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Essential role of Notch signaling in effector memory CD8+ T cell–mediated airway hyperresponsiveness and inflammation

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Adoptive transfer of in vivo–primed CD8+ T cells or in vitro–generated effector memory CD8+ T (T_{EFF}) cells restores airway hyperresponsiveness (AHR) and airway inflammation in CD8-deficient ($CD8^{-/-}$) mice. Examining transcription levels, there was a strong induction of Notch1 in T_{EFF} cells compared with central memory CD8+ T cells. Treatment of T_{EFF} cells with a γ-secretase inhibitor (GSI) strongly inhibited Notch signaling in these cells, and after adoptive transfer, GSI-treated T_{EFF} cells failed to restore AHR and airway inflammation in sensitized and challenged recipient $CD8^{-/-}$ mice, or to enhance these responses in recipient wild-type (WT) mice. These effects of GSI were also associated with increased expression of the Notch ligand Delta1 in T_{EFF} cells. Treatment of sensitized and challenged WT mice with Delta1-Fc resulted in decreased AHR and airway inflammation accompanied by higher levels of interferon γ in bronchoalveolar lavage fluid. These results demonstrate a role for Notch in skewing the T cell response from a T helper (Th)2 to a Th1 phenotype as a consequence of the inhibition of Notch receptor activation and the up-regulation of the Notch ligand Delta1. These data are the first to show a functional role for Notch in the challenge phase of CD8+ T cell–mediated development of AHR and airway inflammation, and identify Delta1 as an important regulator of allergic airway inflammation.

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Abbreviations used: AHR, airway hyperresponsiveness; BAL, bronchoalveolar lavage; GSI, γ-secretase inhibitor; MCh, methacholine; MNC, mononuclear cell; NICD, Notch intracellular domain; RL, lung resistance; T_{CM}, central memory CD8+ T; T_{EFF}, effector memory CD8+ T.

Asthma is a multifactorial inflammatory disorder arising as a result of the cellular and molecular responses induced by allergen exposure in sensitized hosts. Allergic asthma is characterized by persistent airway inflammation and airway hyperresponsiveness (AHR) (1). From several clinical and experimental investigations (2–5), antigen-specific memory T cells, especially CD4+ T cells, were shown to play an integral role in orchestrating the disease process through the secretion of a variety of Th2 cytokines, including IL-4, IL-5, and IL-13, which induce the development of AHR and eosinophilic inflammation. It has also been reported that the transfer of Th2-type cells in mice induces airway eosinophilia and AHR (6).

In addition, there is now increasing evidence for the role of CD8+ T cells in these responses as well. Increased numbers of CD8+ T cells have been shown in the lungs of asthmatic patients (7) and in animal models of allergic asthma (8). We demonstrated that allergen-primed CD8+ T cells were essential for the full development of AHR and airway inflammation through IL-13 production (9). Subsequently, we also reported that in vitro–generated allergen-specific effector memory CD8+ T (T_{EFF}) cells contributed to these responses in the challenge phase through their migration into lung tissue and local production of IL-13 in sensitized and challenged mice (10). We recently demonstrated the critical role of CD4+ T cells in the sensitization phase for the development of CD8+ T cell–mediated AHR and airway inflammation (11). There are numerous articles addressing

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the molecules that regulate effector functions or activation of $CD8^+$ T cells (12, 13).

The Notch signaling pathway plays a fundamental role in cell fate decisions in all organisms (14). In mammals, there are four identified Notch receptors (Notch1–4) and five ligands of the Delta-like families (Delta1, Delta3, and Delta4) and Jagged families (Jagged1 and Jagged2) (14). Notch receptors and their ligands are also expressed on the surface of mature lymphocytes and APCs. Notch proteins are transcriptional activators expressed first as transmembrane heterodimeric surface receptors. After ligation, Notch undergoes proteolytic processing, including a final cleavage by γ-secretase to release the Notch intracellular domain (NICD), which translocates to the nucleus and binds to CSL/RBP-J transcription factor, converting it from a repressor to an activator of gene transcription (14–16). Several target genes of Notch, including Hes1, Hes5, and pT have been identified (17, 18). γ-secretase inhibitors (GSIs) can effectively prevent the enzymatic cleavage of the cytoplasmic domain of Notch receptors, thereby inhibiting the downstream signaling events triggered by activation of these receptors (19).

Recently, studies have implicated Notch in activation (20–23) and differentiation (24–26) of cells of the peripheral immune system. The role of Notch signaling, especially in $CD8^+$ T_{EFF} cells, and its involvement in allergen-induced AHR and airway inflammation have not been defined. In this study, we demonstrated Notch1 expression on $CD8^+$ T_{EFF} cells and that inhibition of Notch signaling using GSI reversed their effects on development of AHR and airway inflammation, in part due to the up-regulation of IFN-γ production. Further, we showed that Delta1, a Notch ligand, is an effective inhibitor of allergen-induced AHR.

RESULTS

Notch1 is expressed in T_{EFF} cells but not central memory $CD8^+$ T (T_{CM}) cells

We previously showed that the development of AHR and eosinophilic inflammation in $CD8^{-/-}$ mice was lower than in WT mice but could be fully restored after transfer of $CD8^+$ T cells from antigen-primed donors or after transfer of in vitro–generated $CD8^+$ T_{EFF} cells but not $CD8^+$ T_{CM} cells (10). Reconstitution of heightened airway responsiveness by T_{EFF} cells was paralleled by restoration of bronchoalveolar lavage (BAL) and tissue eosinophilia, BAL IL-13 levels, and goblet cell metaplasia. To determine if there were differences in gene expression between T_{EFF} and T_{CM} cells, microarray analysis was performed (the microarray data have been deposited in the GEO database under accession number GSM8632). In the analysis of T_{EFF} and T_{CM} cell total RNA, an up-regulation of Notch1 was detected in T_{EFF} cells, which was >1,000-fold higher than in T_{CM} cells ($P < 0.05$) (Fig. 1 A). In contrast, the expression of Notch2 and Notch3 was only minimally higher in T_{EFF} cells.

Because Notch is not up-regulated and NICD is not detected until T_{EFF} cells are activated through the antigen receptor (or TCR) (Fig. 1 B), Notch1 expression is not retained on the surface after stimulation and is down-regulated by 72 h (28). To effectively maintain GSI interference in transferred cells, we used a secondary challenge protocol in which the analysis of AHR was completed within 48 h of a single provocative secondary allergen challenge (27) and transfer of GSI-transferred $CD8^+$ T_{EFF} cells.

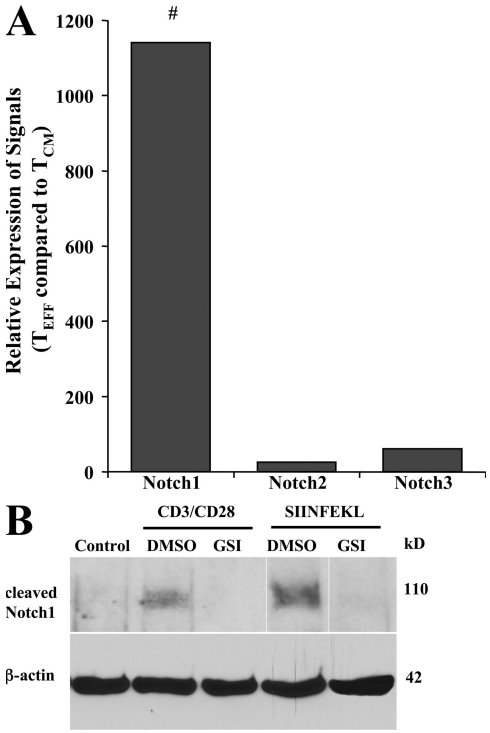

Figure 1. Notch receptor expression and signaling in $CD8^+$ T_{EFF} cells. (A) Relative gene expression of Notch family members in T_{EFF} versus T_{CM} cells. The expression of Notch on T_{EFF} and T_{CM} cells was determined by gene chip analysis. The relative gene expression difference for the hybridization signal is depicted as a ratio. #, $P < 0.05$ summarizing the results of three separate experiments performed in duplicate. (B) Notch signaling is activated in T_{EFF} cells. T_{EFF} cells were stimulated with anti-CD3 and anti-CD28 or SIINFEKL for 24 h after incubation of the cells with DMSO or GSI. Cell lysates were analyzed for the expression of Notch1 by Western blotting, and β-actin was used as a loading control. One representative of three similar experiments is shown.

Notch1 signaling is activated in T_{EFF} cells after engagement of the TCR

To confirm Notch1 signaling in $CD8^+$ T_{EFF} cells, we examined Notch1 protein cleavage. Engagement of Notch by any of its ligands results in proteolytic cleavage at an intracellular site between glycine 1743 and valine 1744. This cleavage event, which results in the liberation of NICD, is dependent on the enzymatic activity of the γ-secretase complex that contains presenilins and nicastrin (29, 30). $CD8^+$ T_{EFF} cells isolated from the lymph nodes or spleens of OT-1 mice were stimulated with anti-CD3 plus anti-CD28 or SIINFEKL peptide in the presence of DMSO (vehicle) or 20 μM GSI for 24 h. The cell lysates were analyzed by Western blotting for the

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cleaved form of Notch1 using a cleavage-specific antibody (Fig. 1 B). Cleaved Notch1 was detected in T_{EFF} cells cultured with either anti-CD3 plus anti-CD28 or SIINFEKL. Treatment with GSI dramatically reduced the levels of cleaved Notch1 protein after TCR or antigen-specific activation. Examination of cell proliferation and cell survival showed no effect of GSI treatment on these parameters (unpublished data). These data demonstrate that Notch1 signaling can be inhibited pharmacologically in $CD8^+$ T_{EFF} cells activated through the TCR.

Effects of transfer of GSI-T_{EFF} cells on allergen-induced AHR and airway inflammation in WT mice

To assess the effects of the Notch signaling pathway on the enhancement of the functional activity of $CD8^+$ T_{EFF} cells in vivo, GSI-T_{EFF} cells or DMSO-T_{EFF} cells were transferred into sensitized and challenged recipient WT mice before secondary OVA challenge. Secondary challenge of sensitized and challenged mice led to the development of increased AHR in WT mice, illustrated by significant increases in lung resistance (RL) (Fig. 2 A), as described previously in this model (31). Fig. 2 A also illustrates the changes in RL in WT recipients of GSI-T_{EFF} cells or DMSO-T_{EFF} cells undergoing secondary challenge. AHR to methacholine (MCh) was significantly increased in recipients of DMSO-T_{EFF} cells, but recipients of GSI-T_{EFF} cells failed to increase AHR over that seen in WT mice not receiving T_{EFF} cells. Indeed, transfer of DMSO-T_{EFF} cells significantly increased AHR, whereas transfer of GSI-T_{EFF} cells inhibited the response to a modest degree. In parallel to the assessment of lung function, the inflammatory cell composition of BAL fluid was examined (Fig. 2 B). Eosinophil numbers in BAL fluid were significantly increased in the DMSO-T_{EFF} cell recipient mice, whereas GSI-T_{EFF} cell recipient mice did not show such increases. In contrast, neutrophil numbers were increased in GSI-T_{EFF} cell recipients. Transfer of either DMSO-T_{EFF} cells or GSF T_{EFF} cells increased lymphocyte numbers in BAL fluid compared with WT control mice after secondary allergen challenge.

Cell composition of BAL fluid was examined in WT recipients of GSI-T_{EFF} cells or DMSO-T_{EFF} cells by flow cytometry 48 h after the secondary challenge. Previous studies demonstrated that adoptively transferred T_{EFF} cells preferentially migrated into allergen-challenged airways via BLT1 (32). The numbers of $CD8^+$ T cells in the BAL fluid were increased to a similar extent in recipients of either DMSO-T_{EFF} cells or GSI-T_{EFF} cells compared with secondary challenged WT control mice (Fig. 2 C). In addition, the percentages of $CD8^+$ T cells among total BAL cells were higher in both recipients of DMSO-T_{EFF} cells (20.4 ± 1.7%) and GSI-T_{EFF} cells (19.4 ± 1.3%) than in secondary challenged WT control mice (6.6 ± 0.4%). These data demonstrate that the accumulation of $CD8^+$ T cells in the airways after T_{EFF} cell transfer and secondary allergen challenge was not altered by pretreatment with GSI. The results from lung cell digestion were similar to those observed in BAL fluid (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20072200/DC1).

Cytokine levels in BAL fluid of WT mice after transfer of GSI-T_{EFF} cells or DMSO-T_{EFF} cells

The relative levels of Th1 and Th2 cytokines have been proposed to play an important role in the development of allergic airway inflammation (33). After transfer of T_{EFF} cells pretreated with DMSO into secondary challenged WT mice, IL-4 and IL-13 levels in BAL of recipients of DMSO-T_{EFF} cells were increased, whereas GSI-T_{EFF} cell recipients showed smaller increases in IL-4 and IL-13 but markedly increased levels of IFN-γ (Fig. 2 D). Similar results on cytokine production were observed when lung cells were assayed (Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20072200/DC1).

The expression of T-bet and GATA-3 in homogenized lung

Because the balance between the transcription factors T-bet and GATA-3 has been associated with the predominance of Th1 and Th2 responses, respectively (12), we analyzed levels of these transcription factors using real-time PCR in homogenized lung samples from mice that received GSI-T_{EFF} cells or DMSO-T_{EFF} cells. In parallel to the results of BAL cytokine levels, the expression level of T-bet in recipients of GSI-T_{EFF} cells was significantly higher than in the other groups; the expression level of GATA-3 in GSI-T_{EFF} cell recipients was significantly decreased (Fig. 2 E).

IFN-γ production in $CD4^+$ or $CD8^+$ T cells

To identify the source of IFN-γ production in the recipients of GSI-T_{EFF} cells, mononuclear cells (MNCs) were obtained from the lung tissue of recipient WT mice after transfer of either GSI-T_{EFF} cells or DMSO-T_{EFF} cells 48 h after secondary OVA challenge. As shown in Table I, the number of IFN-γ^+ cells in the $CD4^+$ fraction of GSI-T_{EFF} cell recipients was significantly higher than in the DMSO-T_{EFF} cell recipients. However, the number of IFN-γ–producing lung $CD8^+$ T cells was significantly decreased in GSI-T_{EFF} cell recipients compared with DMSO-T_{EFF} cell recipients (Table I).

Effects of GSI-T_{EFF} cell transfer on allergen-induced AHR and airway inflammation in $CD8^{-/-}$ recipients

T_{EFF} cells transferred into primary challenged $CD8^{-/-}$ mice restored the full development of AHR and airway inflammation (10). To analyze the role of Notch signaling in the

Table I. Number of IFN-γ^+ $CD3^+CD4^+$ or IFN-γ^+ $CD3^+CD8^+$ cells in the lungs of sensitized and challenged WT mice that received DMSO-T_{EFF} or GSI-T_{EFF} cells

	$CD4^+$IFN-γ^+ ($\times 10^4$)	$CD8^+$IFN-γ^+ ($\times 10^4$)
WT	28.8 ± 1.9	13.2 ± 1.9
DMSO-T_{EFF}	40.8 ± 4.8	32.3 ± 3.0
GSI-T_{EFF}	69.7 ± 5.5[a]	25.3 ± 3.2[b]

Mean values ± SEM are given. WT, OVA-sensitized and challenged WT mice; DMSO-T_{EFF}, DMSO-treated T_{EFF} cell recipient WT mice that were OVA sensitized and challenged; GSI-T_{EFF}, GSI-treated T_{EFF} cell recipient WT mice that were OVA sensitized and challenged.
[a]$P < 0.05$ compared between GSI-T_{EFF} cells versus WT and DMSO-T_{EFF} cells.
[b]$P < 0.05$ compared between GSI-T_{EFF} cells versus DMSO-T_{EFF} cells.

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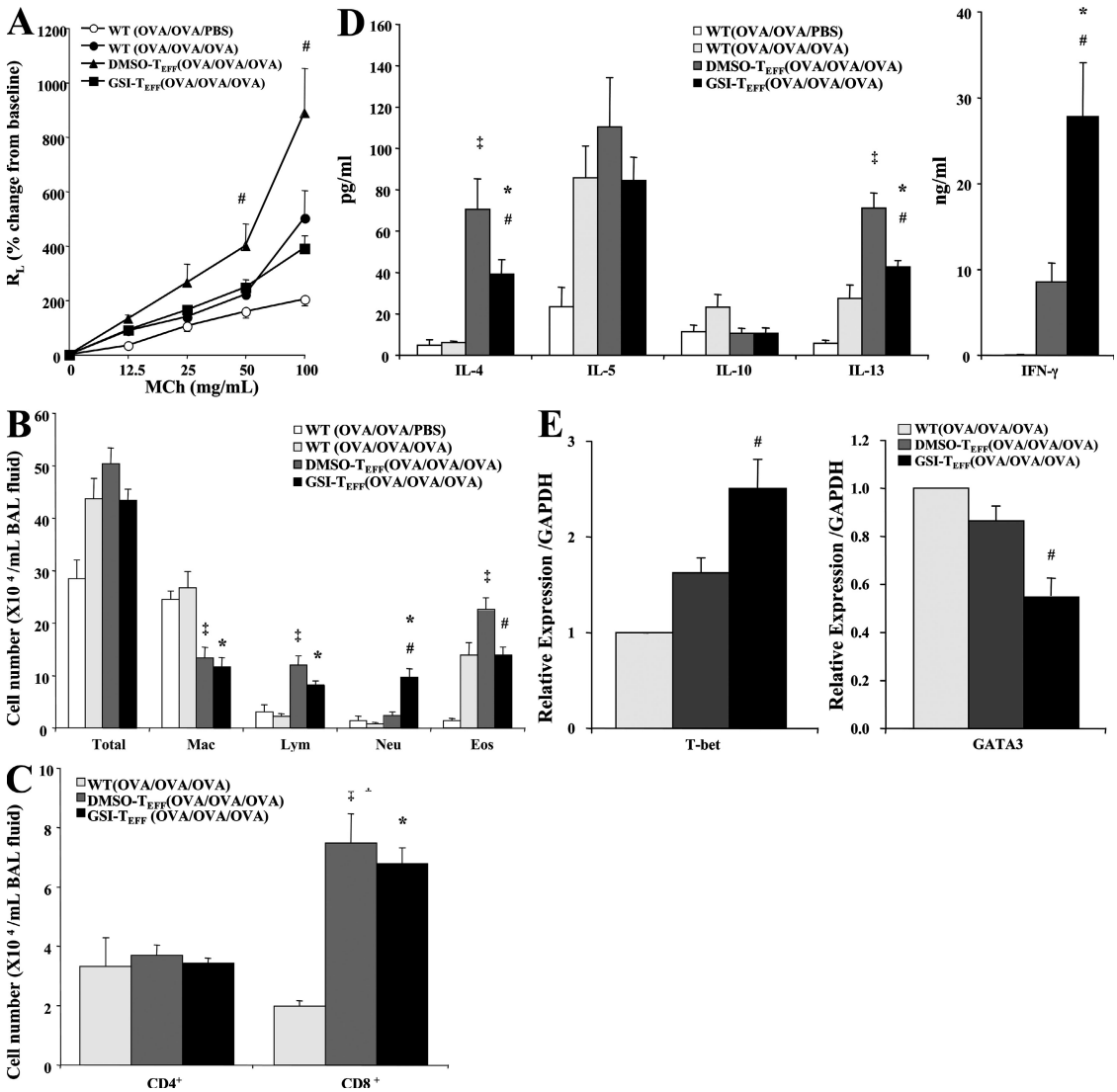

Figure 2. Transfer of GSI-T_{EFF} cells fails to enhance lung allergic responses in WT recipients. Sensitized and challenged WT mice received GSI-T_{EFF} or DMSO-T_{EFF} cells before secondary challenge (OVA/OVA/OVA). Control mice were those that were sensitized and challenged but received PBS at the time of secondary challenge (OVA/OVA/PBS). (A) RL values were obtained in response to increasing concentrations of inhaled MCh, as described in Materials and methods. Data represent the mean ± SEM (n = 12 in each group). #, significant difference ($P < 0.05$) between DMSO-T_{EFF} cell recipients and WT control mice or GSI-T_{EFF} cell recipients. (B) Cellular composition of BAL fluid. #, significant difference ($P < 0.05$) between GSI-T_{EFF} cell recipients and DMSO-T_{EFF} cell recipients; ‡, significant difference ($P < 0.05$) between DMSO-T_{EFF} cell recipients and WT control mice; *, significant difference ($P < 0.05$) between GSI-T_{EFF} cell recipients and WT mice. (C) $CD4^+$ and $CD8^+$ T cells in BAL fluid. ‡, significant difference ($P < 0.05$) between DMSO-T_{EFF} cell recipients and WT control mice; *, significant difference ($P < 0.05$) between GSI-T_{EFF} cell recipients and WT mice. (D) Cytokine levels in BAL fluid of WT mice that received GSI-T_{EFF} or DMSO-T_{EFF} cells. #, significant difference ($P < 0.05$) between GSI-T_{EFF} cell recipients and DMSO-T_{EFF} cell recipients; ‡, significant difference ($P < 0.05$) between DMSO-T_{EFF} cell recipients and WT control mice; *, significant difference ($P < 0.05$) between GSI-T_{EFF} cell recipients and WT control mice. (E) Relative expression levels of GATA-3 and T-bet in the lung were determined by quantitative real-time PCR. Lung cells are from the same groups as described in A. They were isolated using collagenase digestion, and RNA was prepared. Results are representative of three independent experiments. The results for each group are expressed as mean ± SEM. #, significant difference ($P < 0.05$) between GSI-T_{EFF} cell–transferred mice and DMSO-T_{EFF} cell–transferred mice or WT control mice.

reconstitution of $CD8^{-/-}$ mice after secondary allergen challenge, GSI-T_{EFF} cells or DMSO-T_{EFF} cells were transferred into sensitized and challenged $CD8^{-/-}$ mice before secondary allergen challenge. As shown in Fig. 3 (A and B), $CD8^{-/-}$ mice developed significantly lower responses after secondary allergen challenge. Transfer of DMSO-T_{EFF} cells reconstituted the development of AHR and eosinophilic airway inflammation. The development of AHR in $CD8^{-/-}$ recipients of DMSO-T_{EFF} cells was not significantly different from WT recipients of DMSO-T_{EFF} cells. Transfer of GSI-T_{EFF} cells into $CD8^{-/-}$ failed to increase AHR or airway eosinophilia, but it did increase the numbers of neutrophils

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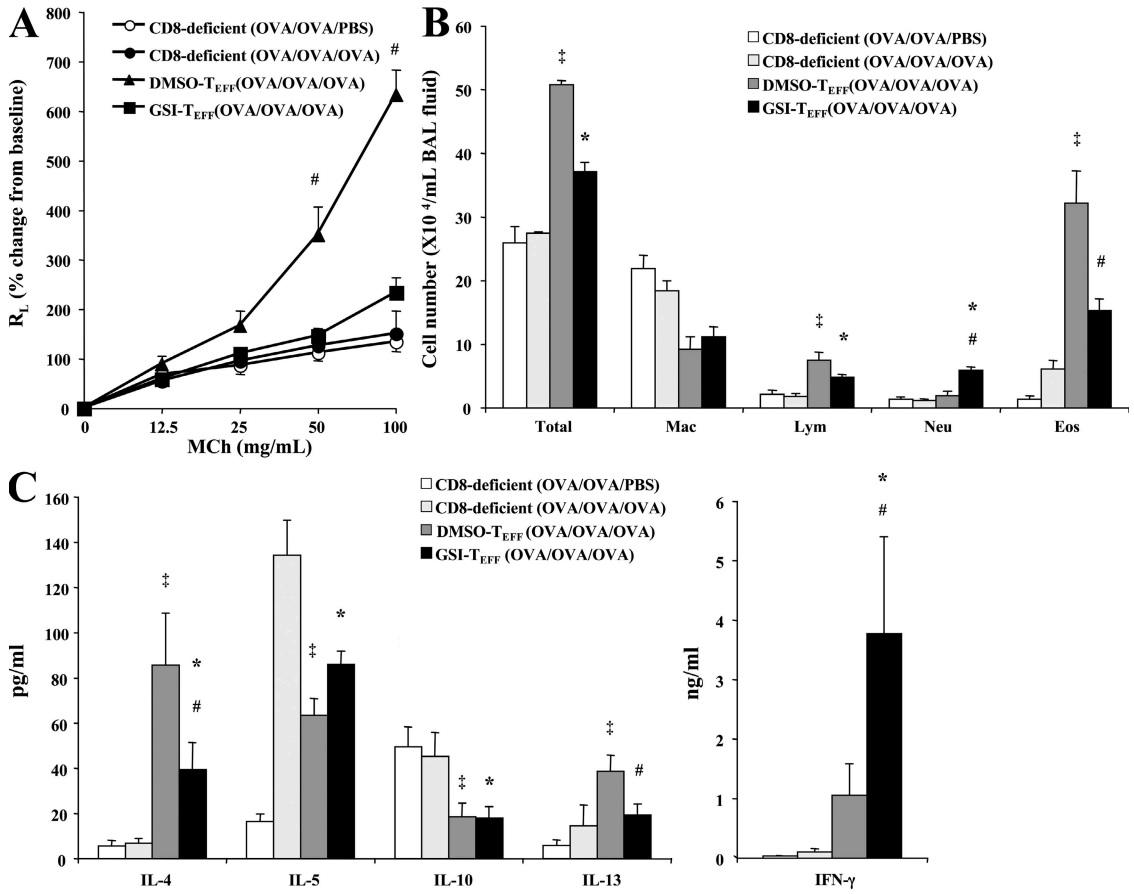

Figure 3. Allergen-induced AHR and airway inflammation are restored in CD8$^{-/-}$ mice that received DMSO-T_{EFF} cells but not GSI-T_{EFF} cells. Before secondary challenge, sensitized and challenged (primary) CD8$^{-/-}$ mice received 5 × 10^6 GSI-T_{EFF} or DMSO-T_{EFF} cells via the tail vein. Control mice were sensitized and challenged and received PBS on secondary challenge. (A) AHR. #, significant difference (P < 0.05) between DMSO-T_{EFF} cell recipients and controls or GSI-T_{EFF} cell recipients. (B) Cell composition in BAL fluid and (C) BAL cytokine levels. The results for each group are expressed as the mean ± SEM (n = 12). #, significant difference (P < 0.05) between GSI-T_{EFF} cell recipients and DMSO-T_{EFF} cell recipients; ‡, significant difference (P < 0.05) between DMSO-T_{EFF} cell recipients and controls; *, significant difference (P < 0.05) between GSI-T_{EFF} cell recipients and controls.

in BAL fluid. IFN-γ levels in BAL fluid were also increased in GSI-T_{EFF} cell recipients compared with recipients of DMSO-T_{EFF} cells, whereas the opposite was true for IL-4 and IL-13 (Fig. 3 C).

MNCs were obtained from the lung tissue of recipient CD8$^{-/-}$ mice after the transfer of either GSI-T_{EFF} cells or DMSO-T_{EFF} cells 48 h after secondary challenge and assessed by flow cytometry (Table II). The number of CD4$^+$IFN-γ$^+$ T cells in GSI-T_{EFF} cell recipients was significantly higher than in the DMSO-T_{EFF} cell recipients. However, the number of IFN-γ–producing lung CD8$^+$ T cells was lower in GSI-T_{EFF} cell recipients than in DMSO-T_{EFF} cell recipients. Based on these results, it appeared that the source of increased IFN-γ production and Th1 polarization was lung CD4$^+$ T cells in the recipients of GSI-T_{EFF} cells.

Expression of Delta1 in T_{EFF} cells from CD8$^{-/-}$ recipients of GSI-T_{EFF} cells or DMSO-T_{EFF} cells

To determine if Notch ligand expression was involved in the response of the transferred cells, CD8$^+$ T_{EFF} cells were isolated from the lungs of secondary challenged CD8$^{-/-}$ recipients of either GSI-T_{EFF} cells or DMSO-T_{EFF} cells and analyzed by real-time PCR. T cells are reported to express the Notch ligands Delta1 (34), Jagged1 (35), and Jagged2 (36). In parallel to the data on BAL (and lung cell) cytokine production and the induction of T-bet expression in the lung, we showed that the level of Delta1 expression was higher in isolated GSI-T_{EFF} cells compared with isolated DMSO-T_{EFF} cells (Fig. 4). Baseline levels of Delta1 expression in either GSI-T_{EFF} cells or DMSO-T_{EFF} cells before transfer were extremely low in the absence of activation. There were no significant differences in the levels of expression of the other Notch ligands (Jagged1 and Jagged2).

Effects of administration of Delta1-Fc to WT mice on allergen-induced AHR and airway inflammation

To directly test whether Delta1 regulates AHR and airway inflammation, sensitized and challenged WT mice were treated with Delta1-Fc or human IgG as a control before secondary OVA challenge (OVA/OVA/OVA) (Fig. 5 A). Administration

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Table II. Number of IFN-γ+ CD3+CD4+ or IFN-γ+ CD3+CD8+ cells in the lungs of sensitized and challenged CD8−/− mice that received DMSO-T_{EFF} or GSI-T_{EFF} cells

	CD4+IFN-γ+ (×10⁴)	CD8+IFN-γ+ (×10⁴)
CD8-deficient	18.0 ± 1.2	–
DMSO-T_{EFF}	35.0 ± 5.6	21.6 ± 4.2
GSI-T_{EFF}	50.7 ± 3.8[a]	14.3 ± 1.3[b]

Mean values ± SEM are given. CD8-deficient, OVA-sensitized and challenged CD8-deficient mice; DMSO-T_{EFF}, DMSO-treated T_{EFF} cell recipient CD8-deficient mice that were OVA sensitized and challenged; GSI-T_{EFF}, GSI-treated T_{EFF} cell recipient CD8-deficient mice that were OVA sensitized and challenged.
[a]$P < 0.05$ compared between GSI-T_{EFF} cells versus CD8-deficient and DMSO-T_{EFF} cells.
[b]$P < 0.05$ compared between GSI-T_{EFF} cells versus DMSO-T_{EFF} cells.

of Delta1-Fc markedly reduced AHR compared with administration of (control) human IgG in response to secondary allergen challenge (Fig. 5 B). Neither Delta1-Fc nor human IgG altered the response in control mice (OVA/OVA/PBS). In parallel, administration of Delta1-Fc to secondary challenged mice markedly reduced the numbers of eosinophils and levels of IL-13 in the BAL fluid without affecting these responses in controls (Fig. 5, C and D).

Administration of Delta1-Fc to secondary challenged mice resulted in significantly increased levels of IFN-γ in the BAL fluid. To confirm these effects of Delta1-Fc and IFN-γ production, MNCs were obtained from the lung tissue of secondary challenged mice or control mice. As shown in Fig. 5 E, the number of CD4+IFN-γ+ T cells in mice treated with Delta1-Fc was significantly higher than in those that received human IgG (or controls that received Delta1-Fc or human IgG). However, the number of CD8+IFN-γ+ lung CD8+ T cells was not significantly different among any of

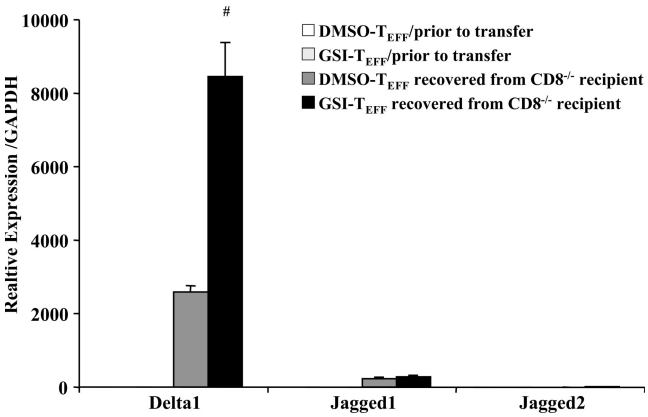

Figure 4. Real-time PCR analysis of Notch ligand expression in T_{EFF} cells. Before and after transfer of GSI-T_{EFF} or DMSO-T_{EFF} cells, CD8+ T_{EFF} cells from the lungs of CD8−/− mice were isolated using MACS beads and RNA was prepared. The relative expression of Notch ligands (Delta1, Jagged1, and Jagged2) was determined by quantitative real-time PCR. Results are from three independent experiments. The results for each group are expressed as mean ± SEM. #, significant difference ($P < 0.05$) between GSI-T_{EFF} cell recipients and DMSO-T_{EFF} cell recipients.

these groups. The results in BAL fluid were confirmed using lung cells (Fig. S3, available at http://www.jem.org/cgi/content/full/jem.20072200/DC1).

DISCUSSION

The Notch signaling pathway is a highly conserved program for cell fate decisions in all organisms, influencing apoptosis or cell cycle arrest (37, 38). The signal induced by ligand binding is conveyed intracellularly by a process involving proteolytic cleavage of the receptor and nuclear translocation of the intracellular domain of the Notch family protein. The GSIs prevent the generation of the NICD and suppress Notch activity. In the immune system, several studies have implicated Notch signaling in hematopoiesis, T–B lineage commitment, and thymic T cell development (16, 39–41). Notch gene expression is induced and Notch1 is activated after CD4+ T cell activation, possibly through a "positive feedback loop" in adjacent cells (21). Studies have also suggested that Notch may influence both Th1 and Th2 polarization (25, 26).

In contrast to several reports that analyzed the relationship between CD4+ T cells and Notch in the peripheral immune system, there is a paucity of information regarding a role for Notch signaling in CD8+ T cells. Several studies have suggested that the activation of Notch suppresses CD8+ T cell effector functions (23, 42). On the other hand, Palaga et al. (20) reported that the number of IFN-γ–producing CD8+ T cells was significantly reduced by inhibition of Notch signaling, indicating that the activation of Notch may be required for CD8+ T cell function.

There is now increasing evidence that in addition to CD4+ T cells, CD8+ T cells contribute to the development of allergic disease (7–10, 43–45). In the development of CD8+ T cell–mediated AHR and eosinophilic inflammation, CD4+ T cells play an essential role in the sensitization phase (11). We previously showed that CD8−/− mice develop lower levels of AHR and eosinophilic inflammation compared with WT mice, and these responses can be restored by transfer of in vivo–activated CD8+ T cells from antigen-primed donors or by transfer of in vitro–generated CD8+ T_{EFF} cells, but not CD8+ T_{CM} cells, before challenge (9, 10). Recently, we showed that T_{EFF} cells upregulate BLT1 (32), the high affinity receptor for LTB4 (46), and BLT1 plays a critical role in the recruitment of T_{EFF} cells into allergen-challenged lungs resulting in AHR and airway inflammation (32).

In this study, we used transcript expression profile analysis to define gene differences between T_{EFF} and T_{CM} cells. In light of the findings that Notch1 expression was much higher in CD8+ T_{EFF} than T_{CM} cells, we investigated the role of this signaling molecule in CD8-mediated allergic airway responses. We first demonstrated that Notch can signal T_{EFF} cells and determined the role of this signaling pathway using a pharmacological approach. We showed that after incubation of CD8+ T_{EFF} cells with anti-CD3/anti-CD28 or the addition of SIINFEKL, cleaved Notch1 could be detected confirming Notch signaling, and incubation of T_{EFF} cells with the GSI prevented this cleavage in vitro.

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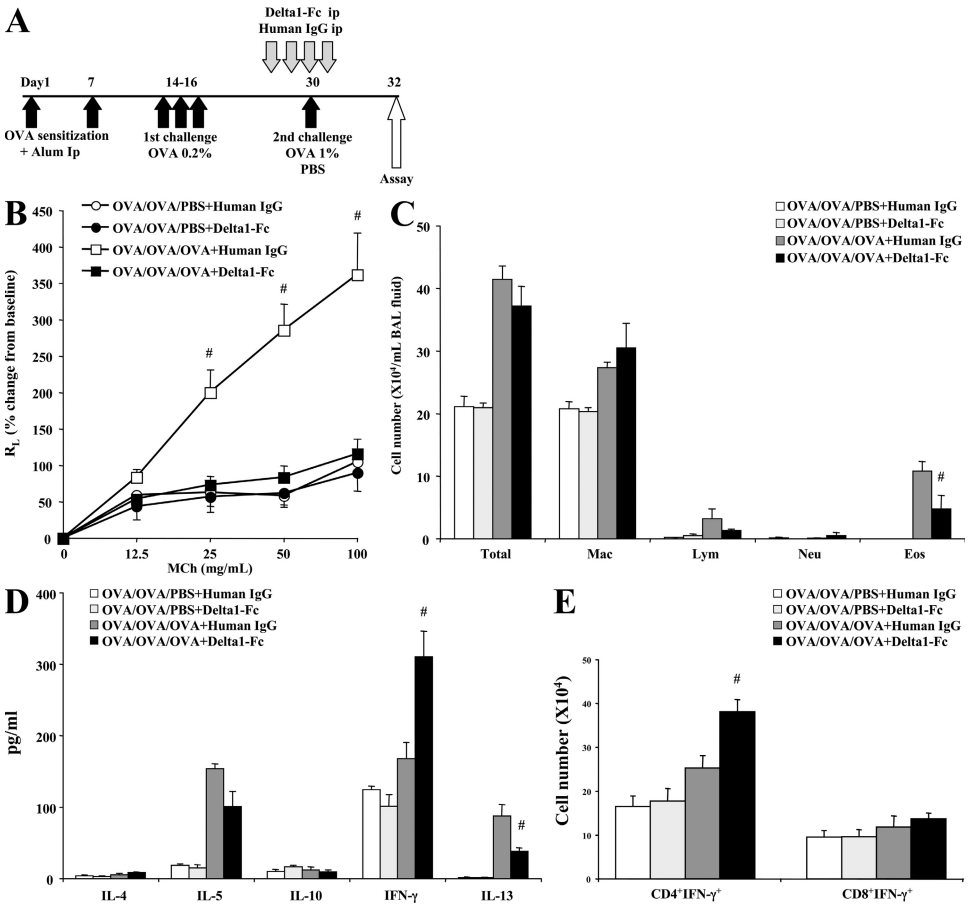

Figure 5. Allergen-induced AHR and airway inflammation are prevented by administration of Delta1-Fc. Secondary challenged (OVA/OVA/OVA) or control (OVA/OVA/PBS) WT mice received Delta1-Fc or human IgG. (A) Experimental protocol, (B) AHR, (C) cell composition in BAL fluid, (D) BAL cytokine levels, and (E) number of IFN-γ⁺-producing CD4⁺ or CD8⁺ T cells in the lung. The results for each group are expressed as the mean ± SEM (n = 8 in each group). #, significant difference ($P < 0.05$) between Delta1-Fc-treated secondary challenged mice and human IgG-treated secondary challenged mice or control mice.

These observations were extended to assess the effects of preventing Notch cleavage in vivo. To effectively maintain GSI interference, we used a secondary challenge protocol in which the analysis of AHR was completed within 48 h of transfer of GSI-treated $CD8^+$ T_{EFF} cells (27). In sensitized and challenged WT recipients, secondary challenge elicited AHR and airway inflammation. Transfer of DMSO (control)-treated T_{EFF} cells led to further increases in AHR, airway eosinophilia, and BAL IL-4 and IL-13 levels. In contrast, transfer of GSI-treated T_{EFF} cells failed to enhance the responses over those seen in WT mice. Notably, levels of BAL IFN-γ were markedly increased in these recipients as were the levels of expression of T-bet with a decrease in GATA-3 expression. These results were not attributable to differences in the ability of GSI-T_{EFF} cells to accumulate in the lung, as the numbers of GSI- and DMSO-treated cells in the lung were the same. The failure of GSI-T_{EFF} cells to enhance AHR or eosinophilic airway inflammation could be attributed to the increases in IFN-γ production. We previously demonstrated that IFN-γ has the potential to inhibit AHR (47, 48) and that intranasal administration of IFN-γ effectively inhibited goblet cell metaplasia (49). Additionally, IFN-γ has been shown to inhibit airway smooth muscle contraction (50) and proliferation (51).

Differentiation of Th1 and Th2 cells is tightly cross-regulated so that development of one subset is inhibited by cytokines produced by the other (52). T-box expressed in T cells (T-bet) plays a central role in Th1 development by activating Th1 genetic programs, IFN-γ production, and repressing Th2 cytokine synthesis (52). In contrast, GATA-3 serves as a Th2 cytokine-specific transcription factor selectively expressed in Th2 cells (53, 54) and leads to inhibition of IFN-γ production (55). In the secondary challenge model, transfer of GSI-treated T_{EFF} cells shifted the balance with modest increases and decreases in T-bet and GATA-3, respectively, accompanied by failure to enhance lung allergic responses. However, the balance was not shifted to the extent that the transfer of GSI-treated T_{EFF} cells prevented sensitized and challenged host cells from contributing to the lung allergic responses.

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A similar pattern was seen in $CD8^{-/-}$ recipients where transfer of GSI-treated T_{EFF} cells, unlike DMSO-treated T_{EFF} cells, failed to reconstitute AHR, airway eosinophilia, or BAL IL-13 levels. As in WT recipients, transfer of GSI-treated T_{EFF} cells resulted in increased IFN-γ levels in BAL. In vitro, incubation of GSI-treated T_{EFF} cells with anti-CD3/anti-CD28 or SIINFEKL did lead to decreased expression of Notch1 mRNA (unpublished data), in keeping with the reduced levels of Notch1 cleavage protein determined by Western blotting. In parallel, activation through the TCR of the GSI-treated T_{EFF} cells led to increases in the expression of Delta1 mRNA. However, these changes were only seen when GSI-T_{EFF} cells were co-cultured with $CD8^+$ T_{EFF} cells during the activation period, but not when the GSI-T_{EFF} cells were cultured alone (Fig. S4, available at http://www.jem.org/cgi/content/full/jem.20072200/DC1). After GSI-T_{EFF} cell transfer and recovery from the lungs of secondary challenged $CD8^{-/-}$ mice, we noted a marked up-regulation of Delta1 mRNA compared with recovered DMSO-treated T_{EFF} cells, with little or no difference between the two in levels of Jagged1 or Jagged2 mRNA.

Notch–Notch ligand interactions govern cell fate decisions in T cells. Notch signaling can direct Th2 differentiation via GATA-3 (56, 57). In vertebrates, Notch can bind to two different families of ligands, Delta-like (34) and Jagged1 and Jagged2 (35, 36). Stimulation of naive $CD4^+$ T cells with Delta1 involving interactions with Notch3 promotes the differentiation toward the Th1 pathway (25), whereas Jagged1-mediated Notch1 activation is critical in driving Th2 differentiation (26). As a rule, the ligands tend to be expressed in a more highly restricted pattern than their receptors.

These effects of GSI on $CD8^+$ T_{EFF} cells decreasing Notch1 expression, reducing Notch1 cleavage, and increasing T-bet and Delta1 expression, and the associated inhibition of enhancement or failure to restore lung allergic responses together with increased IFN-γ production, indicated that Notch–Delta1 pathways were critical regulators of AHR and Th2 lung allergic responses. The GSI reduced the amount of Notch signaling to potentially mimic a Notch1 loss of function phenotype. It appears that activated T cells, and $CD8^+$ T cells in particular, pretreated with GSI produce little IFN-γ (20). We analyzed IFN-γ secretion in TCR-activated GSI-T_{EFF} cells and found production to be significantly decreased compared with similarly treated DMSO-T_{EFF} cells (Fig. S5, available at http://www.jem.org/cgi/content/full/jem.20072200/DC1). After transfer, the majority of IFN-γ–producing cells were localized to the $CD4^+$ subset. The data imply that the increase in $CD4^+IFN\text{-}\gamma^+$ T cells resulted from the changes in Notch signaling ligands expressed on the $CD8^+$ GSI-T_{EFF} cells, i.e., up-regulation of Delta1 on GSI-T_{EFF} cells interacting with Notch receptors on $CD4^+$ T cells. The total numbers of $CD4^+$ T cells in BAL fluid (Fig. 2 C) and lung (Fig. S1) were the same in recipients of GSI-T_{EFF} cells and DMSO-T_{EFF} cells. However, only after the interactions mediated through Notch receptors and Notch ligands on GSI-T_{EFF} cells was attenuation of CD8-mediated AHR and inflammation observed and associated with increased numbers of $CD4^+IFN\text{-}\gamma^+$ T cells in secondary challenged mice. These data are consistent with reports that Delta1 interacts with $CD4^+$ T cells and enhances IFN-γ production in antigen-stimulated $CD4^+$ T cells (25, 26).

To further extend these observations, we examined the consequences of administering a Delta1-Fc protein before secondary challenge. Administration of Delta1-Fc resulted in increased IFN-γ production from $CD4^+$ T cells, and this was associated with a significant inhibition of the development of AHR, airway eosinophilia, and BAL IL-13 levels. At the same time, BAL IFN-γ levels were increased. These results are similar to recent observations in experimental autoimmune encephalomyelitis, where treatment with Delta1-Fc increased the number of Th1 cells in the central nervous system (58). Control mice that received Delta1-Fc did not increase the number of IFN-γ–producing lung $CD4^+$ T cells, suggesting that in the absence of activation, the expression of Notch is not up-regulated and that interactions between Notch receptor and Delta1-Fc do not take place. In recipients of Delta1-Fc, we detected the highest numbers of $CD4^+IFN\text{-}\gamma^+$ T cells and levels of IFN-γ, accompanied by markedly reduced levels of IL-4 and IL-13 in secondary challenged mice (Fig. S3).

After adoptive transfer, $CD8^+$ T_{EFF} cells in the lung exhibit a Th2 phenotype (10, 32). Thus, the phenotype of predominant Th1-type cytokine-producing $CD8^+$ T cells could be redirected toward Th2-type cytokine production in the lungs of sensitized and challenged mice, a plasticity previously emphasized in $CD4^+$ T cells (59). As a result of the inhibition of Notch signaling by GSI, Th2 cytokine production by these lung $CD8^+$ T_{EFF} cells was superseded by Notch receptor–Notch ligand interactions and increased IFN-γ production in $CD4^+$ T cells. Collectively, these data identify a new pathway involved in the regulation of $CD4^+$–$CD8^+$ T cell interactions and the development of Th2-mediated allergic responses through Notch signaling. The pathway appears tightly regulated by the expression pattern of Notch receptor and the Delta1 Notch ligand, which are in turn dependent on the activation of T cells. The data reveal the therapeutic potential of Delta1-Fc in the regulation of allergen-induced AHR.

MATERIALS AND METHODS

Mice. WT C57BL/6 and OT-1 mice (C57BL/6 strain) expressing a transgenic TCR that is specific for $OVA_{257–264}$ (SIINFEKL) peptide (60) were purchased from The Jackson Laboratory. Homozygous CD8-deficient ($CD8^{-/-}$) mice, generated by targeting the *CD8α* chain gene in C57BL/6 mice (61), were obtained from P. Marrack (National Jewish Medical and Research Center, Denver, CO). Each experiment was independently performed at least three times with four mice/group ($n = 12$). Controls were matched with the deficient mice with regard to both age and gender in each experimental group. All studies were conducted under a protocol approved by the Institutional Animal Care and Use Committee of the National Jewish Medical and Research Center.

Cell (CD8 T cell) preparation and culture. Differentiation of T_{EFF} and T_{CM} cells in vitro was performed as described previously (62, 63). For proliferation assays, T_{EFF} cells on day 6 were cultured with GSI or DMSO and IL-2 for 24 h, followed by cell count analysis (Coulter Counter). For the protein and RNA assays, isolated T_{EFF} cells were stimulated with 2 μg/ml of plate-bound anti-CD3 plus 2 μg/ml anti-CD28 (R&D Systems) or 1 μg/ml SIINFEKL peptide in the presence of DMSO (0.1% final concentration) or

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20 μM GSI (dibenzapine; EMD) for 24 h. These T_{EFF} cells were washed for immunoblot analysis, or total RNA was extracted from these T_{EFF} cells and analyzed by real-time PCR. All data were representative of at least three independent experiments.

Preparation of RNA for microarray analysis. RNA was extracted from T_{EFF} and T_{CM} cells using the TRIzol reagent as per the manufacturer's instructions, and microarray hybridizations were performed using the murine MGU74Av2 Gene Chip (Affymetrix). GENESPRING 5.1 was used to analyze resulting data. Gene lists were generated by comparing raw data values of each cell using one-way analysis of variance ($P < 0.05$ with a Benjamini-Hochberg correction).

Western blot analysis. Proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were treated as recommended by the antibody manufacturer for cleaved Notch1 (Val1744; Cell Signaling Technology). For detection of the specific protein, a sensitive chemiluminescence method was used with an appropriate IgG antibody linked to horseradish peroxidase antibody (Thermo Fisher Scientific).

Sensitization and challenge. The experimental protocol for sensitization and challenge to allergen was described previously (27). Mice were challenged (primary) via the airways with OVA (0.2% in saline) for 20 min on days 14, 15, and 16 using an ultrasonic nebulizer (model NE-U07; Omron Healthcare). On day 30, mice received a single secondary challenge via the airways with 1% OVA for 20 min (OVA/OVA/OVA). Control mice were sensitized and challenged to OVA, and on day 30 they received PBS (OVA/OVA/PBS). On day 32, airway function was measured as described below, followed by collection of samples for further analyses.

Assessment of airway function. Airway function was assessed as described previously by measuring changes in RL in response to increasing doses of inhaled MCh (31). Data are expressed as percent change from baseline RL values obtained after inhalation of saline.

BAL. Immediately after measurement of AHR, lungs were lavaged with HBSS and total leukocyte numbers were analyzed. BAL fluid was collected, and numbers of $CD4^+$ and $CD8^+$ T cells were analyzed by flow cytometry.

Adoptive transfer of GSI-treated T_{EFF} cells and administration of Delta1-Fc. For adoptive transfer, 5×10^6 T_{EFF} cells pretreated with GSI (GSI-T_{EFF} cells) or DMSO (DMSO-T_{EFF} cells) were administered intravenously through the tail vein to OVA primary sensitized/challenged WT and $CD8^{-/-}$ mice 2 wk after the last challenge (day 30). After transfer, the mice were exposed to a single (secondary) allergen challenge via the airways on day 30. All assays were performed on day 32. The nontransferred WT or $CD8^{-/-}$ mice were assigned as controls. In the Delta1-Fc protocol, soluble Delta1-Fc (25, 64) was injected intraperitoneally at a dose of 50 μg each day beginning 4 d before and through the day after secondary challenge in WT mice that had undergone primary sensitization and challenge with OVA. As a control, 50 μg of human IgG was administered in the same manner. 48 h after the single (secondary) allergen challenge, all assays were performed.

Measurement of cytokines. Cytokine levels in the BAL fluid and cell culture supernatants were measured by ELISA as described previously (65). The limits of detection were 4 pg/ml for IL-4, IL-5, and IL-13 and 10 pg/ml for IL-10 and IFN-γ.

Lung cell isolation and flow cytometry. MNCs from the left lung were isolated as described previously using collagenase digestion (66). BAL cells and enriched lung MNCs were labeled with anti-CD3, anti-CD4, and anti-CD8 (BD Biosciences). The number of $CD4^+$ and $CD8^+$ T cells per BAL was derived by multiplying the percentage of stained cells by the total number of BAL cells isolated. For intracellular staining, lung MNCs were stimulated with 5 ng/ml PMA and 500 ng/ml ionomycin (Sigma-Aldrich) for 6 h in the presence of 10 μg/ml brefeldin A (Sigma-Aldrich). After staining for cell surface markers, cells were fixed with 4% paraformaldehyde in PBS, permeabilized in 0.1% saponin, and stained for intracytoplasmic IFN-γ (BD Biosciences). The number of IFN-γ–producing $CD4^+$ or $CD8^+$ T cells per lung was calculated from the percentage of total cells. Stained cells were analyzed on a FACSCalibur (BD Bioscience) using CELLQuest software.

Isolation of GSI-T_{EFF} or DMSO-T_{EFF} cells from the lungs of sensitized and challenged recipients. Lung MNCs were isolated from sensitized and challenged $CD8^{-/-}$ mice that received GSI-T_{EFF} or DMSO-T_{EFF} cells before secondary challenge. $CD8^+$ T cells were positively selected using magnetic beads coated with anti-CD8 antibody (Miltenyi Biotec). RNA was extracted from these isolated $CD8^+$ GSI-T_{EFF} or DMSO-T_{EFF} cells and analyzed by real-time PCR.

Real-Time PCR. Real-time cDNA primers and probes for murine T-bet, GATA-3, Delta1, Jagged1, Jagged2, and GAPDH were obtained from Applied Biosystems. The Delta Delta cycle threshold method was performed for relative quantification of mRNA expression.

Statistical analysis. All results were expressed as the mean ± SEM as a standard method of presentation for this type of data. The Tukey-Kramer test was used for comparisons between multiple groups. Nonparametric analysis using the Mann-Whitney *U* 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.

Online supplemental material. Fig. S1 shows the number of $CD4^+$ and $CD8^+$ T cells in the lungs of WT recipients of DMSO-T_{EFF} or GSI-T_{EFF} cells. Fig. S2 illustrates cytokine production from the lung cells of WT recipients of DMSO-T_{EFF} or GSI-T_{EFF} cells. Fig. S3 illustrates cytokine production from lung cells in the recipients of Delta1-Fc. Fig. S4 shows expression levels of Delta1 on DMSO-T_{EFF} or GSI-T_{EFF} cells cultured together with T_{EFF} cells. Fig. S5 shows IFN-γ production in GSI-T_{EFF} cells. Figs. S1–S5 are available at http://www.jem.org/cgi/content/full/jem.20072200/DC1.

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