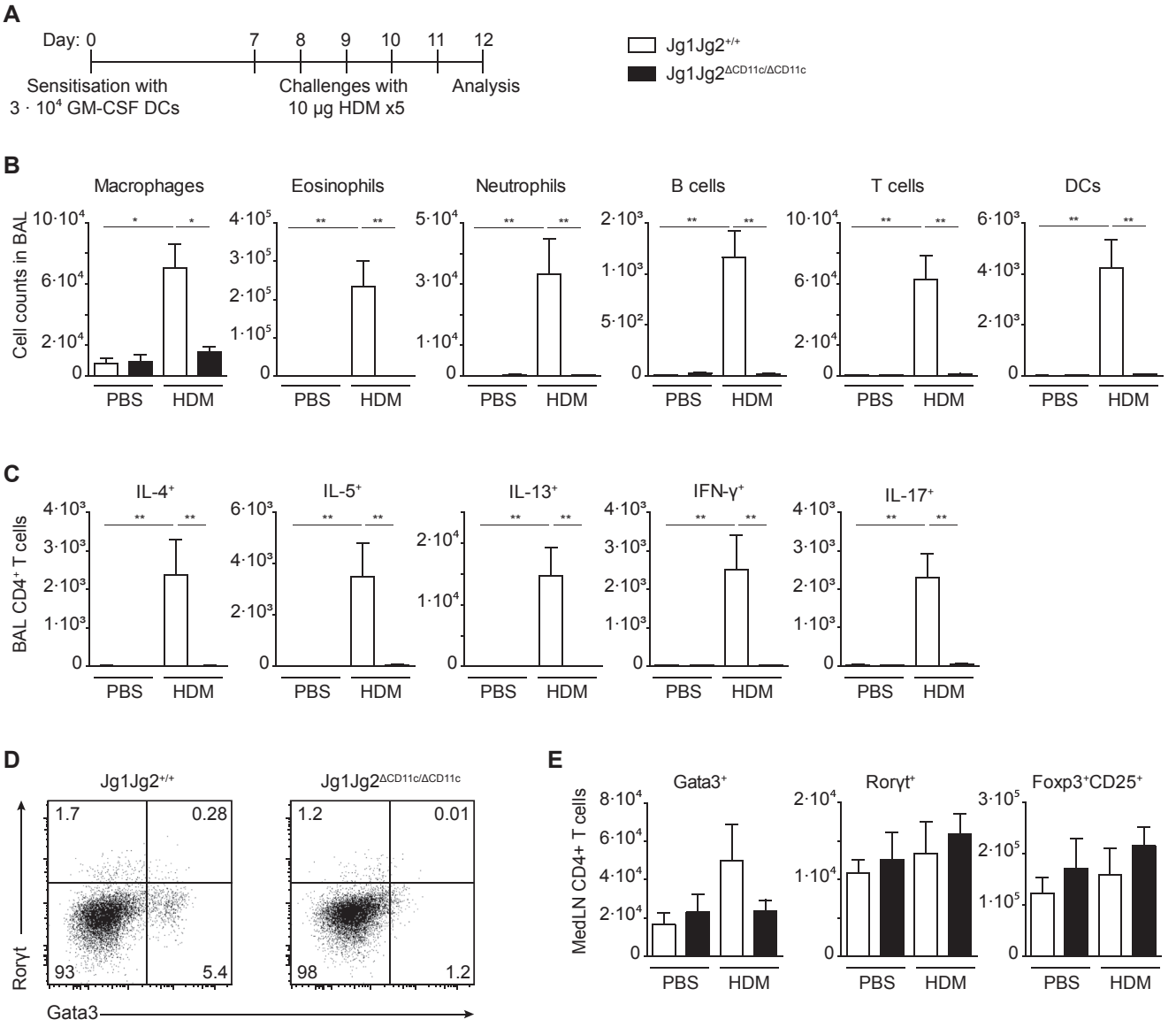


FIG 2. Jagged 1 and Jagged 2 are crucial during the sensitization phase when using GM-CSF BMDCs to induce AAI. **A,** Sensitization and challenge scheme of HDM-driven AAI in mice using cultured total BMDCs. **B,** Numbers of macrophages ($FSC^{high}SSC^{high}$Autofluorescent$^+CD11c^+$Siglec-F^+), eosinophils ($FSC^{int}SSC^{high}$Siglec-F^+), neutrophils (Ly-$6G^+$), B cells ($CD19^+$), T cells ($CD3^+$), and DCs ($CD11c^+$MHC class II^{hi}) in BAL fluid in mice treated with either PBS-pulsed or HDM-pulsed BMDCs from *Jg1Jg2*$^{\Delta CD11c/\Delta CD11c}$ or *Jg1Jg2*$^{+/+}$ mice. *FSC*, Forward scatter; *SSC*, side scatter. **C,** Numbers of IL-4^+, IL-5^+, IL-13^+, IFN-γ^+, and IL-$17A^+$ $CD3^+CD4^+$ T cells in BAL fluid in mice treated with either PBS-pulsed or HDM-pulsed BMDCs. **D,** Flow cytometric profile of Gata-3/Rorγt expression in $CD3^+CD4^+$ T cells in mice treated with HDM-pulsed BMDCs from *Jg1Jg2*$^{\Delta CD11c/\Delta CD11c}$ or *Jg1Jg2*$^{+/+}$ mice. **E,** Total numbers of Gata-3^+, Rorγt^+, and $Foxp3^+CD25^+$ $CD3^+CD4^+$ T cells in MedLNs from mice treated with PBS- or HDM-pulsed BMDCs from *Jg1Jg2*$^{\Delta CD11c/\Delta CD11c}$ or *Jg1Jg2*$^{+/+}$ mice, as indicated. Data are shown as means + SEMs of 4 (PBS) or 6 (HDM) mice per group in 1 experiment. **P* < .05 and ***P* < .01, Mann-Whitney *U* test.

comparable AAI induction in *Jg1Jg2*$^{\Delta CD11c/\Delta CD11c}$ and *Jg1Jg2*$^{+/+}$ mice (not shown), indicating that for AAI induction, Jagged expression is only required on GM-CSF BMDCs during the sensitization phase and not during HDM challenge.

Taken together, these findings confirm that expression of Jagged 1 and Jagged 2 is crucial during the sensitization phase in a model in which GM-CSF BMDCs are used to induce HDM-driven AAI.

Jagged 1 is highly upregulated on *in vivo* migratory $CD11b^+$ conventional DCs on HDM exposure

To analyze the role of Jagged expression in a more physiologic HDM-driven airway inflammation model, we first aimed to establish which *in vivo* DC subsets express crucial Notch ligands during HDM exposure. In this context $CD11b^+$ conventional dendritic cells (cDCs) were shown to be the main DC subset involved in induction of T_H2 cells in the draining lymph nodes,

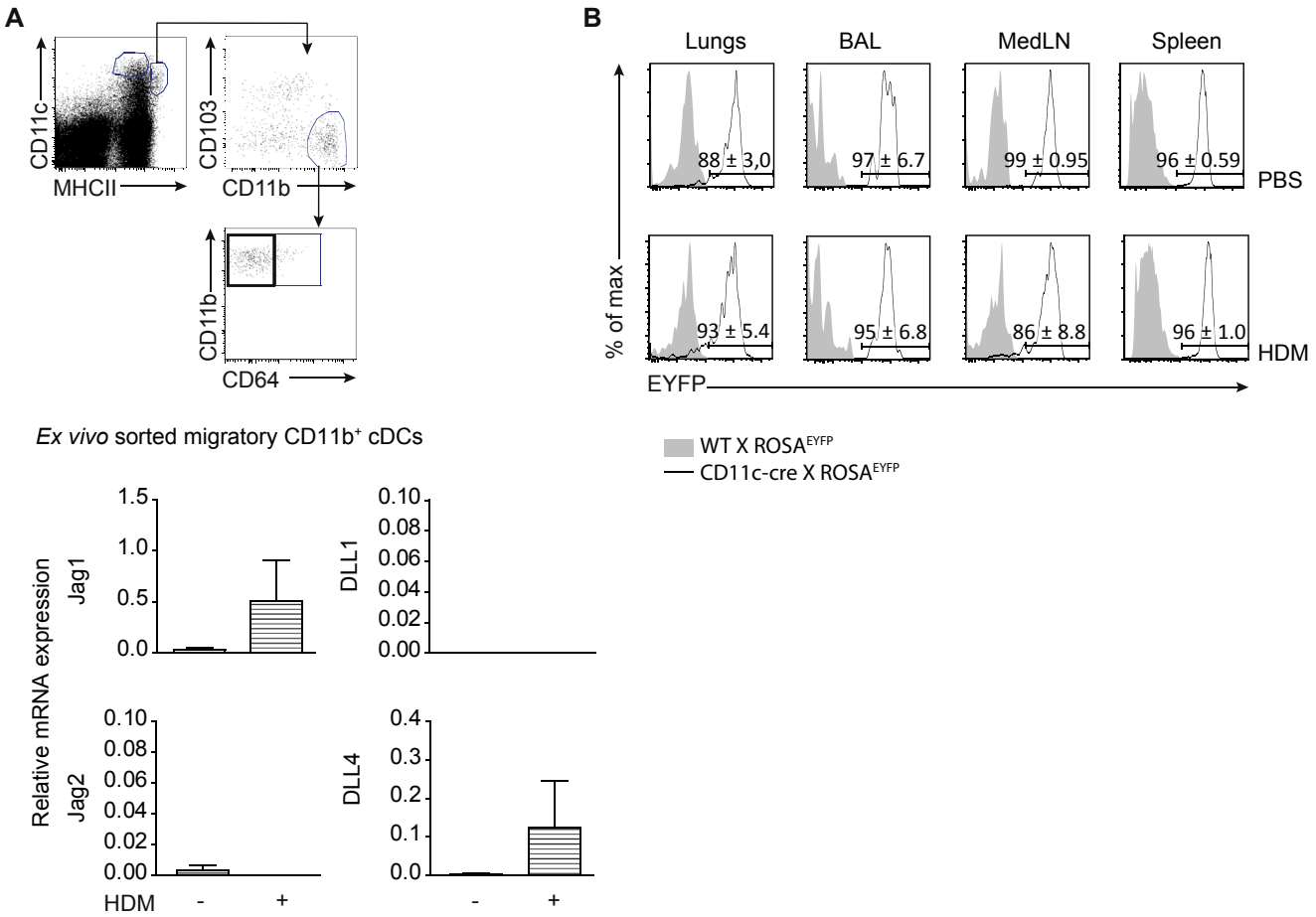


FIG 3. Jagged 1 is upregulated on migratory CD11b$^+$ cDCs on HDM exposure, and CD11c-Cre is effective in *in vivo* DCs. **A,** Gating strategy of *ex vivo*–sorted DC subsets from C57BL/6 mice intranasally treated with 50 μg of HDM or PBS *(top)*. mRNA expression of the indicated Notch ligands, as determined by using qRT-PCR, in DAPI$^-$MHC class II^{hi}CD11b$^+$CD103$^-$CD64$^-$ (migratory) DCs from MedLNs after 72 hours of *in vivo* stimulation *(bottom)*. Six mice were pooled per sample. Data are shown as means + SEMs of 3 samples per group in 1 experiment. **B,** EYFP expression in CD11c$^+$MHC class II^{hi} DCs in the indicated tissues from WT×ROSA^{EYFP} and CD11c-Cre×ROSA^{EYFP} mice after 72 hours of *in vivo* stimulation with 50 μg of HDM or PBS. Data are shown as histogram overlays of EYFP expression in the indicated mice. Samples were concatenated, and data are shown as means + SDs of 4 mice (CD11c-Cre×ROSA^{EYFP}) or 2 to 3 mice (WT×ROSA^{EYFP}) per group in 1 experiment.

whereas MoDCs play a crucial role during the challenge phase.[25] We sorted resident MoDCs, migratory MoDCs, resident CD11b$^+$ cDCs, and migratory CD11b$^+$ cDCs from MedLNs of WT mice intranasally treated with HDM or PBS for 72 hours. In migratory CD11b$^+$ cDCs, both Jagged 1 and DLL4 were expressed at baseline and significantly upregulated on exposure to HDM, whereas Jagged 2 and DLL1 were not detected (Fig 3, *A*). Resident MoDCs, migratory MoDCs, and resident CD11b$^+$ cDCs expressed very low levels of *Jag1* mRNA, and expression of other Notch ligands was not detected (data not shown).

Jag1 and *Jag2* are effectively deleted in DCs from *Jg1Jg2*$^{\Delta CD11c/\Delta CD11c}$ mice

To check the efficacy of CD11c-Cre–mediated *in vivo* gene deletion, we analyzed DCs from CD11c-Cre×ROSA^{EYFP} and control mice. EYFP was expressed in 88% to 97% of CD11c$^+$MHC class II^{high} DCs in lungs, BAL fluid, MedLNs, and spleens and was unaltered when mice were challenged with 50 μg of HDM 72 hours before analysis (Fig 3, *B*, and see Table E2 in this article's Online Repository at www.jacionline.org for a detailed analysis of EYFP expression in DC subsets and other immune cells). In accordance with the EYFP data, *Jag1* and *Jag2* mRNA expression was not detected in migratory CD11b$^+$ cDCs sorted from MLNs from *Jg1Jg2*$^{\Delta CD11c/\Delta CD11c}$ mice (data not shown).

Together, these data show that Jagged 1, but not Jagged 2, is substantially upregulated on migratory CD11b$^+$ cDCs on stimulation with HDM. In addition, DCs from *Jg1Jg2*$^{\Delta CD11c/\Delta CD11c}$ mice show almost complete *in vivo* deletion of both Jagged 1 and Jagged 2.

Mice lacking Jagged expression on DCs have AAI similar to that seen in WT animals

Next, we used an acute AAI model by sensitizing and challenging *Jg1Jg2*$^{\Delta CD11c/\Delta CD11c}$ and *Jg1Jg2*$^{+/+}$ mice with HDM. Four days after the last challenge, mice were analyzed (Fig 4, *A*). Surprisingly, after HDM exposure, both

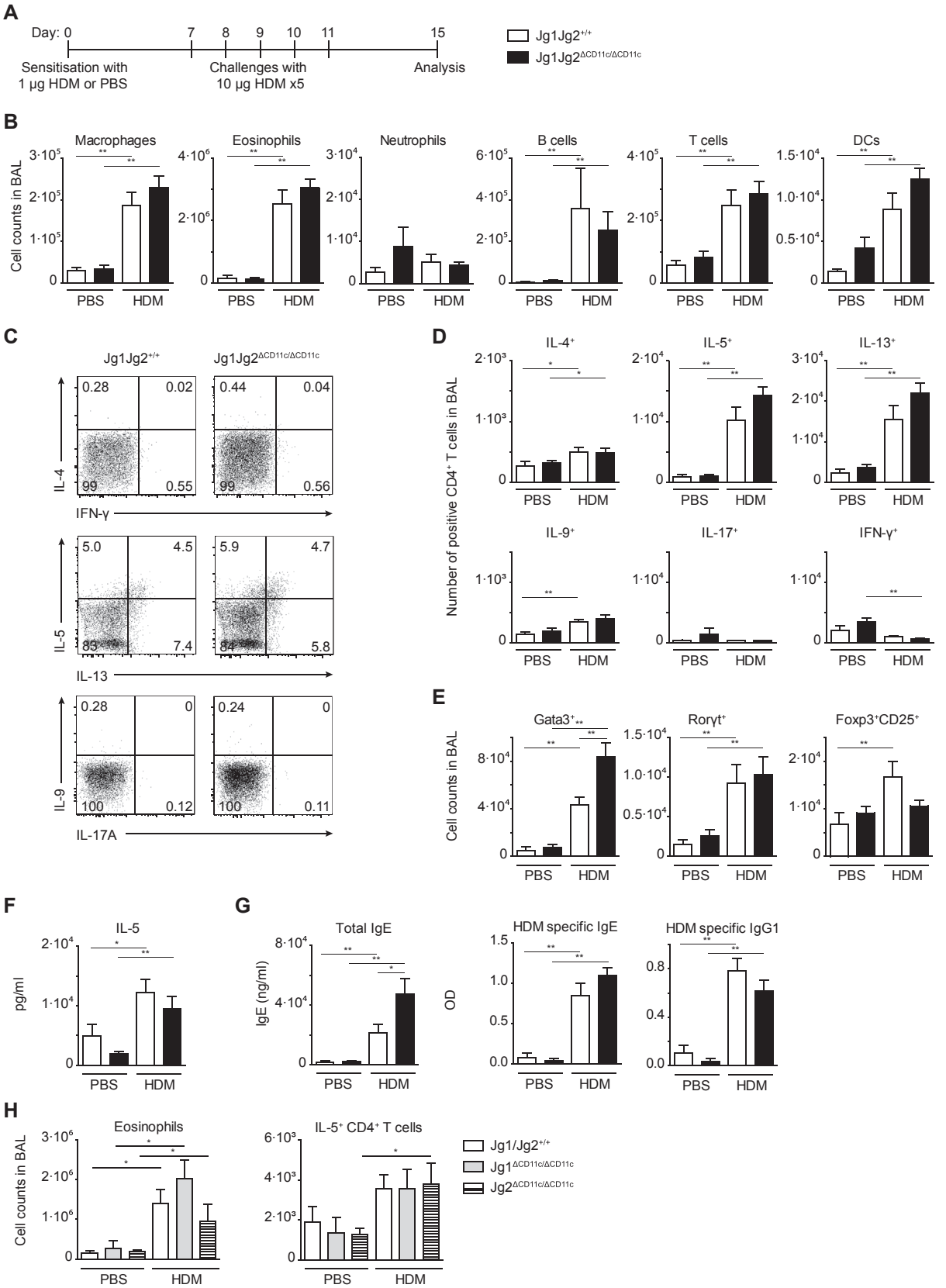


FIG 4. Jagged 1 and Jagged 2 expression on DCs is dispensable for the development of AAI *in vivo*. **A,** Scheme of HDM-mediated AAI induction in mice. **B,** Total numbers of indicated cell populations in BAL fluid from PBS- or HDM-treated *Jg1Jg2*$^{\Delta CD11c/\Delta CD11c}$ or *Jg1Jg2*$^{+/+}$ mice. **C** and **D,** Intracellular flow cytometric analysis of cytokine production by $CD3^+CD4^+$ T cells in BAL fluid from the indicated mice

$Jg1Jg2^{\Delta CD11c/\Delta CD11c}$ and $Jg1Jg2^{+/+}$ mice had similar AAI inflammation characterized by increased numbers of macrophages, eosinophils, neutrophils, B cells, and T cells in BAL fluid compared with those in PBS-sensitized mice (Fig 4, *B*). $Jg1Jg2^{\Delta CD11c/\Delta CD11c}$ and $Jg1Jg2^{+/+}$ mice showed similar increases in IL-4–, IL-5–, IL-13–, and IL-9–expressing $CD4^+$ T cells, and numbers of IFN-γ or IL-17A helper T cells were similar (Fig 4, *C* and *D*). Accordingly, restimulated MedLN cells from $Jg1Jg2^{\Delta CD11c/\Delta CD11c}$ and $Jg1Jg2^{+/+}$ mice showed no difference in HDM-induced IL-5 production (Fig 4, *E*). In addition, numbers of Gata-3^+ T cells were higher in HDM-treated $Jg1Jg2^{\Delta CD11c/\Delta CD11c}$ mice compared with those in $Jg1Jg2^{+/+}$ control mice. In these experiments the numbers of Rorγt^+ and Foxp3^+ T cells were not different between the 2 groups (Fig 4, *F*). T box–containing protein–positive T cells were not detected (data not shown). Although total serum IgE levels were higher in $Jg1Jg2^{\Delta CD11c/\Delta CD11c}$ mice compared with those in $Jg1Jg2^{+/+}$ mice, HDM-specific IgE and IgG_1 levels in serum were similar in the 2 HDM-treated mouse groups (Fig 4, *G*). When we analyzed single-gene conditional knockouts, we found, as expected, that $Jg1^{\Delta CD11c/\Delta CD11c}$ and $Jg2^{\Delta CD11c/\Delta CD11c}$ mice had AAI similar to that seen in WT littermates on HDM exposure (Fig 4, *H*).

To verify that DC migration and responsiveness were comparable between $Jg1Jg2^{\Delta CD11c/\Delta CD11c}$ and $Jg1Jg2^{+/+}$ mice, the DC response to HDM was analyzed 24 hours after intranasal administration of either PBS, 10 μg of HDM, or 50 μg of HDM (see Fig E3, *A*, in this article's Online Repository at www.jacionline.org). We did not detect differences in the numbers of cells of individual DC subsets (see Fig E3, *B* and *C*) or in the expression of costimulatory molecules on total DCs (see Fig E3, *D*) or separate DC subsets (data not shown) in the MedLNs or lungs between $Jg1Jg2^{\Delta CD11c/\Delta CD11c}$ and $Jg1Jg2^{+/+}$ mice. We noticed a small but significant increase in DLL4 expression on DCs in the MedLNs of $Jg1Jg2^{\Delta CD11c/\Delta CD11c}$ compared with $Jg1Jg2^{+/+}$ mice.

Taken together, our analysis demonstrates that in the HDM-driven asthma model there is no evidence for a role for Jagged 1 or Jagged 2 expression on DCs.

Conditional Jagged 1 and Jagged 2 knockout mice have normal T_H1 responses *in vivo*

Although T_H2 responses still developed in the HDM model in mice with Jagged-deficient DCs, it remained possible that these mice had a shift in T_H1/T_H2 balance. However, when we analyzed *in vitro* recall responses to OVA, there was no difference in T-cell activation, T_H1 cells, or T_H2 cells (see Fig E4, *A-D*, in this article's Online Repository at www.jacionline.org) or IL-4^+ and IFN-γ^+ T cells (not shown) between *in vitro* OVA-restimulated lymph node cells from $Jg1Jg2^{\Delta CD11c/\Delta CD11c}$ and $Jg1Jg2^{+/+}$ mice. Likewise, no differences were found in T cell–dependent B-cell responses because total or high affinity tri-nitrophenol keyhole limpet hemagglutinin–specific IgM, T_H2-driven IgG_1, and T_H1-driven IgG_{2c} levels were similar in $Jg1Jg2^{\Delta CD11c/\Delta CD11c}$ and $Jg1Jg2^{+/+}$ mice (see Fig E4, *F* and *G*). Therefore the absence of Jagged expression on DCs does not affect the T_H1/T_H2 balance *in vivo*.

Canonical Notch signaling through RBPJκ in $CD4^+$ T cells is required for AAI development

Mice with T cell–specific conditional deletion of the downstream transcription factor RBPJκ[6] were studied to establish whether Notch signaling in T cells is critical for induction of T_H2 differentiation. We exposed CD4-Cre transgenic $RBPJ\kappa^{fl/fl}$ mice (termed $RBPJ\kappa^{\Delta CD4/\Delta CD4}$) and non–CD4-Cre–expressing $RBPJ\kappa^{fl/fl}$ littermates (termed $RBPJ\kappa^{+/+}$) to our HDM-driven AAI model (Fig 5, *A*). Strikingly, in the absence of RBPJκ in T cells, mice displayed a significant decrease in numbers of macrophages, eosinophils, neutrophils, B cells, T cells, and DCs in BAL fluid compared with WT littermates (Fig 5, *B*). Also, the numbers and percentages of IL-4^+, IL-5^+, and IL-13^+ T cells were lower in $RBPJ\kappa^{\Delta CD4/\Delta CD4}$ than in $RBPJ\kappa^{+/+}$ mice, whereas we found similar numbers and increased percentages of IFN-γ^+ and IL-$17A^+$ T cells in BAL fluid, MedLNs, and lungs (Fig 5, *C* and *D*, and data not shown). Moreover, the ratio of cytokine-producing T cells shifted from a predominant T_H2 phenotype to a more equal $T_H1/T_H2/T_H17$ phenotype in the absence of RBPJκ in T cells (Fig 5, *E*). In addition, induction of Gata-3 was particularly impaired in $RBPJ\kappa^{\Delta CD4/\Delta CD4}$ mice in $CD4^+$ cells in BAL fluid, MedLNs, and lungs (Fig 5, *F* and *G*, and data not shown), Furthermore, serum IgE levels (Fig 5, *H*) and airway resistance to methacholine were significantly lower in $RBPJ\kappa^{\Delta CD4/\Delta CD4}$ mice compared with values in $RBPJ\kappa^{+/+}$ mice (Fig 5, *I*).

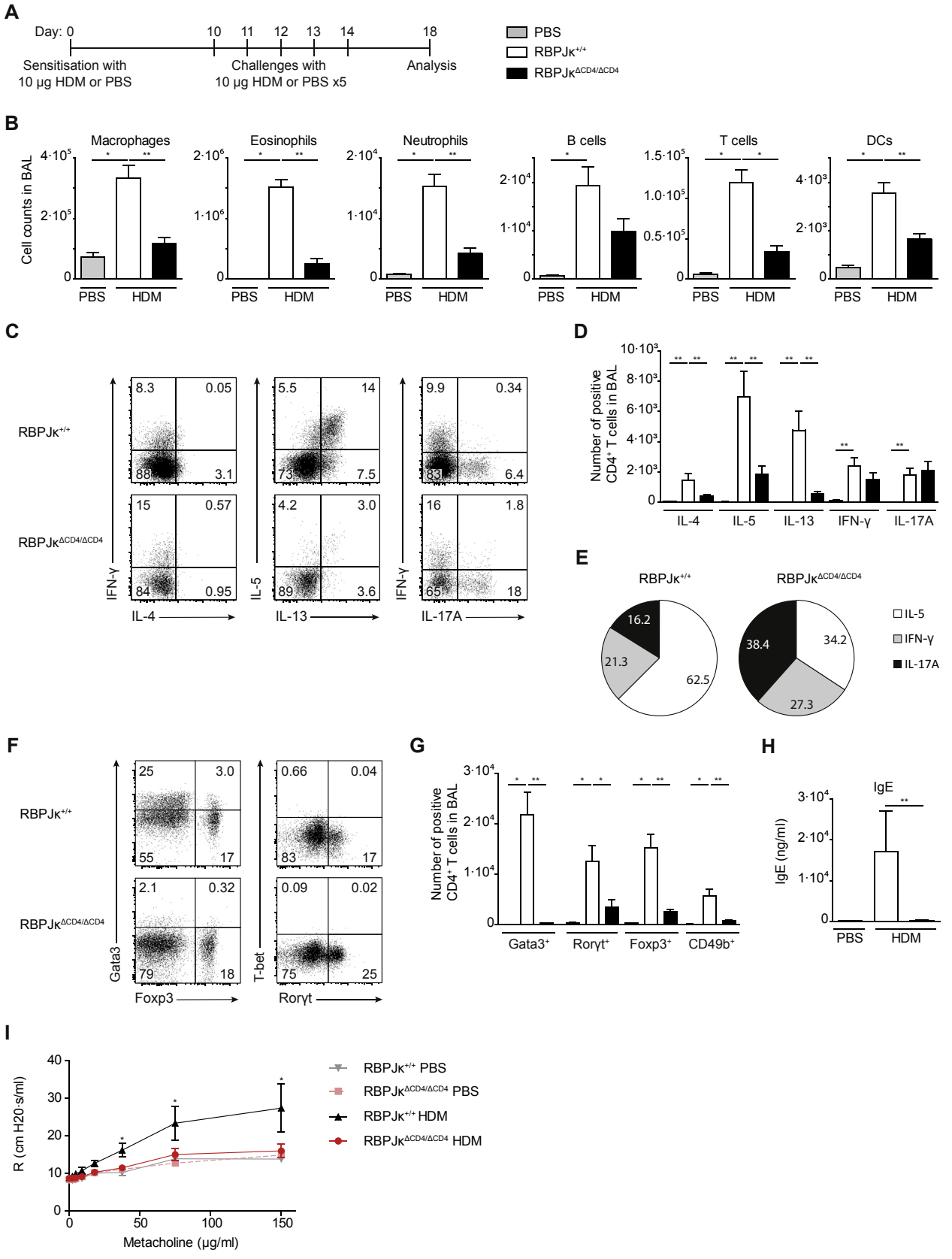
In summary, these results demonstrate that canonical RBPJκ-mediated Notch signaling in $CD4^+$ T cells is crucial for the induction of AAI and airway hyperreactivity *in vivo*.

DISCUSSION

Notch signaling in T cells is crucial to induce a T_H2 response. This was shown earlier in mouse models using parasite antigens[4,7] and in asthma models using OVA.[8] In line with these reports, we found that mice with T cell–specific RBPJκ deficiency did not mount a T_H2 response in an HDM-induced mouse AAI model. However, the role of the Notch ligands Jagged 1 and Jagged 2 in T_H2 induction remains more elusive. Here we show that on HDM exposure, Jagged 1 is specifically upregulated on migratory $CD11b^+$ cDCs in MedLNs, but expression of Jagged 1 and Jagged 2 on DCs is dispensable for the induction of HDM-induced AAI *in vivo*.

Although we found a substantial increase of Jagged 1 expression on HDM stimulation both *in vivo* and *in vitro*, Jagged

(Fig 4, *C*) and quantification of total numbers of cytokine-positive $CD3^+CD4^+$ T cells in BAL fluid (Fig 4, *D*). **E,** Quantification of IL-5 production *in vitro* by MedLN cells restimulated with 15 μg/mL HDM for 7 days, as quantified by means of ELISA. **F,** Numbers of Gata-3^+, Rorγt^+, and Foxp3^+CD25^+ $CD3^+CD4^+$ T cells in BAL fluid from PBS- or HDM-treated $Jg1Jg2^{\Delta CD11c/\Delta CD11c}$ or $Jg1Jg2^{+/+}$ mice. **G,** Total IgE levels and levels of HDM-specific IgE and IgG_1 in serum of indicated mice. **H,** Cell counts of eosinophils and IL-$5^+CD3^+CD4^+$ T cells in BAL fluid from PBS- or HDM-treated $Jg1^{\Delta CD11c}$ and $Jg2^{\Delta CD11c}$ mice with WT littermates. Data are shown as means + SEMs of 6 to 7 mice per group and are representative of 3 independent experiments. $*P < .05$ and $**P < .01$, Mann-Whitney *U* test.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Neutrophils
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DCs
Cell counts in BAL
PBS
HDM
C
RBPJk+/+
RBPJkΔCD4/ΔCD4
IFN-γ
IL-4
IL-5
IL-13
IL-17A
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Number of positive CD4+ T cells in BAL
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IL-5
IFN-γ
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Foxp3
T-bet
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Gata3+
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Foxp3+
CD49b+
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Metacholine (µg/ml)
RBPJk+/+ PBS
RBPJkΔCD4/ΔCD4 PBS
RBPJk+/+ HDM
RBPJkΔCD4/ΔCD4 HDM

2 expression was low and remained unaltered. In addition, Jagged 1 was shown to be crucial in the induction of a T_H2 response in an AAI model by using OVA-pulsed *in vitro*–cultured GM-CSF BMDCs,[14] whereas Jagged 2 is not required for T_H2 induction *in vivo*.[15,16] Therefore we hypothesized that Jagged 1, but not Jagged 2, would be critical in the induction of AAI *in vivo*. However, in our physiologic model using HDM to sensitize and challenge mice, we found that expression of Jagged 1 and Jagged 2 on DCs was dispensable. Nevertheless, our data based on transfer of *in vitro* HDM-activated GM-CSF BMDCs confirmed earlier literature showing that Jagged-deficient GM-CSF BMDCs are incapable of inducing AAI *in vivo*[14] in an OVA-based model. Thus the requirement for Jagged expression on GM-CSF BMDCs for their capacity to induce AAI does not appear to be dependent on the nature of the allergen (HDM or OVA) but is likely related to the use of GM-CSF BMDCs to sensitize the mice. In particular, it was recently shown that GM-CSF BMDCs comprise a heterogeneous cell population, consisting of both cDC-like cells and monocyte-derived macrophages.[24] These findings indicate that data obtained by using *in vivo* transfer of GM-CSF BMDCs should be interpreted with care.

Although there is no doubt that Notch is required to induce proper effector T-cell responses, it is currently under debate whether Notch ligands have an instructive role in helper T-cell differentiation or whether Notch signaling acts as an amplifier of helper T-cell responses.[9] The results obtained after instillation of Jagged-deficient DCs would appear to support a general role for Notch in promoting helper T-cell responses. In contrast, in RBPJκ-deficient mice treated with HDM, we clearly observed a selective defect in T_H2 cell responses, whereas numbers of T_H1 and T_H17 cells were similar to those in WT mice, arguing for a role for Notch as a T_H2-instructive signal. We speculate that Notch can perform both roles, enhance general T-cell activation, and function as a more specific promoter of T_H2 responses, depending on the repertoire of signals mobilized. Thus when HDM-treated DCs are used to prime the response, the repertoire of additional T cell–activating signals might be limited. In that case T-cell activation would become more dependent on Notch activation. When, on the other hand, HDM is inhaled, many cell types (innate lymphocytes, epithelial cells, and tissue-resident myeloid cells) will contribute to the generation of activating signals that might override the requirement for Notch in T-cell priming. In this latter scenario only the T_H2-promoting function of Notch would be critical.

It has previously been suggested that the Notch ligands DLL and Jagged instruct T_H1 and T_H2 responses, respectively.[6] However, we found that mice with a conditional deletion for Jagged 1 and Jagged 2 in DCs had T_H2 responses to HDM to a similar extent as their WT littermates. These findings indicate either (1) a critical role for other Jagged-expressing cells, implying an instructive role for Notch signaling, or (2) redundancy between various Notch ligands (Jagged 1, Jagged 2, DLL1, and DLL4) on DCs during induction of T_H2 responses, which would argue for a role for Notch as an unbiased amplifier.

One explanation for the induction of a T_H2 response in the absence of Jagged 1 and Jagged 2 on DCs could be that there is a redundancy of other Jagged-expressing cells. It is not likely that Jagged expression on alveolar macrophages is required for T_H2 priming. First, although macrophages can take up HDM, they have been reported to lack the capacity to induce T-cell proliferation.[25] Second, our finding of greater than 94% EYFP expression in alveolar macrophages from CD11c-Cre×ROSA^{EYFP} mice (see Fig E2, *B*) would indicate that these cells are Jagged deficient in the *Jg1Jg2*$^{\Delta CD11c/\Delta CD11c}$ mice also. Another candidate would be B cells, which have been implicated in induction of T_H2-mediated AAI.[26-28] Also, B cells are important in the development and maintenance of follicular helper T cells,[29] which play an important role in AAI by secreting IL-4 and IL-21.[27,30-32] However, in fluorescence-activated cell-sorted activated and nonactivated B cells from HDM-treated and control mice, Jagged 1 was not detected, and levels of Jagged 2 were very low (I. Tindemans, unpublished findings), which is inconsistent with a role for Jagged expression on B cells in T_H2 cell induction.

On stimulation with HDM, we found that DLL4 expression was increased on migratory CD11b$^+$ cDCs *in vivo* (Fig 1, *B*). In the absence of Jagged 1 and Jagged 2 on DCs, DLL4 expression was increased (see Fig E4, *D*), raising the possibility that DLL4 compensates for the absence of Jagged 1 and Jagged 2. DLL4 signaling was originally thought to be associated with T_H1 response induction.[6,18] Indeed, DLL4 is upregulated on DCs in response to T_H1 stimuli, including bacterial LPS, respiratory syncytial virus (RSV), and dengue virus.[18,33,34] However, later studies showed that it is also induced by certain T_H2 stimuli, including cockroach allergen, low-dose LPS, and RSV-mediated allergic asthma exacerbations.[12,35,36] Furthermore, a regulatory role for DLL4 was demonstrated in T_H2 responses to cockroach allergen[36] and when DLL4-pretreated BMDCs stimulated with OVA were adoptively transferred to induce AAI.[37] On the other hand, T_H2 responses were decreased when DLL4 was neutralized *in vivo* in a mouse model for RSV-mediated allergic asthma exacerbations.[35] Therefore it is unclear whether DLL4 compensates for the absence of Jagged molecules on DCs or whether DLL4 has a regulatory role in this setting. Further studies targeting both Jagged 1 and DLL4 Notch ligands are required to resolve this question.

In summary, we showed that Notch signaling is crucial for induction of HDM-mediated eosinophilia, T_H2 responses, and

FIG 5. Notch signaling in CD4$^+$ T cells is crucial for AAI induction. **A,** Scheme of HDM-mediated AAI induction in *RBPJκ*$^{\Delta CD4/\Delta CD4}$ and *RBPJκ*$^{+/+}$ mice. **B,** Total numbers of the indicated cell populations in BAL fluid from PBS- or HDM-treated mice. **C-E,** Intracellular flow cytometric analysis of cytokine production by CD3$^+$CD4$^+$ T cells in BAL fluid from the indicated mice (Fig 5, *C*), quantification of total numbers of cytokine-positive CD3$^+$CD4$^+$ T cells in BAL fluid (Fig 5, *D*), and distribution of T_H1, T_H2, and T_H17 cells (Fig 5, *E*), as signified by the key cytokines IL-5, IFN-γ, and IL-17A in BAL fluid of indicated mice. **F,** Flow cytometric profile of transcription factor expression in CD3$^+$CD4$^+$ T cells in mice treated with HDM. **G,** Quantification of Gata-3$^+$, Rorγt$^+$, and Foxp3$^+$ CD4$^+$ T cells and CD49b$^+$ CD4$^+$ natural killer T cells in BAL fluid. **H,** Total IgE levels in serum, as determined by means of ELISA. **I,** Airway resistance, as measured directly after administration of increasing doses of methacholine by using flexiVent in indicated mouse groups. Data are shown as means + SEMs of 4 to 6 mice per group (Fig 5, *B-I*) and are representative of 6 independent experiments (Fig 5, *B-H*). **P* < .05 and ***P* < .01, Mann-Whitney *U* test.

airway hyperreactivity *in vivo*, indicating that Notch on T cells could be a potential therapeutic target in patients with allergic asthma. In addition, our data indicate that there is redundancy, either between various Jagged-expressing cells or between Jagged and DLL on DCs. Therefore further studies are required to identify which cells and which ligands provide the Notch signals that are essential for T_H2 induction in patients with allergic asthma.

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Clinical implications: The Notch signaling pathway in T cells is critical for development of HDM-driven AAI in mice, indicating it could be a potential therapeutic target in asthmatic patients.

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