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STAT5-Induced Lunatic Fringe during Th2 Development Alters Delta-like 4–Mediated Th2 Cytokine Production in Respiratory Syncytial Virus–Exacerbated Airway Allergic Disease

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Notch activation plays an important role in T cell development and mature T cell differentiation. In this study, we investigated the role of Notch activation in a mouse model of respiratory syncytial virus (RSV)–exacerbated allergic airway disease. During RSV exacerbation, in vivo neutralization of a specific Notch ligand, Delta-like ligand (Dll)-4, significantly decreased airway hyperactivity, mucus production, and Th2 cytokines. Lunatic Fringe (Lfng), a glycosyltransferase that enhances Notch activation by Dll4, was increased during RSV exacerbation. *Lfng* loss of function in Th2-skewed cells inhibited Dll4–Notch activation and subsequent IL-4 production. Further knockdown of *Lfng* in T cells in CD4Cre⁺*Lfng*^{fl/fl} mice showed reduced Th2 response and disease pathology during RSV exacerbation. Finally, we identified STAT5-binding *cis*-acting regulatory element activation as a critical driver of *Lfng* transcriptional activation. These data demonstrate that STAT5-dependent amplification of Notch-modifying *Lfng* augments Th2 response via Dll4 and is critical for amplifying viral exacerbation during allergic airway disease. *The Journal of Immunology*, 2014, 192: 000–000.

Respiratory syncytial virus (RSV) infects nearly all infants by age 2 and is the leading cause of bronchiolitis in children worldwide (1). Whereas RSV is especially detrimental in very young infants whose airways are small and easily occluded, RSV is also becoming recognized as an important pathogen in the elderly, transplant recipients, patients with chronic obstructive pulmonary disease, as well as other patients with chronic lung disease, especially asthma (2). Thus, RSV infections impose severe threats for exacerbating chronic lung disease, and this has been hypothesized to be associated with the development of an altered Th2 immune environment. Respiratory virus–induced asthma exacerbations are the most common trigger for induction of worsening airways disease, and understanding the etiology in allergic airway disease may provide better treatments.

Notch signaling regulates a wide range of cellular developmental processes in multicellular organisms, including self-renewal, cell differentiation, and cell fate decisions (3). In mammals, there are five membrane-bound Notch ligands (Jagged 1 and 2, Delta-like ligand [Dll]-1, 3, and 4) and four Notch receptors (N1–4). Notch signaling is activated following ligand–receptor interaction and γ secretase–mediated cleavage of the Notch receptor, leading to release of the Notch intracellular domain. Once activated, Notch intracellular domain translocates to the nucleus, where it interacts with Mastermind-like (MAML) proteins and the transcription factor CSL/RBP-Jk (encoded by *Rbpj*) to induce target gene transcription HES genes in mammals. The HES proteins are basic helix–loop–helix type of transcriptional repressors and negatively regulate expression of downstream target genes such as tissue-specific transcription factors. Furthermore, HES1 and HES5 are involved in T cell commitment in the thymus (4, 5). To this end, Notch was shown to be involved in T cell lineage development in the thymus over a decade ago (6). More recently, roles for Notch in the differentiation of distinct Th cell subsets (Th1, Th2, Th9, Th17, and T regulatory cells) have been identified (7–10).

The importance of Notch receptor and Notch-dependent downstream signaling in Th2 regulation has been shown using T cell–specific gene ablation of *Notch1/2* receptor or *Rbpjk* genes, as well as T cell–specific expression of the pan-Notch inhibitor DNMAML (11, 12) via a *GATA3*-mediated mechanism (11, 13). In contrast, additional reports have demonstrated that individual Notch ligands such as Dll4 regulate primary Th2 responses (14–16). Alternatively, Jagged1 expression on dendritic cells (DC) in response to TLR or IL-25 signaling has been associated with the development of allergic lung disease (17, 18). Thus, these emerging findings with individual Notch ligands indicate differential functions of Notch that are context dependent.

Several proteins modulate Notch signaling by altering extracellular Notch ligand–receptor interactions (19, 20). Of prime

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Abbreviations used in this article: AHR, airway hyperactivity; Cab, control Ab; ChIP, chromatin immunoprecipitation; CRA, cockroach allergen; DC, dendritic cell; Dll4, Delta-like 4; PAS, periodic acid–Schiff; PNU, protein nitrogen unit; RSV, respiratory syncytial virus; siRNA, small interfering RNA; WT, wild-type.

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importance are the Fringe proteins, a highly conserved family of glycosyltransferases that add N-acetyl glucosamine to *O*-fucose on the epidermal growth factor-like repeats in the extracellular domain of Notch. In vertebrates, three Fringe proteins have been identified, as follows: Lunatic (Lfng), Manic (Mfng), and Radical Fringe (Rfng). Fringe-mediated extension of *O*-fucose has been shown to differentially influence cell fate decisions by inhibiting Jagged1-mediated Notch signaling and potentiating Dll-mediated Notch signaling (3). In the thymus, Lfng plays a key role in the early stages of T-lymphocyte development, critically enhancing Dll/Notch1-dependent suppression of alternative B-lineage potential and promoting T-lineage specification (21, 22). In peripheral T cells, Lfng expression is low in naive CD4 T cells from allergic mice and may regulate Th1/Th2 responses (23). However, the role of Lfng in regulating already established Th2 cell responses is unknown.

In this study, a mechanism involved in RSV exacerbation of existing Th2-associated allergic airway disease was addressed utilizing an animal model that reflects several aspects of clinical disease pathology. Our data demonstrate a pathogenic role for Lfng and Dll4 during viral exacerbation in Th2-mediated allergic airway disease. These findings further demonstrate that increased levels of Lfng during Th2 development augment Dll4 signaling during viral exacerbation in a STAT5-dependent manner.

Materials and Methods

Mice

Six- to 8-wk-old female BALB/c, C57BL/6J, 4-get, and OT-II mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The Lfng^{fl/fl} mice (24, 25) were a gift of S. Egan (Hospital for Sick Children Research Institute, University of Toronto). All mice were maintained in specific pathogen-free facilities in the Unit for Laboratory Animal Medicine at the University of Michigan. The University Committee of Use and Care of Animals (University of Michigan) approved all animal experimental protocols, and experiments were conducted according to the guidelines provided by the University Committee of Use and Care of Animals review committee.

Cockroach allergen sensitization, RSV infection, and in vivo neutralization of Dll4

BALB/c mice were sensitized with cockroach allergen (CRA), as described previously (26). Briefly, mice were sensitized with a 1:1 mixture of clinical-grade CRA extract (Hollister-Stier Laboratories) and IFA (Sigma-Aldrich), administered s.c. and i.p. a total of 1000 protein nitrogen unit (PNU) per mouse on day 0. At day 14, mice were sensitized by intranasal challenges of CRA, 150 PNU per mouse, followed by RSV (line 19 clinical isolate) infection on day 16. RSV infection was carried out as previously described (26). In animals treated with Abs, control or anti-Dll4-purified IgG (2.5 mg) was given 2 h before RSV infection on day 16. Two doses of control or anti-Dll4-purified IgG were also given on days 18 and 20. The mouse model is described in Supplemental Fig. 1A. The generation and specificity of the anti-Dll4 Ab have been previously described (15). After a final intranasal challenge of CRA, 150 PNU per mouse, on day 21, the mice were euthanized and airway hyperreactivity (AHR) studies were conducted on day 22. The lungs and draining lymph nodes of these mice were isolated for further characterization.

Airway response and lung histology

Airway hyperreactivity was assessed, as described previously (15). Briefly, anesthetized mice were intubated via cannulation of the trachea and ventilated with a Harvard pump ventilator (0.3 ml tidal volume; 120 breaths/min). Airway hyperreactivity was measured by direct ventilation methodology specifically designed for low tidal volumes (Buxco Research Systems). After baseline measurements, mice were injected i.v. with 7.5 g methacholine (Sigma-Aldrich), and the peak airway resistance was recorded as a measure of AHR. Following measurement of AHR, the left lobe of the lungs was formalin fixed, and paraffin-embedded sections were stained with H&E or periodic acid-Schiff (PAS).

RT-PCR

Total RNA was extracted from tissue or cells using TRIzol reagent (Invitrogen) and was reverse transcribed to cDNA. Real-time PCR was per-

formed with the cDNA using gene-specific primers. Murine primers for *Il4*, *Hes5*, *Lfng*, *Rfng*, *Mfng*, *Stat5a*, and *Gapdh* were purchased from Applied Biosystems (Carlsbad, CA). Gene expressions of *Muc5ac* and *Gob5* were determined using primer/probe detection sets designed by PE Biosystems (Foster City, CA) and purchased from Sigma-Aldrich. All results were normalized to *Gapdh* expression.

CD4 T cell transfection and RNA interference

MACS-enriched, 2×10^6 CD4 T cells were transfected with 300 nM control small interfering RNA (siRNA; nontargeting) or siRNA specific for *Lfng* or *Stat5a* using the Amaxa Nucleofector System following the manufacturer's protocol. The cells were immediately transferred and seeded in 24-well plates with T cell-specific medium provided by the manufacturer. Twenty-four hours later, knockdown efficiency was verified using RT-PCR or Western blotting. Control and On-Target Plus gene-specific siRNA were purchased from Dharmacon.

Quantification of cytokine production

Supernatants from both in vitro skewed T cells and single-cell suspensions of lymph nodes that were seeded ex vivo at a concentration of 2×10^5 cells/200 μ l in 96-well plates and stimulated with RSV for 48 h were analyzed for cytokine production and quantified using a Bio-Plex bead-based (Luminex) cytokine assay purchased from Bio-Rad Laboratories.

Flow cytometry and FACS

Single-cell suspensions of lung cells after collagenase dispersion were prepared, as described previously (26). Cells were stained with CD3, CD11c, and Dll4 (clone HMD4-1) Abs or CD4, CD8, N1 (clone 22ES), and N2 (clone 16F11) purchased from eBioscience, after 10 min of preincubation with Fc block (BD Biosciences). Following two washes, the cells were fixed overnight in 4% formalin. The samples were processed through a flow cytometer (LSRII; BD Biosciences). The data were analyzed using FlowJo software version 9.9.3 (Tree Star). FACS sorting of Th2-skewed GFP⁺ T cells isolated from 4-get mice was carried out using a BD Biosciences FACS-Aria sorter.

DC/T cell coculture

Bone marrow-derived dendritic cells were generated in the presence of GM-CSF. For DC/T cell cocultures, bone marrow-derived DCs were first plated in a flat-bottom 96-well plate and pulsed with OVA (150 μ g/ml) for 2 h. Bone marrow-derived DCs were washed and resuspended in fresh media. Th2-skewed CD4⁺ T cells from OT-II mice were added to wells at a DC:CD4⁺ cell ratio of 1:10, and supernatants were harvested 48 h later for cytokine protein analysis.

In vitro CD4 T cell isolation and skewing

CD4 T cells were sorted from the spleen by negative selection using magnetic beads (Miltenyi Biotec) and plated in 96-well plates at a concentration of 2×10^5 cells/well in RPMI 1640 complete medium (Lonza). The cells were stimulated with plate-coated anti-CD3 and soluble anti-CD28 (eBioscience) each at 2 μ g/ml to obtain Th0 cells. Th2 cells were obtained by skewing the T cells using a mixture of plate-bound anti-CD3, soluble anti-CD28, anti-IL-12 (20 μ g/ml), anti-IFN- γ (20 μ g/ml), and rIL-4 (10 ng/ml) for 4 d. The cells were rested for 3 d and then restimulated with plate-coated anti-CD3 and soluble anti-CD28 in the presence or absence of Dll4. For gene expression studies, the secondary stimulation was only for 6 h, and for protein quantification in supernatants, the cells were stimulated for 48 h.

Western blot

After single-cell isolation from lungs, as described previously, CD4 T cells were MACS enriched by negative selection. Next, the cells were lysed and whole-cell lysate was subjected to SDS-PAGE and probed with Lfng (Santa Cruz) primary Ab. For T cells cultured in vitro, whole-cell lysates were used for Western blotting. The primary Abs used in this case were against Lfng and STAT5 (Cell Signaling) (27, 28). Following transfer and incubation with appropriate secondary Ab, membranes were developed with ECL Western blotting Detection Reagent (Pierce), and pictures of the blot were taken using the ChemiDoc XRS⁺ from Bio-Rad. The data were analyzed with Image Lab software (Bio-Rad). In all the experiments, β -actin was used as the loading control.

Analysis of STAT5 binding sites in Lfng locus and chromatin immunoprecipitation

Alignment and conservation histograms for the murine *Lfng* locus were generated with the University of California, Santa Cruz Genome Browser

and BLAT (<http://genome.ucsc.edu>) on the NCBI37/mm9 mouse genome assembly, using phyloP for basewise mammalian conservation and 30-way Multiz alignment for vertebrate genomes. The GATA3-binding sequence in *Lfng* was derived from the SA Bioscience bioinformatics tool for identification of transcription factor-binding sequences, and the binding site was 8.6 kb downstream of the transcription start site on *Lfng* loci. The chromatin immunoprecipitation (ChIP) assay was carried out, as described previously (9). Briefly, Th0 cells or Th2-skewed cells were cross-linked using 1% formaldehyde, lysed, and immunoprecipitated with anti-STAT5 or anti-GATA3 Ab (Santa Cruz) or control Ab overnight at 4°C with gentle rotation. Cross-linking was reversed for 4 h at 65°C and was followed by proteinase K digestion. DNA was purified by standard phenol/chloroform and ethanol precipitation and was subjected to Sybr Green real-time PCR. *Lfng* primers for STAT5a binding to site 3 (catalogue GPM1037616*03A) and Gata3 binding (catalogue GPM1037616*09A) were purchased from SA BioSciences.

Statistical analysis

Data are reported as mean $\pm$ SE. Statistical significance was determined by Student *t* test or one-way ANOVA with Newman-Keuls post hoc test. A *p* value <0.05 was considered significant.

Results

In vivo neutralization of Dll4 blocks pulmonary pathology and Th2 cytokine production during viral exacerbation

Although RSV disease pathogenesis is becoming better understood, the underlying mechanisms of viral-induced exacerbations of allergic asthma are unknown. Using a mouse model of CRA-induced allergic asthma (Supplemental Fig. 1A), we observed that RSV-induced exacerbation of allergic disease significantly increased AHR compared with controls (Fig. 1A). CRA and RSV together significantly increased the total number of Dll4-expressing CD11c⁺ DCs in the lungs compared with CRA- or RSV-alone groups (Fig. 1B, Supplemental Fig. 1B, 1C). To investigate the role of Dll4 during viral exacerbation of CRA-induced asthma, a mouse polyclonal anti-Dll4 Ab (α Dll4) or control Ab (Cab) was administered, as shown in Supplemental Fig. 1A. Our data indicate that there was a significant decrease in AHR with α Dll4 treatment compared with the Cab-treated group (Fig. 1C). Furthermore, Dll4 blockade reduced peribronchiolar infiltration of inflammatory cells, airway epithelial sloughing, mucus production, and expression of mucus-associated gene *gob5* compared with Cab-treated mice (Fig. 1D, 1E). Neutralization of Dll4 also significantly reduced Th2 cytokine expression compared with the Cab-treated group in the lungs (Fig. 1F). A similar reduction of RSV-reactivated Th2 cytokines was observed in the draining lymph nodes with anti-Dll4 treatment, with complete absence of IL-13 (Fig. 1G). These data indicate reduced Th2 cytokine production during viral exacerbation was augmented by Dll4-mediated activation.

Increased expression of Lfng in Th2-skewed T cells augments Th2 cytokine production in the presence of exogenous Dll4

Previous studies have shown that modification of the Notch by Fringe glycosyltransferases increases binding of Delta-like (3, 29). We observed that CRA plus RSV significantly increased expression of *Lfng* mRNA in the lungs of infected mice compared with mice challenged with CRA or RSV alone. In contrast, *Mfng* and *Rfng* mRNA were similarly expressed among the different groups (Fig. 2A). *Lfng* protein was also expressed at higher levels in CD4 T cells from CRA plus RSV-treated mice compared with CRA- and RSV-only groups (Fig. 2B). *Lfng* expression was below our limit of detection in CD4 T cells from naive mice, suggesting that *Lfng* expression increased during the course of viral exacerbation.

Given that *Lfng* was expressed at higher levels in CD4 T cells from CRA plus RSV-treated mice, we hypothesized that Th2 cytokines upregulate *Lfng* expression. To test this idea, we assessed *Fringe* expression in CD4 T cells under in vitro Th2-skewed con-

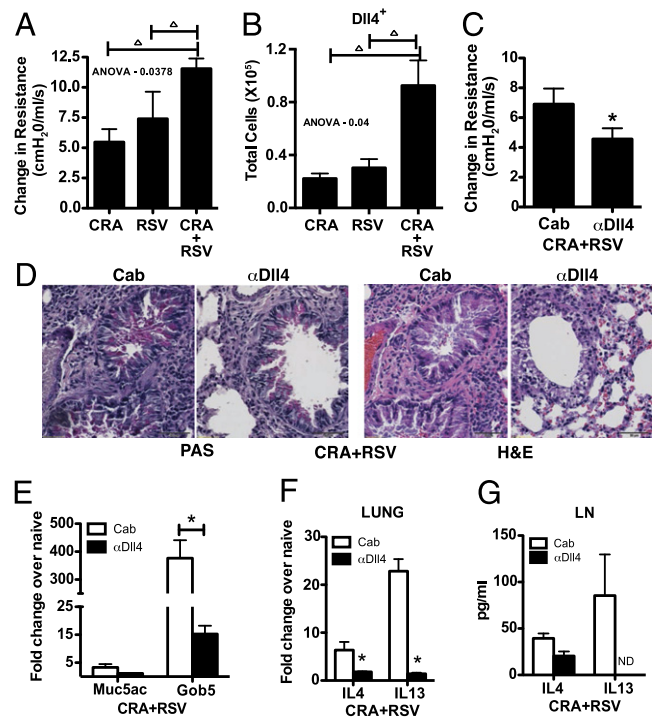


FIGURE 1. Neutralization of Dll4 in vivo inhibits mucus production and Th2 cytokine production during viral exacerbation of existing allergic airway disease. (A) Airway hyperreactivity was measured in BALB/c mice sensitized to CRA, RSV, or both CRA and RSV by plethysmography, and (B) total Dll4-expressing DCs (autofluorescence^{low}CD3⁻CD11c⁺ cells) in the lungs of different mice groups were measured by flow cytometry. CRA-sensitized BALB/c mice were infected with RSV and at the same time treated with Cab or anti-Dll4 Ab (α Dll4). Six days post-RSV infection and 24 h after final challenge with CRA, (C) AHR was measured; (D) lung sections were analyzed by PAS staining for mucus production and H&E staining for inflammation; (E) RNA expression of *Muc5ac* and *Gob5*; (F) IL-4 and IL-13 in the lung were quantified by RT-PCR; (G) the draining lymph nodes were reinfected with RSV ex vivo for an additional 48 h, and cytokine production was measured in the supernatants by bead-based luminex system. Data are expressed as mean $\pm$ SE, and a representation of four independent experiments [except (B), which was repeated twice] is shown. Statistical analysis was done using ANOVA and Student *t* test. *n* = 5–6 mice in each group. $\Delta p < 0.05$, $*p < 0.01$. ND, Not detected.

ditions. We observed significantly higher levels of *Lfng* mRNA and protein in Th2-skewed cells relative to unskewed Th0 cells (Fig. 2C, 2D). In contrast, *Mfng* or *Rfng* mRNA was expressed at slightly higher levels in Th0 cells versus Th2-skewed cells. These data demonstrate a novel and significant upregulation of *Lfng* in T cells in Th2-skewed CD4 cells, suggesting that Th2 cytokines upregulate *Lfng* expression.

We next tested for activation of Notch signaling and subsequent Th2 cytokine production using in vitro skewed Th2 cells in the presence of Dll4. Interestingly, Dll4 increased Notch target gene *Hes5* expression in Th2 but not Th0 cells (Fig. 3A), indicating that sensitivity to Dll4-induced Notch activation is specific for Th2-skewed CD4 cells. Furthermore, Dll4 increased IL-4 mRNA (Fig. 3B) and protein production during in vitro activation of Th2 cells (Fig. 3C). To determine whether Dll4-Notch activation and subsequent IL-4 production were *Lfng* dependent, we knocked down *Lfng* in Th2 cells using *Lfng*-specific siRNA. Western blot analysis showed significant loss of *Lfng* protein expression in Th2 cells transfected with *Lfng* siRNA compared with control siRNA (Fig. 3D, upper panel). Loss of *Lfng* in Th2 cells decreased *Hes5*

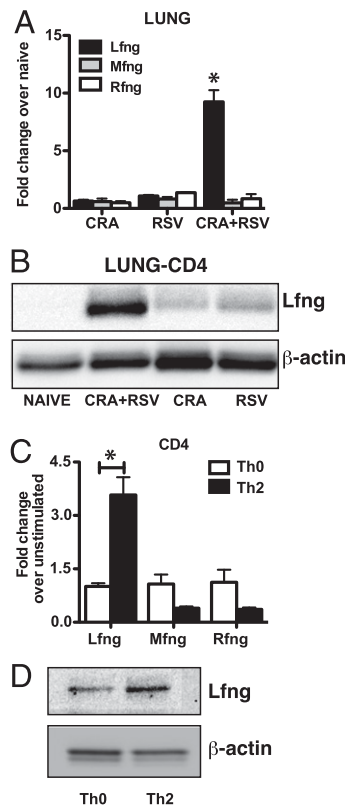


FIGURE 2. Increased expression of Lfng in CD4 T cells during viral exacerbation and in vitro Th2-skewed cells. **(A)** RT-PCR analysis of *Lfng*, *Mfng*, and *Rfng* transcripts in the lungs during viral exacerbation. **(B)** Western blot analysis of Lfng in CD4 T cells in the lungs. **(C)** RT-PCR analysis of *Lfng*, *Mfng*, and *Rfng* mRNA in unskewed Th0 cells or Th2-skewed T cells. Gene expression was normalized to *GAPDH*, and fold change was compared with unstimulated CD4 T cells. **(D)** Western blot analysis of Lfng in Th0- and Th2-skewed cells. β -actin was used as a loading control. Each experiment was repeated three times, and the data are expressed as mean $\pm$ SE. Statistical analysis was analyzed by Student *t* test and $*p < 0.01$.

induction in the presence of exogenous Dll4 (Fig. 3D, lower panel). In addition to attenuating *Hes5* expression, loss of Lfng also resulted in a concomitant and significant reduction of IL-4 production in Th2 cells in the presence of exogenous Dll4 (Fig. 3E).

To test Ag-specific responses during Lfng-mediated regulation of Notch activation by Dll4 and subsequent Th2 cytokine production, we next used Th2-skewed CD4⁺ OT-II cells that have a TCR specific for the chicken OVA peptide (ISQAVHAAHAEI-NEAGR). Lfng knockdown in Th2-skewed OTII cells resulted in a reduction of *Hes5* expression in the presence of Dll4 (Supplemental Fig. 1D, 1E). To directly evaluate Ag-specific responses, OVA-pulsed bone marrow-derived congenic DCs were cocultured with Th2-skewed OT-II cells in which Lfng was transiently knocked down using siRNA. These coculture cells were further stimulated in the presence or absence of Dll4. OT-II cells with reduced Lfng expression were unable to sustain IL-4 production even in the presence of exogenous Dll4 (Fig. 3F). This was confirmed by using CD4-T cells from 4-get mice that carry a bicistronic IL-4-GFP promoter and express GFP when the *Il4* gene is activated, thus making it possible to purify T cells that are differentiating toward a Th2 phenotype. Lfng loss significantly attenuated IL-4 production in the skewed Th2 cells, and addition of exogenous Dll4 to the culture was unable to rescue IL-4 production in the Lfng knocked-down cells (Fig. 3G). Altogether, these data suggested that Dll4-

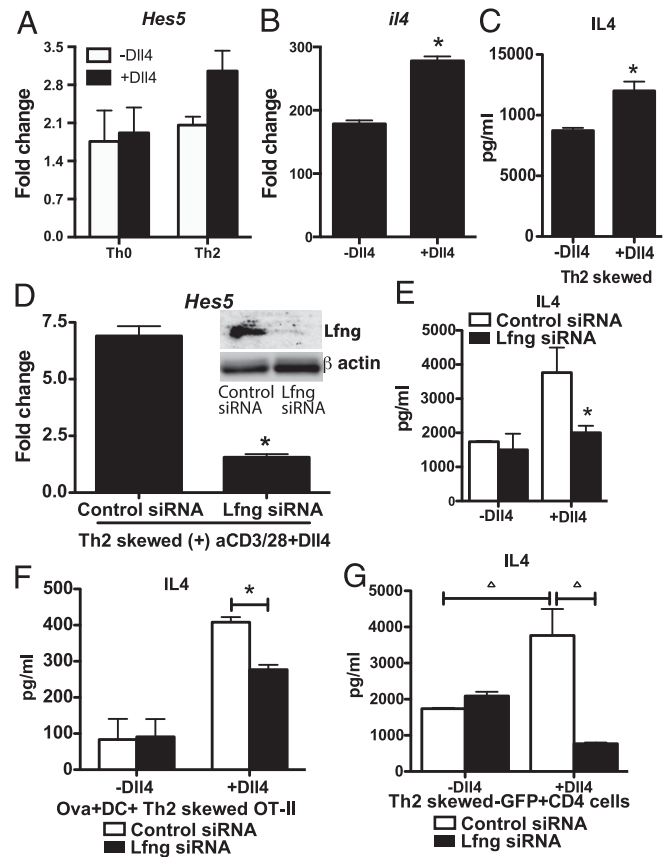


FIGURE 3. Dll4-mediated Notch activation and augmentation of IL-4 production in Th2-skewed cells are Lfng dependent and Ag specific. **(A)** RT-PCR quantification of *Hes5* expression in Dll4-activated Th0 and Th2 cells after 6 h. **(B)** RT-PCR quantification of *il4* gene after 6 h of activation with Dll4 in Th2 cells. **(C)** Supernatants from Dll4-activated Th2 cells were measured for IL-4 production by bead-based luminex system. **(D)** Western blot analysis (upper right panel) of siRNA knockdown of Lfng in Th2 cells in vitro and RT-PCR analysis of *Hes5* in Th2 cells transfected with control siRNA or Lfng-specific siRNA and activated in the presence of Dll4 (lower panel). **(E)** IL-4 production in Th2 cells lacking Lfng and stimulated in the presence or absence of Dll4. **(F)** Bone marrow-derived DCs pulsed with OVA were cocultured with Th2-skewed OT-II cells transfected with control or Lfng siRNA and stimulated with or without Dll4 for 48 h before IL-4 production was measured. **(G)** FACS-sorted splenic CD4 T cells from 4-get mice were transfected with control or Lfng siRNA and stimulated with or without Dll4 for an additional 48 h. The supernatants were analyzed for IL-4 production. Data are expressed as mean $\pm$ SE, and a representation of three independent experiments is shown. Statistical analysis was done using Student *t* test. $\Delta p < 0.05$, $*p < 0.01$.

mediated Notch activation and augmented Th2 cell-derived IL-4 production are Lfng dependent.

Conditional knockdown of Lfng reduces Th2-associated pathology during viral exacerbation of existing allergic disease in vivo

To investigate the specific role of Lfng and subsequent modulation of Dll4-Notch activation in regulating Th2 responses during viral exacerbation of existing allergic disease in vivo, we generated CD4Cre⁺Lfng^{fl/fl} conditional knockout mice (*Lfng* mice). For the generation of T cell-specific Lfng^{-/-} mice, CD4Cre⁺ mice were bred with Lfng^{fl/fl} and the F₁ mice were bred with each other to achieve *Lfng* mice. CRA-induced allergic *Lfng* mice were resistant to RSV-induced exacerbation of lung pathology as compared with wild-type (WT) (Cre⁻Lfng^{fl/fl}) littermate control mice. There was marked reduction in mucus production in the airways and peribronchial

inflammation in the lungs of *Lfng* mice compared with the WT mice (Fig. 4A). This was associated with reduced expression of *Muc5ac* and *Gob5* in the *Lfng* mice (Fig. 4B). Th2 cytokines IL-4 and IL-13 were significantly reduced in total lung tissue and lymph nodes of the *Lfng* mice compared with the WT mice (Fig. 4C, 4D). These changes in pathology and immune responses in CRA plus RSV-infected *Lfng* mice were reminiscent of mice treated with anti-Dll4 Ab. Also, no significant difference was observed in Notch1 (N1) or Notch 2 (N2) expression on CD4 T cells in the CRA plus RSV-infected *Lfng* mice compared with the WT mice, suggesting that the alteration in the phenotypic responses between the WT and *Lfng* mice was exclusively due to Notch modification by *Lfng* (Fig. 4E, Supplemental Fig. 2).

To further confirm that genetic ablation of *Lfng* alters Notch signaling to Dll4 and to recapitulate our in vitro data with knockdown of *Lfng* with specific siRNA, we used the *Lfng* conditional knockout T cells in vitro. We demonstrate that in the presence of Dll4 there was a significant decrease in *Hes1* and *Hes5* expression in the T cells lacking *Lfng* compared with the WT cells (Fig. 4F). Fur-

thermore, *Lfng* deficiency impaired induction of *Il4* and *Il13* mRNA under Th2-skewing conditions (Fig. 4G, 4H). Taken together, these data demonstrate that genetic ablation of *Lfng* significantly attenuates Dll4-induced Notch signaling in CD4 T cells, and thereby impairs production of Th2 cytokines to Dll4 during acute viral exacerbation of allergic lung disease in vivo.

STAT5 regulates *Lfng* expression during Th2 cell differentiation

The data in these studies suggest that Th2-skewing conditions increased *Lfng* expression in CD4⁺ T cells; however, molecular mechanisms regulating expression of *Lfng* in peripheral T cells have not been identified. IL-2 is an important cytokine in augmenting Th2 differentiation (30). Furthermore, previous reports suggest that STAT5 drives Th2 cell differentiation in both the presence and absence of IL-2 (31). In addition to IL-2-STAT5, Th2 differentiation is also driven by GATA-3 (32–34). We identified a conserved GATA3 binding site on the 5' untranslated region of *Lfng* that was 8.6 kb downstream of the transcription start site (Supplemental Fig. 3A). Although we observed a trend toward increased GATA3 binding on the 5' untranslated region site of *Lfng* in Th2 cells, it was not significantly different from control Ig (Supplemental Fig. 3B). Furthermore, in our studies, we observed a significant increase in IL-2 production in the draining lymph nodes and the total number of IL-4⁺ Th2 cells in CRA plus RSV-infected mice compared with CRA- or RSV-only groups (Fig. 5A, Supplemental Fig. 3C). In addition, we showed that exogenous IL-2 further increased *Lfng* expression during Th2 skewing in vitro (Fig. 5B). Finally, total STAT5 was upregulated during Th2 development but not under Th0 conditions (Fig. 5C).

We therefore postulated that upregulation of *Lfng* expression under Th2-skewing conditions could be STAT5 dependent. We identified three consensus STAT5-binding motifs (TTCNmGAA, where *m* = 3 or 4) near the promoter region and in intron 1 of the *Lfng* locus (Supplemental Fig. 3D). Site 1 (–660 to –642) is upstream of the transcriptional start site within a larger region of conserved noncoding sequence, but, because *m* = 4, it would most likely be suboptimal for STAT5 binding (35). Site 2 (+1726 to +1744) and site 3 are in intron 1, but only site 3 is in an evolutionarily conserved region. Furthermore, site 3 includes two adjacent *m* = 3 sites (+2371 to +2389) separated by 6 bp. Such tandem sites efficiently bind STAT5a tetramers (35), and STAT5 tetramerization is critical for normal cytokine responses in vivo (36). Using a ChIP assay, we detected STAT5 binding to site 3 in intron 1 of *Lfng* at site 3 by ChIP in Th2 but not in Th0 cells (Fig. 5D). Thus, these data suggest that IL-2-dependent STAT5 activation directly augments *Lfng* expression during Th2 differentiation.

To confirm that STAT5 is required for *Lfng* induction during Th2 differentiation, we used siRNA to transiently knock down STAT5a expression (Supplemental Fig. 3E). This treatment significantly reduced *Lfng* mRNA and protein compared with control siRNA-transfected cells (Fig. 5E). Importantly, exogenous Dll4 could not rescue IL-4 production in the Th2 cells transfected with STAT5a siRNA compared with control siRNA group (Fig. 5F). These data suggest that STAT5 induced during Th2 differentiation directly augments *Lfng* transcription. Altogether, these data suggest that STAT5-dependent augmentation of *Lfng* expression during Th2 differentiation enhances Notch activation by Dll4, which further increases production of IL-4. This is summarized in a schematic (Fig. 6) in the context of viral exacerbation of allergic asthma.

Discussion

In this study, we found both in vitro and in vivo that a skewed Th2 environment increased *Lfng* expression in CD4 T cells. Impor-

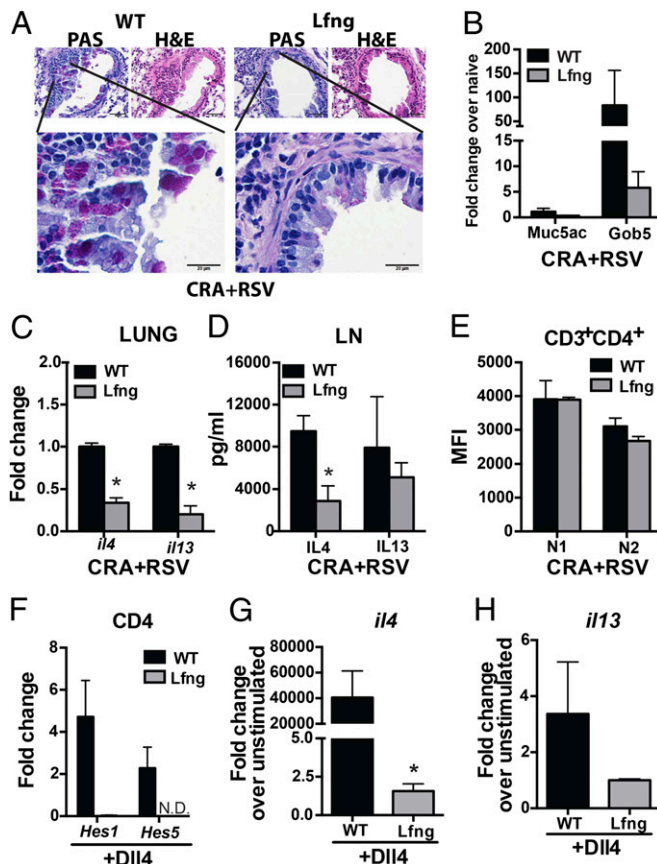


FIGURE 4. Loss of *Lfng* reduces lung pathology and Th2 cytokine response during RSV exacerbation of allergic airway disease in vivo. Lung tissue from WT and *Lfng* mice sensitized with CRA and infected with RSV was (A) stained with PAS and H&E and represented at original magnification $\times 400$. (B and C) *Muc5ac*, *Gob5*, and *Il4* and *Il13* expression in the lungs by RT-PCR. (D) Lymph nodes from CRA- and RSV-infected WT and *Lfng* mice were restimulated with RSV, ex vivo for 48 h. Levels of IL-4 and IL-13 in the supernatant were measured by bioplex. (E) Mean fluorescence intensity (MFI) of N1 and N2 on CD4⁺ T cells in the lungs of WT and *Lfng* mice sensitized with CRA and infected with RSV was analyzed by flow cytometry. RT-PCR analysis of (F) *Hes1* and *Hes5*, (G) *Il4*, and (H) *Il13* transcripts in in vitro Th2-skewed cells in the presence of Dll4. Data are expressed as mean $\pm$ SE, and a representation of two independent experiments is shown. $n = 3$ –4 mice in each group. Statistical analysis was done using Student *t* test, and $*p < 0.01$.

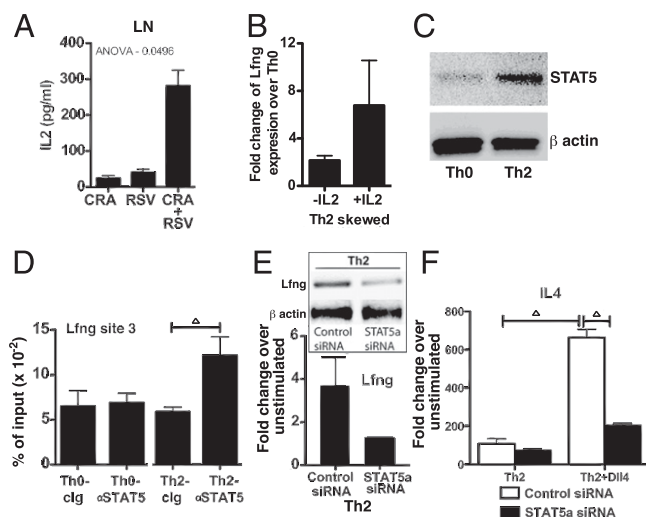


FIGURE 5. STAT5 regulates Lfng expression during Th2 skewing. **(A)** Levels of IL-2 in the lungs of mice sensitized to CRA, RSV, or both CRA and RSV. **(B)** RT-PCR analysis of Lfng expression in Th2-skewed cells in the presence or absence of exogenous IL-2 (10 ng/ml). **(C)** Immunoblot of total STAT5 in Th0- and Th2-skewed cells. β-actin used as loading control. **(D)** ChIP analysis of STAT5 binding to *Lfng* site 3 in Th0 and Th2 cells. **(E)** RT-PCR (lower panel) and Western blot (upper panel) analysis of loss of expression of Lfng in STAT5a-specific siRNA-transfected T cells that were Th2 skewed in vitro. **(F)** RT-PCR analysis of il4 expression in Th2 cells transfected with control siRNA or STAT5A siRNA and activated in the presence or absence of Dll4. Data are expressed as mean ± SE, and each experiment is a representation of three independent repeats. Statistical analysis was done using Student *t* test, and ^Δ*p* < 0.05.

tantly, Lfng expression exacerbated lung pathology and Th2 immune response during RSV-mediated exacerbation of allergic airway disease by enhancing Dll4-induced Notch activation. These findings are in accordance with previous work demonstrating that Lfng-mediated glycosylation of Notch1 enhances its capacity to be activated by Delta-like Notch ligands (3). Interestingly, a STAT5-dependent mechanism regulated Lfng expression during Th2 differentiation, and, in a positive feedback manner, Lfng further augmented Dll4-induced Notch activation and production of Th2 cytokines.

Mechanisms that regulate the immune response and associated pathology during RSV exacerbation of pulmonary airway disease are largely unknown. RSV exacerbation exploits the allergen-driven Th2 cytokine environment to further enhance Th2 cell development and worsen the inflammatory response. In this study, we demonstrate that neutralization of Dll4 in vivo reduced Th2 cytokine

production and Th2-associated pathology during RSV exacerbation of allergic airway disease. This is consistent with previous reports demonstrating that RBPj-dependent canonical Notch1 and Notch2 signaling are required for Th2 responses in vivo (12), at least in part due to Notch-induced transcription of Gata3, a Th2-specific transcription factor (11, 13). The activation of Rbpj-containing hypersensitive site 5 within the 3' enhancer is also critical for IL-4 expression and thus Th2 differentiation (37). Although these studies suggest a positive role of Notch in Th2 development, it is also reported that Notch ligands Dll1 or Jagged1 could not instruct or redirect Th fate specification (38). These observed differences in the regulation of Th2 responses to Notch are associated with the different in vitro T cell activation conditions and platforms that were used to identify Notch regulation of Th2 differentiation. Furthermore, sustained T cell responses are observed during coactivation of Notch with TCR activation by protecting activated CD4⁺ T cells against apoptosis after an initial phase of clonal expansion, suggesting that Notch controls the magnitude of CD4⁺ T cell responses by promoting cellular longevity (39). Together these studies (11–13, 37–39) suggest that Notch plays an enhancing role to optimize Th cell differentiation and survival and elicits complex context-specific T cell responses. To this end, previous reports from our laboratory have shown that blocking Dll4 in primary RSV infection or allergen-induced responses increased Th2 responses (14, 15), but the data from these studies demonstrated that during viral exacerbation Dll4-mediated Notch activation further enhances the Th2 cell activation. The allergen-induced Th2 inflammatory environment is used by the virus to further exacerbate the allergic condition most likely due to RSV-induced pro-Th2 factors such as IL-25 (17) and thymus- and activation-regulated chemokine (40), resulting in amplification of Th2 cell development and increased recruitment of Th2 cells in the lung. This in turn leads to an enhanced Dll4-mediated Th2 cytokine response in the presence of Th2 cytokine-induced Lfng. Thus, the current study further describes a context-specific Dll4-mediated Notch regulation of Th2 cytokines.

In this study, the capacity of Dll4-induced Notch signaling to augment the Th2 response was specifically Lfng dependent. Expression of other Fringe family members, Mfng and Rfng, did not change during Th2 development. Interestingly, a recent study in rats suggested that Rfng was prominently expressed, whereas Mfng and Lfng were not increased during induction of Th2 cell responses, suggesting differential utilization in Fringe depending upon mammalian species (23). However, Lfng is a more efficient N-acetyl glucosamine transferase than Mfng or Rfng (3), and it has also been shown to maximize Dll4-dependent Notch activation during discrete developmental processes such as T cell development in the thymus and in angiogenesis (22, 41, 42).

In this study, we provide genetic evidence that Lfng regulation of Dll4-Notch signaling critically regulates Ag-induced Th2 differentiation in vitro. Furthermore, we show that T cell-specific deletion of Lfng reduced Th2-associated cytokines and lung pathology induced during RSV-mediated exacerbation of allergic airway disease. Notably, to our knowledge, these data provide in vivo genetic evidence for the first time demonstrating the critical role of Lfng in regulating Notch signaling in a clinically relevant Th2-associated infectious disease model. In addition, these findings identify a therapeutic potential of targeting Lfng and/or Dll4 during viral exacerbation to restrict the development of Th2 inflammation during the course of disease pathogenesis.

In the last two decades, the transcription factors involved in Th2 differentiation have been studied comprehensively, and the two most important transcription factors are GATA3 and STAT5 (33). Although early Th2 differentiation requires GATA3, the

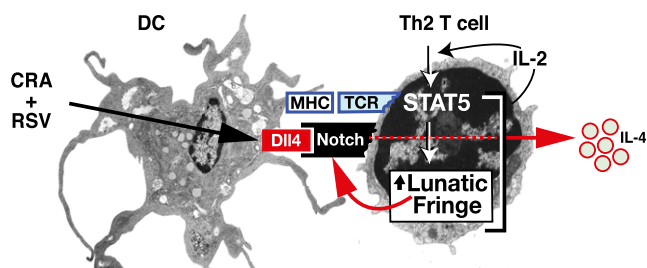


FIGURE 6. STAT5-dependent Lfng expression in a Th2 cell augments Dll4-Notch-mediated IL-4 release during viral exacerbation of allergic asthma. The schematic highlights STAT5-dependent mechanism of Lfng expression during Th2 differentiation and how Lfng contributes to the augmented Th2 response to Dll4-Notch activation during viral exacerbation of existing airway allergic disease.

IL-2–STAT5 axis is involved in priming Th2 differentiation and in maintaining Th2 commitment (32, 43). Furthermore, this latter mechanism seems more relevant in cases of allergen-induced existing Th2 environment, which is then exacerbated by RSV, leading to more IL-2 production, Th2 development, and Th2-associated inflammation. Interestingly our data demonstrate that upregulation of *Lfng* during Th2 differentiation is STAT5 dependent. Whereas we have not exhaustively examined the role of GATA3, our studies have not yet uncovered a direct role of GATA3 on *Lfng* expression. Although our data indicate the importance of STAT5 in upregulating *Lfng* during Th2 differentiation, it remains to be determined whether STAT5 increases *Lfng* expression and Dll4-dependent Notch activation in other IL-2–dependent Th cells, including Th1 and regulatory T cells (44). It is also possible that *Lfng* expression is further regulated in other contexts by additional factors involved in STAT5 signaling, including GM-CSF, thymic stromal lymphopoietin (45), and other IL-2 family members such as IL-7 and IL-15 that regulate Th2 differentiation (43, 46). Furthermore, it will be interesting to determine whether *cis*-acting regulatory elements essential for cyclic *Lfng* expression in presomitic mesoderm also regulate *Lfng* expression in CD4 T cells (47). However, we predict that these findings are not limited to Th2-skewed RSV exacerbations but are critical to overall Th2 humoral immunity and host defense.

In summary, to our knowledge, we provide the first description of *Lfng* regulation in peripheral T cells and show how STAT5 increases its expression under Th2-skewed conditions to modulate Dll4–Notch activation and the subsequent Th response to Dll4. Our study provides novel data demonstrating that *Lfng* controls Notch sensitivity to Dll4 during development and exacerbation of a Th2-mediated allergic airway disease. However, extending our results to other aspects of Notch-mediated T cell and hematopoietic cell differentiation should be examined in a context-specific manner, as Notch activation and *Lfng* expression most likely play a role in the fine-tuning of immune responses, rather than being an all or none immune regulator.

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Disclosures

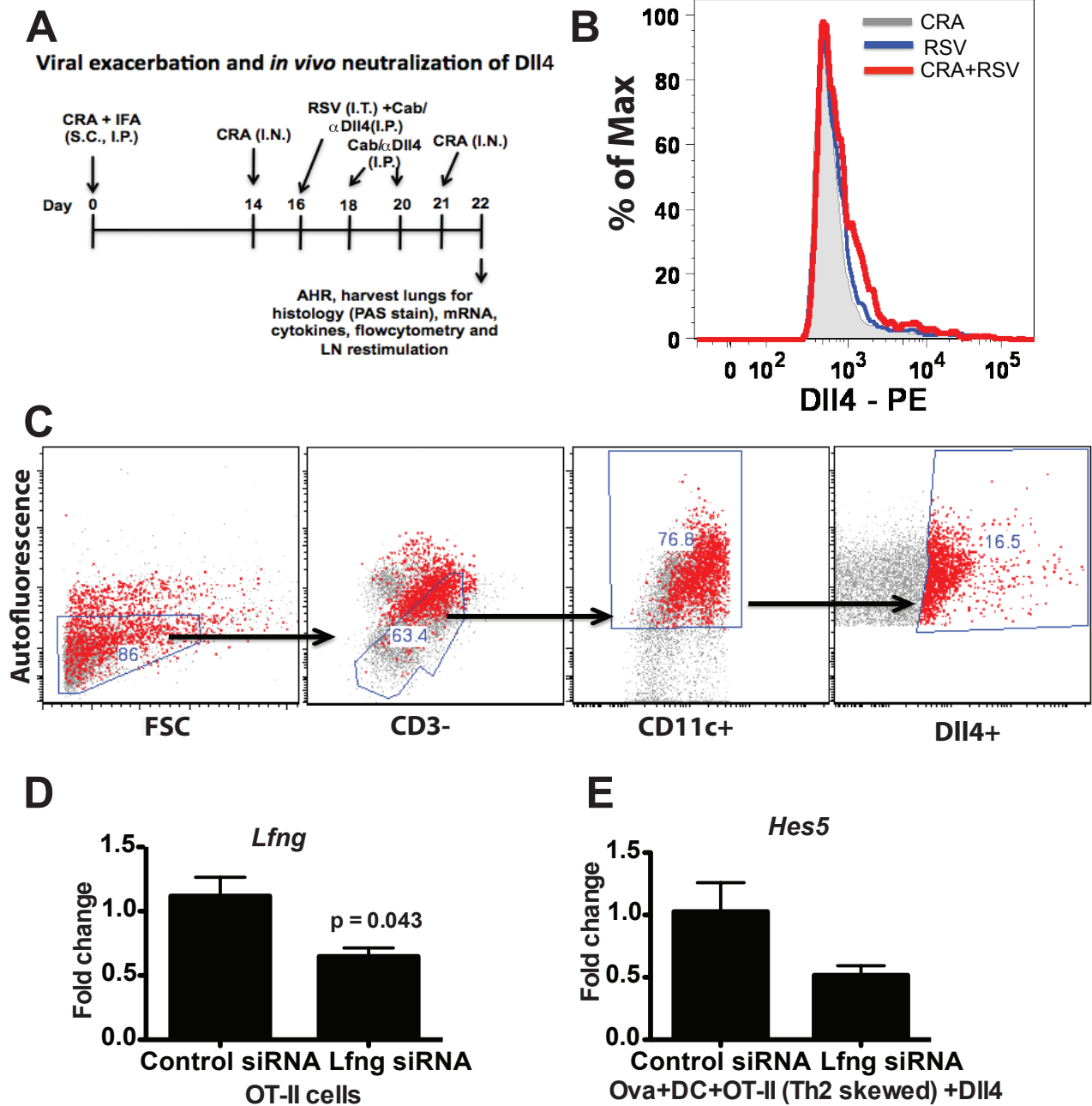
The authors have no financial conflicts of interest.

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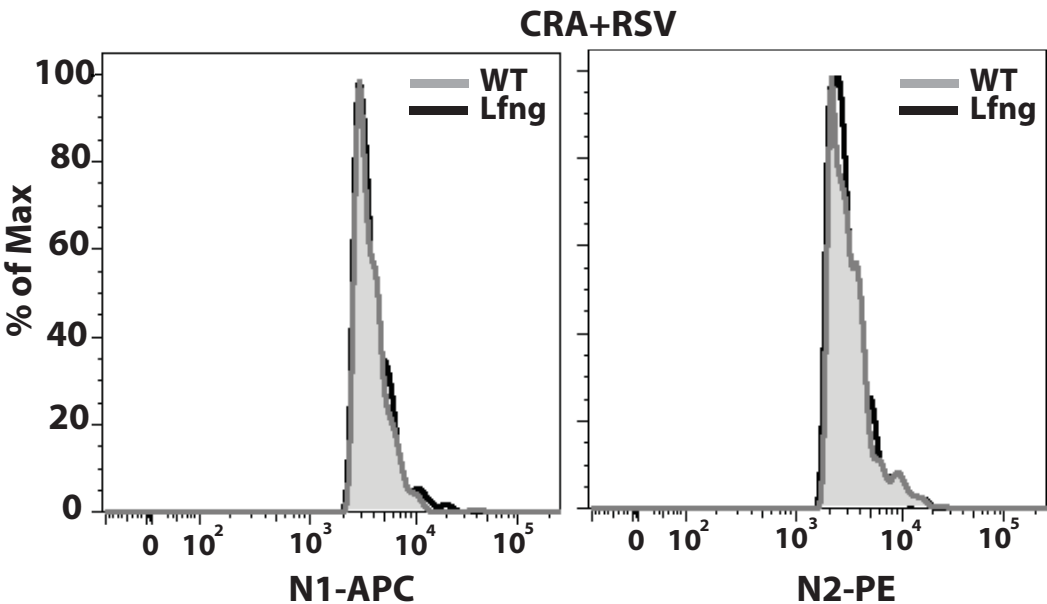
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Figure S1



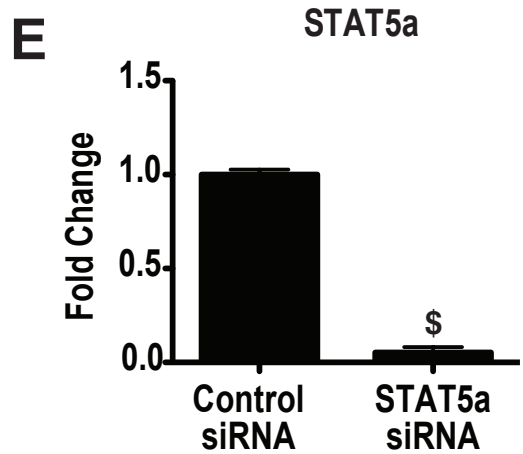
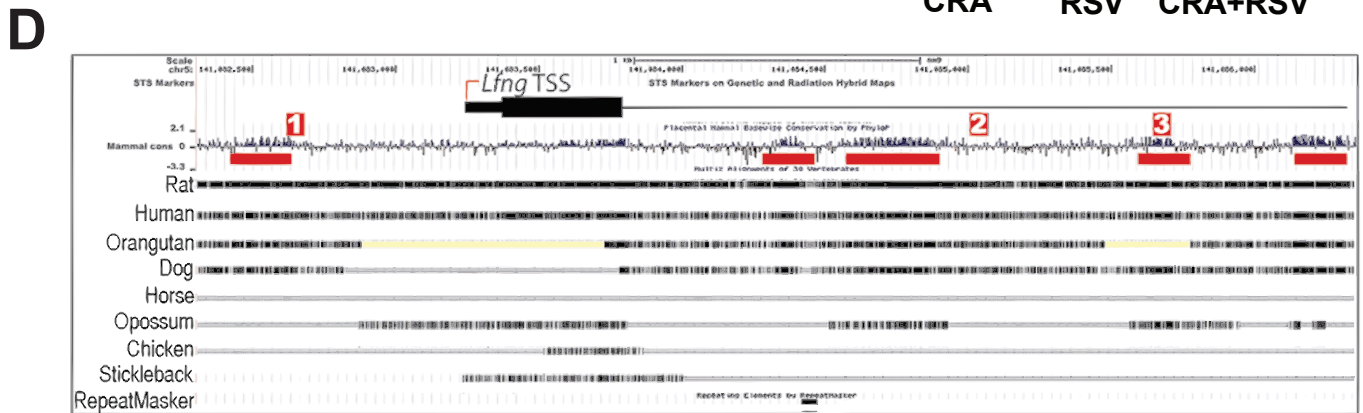
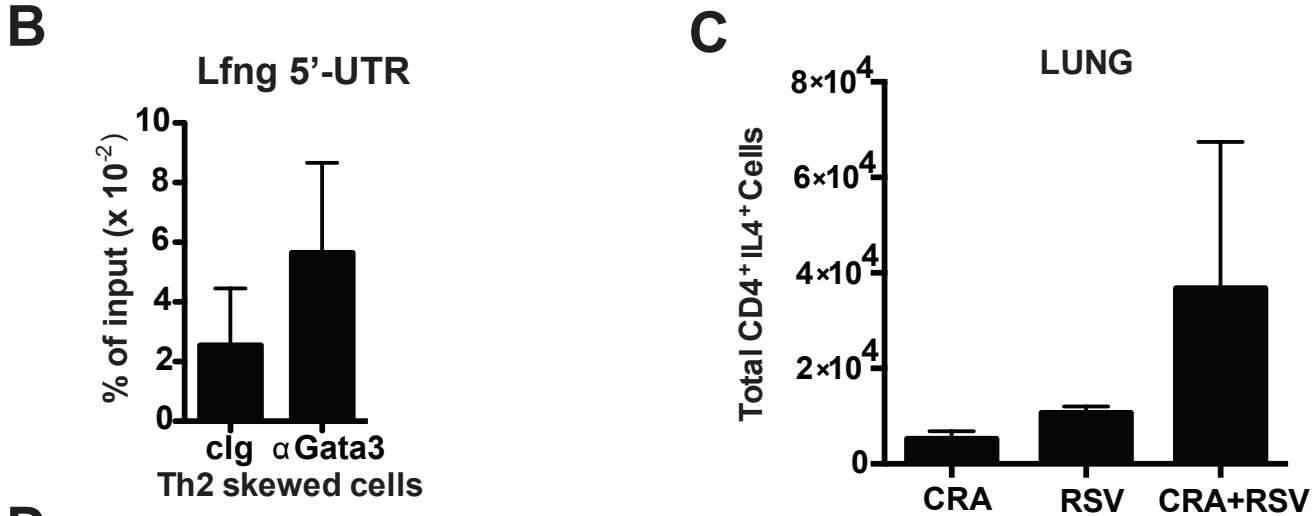
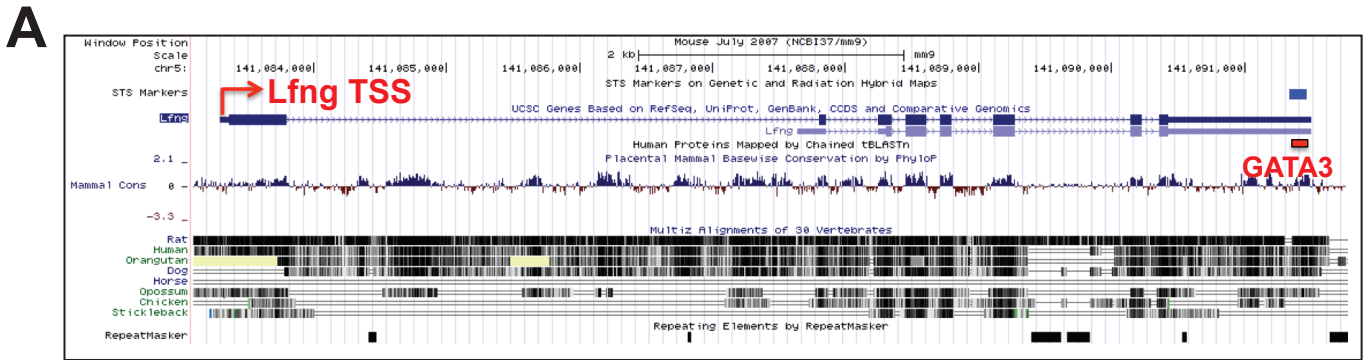
S1. A) *In vivo* neutralization of Dll4 during viral exacerbation of existing airway allergic disease. **B&C)** Mean Fluorescence Intensity (MFI) of Dll4 expression in CD11c⁺ (DCs) in the lungs of CRA, RSV and CRA+RSV infected mice analyzed by Flow cytometry and the gating strategy for identifying the Dll4 expressing DCs. **D)** RT-PCR analysis of Lfng knockdown in OT-II cells transfected with control siRNA or Lfng siRNA. Statistical analysis was done using student t-test and Δ represents $p < 0.05$ **E)** RT-PCR analysis of bone marrow-derived DCs pulsed with ova and co-cultured with Th2 skewed OT-II cells that were transfected with control or Lfng siRNA.

Figure S2



S2. A) Flow cytometry analysis of MFI of N1 and N2 on CD3+CD4+ T cells in the lungs of CRA+RSV infected mice

Figure S3



S3. A) Identification of GATA3 binding region on *Lfng* 5'UTR (in red) 8.6kb downstream of TSS using the UCSC Genome Browser **B)** ChIP analysis of GATA3 binding to 5'UTR on *Lfng* loci in Th2 cells. **C)** Total number of Th2 cells identified by intracellular staining for IL4 in the lungs of mice from CRA only, RSV only and CRA+RSV infected groups. **D)** Identification of consensus STAT5 binding motifs relative to blocks of conserved non-coding sequence (red lines) in promoter and 5' half of intron 1 of *Lfng* locus. All numbers are relative to the TSS of *Lfng*. **E)** RT-PCR analysis of STAT5A knockdown with siRNA in CD4 T cells. Statistical analysis was done using student t-test - \$ represents $p < 0.001$.