

Notch signaling mediates granulocyte-macrophage colony-stimulating factor priming-induced transendothelial migration of human eosinophils

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Abstract

Background: Priming with cytokines such as granulocyte-macrophage colony-stimulating factor (GM-CSF) enhances eosinophil migration and exacerbates the excessive accumulation of eosinophils within the bronchial mucosa of asthmatics. However, mechanisms that drive GM-CSF priming are incompletely understood. Notch signaling is an evolutionarily conserved pathway that regulates cellular processes, including migration, by integrating exogenous and cell-intrinsic cues. This study investigates the hypothesis that the priming-induced enhanced migration of human eosinophils requires the Notch signaling pathway.

Methods: Using pan Notch inhibitors and newly developed human antibodies that specifically neutralize Notch receptor 1 activation, we investigated a role for Notch signaling in GM-CSF-primed transmigration of human blood eosinophils *in vitro* and in the airway accumulation of mouse eosinophils *in vivo*.

Results: Notch receptor 1 was constitutively active in freshly isolated human blood eosinophils, and inhibition of Notch signaling or specific blockade of Notch receptor 1 activation during GM-CSF priming impaired priming-enhanced eosinophil transendothelial migration *in vitro*. Inclusion of Notch signaling inhibitors during priming was associated with diminished ERK phosphorylation, and ERK-MAPK activation was required for GM-CSF priming-induced transmigration. *In vivo* in mice, eosinophil accumulation within allergic airways was impaired following systemic treatment with Notch inhibitor, or adoptive transfer of eosinophils treated *ex vivo* with Notch inhibitor.

Conclusions: These data identify Notch signaling as an intrinsic pathway central to GM-CSF priming-induced eosinophil tissue migration.

A characteristic attribute of some phenotypes of asthma is eosinophil accumulation within the bronchial mucosa, associated with mucus production and tissue remodeling in acute and chronic disease, respectively. Current treatments for tissue eosinophilia are limited to glucocorticosteroids. Therefore, delineating cell-intrinsic pathways that control eosinophil migration into inflamed tissues is a central goal in developing targeted therapeutics.

Concentrations of IL-5 family cytokines (i.e., GM-CSF, IL-5, and IL-3) are increased in asthmatic airways and serum, and prime eosinophils by enhancing their basal migratory capacity (1). Granulocyte-macrophage colony-stimulating factor (GM-CSF) is central to the recruitment *in vitro* and *in vivo* of human

eosinophils (2–6), a phenomenon recapitulated in mouse models (7–10), and the *in vivo* primed phenotype of eosinophils from asthmatics can be induced in eosinophils from healthy donors *in vitro* by incubation with GM-CSF (2, 3). However, molecular mechanisms that drive GM-CSF priming effects on eosinophils are incompletely defined.

Notch signaling is an evolutionarily conserved pathway that regulates cell fate decisions throughout organogenesis and hematopoiesis (11), and aberrant Notch signaling is implicated in tumor pathophysiology (12–14). In the canonical pathway, ligand binding to any of the four mammalian Notch receptors (Notch 1–4) induces sequential α - and γ -secretase-dependent cleavages that release a fragment of the

receptor intracellular domain (NICD) within the cytoplasm. Freed NICD undergoes nuclear translocation and turns on transcription of a number of Notch-responsive genes (11). Inhibition of Notch signaling is most commonly achieved, both in experimental studies and in human therapeutic approaches (i.e., in cancer clinical trials (15, 16)), by blockade of the final cleavage using γ -secretase inhibitors (GSIs), simultaneously inhibiting all four Notch receptors, and producing the pharmacological equivalent of a loss of Notch function.

We previously demonstrated that mature human blood eosinophils express Notch receptors and ligands (17). Here, we build on these studies to investigate a requirement for Notch signaling in GM-CSF priming-induced transendothelial migration of human eosinophils *in vitro* and the bronchoalveolar accumulation of mouse eosinophils in allergic airway inflammation *in vivo*.

Materials and methods

Human eosinophils

Blood eosinophils were isolated from mild allergic or normal donors by negative selection as described (17). Eosinophil purity (as determined by cyto-spin analysis of ≥ 350 cells from random fields) was $>98\%$ and viability $>98\%$. Informed written consent was obtained in accordance with the Declaration of Helsinki, and Institutional Review Board approval was obtained from Beth Israel Deaconess Medical Center.

Transmigration

Human eosinophils were primed for 18 h in complete culture medium alone or supplemented with 10 pM recombinant GM-CSF in the presence of pan Notch signaling inhibitors or Notch receptor 1 neutralizing Abs as described in Data S1. Primed eosinophils were overlaid onto confluent monolayers of resting or preactivated human umbilical vein endothelial cells (HUVECs) grown on transwell inserts. Transmigrated eosinophils collected from lower wells are expressed as follows: (number of migrated eosinophils/total input eosinophils) $\times 100$.

Western blotting

Freshly isolated human eosinophils were lysed and probed with Abs against Notch 1 intracellular domains or activated Notch 1 intracellular domains as detailed in Data S1.

Flow cytometry

As detailed in Data S1, cleaved Notch 1 intracellular domains were detected in saponin-permeabilized human eosinophils. Surface-expressed Notch receptors were detected on nonpermeabilized mouse eosinophils purified from spleens of IL-5 transgenic BALB/c mice as described (18), using Abs against extracellular regions of Notch receptor 1 or Notch receptor 2, or appropriate isotype controls.

ERK phosphorylation

Human eosinophils were incubated with complete cell culture medium alone or containing 100 pM GM-CSF, with or without GSI II, clone A6, or appropriate vehicle or isotype Ab control. At the indicated time points, cell lysates or intact cells were assayed for levels of phosphorylated ERK by multiplex analysis or flow cytometry, respectively, as described in Data S1.

In vivo model of eosinophil recruitment

Wild-type female BALB/c mice (6–8 weeks old) were sensitized intraperitoneally (i.p.) with OVA mixed with alum adjuvant and challenged with aerosolized OVA, as detailed in Data S1. Prior to each aerosolized OVA challenge, mice were treated i.p. with either GSI II or vehicle control. Twenty-four hours after the final airway challenge, BAL fluid was obtained from euthanized mice and assessed for cellular composition, as described in Data S1.

Eosinophil adoptive transfer

Mouse eosinophils were purified from spleens of IL-5 transgenic BALB/c mice as described (18) and treated with GSI II or vehicle control. Treated and control cells were labeled with Dye eFluor 670 or CFSE, respectively. Equal mixtures of labeled GSI or vehicle control-treated eosinophils were injected i.v. into OVA-sensitized and OVA-challenged eosinophil-deficient Δ dblGATA mice immediately before the final OVA aerosol challenge, as detailed in Data S1.

Statistical analysis

Statistical analyses were performed and graphs created with GRAPHPAD PRISM 4 (GraphPad Software, Inc., La Jolla, CA, USA). One-way ANOVA with Bonferroni *post hoc* test was used for selected pairs comparison unless otherwise stated. A *P*-value of <0.05 was considered significant.

Results

γ -secretase activity during GM-CSF priming is required for the priming-induced transendothelial migration of human eosinophils

Our initial strategy to silence Notch signaling was to utilize GSIs. Human blood eosinophils were isolated and primed *ex vivo* with GM-CSF in the presence or absence of GSI II. Following overnight priming, eosinophils were washed and overlaid onto the apical surface of confluent HUVEC monolayers. Granulocyte-macrophage colony-stimulating factor priming enhanced eosinophil transmigration, and inclusion of GSI during priming reversed the priming effect on eosinophil migration (Fig. 1A).

Activated endothelium at the site of inflammation upregulates adhesion molecules that promote leukocyte tethering, adhesion, and diapedesis via interactions with leukocyte-

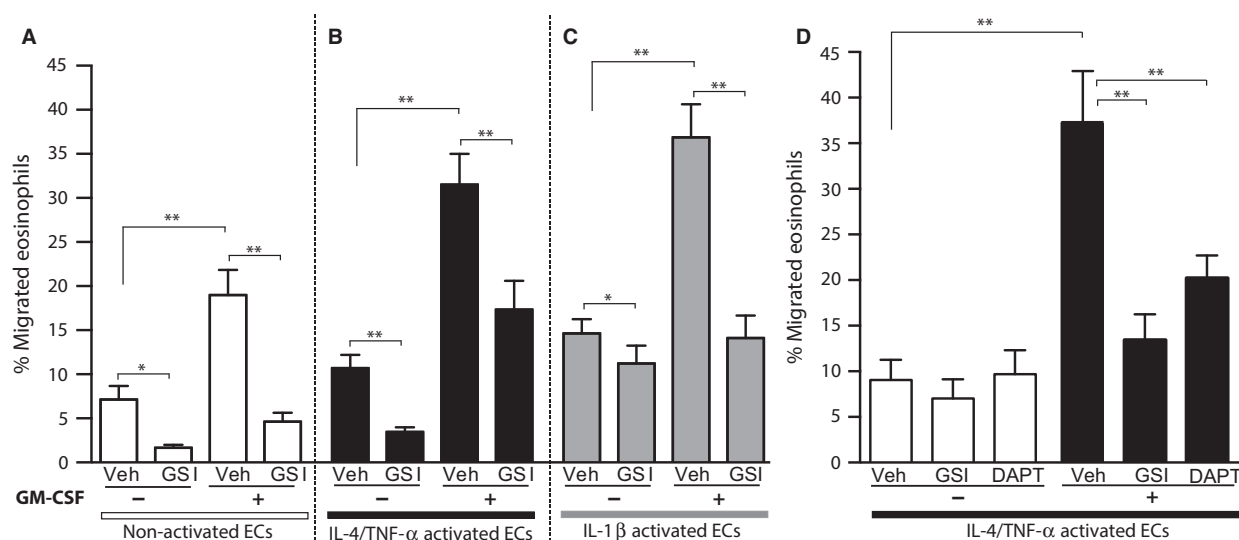


Figure 1 γ -secretase activity is required during granulocyte-macrophage colony-stimulating factor (GM-CSF) priming for enhanced transendothelial migration of human eosinophils. Eosinophils were primed with or without 10 pM GM-CSF in the presence of GSI II, DAPT, or DMSO vehicle control before being overlaid onto nonprimed (A), IL-4/TNF- α (B, D), or IL-1 β (C) activated human umbilical

vein endothelial cell (HUVEC) monolayers. Data are expressed as the combined mean $\pm$ SEM of $N = 7$ independent experiments (A–C) or $N = 5$ independent experiments (D), using different eosinophil donors (* $P < 0.05$; ** $P < 0.01$). EC, endothelial cells; GSI, γ -secretase inhibitor.

expressed integrins. To determine whether Notch-signaling-dependent control of GM-CSF-primed eosinophil transmigration would be overcome by an activated endothelium, eosinophils primed with GM-CSF in the presence or absence of GSI were washed, and layered over endothelial monolayers preactivated with either IL-4 and TNF- α , or IL-1 β . Endothelial cell activation by itself induced eosinophil transmigration above baseline, and the GM-CSF priming effect on eosinophil transendothelial migration remained susceptible to treatment with GSI II (Fig. 1B,C), or another GSI, DAPT (Fig. 1D), in the presence of an activated endothelium. Viability of eosinophils after overnight GM-CSF priming in the presence of vehicle control or GSIs, as measured by exclusion of propidium iodide, was as follows: vehicle = $95 \pm 1\%$; GSI II = $95 \pm 2\%$; and DAPT = $95 \pm 1\%$, suggesting the impaired transmigration of eosinophils primed in the presence of GSI II or DAPT was not caused by differences in eosinophil viability. These data directly implicate γ -secretase activity in GM-CSF priming-induced eosinophil migration.

Notch receptor 1 is constitutively active in isolated human blood eosinophils and mediates GM-CSF priming-induced eosinophil transendothelial migration

Although GSIs are the conventional inhibitors of Notch currently utilized in experimental and clinical settings, approximately 50 cellular substrates exist for γ -secretase in addition to Notch receptors. We previously demonstrated that human blood eosinophils express Notch receptors 1 and 2 (17). Using a combination of antibodies recognizing intracellular epitopes of intact and/or activated Notch 1 (experimental design shown in Fig. 2A), both full-length receptors and cleaved intracellular

domains of activated Notch receptor 1 (NICDs) were detected within freshly isolated blood eosinophils, suggestive of constitutive activation of Notch 1 (Fig. 2B,C). Constitutive expression of cleaved Notch receptor 1 was also detected in eosinophils from whole blood, suggesting Notch 1 activation was not an artifact of eosinophil isolation (Fig. S1).

To determine whether Notch receptor 1 activation contributes to GM-CSF-induced priming effects on eosinophil transmigration, GM-CSF priming was carried out in the presence of two different anti-Notch receptor 1 neutralizing antibodies. Clone A6 has been reported to neutralize Notch receptor 1 activation, presumably by interfering with ligand binding (19). NRR1 antibodies bind an epitope within the negative regulatory region of Notch 1 and inhibit Notch signaling by stabilizing the receptor in a manner that prevents the ligand binding-induced change in structural conformation required to expose the α -secretase-dependent cleavage site (20) (illustrated in Fig. 2D). Consonant with data generated using the pan Notch inhibitors, eosinophil transmigration was inhibited when either clone A6 or NRR1 anti-Notch 1-blocking Abs were present during GM-CSF priming (Fig. 2E,F). Viability of eosinophils after overnight priming with GM-CSF was as follows: IgG2b = $94 \pm 3\%$; clone A6 = $89 \pm 5\%$; IgG1 = $96 \pm 3\%$; and NRR1 = $95 \pm 3\%$, suggesting the impaired transmigration of eosinophils primed in the presence of Notch 1 neutralizing antibodies was not caused by overt differences in eosinophil viability. Antibodies targeting eosinophil cell surface-expressed MHC I had no effect on GM-CSF-primed eosinophil migration, indicating Notch 1 neutralizing antibody-dependent inhibition of eosinophil migration was not caused by a bystander effect of Fc receptor cross-linking (Fig. S2).

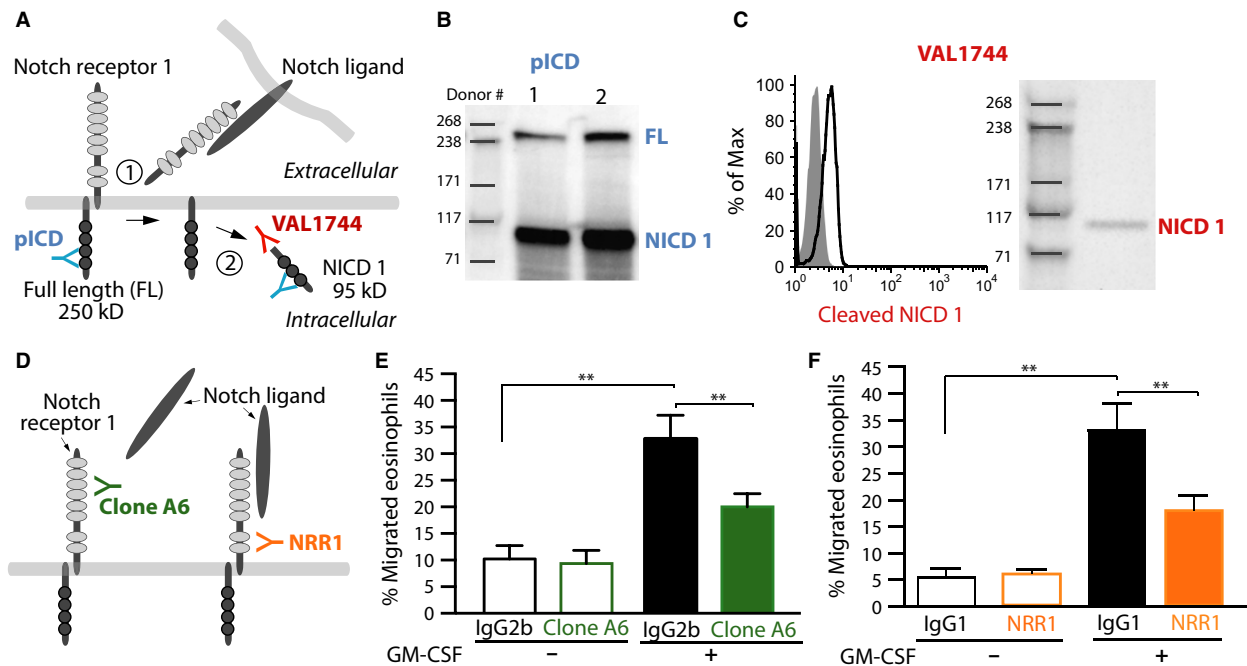


Figure 2 Notch receptor 1 is constitutively active in freshly isolated human blood eosinophils and is required for granulocyte-macrophage colony-stimulating factor (GM-CSF) priming-induced transendothelial migration. (A) Upon binding to its ligand, an α -secretase-dependent cleavage event releases the extracellular portion of Notch receptor 1 (1), exposing a γ -secretase-sensitive cleavage site. Subsequent cleavage by γ -secretase (2) frees a 95-kD Notch intracellular domain (NICD) fragment. A polyclonal antibody recognizing an intracellular epitope of Notch receptor 1 unaffected by receptor activation (pICD) is shown in blue, while VAL1744 antibodies (shown in red) recognize an epitope that is exposed only after γ -secretase-dependent cleavage of activated Notch receptor 1. Lysates were prepared of freshly isolated human eosinophils and probed by Western blot with pICD

(B) or VAL1744 (C, inset). In C, freshly isolated eosinophils were permeabilized and expression of the cleaved NICD assessed by flow cytometry with VAL1744. Shaded histogram = isotype control. Data shown are representative of $N \geq 3$ eosinophil donors. (D) Notch receptor 1 activation was inhibited by blocking ligand binding (Clone A6, green) or by preventing exposure of the α -secretase-sensitive site (NRR1, orange). (E, F) Eosinophils were primed with or without GM-CSF in the presence of clone A6 (E), NRR1 (F), or their respective isotype controls before being overlaid onto IL-4 and TNF- α -activated human umbilical vein endothelial cell (HUVEC) monolayers and assessed for their trans migratory ability. Data shown are the combined mean $\pm$ SEM of $N = 5$ independent experiments, using different eosinophil donors (** $P < 0.01$).

Inhibition of Notch signaling depresses ERK-MAPK signaling

Granulocyte-macrophage colony-stimulating factor priming of eosinophils induces a long-lived response that persists despite receptor desensitization (21). Activation of ERK-MAPK signal transduction pathways are implicated in both early transient events and subsequently in the prolonged effects of GM-CSF priming. Preliminary kinetics experiments using multiplex analysis revealed two peaks (at approximately 5 and 60 min) of GM-CSF-induced ERK phosphorylation. In the presence of GSI II, GM-CSF-induced ERK phosphorylation was diminished at 60 min of stimulation (Fig. 3A). Anti-Notch 1 neutralizing antibodies (clone A6) were included in parallel with some experiments and yielded a similar trend (Fig. 3B). In a complementary approach, intracellular flow cytometry was used to assess ERK phosphorylation on a single cell basis. In support of multiplex analyses, GM-CSF-stimulated eosinophils exhibited a decrease in ERK phosphorylation at 60 min in the presence of either GSI or anti-Notch 1 Abs when compared to vehicle or irrelevant Ab controls, respectively (Fig. 3C,D).

To determine whether diminished ERK phosphorylation in the presence of Notch signaling inhibitors is sufficient to cause the observed defect in transendothelial migration, eosinophils were primed with GM-CSF in the presence of the MEK1/2 inhibitor U0126 or the nonfunctional peptide mimic U0124. Inhibition of MEK1/2 during GM-CSF priming attenuated eosinophil migration, even at suboptimal concentrations (i.e., 2.5 μ M, Fig. 3E). Using the reverse approach, PMA, a robust activator of ERK-MAPK pathways through protein kinase C activation of Raf (22), rescued the GSI-induced phenotype of impaired GM-CSF priming-induced eosinophil transmigration (Fig. 3F).

In vivo administration of GSI attenuates accumulation of eosinophils in allergic airways

Eosinophils isolated from the spleens of naïve IL-5 transgenic mice expressed Notch receptors 1 and 2 (Fig. 4A). Of note, the low surface expression observed for Notch 1 was replicated using NRR1 antibodies (not shown). Upon confirmation that mouse eosinophils expressed Notch receptors, we

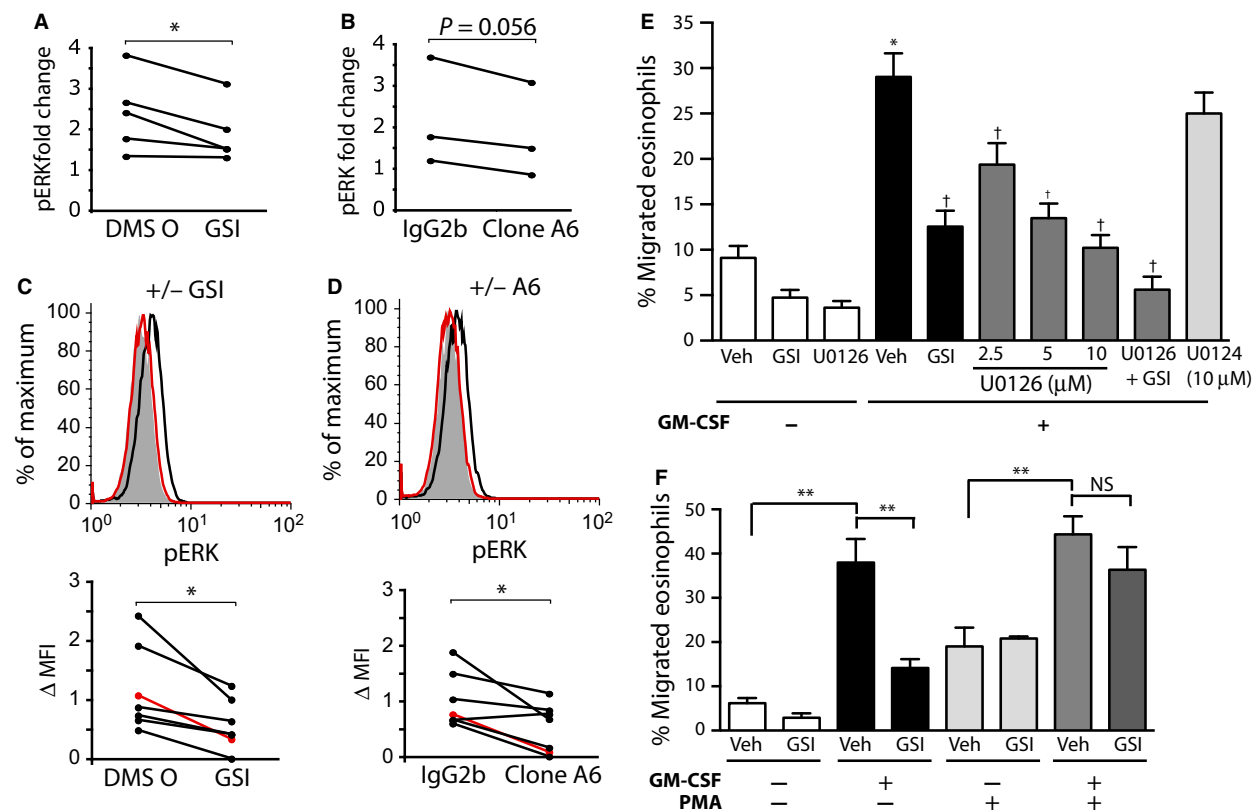


Figure 3 Inhibition of Notch signaling impairs granulocyte-macrophage colony-stimulating factor (GM-CSF)-induced ERK phosphorylation. Eosinophils were cultured in medium alone or containing GM-CSF with γ -secretase inhibitor (GSI), clone A6, or vehicle or isotype control for 60 min. (A, B) ERK phosphorylation was detected from eosinophil lysates by multiplex analysis. Phosphorylated ERK (normalized to total ERK) is expressed as fold change in phospho-ERK expression relative to baseline control cells from the same experiment. Each data point represents an average from duplicate wells, and each line connects vehicle control- and GSI-treated data points from a single donor. In C and D, GM-CSF-induced phosphorylated ERK was detected by intracellular flow cytometry. Upper panels show representative histograms from one representative donor: shaded histogram, nonstimulated eosinophils; solid black line, GM-CSF stimulated in the presence of vehicle or isotype control; red line, GM-CSF stimulated in the presence of GSI (C) or clone A6 (D). Lower panels show data from seven independent experiments, expressed as the delta MFI of phosphorylated ERK over isotype control. Each line connects control- and GSI- or clone A6-treated data points from a single donor. Red lines identify the representative data shown in the respective upper panels. * $P < 0.05$, paired, two-tailed Student's t -test. (E, F) Eosinophils were primed with or without GM-CSF in the presence or absence of GSI or DMSO vehicle control, and +/- MEK inhibitor U0126 ($N = 10$), its inactive analogue control U0124 ($N = 9$) (E), or PMA ($N = 3$) (F). Following priming, eosinophils were overlaid onto endothelial cell monolayers preactivated with IL-4 and TNF- α . Data shown are mean $\pm$ SEM of data from all independent experiments combined (* $P < 0.05$ vs nonstimulated vehicle control; † $P < 0.05$ vs GM-CSF stimulated vehicle control).

investigated a requirement for Notch signaling for eosinophil transmigration *in vivo*. OVA-sensitized mice were exposed to aerosolized OVA as described in Materials and methods and Fig. 4B. This method induces robust eosinophil accumulation within bronchoalveolar lavage fluid (BALF), readily detected by a combination of forward-side scatter characteristics and expression of surface Siglec-F (Fig. 4C). Systemic administration of GSI II 30 min prior to each airway challenge reduced the accumulation of eosinophils within BALF (Fig. 4D). Differences in total BAL cells recovered from GSI- and vehicle-treated mice did not reach statistical significance, and the loss of BAL eosinophils fully accounted for the discrepancy in total numbers of BAL cells recovered, indicating eosinophils were the only or at least the quantitatively dominant cellular

population impacted by GSI treatment (compare Fig. 4D and E). Attenuation of BAL eosinophilia was apparent despite recoveries of statistically similar percentages of bone marrow eosinophils (7.81 ± 1.34 and 8.87 ± 0.18 for control- and GSI-treated mice, respectively, $N = 3$), suggesting the deficiency in BAL eosinophilia was not a consequence of GSI-induced hematopoietic defects.

Ex vivo exposure to GSI impairs the migration of adoptively transferred eosinophils into allergic airways

Notch signaling is implicated in immune polarization of T lymphocytes (23–26). To verify a direct effect of GSI on eosinophil recruitment *in vivo*, distinct from a GSI-depen-

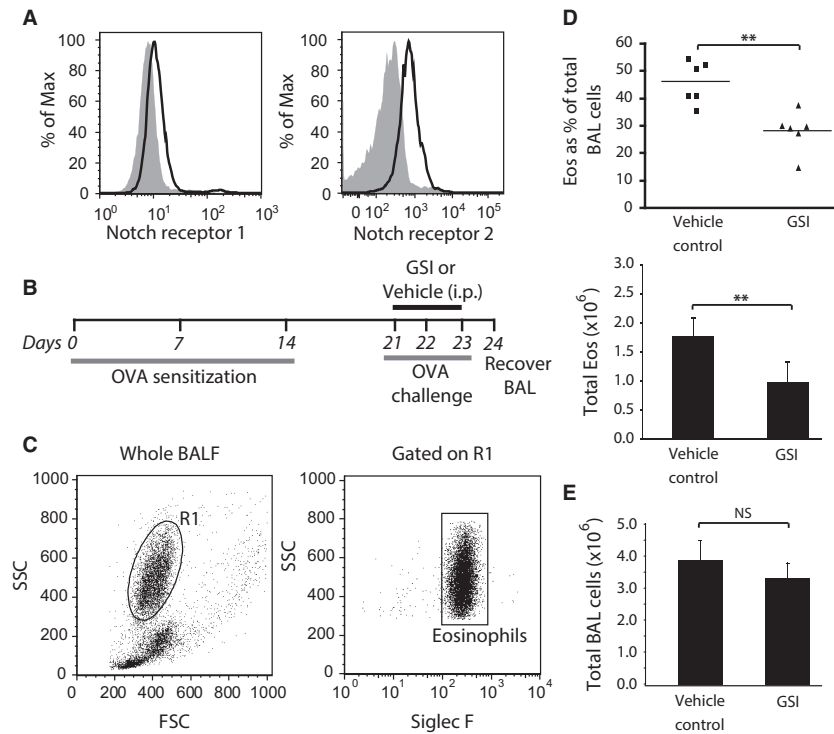


Figure 4 *In vivo* administration of γ -secretase inhibitor (GSI) impairs eosinophil accumulation within allergic airways. (A) Surface expression of Notch receptors 1 and 2 on mouse eosinophils. (B) Experimental design. (C–E) Total BAL cells were counted and stained with PE-labeled rat anti-mouse Siglec-F mAb. BAL eosinophils were gated on their forward and side scatter characteristics

and further identified as Siglec-F⁺/SSC^{high} eosinophils (C). (D) BAL eosinophils were quantified from six mice per group in two independent experiments and expressed as percentage of total BAL cells (top panel), or total cell numbers (bottom panel). (E) Total BAL cells. Data are presented as mean $\pm$ SD. ** P < 0.01 (unpaired two-tailed Student's t -test). Eos, eosinophils; NS, not significant.

dent effect on T-cell-derived eosinophil growth or chemotactic factors, donor eosinophils were treated with GSI or DMSO vehicle control *ex vivo*, mixed together in equal parts, and instilled into eosinophil-deficient recipient mice prior to the final OVA airway challenge (Fig. 5A). Analysis of BALF 24 h after the final OVA airway challenge revealed a nearly threefold reduction in the number of GSI-pretreated donor eosinophils present in the BALF, in comparison with donor eosinophils pretreated with DMSO vehicle control (Fig. 5B).

Discussion

Granulocyte-macrophage colony-stimulating factor priming-enhanced migration of eosinophils plays a key role in the accumulation of eosinophils within inflamed bronchi in allergic asthma. However, cell-intrinsic mechanisms that regulate priming-induced eosinophil migration are poorly defined. Here, we demonstrate that Notch signaling, an evolutionarily conserved pathway that integrates intrinsic and extrinsic cues to direct pleiotropic cellular functions, is required for GM-CSF-primed eosinophil transendothelial migration and airways accumulation in allergic airway disease. Inclusion of GSIs during GM-CSF priming inhibited eosinophil migration across nonactivated or cytokine-acti-

vated endothelial monolayers, directly implicating γ -secretase activity in GM-CSF priming-induced eosinophil transendothelial migration. Freshly isolated human blood eosinophils constitutively express the cleaved fragment of Notch receptor 1, indicating that Notch 1 is constitutively activated in circulating eosinophils. It is unknown how constitutive Notch 1 activation is maintained in circulating eosinophils, but may be due to interactions with Notch ligands expressed on vascular endothelium, myeloid cells, or serum-derived Notch ligand-expressing exosomes. Eosinophils exposed during GM-CSF priming to antibodies that inhibit ligand binding or α -secretase cleavage of Notch 1 exhibited impaired transendothelial migration, implicating Notch signaling, specifically through surface-expressed Notch 1, in the GM-CSF priming effects on transendothelial migration of human eosinophils. Further studies are needed to determine whether or not signaling through Notch receptor 2 might also contribute to GM-CSF-primed eosinophil transmigration and whether combined inhibition of Notch receptors 1 and 2 might be necessary to fully eradicate the priming effects of GM-CSF.

Inhibition of Notch signaling was associated with diminished ERK phosphorylation measured at 60 min of GM-CSF stimulation. ERK-MAPK pathway activation was required for GM-CSF-primed eosinophil transmigration, and partial

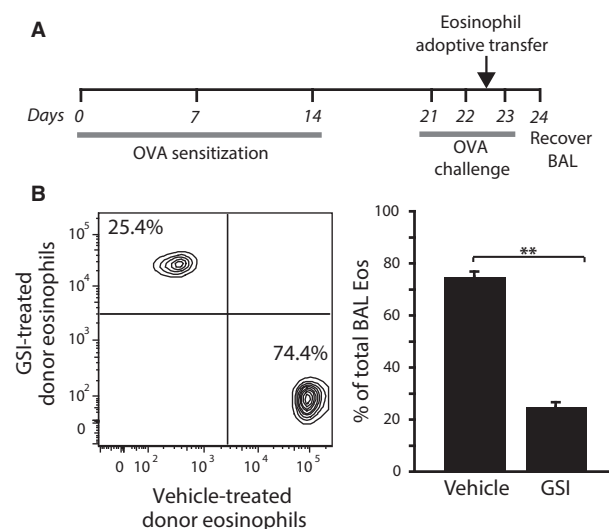


Figure 5 *Ex vivo* treatment with γ -secretase inhibitor (GSI) impairs eosinophil accumulation within allergic airways. (A) Experimental design. (B, left panel) Representative FACS plot is shown of BAL eosinophils recovered from recipient mice 24 h after final airway challenge. Numbers above boxes indicate percentage of labeled eosinophils out of the total BAL eosinophils. (B, right panel) Data from three mice are combined and expressed as mean $\pm$ SD. $**P < 0.002$, Student's two-tailed *t*-test. Eos, eosinophils.

inhibition of MEK phosphorylation was sufficient to impair migration. Moreover, PMA, a strong stimulus for PKC-dependent activation of ERK-MAPK signaling at the level of Raf (thus bypassing Ras) (22), rescued transmigration in GSI-treated eosinophils. Taken together, these data suggest one mechanism whereby Notch signaling may promote GM-CSF-primed transmigration is through enhancing Ras-Raf-MEK-ERK-MAPK signaling and suggest the nodal point between ERK and Notch pathways may be upstream of Raf. Complementary to our findings, Chung et al. found eosinophils differentiated from cord blood progenitors in the presence of GSI to be unable to phosphorylate ERK upon subsequent exposure to the chemokine eotaxin (27), although in that system, eosinophils derived in the presence of GSI exhibited higher basal levels of ERK phosphorylation than their vehicle-treated controls (28). Notch and ERK-MAPK signaling pathways are frequently interdependent throughout development (29). In *Drosophila*, the majority of Ras-responsive genes are also targets of Notch, and Notch directly regulates the transcription of genes encoding proteins of the Ras pathway, and vice versa (30). Noncanonical, cytoplasmic functions for NICDs independent of nuclear translocation are also implicated in Notch-Ras pathway interactions (31, 32). However, it is also plausible that Notch signaling impacts ERK phosphorylation of GM-CSF-primed eosinophils indirectly through modulating another GM-CSF-induced, intermediary step.

To investigate the physiological significance of Notch signaling to eosinophil recruitment *in vivo*, we utilized a standard mouse model of allergic airway inflammation. Our data

show, to our knowledge for the first time, that mouse eosinophils express Notch receptors 1 and 2. Our data further demonstrate impaired recruitment of mouse eosinophils into allergic lungs following whole-body administration of GSI II. Notch signaling has been proposed as a therapeutic target for asthma, and in a mouse model of allergic airway inflammation, intranasally administered GSI attenuated pathological outcomes, including levels of serum IgE and airway hyper-responsiveness (33). In that study, the authors attributed improved clinical outcomes to known effects of Notch signaling on CD4⁺ T-cell differentiation (23–26), while acknowledging Notch-dependent effects on additional cell types could not be excluded. Data presented here would suggest eosinophil recruitment may also have been directly impacted and may have contributed to the attenuation of symptoms in those studies. Importantly, failure of GSI-treated eosinophils adoptively transferred into eosinophil-deficient recipients to migrate into inflamed lungs (Fig. 5) establishes the direct effect of γ -secretase activity on eosinophil recruitment and confirms the biological significance of Notch signaling specifically by mature eosinophils in airway disease.

These data suggest targeting Notch signaling pathways may provide a multipronged approach to asthma therapy. Further studies designed to elucidate specific Notch receptor–ligand pairs and cell-intrinsic molecular mechanisms that differentially regulate Notch signaling outcomes in eosinophils and T cells may provide customizable strategies that manipulate clinical outcomes and limit potential for collateral effects. Moreover, with the growing interest in GSIs for the treatment of cancers and other diseases (12–14), these data are also more broadly relevant, as they anticipate functional consequences of such treatments on eosinophils that might directly or indirectly impact prognoses.

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Author contributions

L.L., H.W., and L.A.S. designed the experiments and analyzed data; L.L., H.W., and J.J.X. performed the experiments; and L.L., H.W., and L.A.S. wrote the manuscript.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Supplemental materials and methods.

Figure S1. NICD detection within eosinophils from unfractionated whole blood indicates NICD detection in isolated eosinophils is not due to Notch 1 activation through the process of eosinophil purification.

Figure S2. Antibodies targeting eosinophil surface-expressed MHC I or CD4 had no impact on GM-CSF-primed eosinophil migration.

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Data Supplement 1

Transmigration

Eosinophils were cultured for 18 h at 2×10^6 cells/ml in complete culture medium (RPMI-1640 containing 10% fetal bovine serum and 1% penicillin/streptomycin) alone or with 10 pM recombinant GM-CSF (R&D Systems, Minneapolis, MN) in the presence of γ -secretase inhibitor-II (GSI II, 15 μ M, Calbiochem, San Diego, CA), -IX (DAPT, 10 μ M, Calbiochem), or DMSO vehicle control, or anti-Notch 1 Abs Clone A6 (Thermo Scientific, Fremont, CA), NRR1 (kind gift of Genentech), or matching isotype controls (10 μ g/ml). Eosinophils were washed before overlaying (0.2×10^6 eosinophils) onto confluent monolayers of human umbilical vein endothelial cells (HUVEC, Lonza, Walkersville, MD), grown on 24-well transwell inserts (polycarbonate, 5.0 μ m pore, Corning Incorporated, Corning, NY). In some wells, HUVEC monolayers were pre-activated overnight with IL-4/TNF- α (100 pM each) or for 6 h with IL-1 β (100 pM), and rinsed before addition of eosinophils. Transmigration occurred for 2 h at 37 $^{\circ}$ C, 5% CO $_2$, and migrated cells in the lower compartments were collected and counted by flow cytometry. Transmigration is expressed as: (number of migrated eosinophils/total input eosinophils) x 100.

Western blotting

Freshly isolated eosinophils were lysed (25×10^6 /ml) in Radio Immuno Precipitation Assay (RIPA) Buffer (Boston Bio Products) with protease inhibitor cocktail (1:1000, Sigma Aldrich). Denatured samples were run on 4-15% Mini-protean TGX precast gels, transferred to polyvinylidene difluoride membranes, and blocked for 1 hour with 5%

milk/TBST before probing with 1 µg/ml human Notch-1 intracellular domain antibodies (Abs) (polyclonal sheep IgG, R&D Systems, Minneapolis, MN) or 2 µg/ml rabbit polyclonal Ab to cleaved Notch1 (VAL1744, Abcam) overnight at 4°C, followed by anti-sheep IgG HRP-conjugated Ab (1:1000, R&D Systems) or anti-rabbit IgG HRP-conjugated Ab (1:1000, Cell Signaling Technology, Danvers, MA). Membranes were developed with Super Signal West Femto substrate (Thermo Scientific, Rockford, IL) per the manufacturer's instructions.

Flow Cytometry

Freshly isolated eosinophils were fixed with 2% paraformaldehyde for 5 min at RT, and permeabilized with 0.1% saponin for 5 min at 4°C, before incubation with VAL1744 or isotype control, and secondary Ab (Alexa 488 conjugated goat anti-rabbit) in the continued presence of saponin. For analysis of surface-expressed Notch receptors, mouse eosinophils were stained without permeabilization using Abs against extracellular regions of Notch receptor 1 (clone 22E5.5, BD Biosciences) or Notch receptor 2 (clone HMN2-35, Biolegend), or appropriate isotype controls. Data was acquired using a FACSCan, and analyses performed using CellQuest (BD Biosciences) or FlowJo (Ashland, OR) software.

ERK phosphorylation

Eosinophils were incubated with complete cell culture medium alone or containing 100 pM GM-CSF, with or without 15 µM GSI, 10 µg/ml clone A6, or appropriate vehicle or isotype Ab control. At the indicated time points, cell lysates or intact cells were assayed

for levels of phosphorylated ERK by multiplex analysis or flow cytometry, respectively. For multiplex analysis, lysates (15×10^6 cells/ml) were prepared with buffer containing 1% Triton X-100, 0.5% CTAB, 10 mM Tris-base, 100 mM NaCl, 1 mM EGTA, 10% glycerol, phosphatase inhibitor cocktails 2 and 3 (1:100, Sigma-Aldrich), and Complete Mini protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany). Total and phosphorylated ERK1/2 were detected by Luminex assay using the Milliplex MAP 2-plex total/phospho ERK kit (EMD Millipore Corp, St. Charles, MI) following manufacturer's guidelines. Data were normalized to total ERK1/2 and expressed as fold changes of the median fluorescence intensity (MFI, GM-CSF treated vs. medium alone). For flow cytometry, cells were fixed with 2% PFO, permeabilized with ice cold 90% methanol, and stained with Alexa488-conjugated pERK1/2 Abs (pT202/pY204, BD Biosciences, San Jose, CA) or mouse IgG1 isotype control Ab.

In vivo model of eosinophil recruitment. Wild-type female BALB/c mice (6-8 weeks old) were sensitized intraperitoneally (i.p.) with 20 μ g OVA (grade VI, Sigma-Aldrich) mixed with 1 mg adjuvant Inject Alum (Thermo Scientific) in 0.2 ml PBS on days 0, 7, and 14. On days 21, 22, and 23 sensitized mice were i.p. injected with 10 μ g GSI in 0.1 ml PBS or 0.1 ml DMSO/PBS as a vehicle control before receiving aerosolized OVA challenges (5% OVA in PBS) for 30 min delivered by a DeVilbiss 646 nebulizer. Twenty-four hours after the final airway challenge, mice were sacrificed by CO₂ inhalation, and lungs lavaged 6 times with 1 ml each PBS containing 1% FBS to obtain BAL fluid (BALF).

Eosinophil adoptive transfer. Splenic eosinophils from naïve IL-5 transgenic mice were treated with GSI (20 μ M) or DMSO in RPMI 1640 medium at 37⁰C for 1 h. GSI-treated eosinophils were stained with Dye eFluor 670 (5 μ M, eBioscience), and DMSO-treated eosinophils stained with CFSE (5 μ M, Invitrogen) for 10 min in PBS at 37⁰ C, and then washed three times with PBS. Equal mixtures of 20 x 10⁶ GSI-eFluor 670 and 20 x 10⁶ DMSO-CFSE eosinophils (total 40 x 10⁶ cells in 200 μ l PBS) were i.v. (tail vein) injected into OVA-sensitized and –challenged eosinophil-deficient Δ dblGATA mice immediately before the final OVA-aerosol challenge. Twenty-four hours after eosinophil transfer, BALF was collected and fluorescently labeled donor eosinophils were detected using an LSR II (BD Biosciences).

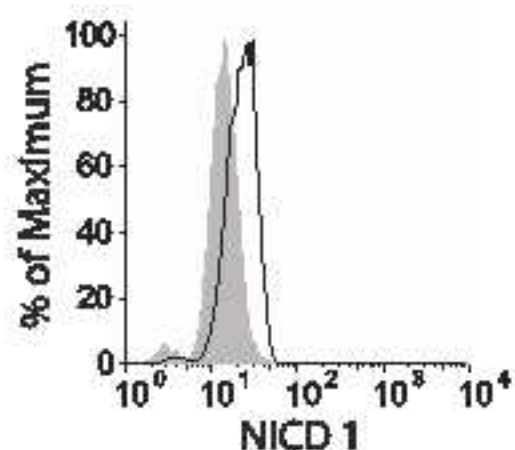


Figure S1. NICD Detection within eosinophils from unfractionated whole blood indicates NICD detection in isolated eosinophils is not due to Notch 1 activation through the process of eosinophil purification. Gray shaded, Isotype control, black line, NICD 1.

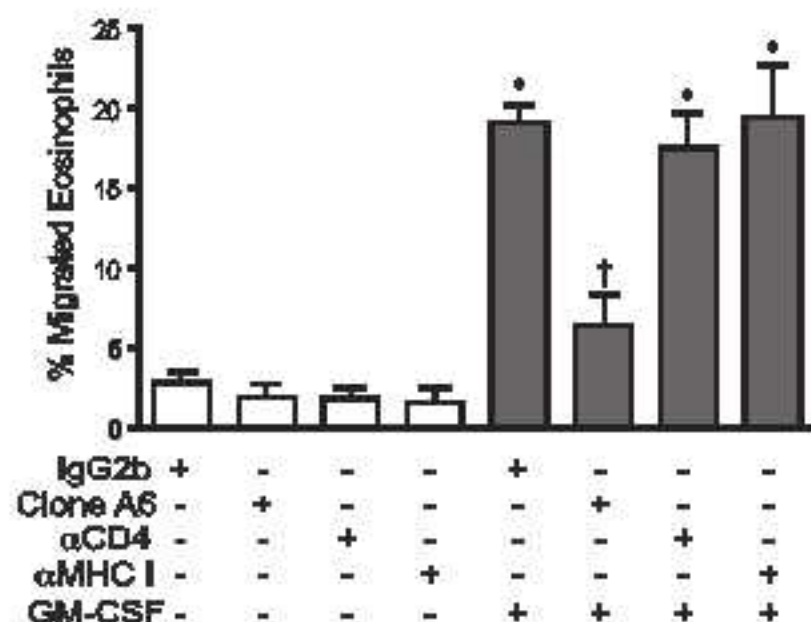


Figure S2. Antibodies targeting eosinophil surface-expressed MHC I or CD4 had no impact on GM-CSF-primed eosinophil migration.

* $p < 0.01$ compared to non-primed; † $p < 0.01$ compared to other treatments of GM-CSF primed cells

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