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Computational and Experimental Analysis Reveals a Requirement for Eosinophil-Derived IL-13 for the Development of Allergic Airway Responses in C57BL/6 Mice

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Eosinophils are found in the lungs of humans with allergic asthma, as well as in the lungs of animals in models of this disease. Increasing evidence suggests that these cells are integral to the development of allergic asthma in C57BL/6 mice. However, the specific function of eosinophils that is required for this event is not known. In this study, we experimentally validate a dynamic computational model and perform follow-up experimental observations to determine the mechanism of eosinophil modulation of T cell recruitment to the lung during development of allergic asthma. We find that eosinophils deficient in IL-13 were unable to rescue airway hyperresponsiveness, T cell recruitment to the lungs, and Th2 cytokine/chemokine production in Δ dblGATA eosinophil-deficient mice, even if Th2 cells were present. However, eosinophil-derived IL-13 alone was unable to rescue allergic asthma responses in the absence of competence of other IL-13-producing cells. We further computationally investigate the role of other cell types in the production of IL-13, which led to the various predictions including early and late pulses of IL-13 during airway hyperresponsiveness. These experiments suggest that eosinophils and T cells have an interdependent relationship, centered on IL-13, which regulates T cell recruitment to the lung and development of allergic asthma. *The Journal of Immunology*, 2011, 186: 000–000.

Allergic asthma is a disease of the lungs and airways that results from overactive immune responses to benign environmental stimuli and is characterized by airway hyperresponsiveness (AHR), wheezing, mucus hypersecretion in

the lungs, and eosinophilia of lungs and airways. Increasing evidence suggest that eosinophils play a major role in the development of allergic asthma, although this conclusion is not without controversy. However, recent reports suggest that exacerbations in a subset of asthmatics can be significantly reduced by treatment with an Ab against IL-5, which affects eosinophils (1, 2). Using murine models of allergic airway inflammation, we and others have recently shown that in C57BL/6 (but not BALB/c) mice, eosinophils modulate chemokine and/or cytokine production in the lung, leading to T cell recruitment (3, 4). This work showed that eosinophils act early in the late phase of allergic airway inflammation by facilitating the recruitment of T cells into the lung (3, 4). However, the mechanism by which these cells modulate this interaction with T cells in the sensitive mouse background is not clear.

Eosinophils can produce a large number of cytokines that have the potential to influence the production of chemokines and subsequent recruitment of T cells to the lung (5). Of these cytokines, knockout mouse models and Ab ablation studies have shown that IL-13 is critical for the development of symptoms of allergic asthma, including AHR, T cell recruitment to the lung, and eosinophilic infiltration of the lung parenchyma (6). Administration of this cytokine alone to the airways of naive mice is sufficient to induce AHR and goblet cell hyperplasia, and severe disease results when administered along with Ag (6). However, these studies do not shed light on temporal needs for this cytokine or which cell type may be critical for the production of IL-13. Differentiated Th2 cells are able to produce large amounts of IL-13 in addition to other cytokines and are considered to be the main purveyors of disease in the pathogenesis of asthma (6, 7). In some animal models, mast cell-derived IL-13 has been shown to be important in the early phase of an allergic asthma response (8), but these cells are not required for induction of a late-phase allergic response (9).

We have synthesized a network of acute allergic responses based on available data from the literature in C57BL/6 and BALB/c

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Abbreviations used in this article: AHR, airway hyperresponsiveness; AL, allergen; Alum, aluminum hydroxide; BAS, basophil; EAL, Ag-IgE; EB, eosinophils in the blood; EBM, eosinophil progenitor cells in bone marrow; EC, epithelial cell; EL, eosinophil resident in the lungs; EL2, eosinophil recruited in the lung; ELA, eosinophils resident in the lungs interact with allergen complex; EO, eotaxin; Eos^{-/-}, Δ dblGATA eosinophil-deficient mice; Eot-1, eotaxin-1; His, histamine; IgEL, IgE transported to the lung; i.n., intranasal; L, lung; LN, lymph node; MC, mast cell; mDC, mature dendritic cell; mDCLN, mature dendritic cell traffic to the lymph node; NKTL, NKT cell transported to the lungs; PAS, periodic acid-Schiff; PIC, proinflammatory cytokine; QRT-PCR, quantitative RT-PCR; TSLP, thymic stromal lymphoprotein; TSLPLN, thymic stromal lymphoprotein that also drains to the draining lymph nodes; WT, wild-type.

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mice and from our own experiments in C57BL/6 mice. The network was subsequently translated into a predictive discrete dynamic computational model to analyze the development of allergic asthma in C57BL/6 mice. We use this computational model, along with murine genetic models dependent on eosinophils for this event, to examine the cell-specific requirements for IL-13 in the generation of allergic asthma. We find that eosinophil production of IL-13 is integral to the development of allergic asthma. However, eosinophil production of IL-13 cannot sustain this response in the absence of T cell (or other cell type)-derived IL-13 to mobilize mass recruitment of inflammatory cells. Thus, IL-13 secretion by eosinophils early in the late phase of allergic asthma is able to facilitate the large-scale infiltration of Th2 T cells into the lung and propagation of the disease.

Materials and Methods

Computational model

We started by synthesizing the available data from the literature on experiments performed in C57BL/6 and BALB/c mice, as well as from our own experiments in C57BL/6 mice (Table I) into an interaction network (Fig. 1) (10). Allergen and the components of the immune system were represented as network nodes, and interactions, regulatory relationships, and transformations among components were represented as directed edges starting from the source (regulator) node and ending in the target node. We incorporated regulatory relationships that modulate a process (or an unspecified mediator of a process) as edges directed toward another edge. The regulatory effect of each edge was classified into activation or inhibition and was represented by an incoming arrow or an incoming blunt segment, respectively (Fig. 1). Dotted lined arrows indicate those observations that differ between C57BL/6 mice and BALB/c mice. As all of our experiments in this report were performed on C57BL/6 mice, we limited our simulations to take into account observations using that strain of mice; however, the model can be adjusted to simulate experiments in BALB/c mice by altering specific parameters.

The initial stimulus to the network is allergen (AL), and the response of the network is measured by airway hyperreactivity and inflammation. Because the trafficking of immune cells and cytokines from lymph node to the lungs is critical in this, we distinguish between certain immune components based on their localization in the lymph node or lungs and indicate them by rectangle and oval, respectively, in the network. Moreover, to represent the development of eosinophils, we introduce a bone marrow compartment depicted by diamonds in the network. In the text, we represent the nodes in different compartments with suffix L (lung), lymph node (LN), and bone marrow. Source nodes are depicted by triangles. AL activates airway epithelial cells (EC), leading to the production of proinflammatory cytokines (PIC) and thymic stromal lymphoprotein (TSLP). Dendritic cells mature (mDC) upon exposure to PIC and traffic to the LN (mDCLN). Under the influence of TSLP that also drains to the draining LN (TSLPLN), DCs induce the activation and differentiation of native T cell (T0) into Th2 cells in the lymph nodes. Th2 cells influence the activation of B cells, leading to their proliferation and class switching to produce IgE Abs under the influence of IL-4 produced by Th2 cells. IgE is then transported to the lungs (IgEL), where it interacts with AL to form the Ag-IgE (EAL) complex. Mast cells (MC) in the lungs are activated by allergen, and degranulate, releasing histamine (His). We note that a role of MC in allergic airway inflammation is dependent on the mouse model being used; however, a role for these cells in early AHR has been demonstrated (11). To dissect the role of IL-13 produced by different cell types, we indicated IL-13 nodes with a suffix. Although controversial, basophils (BAS) have recently been suggested to play a role in the development of Th2 responses, and we include these cells in the model as they are responsive to IgE and AL and can secrete cytokines such as IL-4 and IL-13 (IL13b) (12–15). In addition, these cells can be activated by allergen, and degranulate, releasing His. We also included in the model alveolar macrophages (Macrophages) that can respond to Th2-derived IL-13 (denoted as IL13II), as well as secrete IL-13 (IL13m) (reviewed in Ref. 16). We model the response of these cells to IL-13 as inhibitable by high levels of IFN- γ (16–18).

Th2 cells can be transported to the lungs under the influence of chemokines, represented in the model by eotaxin (EO), becoming Th2L. IL-5 and IL-13II are produced by Th2 cells in LN, whereas EO is activated by IL-13I and IL-13II. Note that we have not designated a specific cellular source for this chemokine in the model, as it could originate from airway EC as well as alveolar macrophages (19–22). When in the lungs, Th2 cells produce IL-

5 (denoted as IL5L) and IL-13II, the latter of which can activate AHR. NKT cells can also be transported to the lungs during allergic airway inflammation, becoming NKTL. IL-4, IL-13, and IFN- γ are produced by NKT cells in the lungs (denoted as IL4L, IL13n, and IFNg) (23).

Eosinophils are represented by four nodes: eosinophils resident in the lungs (EL), eosinophil progenitor cells in bone marrow (EBM), mature eosinophils in the blood (EB), and eosinophils recruited in the lungs (EL2). EL2 have enhanced survival in presence of IL-5L produced by Th2 cells recruited to the lung (24) [note that the role of IL-5 in the development of allergic airway inflammation also differs dependent on the strain used, with requirement demonstrated using C57BL/6 mice and lack of a requirement on a BALB/c background (25, 26). This might be related to the difference in eosinophil requirements for allergic airway response that we have observed between these two strains (3)]. EL and EBM are always active, EB are activated by IL-5, and EL2 are recruited to the lung by the presence of EB and EO. When EL interacts with AL complex, they become active (ELa) and release IL-13 (denoted as IL13I). EL2 can also produce IL13I. The assumption is that IL-13 is produced by resident eosinophils or by the recruited eosinophils upon interaction with AL. As eosinophils have abundant preformed message for and spontaneously secrete IL-13 (Supplemental Fig. 1) (27, 28), we included this function in the model. Finally, inflammation is one of the output nodes that is activated by MC, macrophages, eosinophils, and Th2 cells. There are two independent paths of unequal length between Th2 cells in LN (a source node) and EO (a target node), one running through IL13I and the other through IL13II. The MC-mediated pathway is connected to the rest of the network through IgE Abs.

Given the scarcity of kinetic and quantitative characterization of the immune processes involved in the allergic asthma response, we employed a discrete dynamic modeling approach to simulate the development of allergic asthma (29). In this approach, the network's nodes were assumed to have two qualitative states: 0 (off) and 1 (on), corresponding to a baseline (subthreshold) and high (above-threshold) concentration or activity, respectively. We initiated the simulations with the AL in the ON state; additionally, basal levels of nonactivated airway eosinophils, EBM, and naive T cells (T0) were also in the ON state at the initiation of the simulations (nodes represented with triangles in Fig. 1). The state change of each node was described by a Boolean transfer function F (Table I) that depends on the state of the nodes connected to the node by directed edges and potentially on its own state. The iteration of these transfer functions determines the evolution of the state of the nodes over time. The transfer functions were developed from the knowledge of the nodes directly upstream of each target node in the network, augmented with dynamic information from the literature and basic immunology when available. The state of target nodes having a single activator and no inhibitors follows the state of the activator with a delay. Often the target node is regulated by more than one pathway. We used the AND operator whenever synergy between two (or more) nodes is absolutely necessary to activate the target node. When either of the nodes connected to the target node could activate it, the OR operator was used. For inhibition, we used the AND NOT operator, requiring a low level or inactivity for the inhibitor in order for the activation of the target node. Table I lists the transfer functions of each node and a detailed justification of each transfer function.

We used random asynchronous update in which the time scales of each regulatory process are randomly chosen (30, 31). Time is quantized into regular intervals (time steps). The asynchronous method entails updating the nodes in a randomly selected order during each time step and interprets the time step as the longest duration required for a node to respond to a change in the state of its regulator(s) (also called a round of update) (10). In the asynchronous algorithm, the Boolean updating rules were written as $X_i^{t_c} = F_i(X_a^{t_a}, X_b^{t_b}, X_c^{t_c}, \dots)$, in which F_i is the Boolean transfer function, and t_a, t_b, t_c are the time points corresponding to the last change in the state of the input nodes a, b, c and could be either in a previous or the current time step. The results of the simulations are measured in terms of activity, which described the frequency of node activation as a function of time steps. The frequency is calculated by running the simulations for 1000 times for each time step and averaging the activity of the node for a given time step. Thus, 100% (0%) activation indicates that the node is active (inactive) irrespective of the order of update.

We extended the general Boolean framework to incorporate known quantitative information by introducing decay rates for His, NKTL activation for cytokine production, and ELa nodes. The decay time indicates the number of time steps after which the node is switched off even if the upstream nodes that activate the node are on. His activity is observed immediately in response to AL and subsides after an initial burst. A decay time of one time step correctly models the early burst of His. We also introduced a decay time of one time step for ELa, NKT, and NKTL, representing the known transient activation of NKT cells for cytokine production (32).

Simulations

We simulated the effects of knockouts or additions by changing the affected nodes as described in Table II. The code for the computational model was written in Python 2.5 using the software library BooleanNet (10, 29), available at <http://code.google.com/p/booleannet/>. Simulation results were collected and plotted using GraphPad Prism (GraphPad).

Mice

Wild-type (WT) C57BL/6, OT-II OVA-specific TCR transgenic mice (33) (The Jackson Laboratory, Bar Harbor, ME), Δ dblGATA eosinophil-deficient mice ($Eos^{-/-}$; backcrossed to the C57BL/6 background for seven generations, kind gift of Drs. Stuart Orkin, Craig Gerard and Allison Humbles, Harvard Medical School) (34), C57BL/6 IL-5 transgenic mice (35) (kind gift of Drs. Jamie and Nancy Lee, Mayo Clinic, Scottsdale, AZ), C57BL/6 IL-13 $^{-/-}$ (kind gift of Dr. Alfred Bothwell, Yale School of Medicine, New Haven, CT), and C57BL/6 IL-13 $^{-/-}$ /IL-5 transgenic mice were kept in microisolator cages in the animal facilities at The Pennsylvania State University. They were provided with food and water ad libitum. All experiments were approved by the Office of Research Protection's Institutional Animal Care and Use Committee at The Pennsylvania State University.

OVA-induced allergic asthma model

Groups of mice (WT, Δ dblGATA, or IL-13 $^{-/-}$) were injected i.p. on day 0 and day 5 with 50 μ g/ml OVA complexed with aluminum hydroxide (10 μ g OVA/1 mg Alum; Pierce). Mice were then daily exposed intranasally (i.n.) with OVA (2 mg/ml, 40 μ g total) on days 12–15 and sacrificed 24 h later for analysis as described (3). In the indicated experiments, mice received 1.5×10^6 eosinophils i.v. on day 11 or 12 (purity >90%) and were then challenged with OVA on day 12 or at least 6 h after eosinophil transfer. Note that transferring T cells from the IL-5 transgenic mice does not rescue airway inflammation of AHR, indicating that any potential contaminating IL-5-producing T cells are not responsible for the observed results (3). Although it is possible that eosinophils purified from the IL-5 transgenic mice may behave differently from those in mice not carrying this transgene, during allergic inflammation, eosinophils are similarly exposed to IL-5 and should behave in a similar fashion because Clark et al. (36) have found little difference between blood or tissue eosinophils isolated from WT mice that have developed allergic airway inflammation and IL-5 transgenic mice under the same conditions with regard to activation status at rest or in piecemeal degranulation. The main difference observed was increased piecemeal degranulation in the airways observed in the IL-5 transgenic mice after exposure to AL. Because we purify our eosinophils from the peritoneum of IL-5 transgenic mice and transfer them into non-IL-5 transgenic backgrounds, we expect that they behave similar to eosinophils from a WT background, because in the lungs, they will be in a similar environment. We also note that mere transfer of eosinophils does not rescue allergic airway inflammation in Δ dblGATA mice (3). WT and Δ dblGATA mice primed with OVA/Alum and i.n. challenged with PBS were used as controls in these experiments, and we note that we have previously reported experiments in which mice were challenged with PBS instead of OVA and found that there was no induction of allergic inflammation (3).

Determination of AHR and analysis of airway inflammation

AHR was determined by mechanical ventilation (37) using a Flexivent system (Scireq, Montreal, Canada). To determine airway inflammation, mice were euthanized and lungs collected, fixed in paraformaldehyde, sectioned, and stained with H&E to detect cellular infiltration or periodic acid-Schiff (PAS) to detect mucous (performed by the Animal Diagnostic Laboratories, The Pennsylvania State University).

Determination of T cell recruitment into the lungs and bronchoalveolar lavage fluid

Bronchoalveolar lavage fluid (BALF) was collected from lungs of mice in PBS and analyzed on an Advia Blood Analyzer (Bayer, Wayne, NJ) for cell differentials, or stained with Abs against CD4 (BD Pharmingen) and CCR3 (R&D Systems) and used along with FSC and SSC for detection of T cells and eosinophils. In other mice, whole lungs were collected, digested with Liberase (Roche) in HBSS with Ca^{++} and Mg^{++} , and either enumerated on an Advia Blood Analyzer or hemocytometer. Cells were then stained for flow cytometry on a FC500 Bench Top Cytometer (Beckman-Coulter, Miami, FL) or collected for RNA analysis. Flow cytometry data were analyzed using WinMDI software (Scripps Research Institute).

In vitro differentiation of Th2 OT-II T cells and transfer to Δ dblGATA or WT mice

OT-II lymph node cells were cultured in RPMI complete medium with IL-4, anti-IFN- γ , and 10 μ g/ml OVA for 5 to 6 d. A total of 10×10^6 differentiated T cells were injected i.v. into naive WT or Δ dblGATA mice in PBS. In some experiments, Δ dblGATA mice received OT-II cells and either WT or IL-13 $^{-/-}$ eosinophils as well. Mice were challenged i.n. with OVA and analyzed for AHR as previously described.

In vivo eosinophil migration assay

IL-5 transgenic eosinophils were CFSE labeled and resuspended in PBS. A total of 10×10^6 cells were injected i.v. into Δ dblGATA mice that had been challenged i.n. with OVA 1 d prior to i.v. injection. Mice were then given another i.n. challenge and allowed to rest for 12 h before they were sacrificed and tissues analyzed for presence of CCR3 $^{+}$ CFSE $^{+}$ cells.

Quantitative RT-PCR analysis of gene expression

RNA was isolated from lung tissue using TRIzol reagent (Invitrogen Life Technologies). RT-PCR was performed in triplicate with primers and FAM-labeled probes (Assays on Demand; Applied Biosystems) as described (3).

Statistics

Data was analyzed using GraphPad Prism (GraphPad), two-way ANOVA (mechanical ventilation), or Student *t* test (all other data: MS Excel, Microsoft, Seattle, WA), with one tail distribution (for the expectation of one way change) and two sample equal variance. The *p* values are given in the legends of the appropriate figures, with significance determined to be <0.05. Data are reported as average $\pm$ SEM.

Results

A dynamic computational model of the development of allergic asthma

We developed a dynamic model and tracked the activity of the nodes shown in Fig. 1. We validated our model by systematically perturbing the nodes and recapitulating the experimental observations whenever available (Table I). In particular, we observe an early induction of AHR that is dependent on mast cells, with late development of AHR dependent on Th2 cells and eosinophils (7), thus validating the model (Fig. 2A, see Supplemental Fig. 3A for time-related events among Th2 recruitment, eosinophil recruitment, and development of AHR). Supplemental Fig. 3 also demonstrates that deletion of mast cells (node MC) in the simulations results in the absence of the early AHR (Supplemental Fig. 3B). This simulation represents *c-kit* mutant mice *Sash* that lack mast cells, which exhibit an absence of the early AHR (38). However, in the simulations, MC are not critical for the late AHR, recruitment of Th2 cells (Supplemental Fig. 3C), or eosinophils (Supplemental Fig. 3D) (as seen in mouse models that use OVA/Alum to sensitize mice as in our model) (e.g., see Ref. 39). Blocking the production of His by MC (by turning off the His node), confirmed that MC are not important in the late AHR (Supplemental Fig. 3B–D).

By contrast, Supplemental Fig. 4 demonstrates that deletion of T cells (node T0) results in the absence of AHR (Supplemental Fig. 4A). This represents the finding that TCR $\beta^{-/-}$ mutant mice, which lack all α/β T cells including NKT cells, do not develop AHR (40). In addition, as determined by turning off the mDC node, DC as well as T cells are required for recruitment of eosinophils (Supplemental Fig. 4A–C), which is seen in experimental mouse models (40–42).

IL-13 add back to Δ dblGATA mouse lungs rescues pathology associated with allergic asthma

Using this discrete dynamic computational model, we simulated the effects of adding exogenous IL-13 to mice lacking eosinophils, which are unable to develop AL-induced late-stage AHR (3, 4,



We also simulated the outcome of the effect of IL-13^{-/-} eosinophils on the ability of T cells to be recruited to the lungs of Δ dblGATA mice (Fig. 5A). The results indicated that IL-13-deficient eosinophils would not be able to induce recruitment of T cells to the lungs at levels similar to WT (Fig. 5A). We tested this prediction experimentally by examining the lungs from WT, Δ dblGATA mice, or Δ dblGATA recipients of WT or IL-13^{-/-} eosinophils for the presence of CD4⁺ T cells. We found significantly lower numbers of CD4⁺ T cells in the lungs of Δ dblGATA mice challenged with OVA alone or that had received IL-13^{-/-} eosinophils and OVA challenge compared with the lungs of WT or Δ dblGATA mice that had received WT eosinophils and challenge with OVA (Fig. 5B). This indicates that IL-13 production specifically by eosinophils is required to induce recruitment of T cells to the lungs.

Table I. Network rules for allergic asthma simulation

Rational	Boolean Rule	References ^a
Allergen activation of airway EC and secretion of inflammatory cytokines	EC* = AL PIC*	77
Transient activation of lung-resident eosinophils	^b EL(t+1) = EL(t) $\cap_{t=0}^t$ and not ELa(τ)	78
Lung eosinophils are activated by allergen	ELa* = AL and EL	79, 80
Activated airway EC secrete TSLP, which reaches the draining lymph nodes	TSLP* = EC TSLPLN*	81
Cytokine induced maturation of DC and migration to draining lymph nodes carrying the allergen	mDC* = PIC mDCLN*	82
Activation of B cells and class switch to IgE, followed by IgE trafficking to lung and binding allergen	BCLN* = (AL and Th2) or BCLN IgELN* = BCLN and IL4 IgEL* = IgELN EAL* = IgEL and AL MCA* = EAL and MC BASa* = BAS and IgELN and AL BASL* = BAS and EAL and IL13 ^b His(t+1) = (MCRc(t) and EAL(t)) $\cap_{t=0}^t$ and not His(τ)	83, 84
Allergen-IgE complexes activate mast cells and basophils to induce degranulation and histamine release. This decay represented by $\cap$ and not describes the burst of His release immediately after exposure to allergen and IgE. Basophils are recruited to the lung in response to IL-13	Th2* = T0 and TSLPLN and mDCLN IL4* = Th2 or NKTL or BASa Th2L* = EO and Th2 IL5* = Th2	11
Activation and differentiation of naive T cells to Th2 cells by DCs and TSLP. Th2, activated lung iNKT cells or basophils then secrete IL-4 and IL-5. These Th2 cells can be recruited to the lung under the influence of lung eotaxin	NKTL* = NKT and AL and PIC and IL13I and mDC	32, 85
iNKT cells are recruited into the lung and activated in response to early IL-13, proinflammatory cytokines, and allergen. Note that lung iNKT cells are transiently activated to secrete cytokines reflected in the ($\cap$ and not) rule as for the lung eosinophils that get activated	IFNg* = NKTL ^b NKT(t+1) = (NKT(t) and AL(t) and PIC(t) and IL13I(t)) $\cap_{t=0}^t$ and not NKT(τ)	32
Lung Th2, basophils, and iNKT cells (transiently) secrete IL-4 and IL-13. Macrophages are also able to secrete IL-13 after they develop into alternative macrophages, but this is inhibited if they are exposed to IFN- γ	IL5L* = Th2L IL13II* = Th2L or (Macrophages and not IFNg) or EL2	7, 27, 32
Alveolar macrophages develop into alternative activated macrophages in the presence of IL-13 and are inhibited from doing so by IFN- γ	IL4L* = Th2L or NKTL or BASL Macrophages* = IL13II and not IFNg	16, 18
Eotaxin is produced by epithelial cells following stimulation with IL-13 from any cellular source	EO* = IL13a or IL13II	21, 77, 86
Eosinophils are recruited to the lung by lung eotaxin, where their survival and activation is dependent on lung IL-5	EL2* = EO and EB and IL5L	54
IL-13 is produced by resident eosinophils or recruited eosinophils upon interaction with allergen. IL-13 can also be produced by NKT cells, basophils, or macrophages. These different sources of IL-13 are separated for identification purposes and are collapsed into one source of this cytokine	IL13I* = AL and (ELa or EL2) IL13n* = NKTL IL13b* = BASL IL13m* = Macrophages IL13a* = IL13I or IL13n or IL13b or IL13m	Supplemental Fig. 1; 5, 16, 18, 27, 32
Blood eosinophils are produced from bone marrow precursors by stimulation with IL-5	EB* = IL5 and EBM	5
AHR is induced by histamine or IL-13 exposure	AHR* = His or IL13II	6, 11

^aIn the interest of space, the references are examples that support our rationale for the indicated rules.

^bThe rule is given by using the state of the node on left hand side at the time points 0 (the initial condition), 1, ..., t, t+1. Note that the node's state is actually updated at a randomly selected instance between every two time points.

*, The future state of the node; iNKT, invariant NKT.

Interestingly, these mice also had lower numbers of eosinophils in lungs, prompting the question of whether IL-13^{-/-} eosinophils were able to home to the lung (Fig. 5B). However, when we transferred CFSE-labeled WT or IL-13^{-/-} eosinophils into Δ dblGATA mice, we found that these eosinophils had equal ability to migrate to the lung when mice were exposed to i.n. OVA without prior immunization (i.e., minimal T cell recruitment and inflammation; Supplemental Fig. 2).

Given that there was a decrease in the number of T cells being recruited to the lung, we expected that there would also be a decrease in Th2 cytokine production in the lungs of Δ dblGATA that had received IL-13^{-/-} eosinophils and challenged with OVA. Indeed, analysis of BALF from these mice as well as Δ dblGATA mice challenged with OVA alone revealed that there was a drastic decrease in the amount of IL-4, IL-5, and IL-13 produced in the lungs of these mice compared with WT or Δ dblGATA mice that had received WT eosinophils (Fig. 5C), although there was no

significant difference in the production of IFN- γ (Fig. 5D). This confirmed our expectation that low numbers of T cells in the lungs of these mice would lead to reduced Th2 cytokine production.

Eosinophils are unable to induce responses in the lungs if they are the sole source of IL-13 in an in vivo model of allergic asthma

The finding that eosinophil production of IL-13 is required for the induction of allergic inflammation in the lungs suggests that they play a critical role in early production of this cytokine for subsequent development of allergic airway inflammation. Hence, we computationally determined whether IL-13 production by eosinophils alone is sufficient for the allergic inflammatory response in the lung (conditions listed in Table II). The model predicted that this would be insufficient to generate AHR if other IL-13-producing cells such as T cells, NKT cells, macrophages, and/or BAS, do not secrete significant levels of this cytokine at this

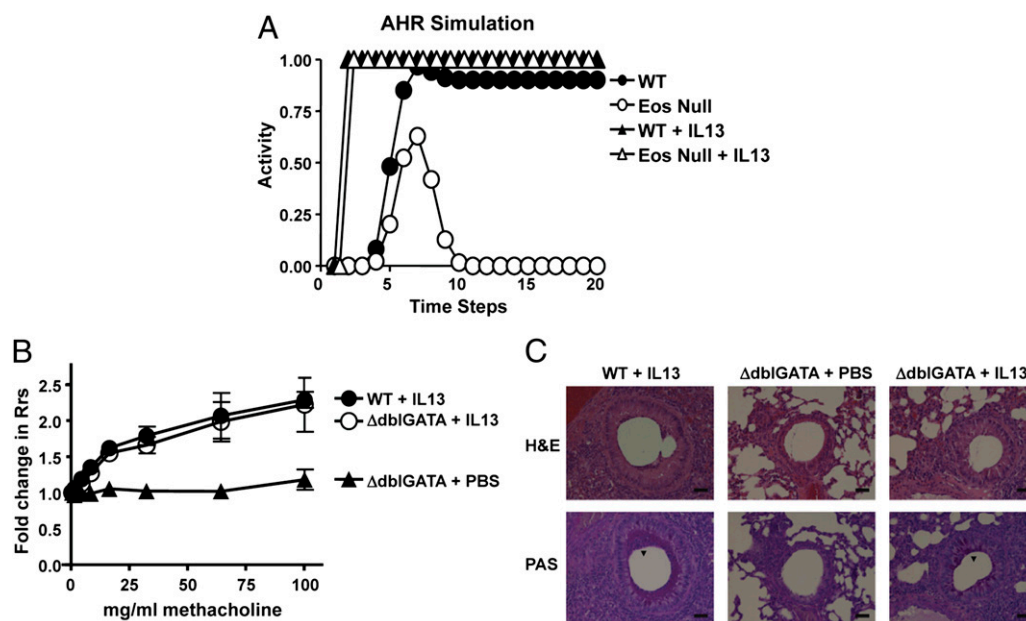


FIGURE 2. IL-13 rescues the development of allergic asthma in Δ dblGATA mice. **A**, Simulation of network shown in Fig. 1. IL-13 rescues bronchoconstriction (Activity, y-axis) in the absence of eosinophils. Simulations were run using WT conditions (●) or WT conditions with extraneous IL-13 (▲), eosinophil null conditions (○), or eosinophil null conditions with extraneous IL-13 (△). Note that WT with extraneous IL-13 (▲) and eosinophil null conditions with extraneous IL-13 (△) overlap on the graph. **B**, WT or Δ dblGATA mice were immunized with OVA, then given OVA + IL-13 (or PBS) i.n. Fold change in airways resistance was determined by mechanical ventilation ($n = 3$ mice, $p < 0.0001$ by two-way ANOVA). **C**, Sections of lungs from the indicated mice in **C** were analyzed by H&E (top panels) or PAS (bottom panels). Original magnification $\times 20$. Scale bars, 50 μ m. Arrowheads indicate mucous.

early time frame (Fig. 6A). We tested this prediction by transferring WT eosinophils into OVA-sensitized IL-13^{-/-} mice followed by i.n. OVA challenge. Note that in these mice, all cells lack the ability to secrete IL-13, and providing them with WT eosinophils would contribute a sole source of IL-13. We found that IL-13^{-/-} mice were unable to develop significant AHR as previously reported (45). Transferring WT eosinophils into these mice did not induce significant AHR over that seen in IL-13^{-/-} mice alone and was statistically lower than AHR responses in WT mice (Fig. 6B). Although we also found increased airway inflammation and infiltration of inflammatory cells into the lung tissue of IL-13^{-/-} recipients of WT eosinophils, this inflammation was lower than that found in WT lungs, and little mucus was found in lungs of either IL-13^{-/-} mice alone or those that received WT eosinophils (Fig. 6C). These data indicate that as predicted by the model, IL-13 production by eosinophils is not sufficient to induce significant symptoms of an allergic asthma response.

We next examined the effect of having eosinophils as the sole source of IL-13 on T cell recruitment to the lungs. Our simulations predicted that although WT conditions would yield the expected high levels of T cell recruitment, and the IL-13-deficient system would have low levels of T cell recruitment, adding back eosinophil-derived IL-13 to IL-13^{-/-} mice would lead to increased T cell recruitment to the lungs, but lower than that seen in WT mice. This prediction supports the view that stable T cell recruitment to the lung itself is not only dependent on eosinophil-derived IL-13, but also T cell (or other cell type)-derived IL-13 (Fig. 6A). We tested this prediction by determining whether T cells could still be recruited to the lungs of IL-13^{-/-} mice that had received WT eosinophils and challenged i.n. with OVA, if those T cells (or in fact all other cells) are intrinsically incapable of producing IL-13. We found that the lungs of IL-13^{-/-} recipients of WT eosinophils had significantly lower numbers of CD4⁺ T cells compared with WT mice, but a trend toward higher

numbers of CD4⁺ T cells than IL-13^{-/-} mice alone (although this did not quite reach statistical significance; Fig. 6D). This correlated with our data from Δ dblGATA mice transferred with IL-13^{-/-} eosinophils that suggests that T cell recruitment to the lung is facilitated by eosinophil-derived IL-13 (Fig. 6B). IL-13^{-/-} mice reconstituted with IL-13 competent eosinophils were able to recruit T cells to the lung, although these T cells were unable to produce or induce cytokines to sustain the response, and the quantity produced by eosinophils is not able to drive the response on its own. In fact, there was no significant difference in the amount of IL-13 mRNA in the lungs of IL-13^{-/-} mice versus those that received WT eosinophils; however, there was a trend toward higher IL-4 message in the latter mice (although this did not quite reach statistical significance), in agreement with the increased T cell recruitment observed (Fig. 6E). However, eosinophil-derived IL-13 can induce CCL11 expression (Fig. 6F), as seen in our simulations (Supplemental Fig. 5).

Eosinophil production of IL-13 is required for recruitment of in vitro-differentiated Th2 T cells to the lungs of Δ dblGATA mice

Because we found that non-eosinophil production of IL-13 appeared to be integral to the development of an allergic response in the lung, and because T cells are major producers of this cytokine, we wanted to determine whether elevated numbers of Th2-differentiated T cells already capable of producing IL-13 could bypass the requirement for eosinophil production of this cytokine in the lung. We therefore simulated conditions in which Th2 cells were delivered to the mice (conditions listed in Table II), and they were exposed to AL (Fig. 7A). Our simulations predicted that similar to the conditions seen when T cells are not transferred, eosinophil-derived IL-13 remains important for the recruitment of these Ag-specific Th2 cells into the lung and the development of AHR. To test this prediction, we differentiated OVA-specific

Table II. States of nodes for simulations

Condition	Node Settings
WT	AL, EBM, T0, MC, BAS, NKT and EL in the ON state, all other nodes initially OFF
Eos ^{-/-}	AL, EBM, T0, MC, BAS, NKT in the ON state, all other nodes initially OFF, and EL and EL2 permanently OFF
IL-13 add back to WT mice	AL, EBM, T0, MC, BAS, NKT, EL and IL13I in the ON state, all other nodes initially OFF
IL-13 add back to Eos ^{-/-} mice	AL, EBM, T0, MC, BAS, NKT and IL13I in the ON state, all other nodes initially OFF, and EL and EL2 permanently OFF
IL-13 ^{-/-} eosinophil add back to Eos ^{-/-} mice	AL, EBM, T0, MC, BAS, NKT and EL in the ON state, all other nodes initially OFF and IL13I permanently OFF
WT eosinophil add back to Eos ^{-/-} mice	AL, EBM, T0, MC, BAS, NKT and EL in the ON state, all other nodes initially OFF
IL-13 ^{-/-} mice	AL, EBM, T0, MC, BAS, NKT and EL in the ON state, all other nodes initially OFF, and IL13I and IL13II permanently OFF
WT eosinophil add back to IL-13 ^{-/-} mice	AL, EBM, T0, MC, BAS, NKT and EL in the ON state, all other nodes initially OFF, and IL13II permanently OFF
Th2 cell transfer to WT mice	AL, EBM, MC, BAS, NKT and EL in the ON state, all other nodes initially OFF, and T0 and IgELN permanently OFF. These rules mimic the fact that this short-term response is not long enough to generate new Th2 cells or an IgE response
Th2 cell transfer to Eos ^{-/-} mice	AL, EBM, MC, NKT, BAS and Th2 in the ON state, all other nodes initially OFF, and T0, EL, EL2 and IgELN permanently OFF
Th2 cell transferred along with WT eosinophils to Eos ^{-/-} mice	AL, EBM, MC, Th2, NKT, BAS and EL in the ON state, all other nodes initially OFF, and T0 and IgELN permanently OFF
Th2 cell transferred along with IL-13 ^{-/-} eosinophils to Eos ^{-/-} mice	AL, EBM, MC, NKT, BAS and Th2 in the ON state, all other nodes initially OFF, and T0, IL13I and IgELN permanently OFF
T cell deficiency	AL, EBM, BAS and MC in the ON state, all other nodes initially OFF, and T0 and NKT permanently OFF
NKT cell deficiency	AL, EBM, BAS and MC in the ON state, all other nodes initially OFF, and NKT permanently OFF
MC deficiency	AL, EBM, BAS, NKT and T0 in the ON state, all other nodes initially OFF, and MC permanently OFF
mDC deficiency	AL, EBM, T0, BAS, NKT and MC in the ON state, all other nodes initially OFF, and mDC permanently OFF

Refer to the *Materials and Methods* section for an explanation of node names.

T cells from OT-II mice to Th2 cells and transferred these cells i.v. into naive WT or Δ dblGATA mice. Some Δ dblGATA mice also received WT or IL-13^{-/-} eosinophils i.v. in a different vein, 6 h

prior to T cell transfer. We challenged all mice with OVA and then analyzed for AHR. We found that, even with the presence of elevated numbers of differentiated OVA-specific Th2 T cells,

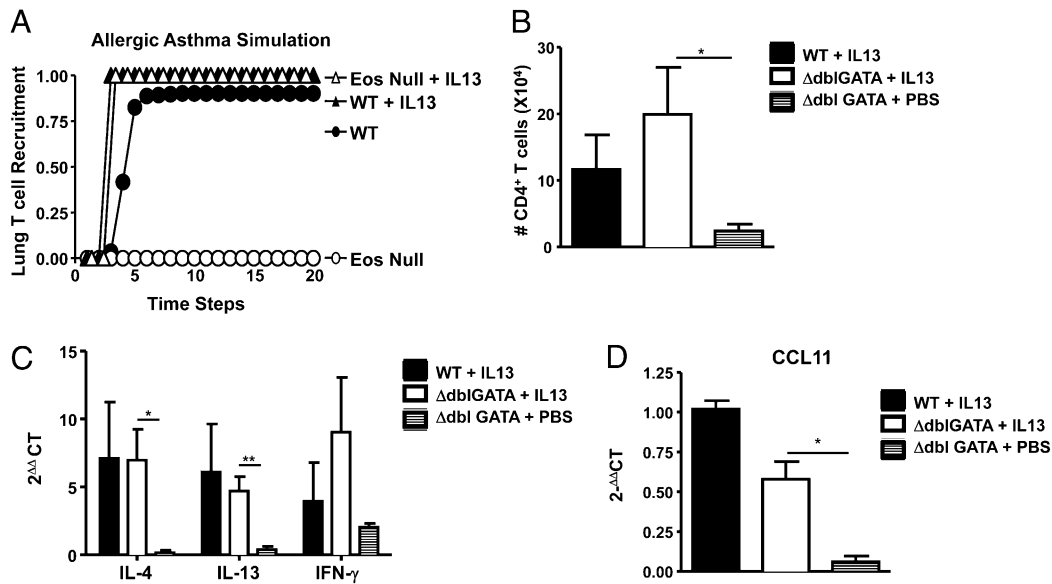
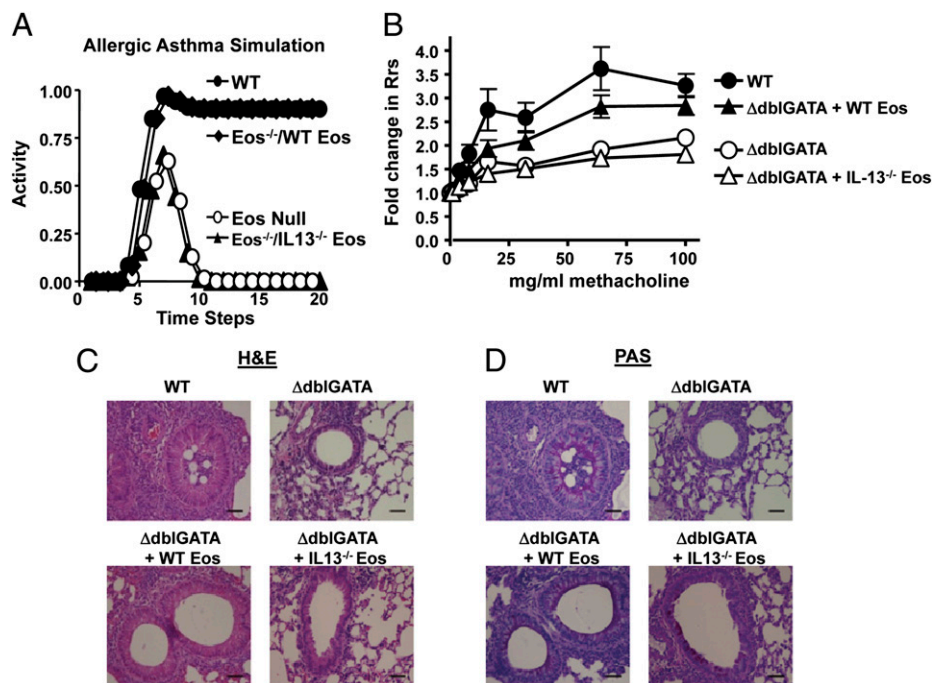


FIGURE 3. IL-13 rescues the recruitment of T cells into the lungs of Δ dblGATA mice. *A*, Simulation of the effect of IL-13 on T cell recruitment to the lungs in mice lacking eosinophils. Simulations were run using WT conditions (●) or WT conditions with extraneous IL-13 (▲), eosinophil null conditions (○) or eosinophil null conditions with extraneous IL-13 (△). *B*, WT or Δ dblGATA mice were immunized with OVA, then given OVA + IL-13 (or PBS) i.n. CD4⁺ T cell recruitment to the lung was determined 24 h after final exposure of OVA/IL-13 or OVA/PBS ($n = 3$ –5 mice, $*p < 0.05$ by Student *t* test). Analysis of IL-4, IL-13, and IFN- γ , ($n = 3$ –5 mice, $*p < 0.05$, $**p < 0.005$ by Student *t* test) (*C*) or CCL11 in the lungs from the indicated mice in *B* by quantitative RT-PCR (QRT-PCR) (*D*) ($n = 3$ –5 mice, $*p < 0.05$ by Student *t* test).

FIGURE 4. Eosinophil-derived IL-13 is critical for the development of allergic asthma. **A**, Simulation of the lack of eosinophil-derived IL-13 on AHR. Simulations were run using WT conditions (●), eosinophil null conditions (○), or eosinophil null conditions with the additions of WT Eos (◆) or IL-13^{-/-} Eos (▲). **B**, WT (●) or ΔdblGATA mice were immunized with OVA. ΔdblGATA mice were reconstituted with WT eosinophils (▲), IL-13^{-/-} eosinophils (△), or left alone (○), followed by i.n. challenges with OVA. Fold change in airways resistance was determined by mechanical ventilation (*n* = 16 mice, *p* < 0.001 by ANOVA). Sections of lungs from the indicated mice in **B** were analyzed by H&E (**C**) or PAS (**D**). Scale bars, 50 μm. Original magnification ×20.



ΔdblGATA mice required eosinophils to generate AHR, as ΔdblGATA mice-provided OT-II Th2 cells alone had significantly reduced AHR compared with WT mice transferred with OT-II Th2 cells (Fig. 7B). Furthermore, although in vitro-differentiated Th2 cells are capable of making their own IL-13 (Supplemental Fig. 1) (46), IL-13 production by eosinophils was still required, as ΔdblGATA mice transferred with IL-13^{-/-} eosinophils along with OT-II Th2 cells had significantly reduced AHR compared with ΔdblGATA mice transferred with WT eosinophils along with OT-II Th2 cells or WT mice transferred with OT-II Th2 cells (Fig. 7B).

ΔdblGATA mice transferred with OT-II Th2 cells and IL-13^{-/-} eosinophils also had significantly reduced airway inflammation and mucus production in the lung compared with ΔdblGATA mice

transferred with OT-II Th2 cells and WT eosinophils or WT mice injected with OT-II Th2 cells (Fig. 7C). ΔdblGATA mice transferred with OT-II Th2 cells alone also had drastically reduced airway inflammation and mucus production, similar to ΔdblGATA mice transferred with OT-II Th2 cells and IL-13^{-/-} eosinophils (Fig. 7C). These results indicated that IL-13 production by eosinophils is required to induce airway inflammation and mucus production in the lung, even when elevated numbers of Ag-specific Th2-differentiated T cells are circulating in the system.

These findings were likely due to the lack of T cell recruitment to the lungs in the absence of eosinophil-derived IL-13, because we found significantly lower numbers of CD4⁺ T cells in the lungs of ΔdblGATA mice transferred with OT-II cells alone or along with

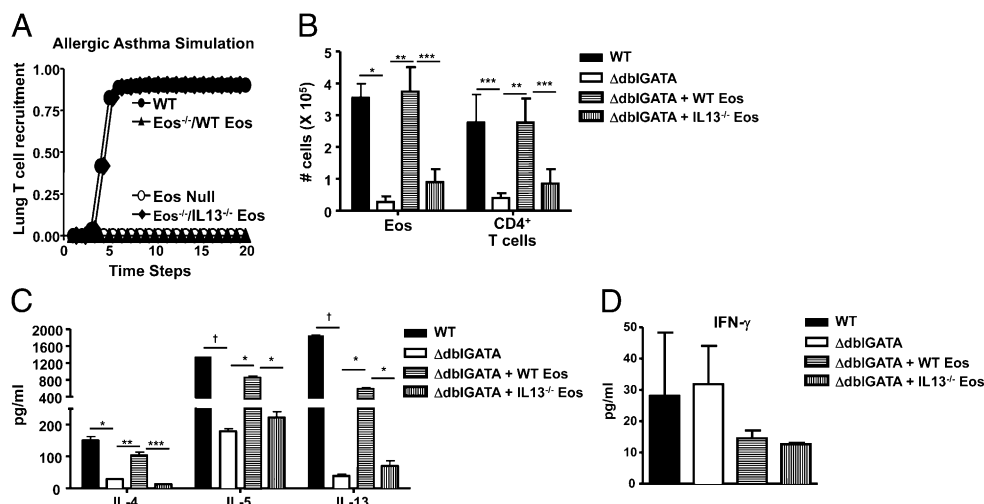


FIGURE 5. Eosinophil-derived IL-13 is critical for the recruitment of T cells into the lung. **A**, Simulation of the lack of eosinophil-derived IL-13 on T cell recruitment to the lungs. Simulations were run using WT conditions (●), eosinophil null conditions (○), or eosinophil null conditions with the additions of WT Eos (◆) or IL-13^{-/-} Eos (▲). **B**, WT or ΔdblGATA mice were immunized with OVA. ΔdblGATA mice were reconstituted with WT eosinophils, IL-13^{-/-} eosinophils, or left alone, followed by i.n. challenges with OVA. Eosinophil and CD4⁺ T cell recruitment to the lung was determined 24 h after final exposure of OVA (*n* = 3–4 mice; **p* < 0.0005, ***p* < 0.005, ****p* < 0.05 by Student *t* test). Analysis of IL-4, IL-5, and IL-13 (*n* = 3–4 mice; **p* < 0.005, ***p* < 0.05, ****p* = 0.0660, †*p* < 0.0005 by Student *t* test) (**C**) or IFN-γ in BAL from the indicated mice in **B** by ELISA (**D**) (*n* = 3–4 mice; *p* > 0.05, NS by Student *t* test).

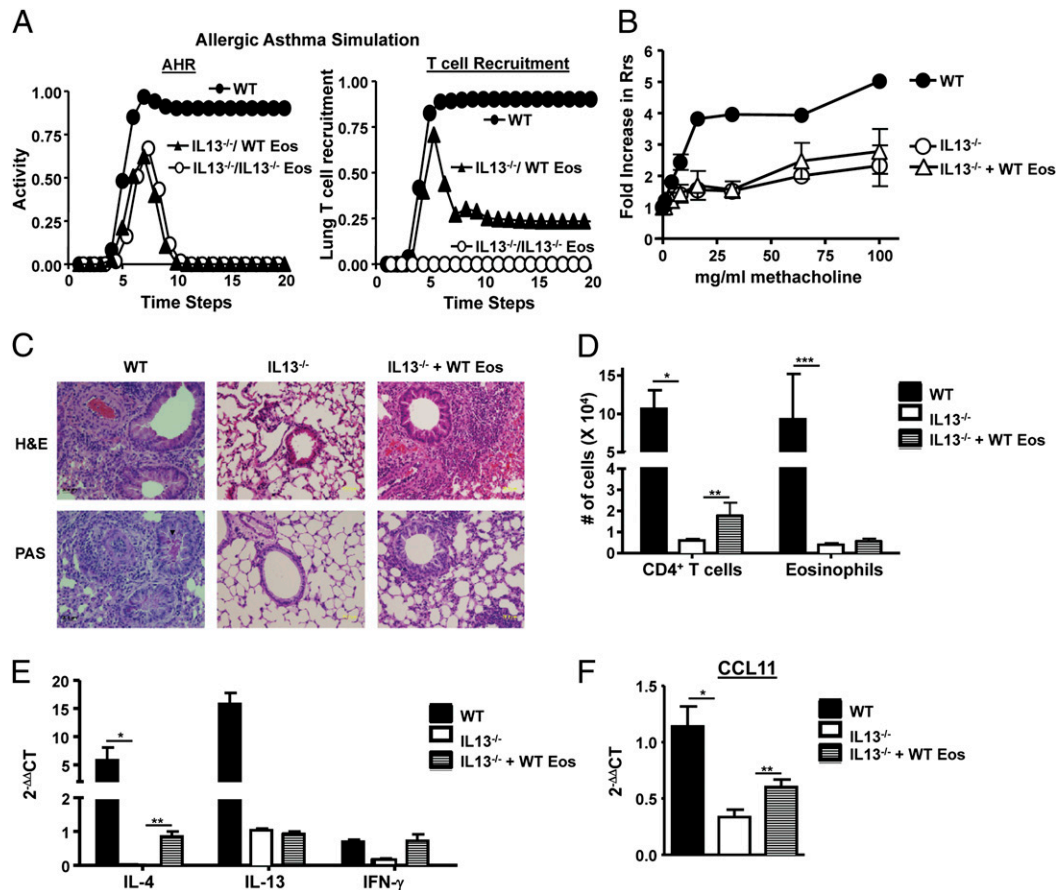


FIGURE 6. Eosinophil-derived IL-13 is not sufficient to drive AHR but can induce recruitment of T cells during the development of allergic asthma. *A*, Simulation of the ability of WT eosinophils capable of secreting IL-13 to rescue airways hyperresponsiveness in IL-13^{-/-} mice (*left panel*) or T cell recruitment to the lungs (*right panel*). Simulations were run using WT conditions (●), IL-13^{-/-} conditions (○), or IL-13^{-/-}/WT eosinophils (▲). *B*, WT or IL-13^{-/-} mice were immunized with OVA. IL-13^{-/-} mice were then either reconstituted with WT eosinophils or left alone, followed by i.n. challenges with OVA. Fold change in airways resistance was determined by mechanical ventilation ($n = 2-5$ mice; $p < 0.001$ by ANOVA). *C*, Sections of lungs from the indicated mice in *B* were analyzed by H&E (*top panels*) or PAS (*bottom panels*). Original magnification $\times 20$. *D*, CD4⁺ T cell recruitment to the lung was determined 24 h after final exposure of OVA in the mice indicated in *B* ($n = 3-4$ mice; $*p < 0.005$, $**p = 0.0999$, $***p < 0.05$ by Student *t* test). *E*, Analysis of IL-4, -13 and IFN- γ ($n = 3-5$ mice; $*p < 0.05$, $**p = 0.06998$ by Student *t* test). Note that mRNA levels in IL-13^{-/-} mice were set at 1 for the IL-13 analysis, whereas IL-13^{-/-}/WT eosinophils were set at 1 for the other cytokines. *F*, CCL11 in the lungs of the indicated mice by QRT-PCR ($n = 3-5$ mice, $*p < 0.005$, $**p < 0.05$ by Student *t* test).

IL-13^{-/-} eosinophils, compared with Δ dblGATA mice that received OT-II Th2 cells and WT eosinophils or WT mice that received OT-II Th2 cells (Fig. 7E). Importantly, we also observed reduced eosinophil recruitment into the lungs of mice that received IL-13^{-/-} eosinophils (Fig. 7D), indicating that inflammation induced recruitment of these cells is affected by the lack of IL-13 secreted by these cells, as seen in Fig. 5B. These results suggested that even when the numbers of Th2 cells are elevated, these cells still have a requirement for the production of IL-13 by eosinophils to migrate to the lung.

We also analyzed the amount of IL-4 and IL-13 mRNA in the lungs of the different groups of mice and found that Δ dblGATA mice transferred with OT-II Th2 cells alone or OT-II Th2 cells and IL-13^{-/-} eosinophils had statistically lower levels of IL-4 and IL-13 in the lungs when compared with WT mice injected with OT-II Th2 cells or Δ dblGATA transferred with OT-II Th2 cells and WT eosinophils (Fig. 7F). These data indicate that lack of T cell migration into the lungs of Δ dblGATA mice transferred with OT-II Th2 cells and IL-13^{-/-} eosinophils also results in reduced Th2 cytokine production in the lungs of these mice. Furthermore, analysis of the levels of CCL11/eotaxin-1 (Eot-1), which we have previously shown can regulate T cell recruitment into the lungs of

Δ dblGATA mice, revealed that IL-13^{-/-} eosinophils could not induce stable expression of this chemokine (Fig. 7G) (3). Overall, these data suggest that regardless of the number of T cells that are able to produce IL-13, they still maintain a requirement for eosinophil-derived IL-13 to migrate to the lung.

Discussion

The role of eosinophils in the development of allergic asthma has been an enigma. Using mouse models, recent work by our group as well as others suggest that these cells are required for the development of this disease in C57BL/6 mice but not in BALB/c mice (3, 4). More importantly, this work suggests that eosinophils are required for T cell recruitment to the lungs and subsequent cytokine secretion in this animal model. In this investigation, we have provided computational and experimental evidence that IL-13 secretion specifically by eosinophils is a key early event required for the later signaling including recruitment of T cells to the lung during the pathogenesis of allergic asthma. Our previous work, as well as that of others, suggesting that eosinophils are required for the recruitment of T cells to the lungs (3, 4), allowed us to construct a network model of allergic asthma development and consequently simulate the dynamics of different components

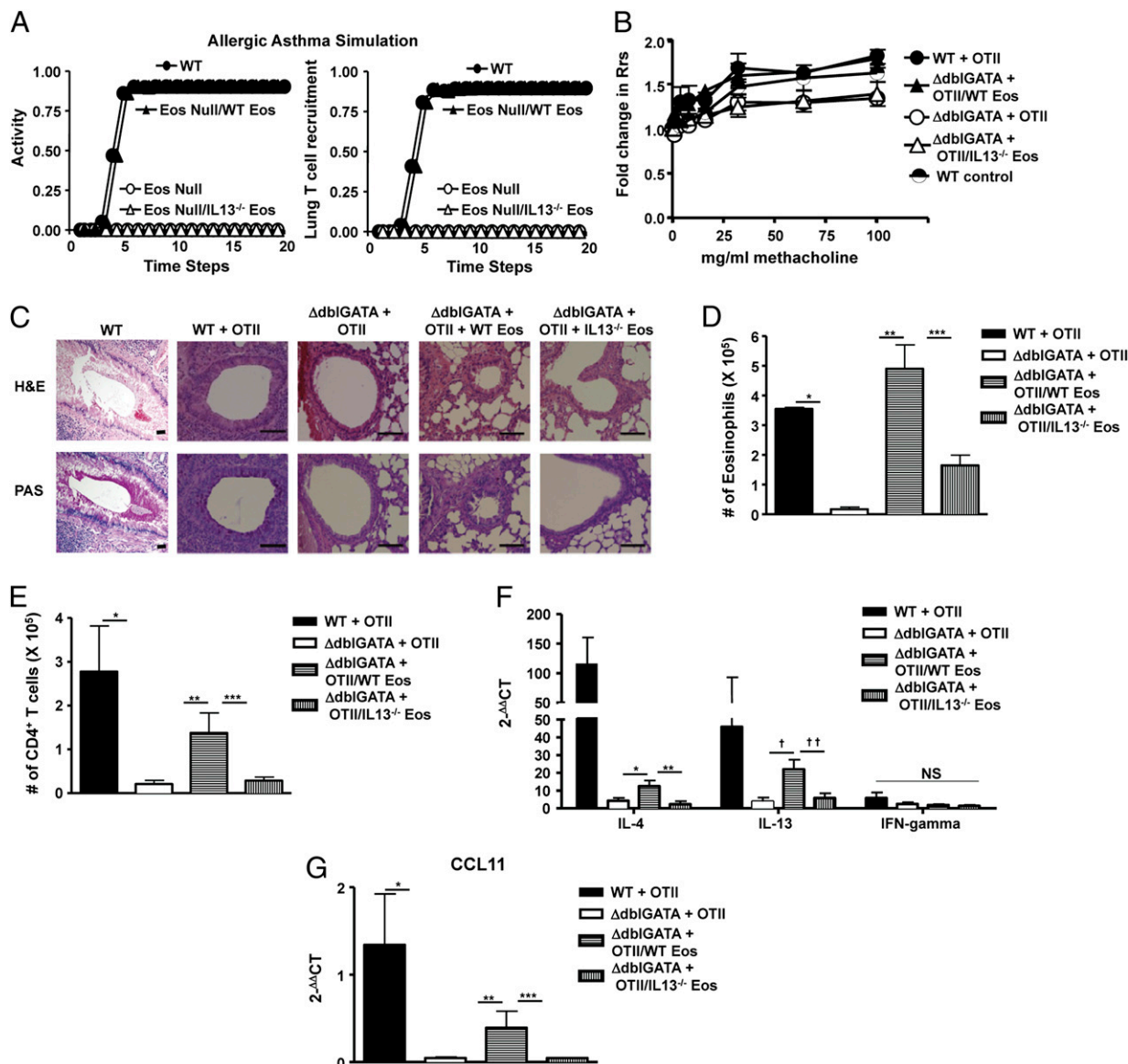


FIGURE 7. Eosinophil production of IL-13 is required for recruitment of in vitro differentiated Th2 T cells to the lungs. **A**, Simulation of the introduction of differentiated Th2 cells into WT or eosinophil null mice, followed by reconstitution with WT or IL-13^{-/-} eosinophils. AHR (left panel) or T cell recruitment to the lungs (right panel) were analyzed. Simulations were run using WT (●), eosinophil null (○), eosinophil null mice receiving WT eosinophils (▲), or eosinophil null mice receiving IL-13^{-/-} eosinophils (△) as recipients of differentiated Th2 cells. **B**, WT or ΔdbiGATA mice were given Th2 differentiated OT-II cells, followed 24 h later by i.n. challenges with OVA. WT (●), ΔdbiGATA reconstituted with WT eosinophils (▲), ΔdbiGATA reconstituted with IL-13^{-/-} eosinophils (△), or left alone (○). Fold change in airways resistance was determined by mechanical ventilation ($n = 3-4$ mice, $p < 0.001$ by ANOVA). Note that WT control mice with OVA overlapped with WT-reconstituted Th2-differentiated OT-II cells in the graph. **C**, Sections of lungs from the indicated mice in **B** were analyzed by H&E (top panels) or PAS (bottom panels). Eosinophil ($n = 3-4$ mice, $*p < 0.00005$, $**p < 0.0005$, $***p < 0.005$ by Student *t* test) (**D**) and CD4⁺ T cell recruitment to the lung (**E**; $*p < 0.05$, $**p < 0.01$, $***p < 0.02$ by Student *t* test) was determined 24 h after final exposure of OVA in the mice indicated in **B** ($n = 3-4$ mice, $*p < 0.05$ by Student *t* test). Analysis of the IL-4, IL-13, and IFN- γ ($n = 3-4$ mice, $*p < 0.05$, $**p < 0.03$, $†p < 0.02$, $††p < 0.03$ by Student *t* test) (**F**) or CCL11 in the lungs of the indicated mice by QRT-PCR ($n = 3-4$ mice, $*p < 0.05$, $**p = 0.0609$, $***p = 0.0607$ by Student *t* test) (**G**). Scale bars, 50 μ m.

of the system. Additionally, the transfer functions we used gave insight into the cooperative regulation of various cytokines and cells, which can be used in other studies in this field. Our experiments support the simulations, validating the model and its predictions. This model will be useful for directing future experiments aimed at increasing our understanding of the allergic airway inflammation.

There is much indirect evidence to support an interdependent relationship between eosinophils and T cells in the generation of an allergic response, although there is some dependence on the animal model. IL-5 and Eot-1 double-deficient mice cannot produce

IL-13 in the lungs when challenged with allergen (47). Whether this is intrinsic to T cells is unclear. PHIL mice have defective T cell migration to the lung and Th2 cytokine production in the absence of eosinophils, despite the delivery of in vitro-differentiated Th2 cells (4). Mice lacking the common β c receptor, required for the development of eosinophils, MC, and BAS, also have defects in T cell migration to the lung as well as Th2 T cell differentiation (48), although this may be due to a role for basophils in Th2 responses (14, 15, 49). At the same time, Ab blockade of CD4⁺ T cells in the lung reduces secretion of chemokines and Th2 cytokines as well as attenuates eosinophilic inflammation in

the lung (7, 8, 50). Rag^{-/-} mice lacking T and B cells also fail to develop significant eosinophilic inflammation in the lungs in response to allergen, as do CD4-deficient mice (51, 52).

Our observations and simulations suggest that eosinophils deficient in IL-13 are unable to generate AHR and airway inflammation due to an inability to recruit additional cell types including T cells producing IL-13 to the lung, leading to defects in subsequent Th2 cytokine production. This is likely due in part to an inability to induce production of chemokines that play a major role in recruiting T cells and other immune cells to the lung as well as Th2 cytokine production during allergen challenge. Eotaxin/CCR3 signals are able to induce eosinophils to release Th2 cytokines from secondary granules that can affect immediate activation of cells such as MC, which can secrete inflammatory mediators that contribute to lung pathology (8, 53). Mice lacking both Eot-1 and Eot-2 have reduced inflammatory cell recruitment to the lung as well as defects in Th2 cytokine production in the lung (54). Similarly, mice double deficient in IL-5 and Eot-1 are unable to recruit inflammatory cells to the lungs due to lack of eosinophils and reduced chemokines; however, transferring eosinophils back into the lungs of these mice restores the ability of inflammatory cells to migrate to the lung and secrete Th2 cytokines (47). Additionally, it is possible that IL-13 production by eosinophils is required for their survival or for their production of other effectors that regulate T cell recruitment to the lungs.

It was also interesting that both naive and OVA-sensitized Δ dblGATA mice provided with IL-13^{-/-} eosinophils had reduced numbers of these cells in the lungs compared with Δ dblGATA mice that were given WT eosinophils. Both types of eosinophils were equally able to migrate to the lungs in Δ dblGATA mice when examined 16 h after i.v. transfer and i.n. OVA challenge. However, when lungs from OVA-sensitized Δ dblGATA mice challenged with OVA were analyzed, we found reduced numbers of IL-13^{-/-} eosinophils. Thus, T cells require eosinophils to migrate into the lungs, and eosinophils may require T cell production of cytokines, such as IL-5, to survive for extended periods in the lungs, or to maintain or recruit these cells into the lung, a point suggested by the simulations of our model (Supplemental Fig. 7). This hypothesis is supported by the fact that even when Δ dblGATA mice received elevated numbers of Th2-differentiated T cells along with IL-13^{-/-} eosinophils, they were still unable to recruit T cells to the lungs. Conversely, when WT eosinophils were transferred into IL-13^{-/-} mice, they were able to induce some migration of T cells to lung, but as these recruited cells were unable to produce IL-13, a large-scale response could not occur.

Our data and simulations also suggest that lung-resident eosinophils may play a role in the initiation of this response by producing IL-13, which leads to T cell recruitment (Supplemental Fig. 8), and then to further recruitment and increases in eosinophils (Supplemental Fig. 4). In the absence of these pools of eosinophils capable of secreting this cytokine, T cells cannot be recruited. Thus, eosinophils may be critical in the early production of IL-13. Furthermore, our simulations suggest that removal of this population of lung-resident eosinophils may be critical in preventing the response. These simulations may also explain the results of some trials in asthmatic patients that attempted to deplete serum IL-5 that lead to little effect on disease, but may not have been effective in reducing lung IL-5 (55–57).

In the bronchial submucosa of asthmatic patients, a large majority of the cells that carry IL-13 mRNA are T cells, whereas a large proportion of those cells that carry IL-13 protein have been identified as eosinophils and, at a lower level, MC (58–60 and as reviewed in Ref. 61). In addition, although developing work suggests that cytokines such as IL-25, IL-33, and TSLP can induce

non-T cells to secrete IL-13, the contribution of such non-T cells to the overall IL-13 being produced or the timing of their expression is unclear (62). Other non-Th2 cell sources of IL-13 such as NKT cells, MC, BAS, and alveolar macrophages likely also make significant contributions to the presence of this cytokine. Our model thus also included some simplifying assumptions about the behavior of IL-13 production by basophils, NKT cells, and macrophages.

With regards to NKT cells, a number of reports have indicated that these cells are critical for the development of AHR in C57BL/6 mice, and these cells are also found in humans with asthma, although these findings are controversial (23, 63–69). We therefore also developed alternative networks that temporally place the ability of NKT cells secreting IL-13 prior to eosinophil-derived IL-13. Simulating these networks reveals that they do not predict the experimental data (i.e., having a requirement for eosinophils for this cytokine in T cell recruitment and development of AHR) (Supplemental Fig. 6). Furthermore, simulations of an alternative model in which NKT cell and eosinophil-derived IL-13 are equally important for these responses also does not predict the results of empirical experiments (Supplemental Fig. 6). Our current simulations therefore also have NKT cells acting downstream of eosinophils, either due to recruitment (as for the T cells) or to activation. Similar effects are observed when we simulate macrophage or BAS production of early IL-13, and we thus modeled these cells as later producers of IL-13. Our model thus includes the assumption that their IL-13 contributions may not be enough at the early stage of the disease, or may be more important later in the disease (i.e., after eosinophils are recruited and concomitant with or after T cell recruitment to the lung). Thus, although the temporal nature of when these cells secrete IL-13 will determine their importance in the early development of the disease, including recruiting T cells to the lungs, we consider eosinophils and T cells (including NKT cells) as major early producers of IL-13 in our models. Our simulations suggest that NKT cells may also contribute to recruitment of T cells to the lung (Supplemental Fig. 9), which we plan to test experimentally in the future. More detailed analysis of the interactions between these cells will be the topic of future work.

Research in an IL-13^{-/-} model of respiratory syncytial virus suggests that prolonged maintenance of eosinophils in the lung is difficult in the absence of IL-13 (70). However, because both T cells and eosinophils, as well as other cell types, can secrete IL-13, the cell type responsible in this system is not clear. These investigators did not examine whether eosinophils are recruited to the lung early during respiratory syncytial virus infection, however, and more studies are needed to determine this. Although other murine models of allergic diseases such as those using helminths have reported variable requirement for eosinophils, it is important to note that dependent on the background of the mice (e.g., C57BL/6 versus BALB/c), eosinophils may or may not be critical for the model. In addition, the magnitude of the inflammation and the requirement for T cell recruitment may also be important in whether these cells are critical.

Qualitative discrete modeling such as ours has been successfully used previously in gene regulatory networks and signal transduction networks for predicting the dynamic trajectory of biological circuits and for accessing the reliability of gene regulatory networks in signal processing (30). In the study of immunological responses, this approach has been implemented in small networks for the analysis of T cell activation and anergy, for the analysis of lymphocyte subsets, and we have used this approach to analyze the pathogenesis of *Bordetella* infections (10, 71–73). In this study, we assembled the literature on the immune components

involved in case of the i.p. sensitization in the form of a comprehensive network. In the absence of the estimates of the rate constants, we decided to use the qualitative discrete model (74, 75). The ingredients (node states, transfer functions) of our dynamic model refer to the node (component) level, and there is no explicit control over pathway-level effects. Moreover, the combinatorial transfer functions we used are, to varying extents, conjectures, informed by the best available experimental information.

Although in the current study we focused on the i.p. sensitization in C57BL/6 mice, AHR is dependent on the route of sensitization and the strain of mice in which the experiments are performed. Similarly, allergic AHR varies in people even in the similar geographical areas and is also partially genetically determined. Through network modeling, we can, for example, construct strain-specific subnetworks on the background of the conserved network showing the pathways that are activated in different strains and via different routes of sensitization. Additionally, HLA information can be integrated to develop patient specific networks. Because in the current study we only performed experiments using C57BL/6 mice, we focused our computation models in the same system because it is challenging to assemble and simulate the strain-specific responses. In this study, we have constructed the network model at the cellular level where the interactions between cells and cytokines are depicted. Based on these questions, a similar approach can be used on fewer cell types but at the molecular level to study the transcriptional regulation.

Collectively, these data and simulations suggest that there is a unique interdependence between eosinophils and T cell during the course of an allergic airway response. T cells require eosinophil secretion of IL-13 to migrate to the lung in response to AL challenge, and eosinophils most likely require T cells to secrete cytokines for sustained eosinophil survival in the lung environment, as well as to induce mass mobilization of eosinophils from the bone marrow. We have used modeling and computer simulations to assist in understanding this complex process. In the development of the computational model, we note the words of David Drubin, writing in the journal *Molecular Cell Biology* on his efforts collaborating with theoreticians to develop relevant models: "A theoretical model should organize the experimental facts, clearly state the assumptions on which it is based, make testable predictions, and present a (hopefully) new conceptual framework for thinking about a biological phenomenon. A model is not meant to be the final word on a subject but a beginning that invites empirical tests of its validity" (76). In building our model, we were able to quickly compare competing hypotheses, and the results suggested critical experiments to test those hypotheses or areas of contention. One of the most important contributions of our model is in the study of the pathogenesis, in which we can actually look at the patterns of all the nodes and decide the treatment time and strategy to reduce the AHR. Putative medical treatments can thus be evaluated in silico by simulating them through adding/removing or activating/inhibiting certain nodes and studying their effect on the AHR. This study addresses the goals of systems biology by effectively encapsulating prior knowledge to generate a mechanistic and predictive understanding at the systems level.

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Disclosures

The authors have no financial conflicts of interest.

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Supplementary Figure Legends:

Supplementary Figure 1. Eosinophils express IL-13. A) Q-RT-PCR of the indicated cytokines from mRNA isolated from purified eosinophils or in vitro differentiated Th2 cells. B) Purified eosinophils were incubated in media, or stimulated with LPS or eotaxin and supernatants analyzed for IL-13.

Supplementary Figure 2. IL-13 null eosinophils migrate to the lung under steady state non-inflammatory conditions. WT or IL-13 null eosinophils were purified and loaded with CFSE. Cells were then transferred into Δ dblGATA mice intravenously. Twenty four hours later, mice were exposed to OVA intranasally and after a further 24 hours, sacrificed and lungs isolated and analyzed for CFSE labeled eosinophils (p=Not Significant, n=3).

Supplementary Figure 3. Validation of network. A) **Simulation of the allergic airway inflammation in WT mice.** Analysis of the development of AHR (●), T cell (◇) and eosinophil (▽) recruitment over time steps. Note the apparent delay in recruitment of eosinophils until after T cells have been recruited. **Simulation of the allergic airway inflammation in WT (●), mast cell null mice (◇), or mice unable to generate histamine (▽).** B) Development of Airways Hyperresponsiveness (AHR), C) T cell recruitment and D) Airway eosinophil recruitment (increase over baseline). Note the reduction in early AHR in the absence of mast cells or histamine (the remainder being due to eosinophils), without any effect on T cell and eosinophil recruitment.

Supplementary Figure 4. Further validation of network. Simulation of the allergic airway inflammation in WT (●), T cell null mice (▽), or mice lacking dendritic cells (mDC) (△). **A)** Development of Airways Hyperresponsiveness (AHR), **B)** T cell recruitment and **C)** Airway eosinophil recruitment (increase over baseline). Note the absence of AHR and eosinophil recruitment in the absence of T cells or dendritic cells.

Supplementary Figure 5. Eosinophil derived IL-13 is required for induction of CCL-11/Eotaxin-1. Simulation of allergic airway inflammation in WT mice or introduction of WT or IL-13^{-/-} eosinophils into eosinophil null mice. Eotaxin expression was analyzed. Simulations were run using WT (●), WT mice reconstituted with IL-13^{-/-} eosinophil null (○), WT reconstituted with WT eosinophils (▲). Note that the triangles overlap with the circles in the figure and have been nudged by 0.4 units for clarity.

Supplementary Figure 6. Simulation of alternate models of allergic airway inflammation. Simulations of allergic airway inflammation in WT (●), Eosinophil null (○) or NKT cell null mice (▽). Alternate models 2 and 3. **A)** Network generated where NKT cell produced IL-13 is independent of eosinophil derived IL-13 (model 2) or is equally important with eosinophil derived IL-13 (model 3). Purple lines indicate the new edges, and dashed purple lines indicate those edges that would no longer exist. **B & C,** **Simulation of alternative model 2:** simulation of network generated with NKT cell produced IL-13 is independent of eosinophil derived IL-13. **B)** Simulation of AHR. **C)** Simulation of T cell recruitment. Note that model does not predict the reduced AHR or T cell recruitment that is observed in the absence of eosinophils. **D-H, Simulation of**

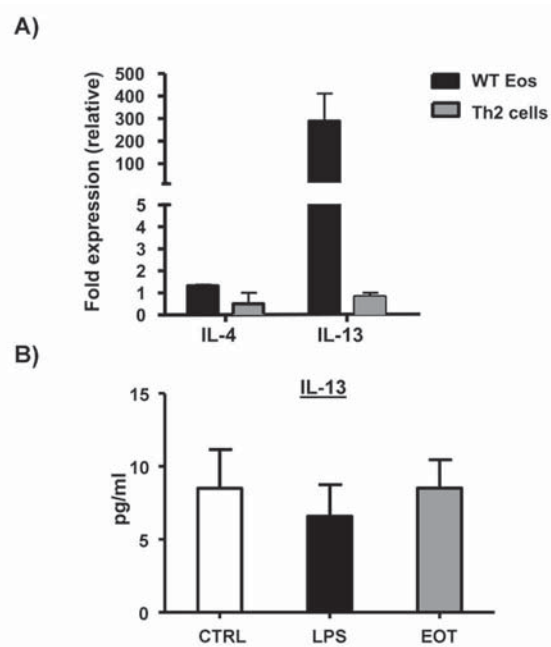
alternative model 3: simulation of network generated with NKT cell produced IL-13 is equally important with eosinophil derived IL-13. **D)** Simulation of AHR. Note three phases of AHR, very early when NKT cells are activated (which matches experimental observations), intermediate AHR that is IgE dependent and late phase AHR. **E)** Simulation of T cell, or **F)** eosinophil recruitment. Note reduced T cell recruitment as well reduced eosinophil recruitment in the absence of NKT cells. **G)** Simulation of T cell recruitment during allergic airways inflammation in WT mice (●), or mice with only eosinophil (○) or NKT cell derived IL-13 (▽). Compare this to figure 5a in parent text for eosinophil derived IL-13. **H)** Simulation of model 3 examining T cell recruitment during allergic airways inflammation in WT mice (●), or IL-13 null mice that have received IL-13 deficient eosinophils (○), IL-13 null mice that have received WT NKT cells (able to secrete IL-13, (▽)), or IL-13 null mice that have received WT eosinophils (able to secrete IL-13, (▼)). Compare this to figure 6a in parent text for eosinophil derived IL-13. Again, note that while model 3 predicts reduced AHR and T cell recruitment in the absence of eosinophils, it does not predict the behavior of T cell recruitment when IL-13 null mice are given WT eosinophils able to make IL-13.

Supplementary Figure 7. Role of IL-5 in recruitment of eosinophil and T cells to the lung. Simulation of allergic airway inflammation in WT mice (●) or serum depleted of IL-5 (○), or IL-5^{-/-} mice (▽). **A)** Airway eosinophil recruitment (increase over baseline), and **B)** T cell recruitment. Note the absence of IL-5 does not affect T cell recruitment to the lung but affects further eosinophil recruitment to the lung. This is due to the assumption in the model that there are resident airway eosinophils that respond to

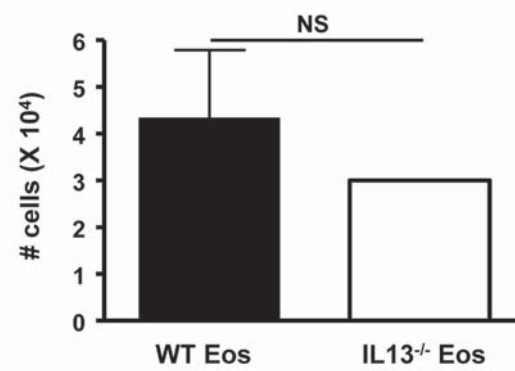
allergen. When this requirement was removed, the simulations did not match the experimental data (see **Supplementary figure 8**). We therefore implicitly modeled the network with a population of airway eosinophils that exist under steady state non-inflammatory conditions (e.g. as in supplementary figure 2).

Supplementary Figure 8. Role of resident airway eosinophils in recruitment of T cells to the lung. Simulation of allergic airway inflammation and T cell recruitment in WT mice (●) or mice that have been selectively depleted of airway eosinophils (○). Note the absence of airway eosinophils prevents the recruitment of T cells during allergic airway inflammation. This supports to model's assumptions for the presence of these cells in the airway, also supported by experimental data in supplementary figure 2.

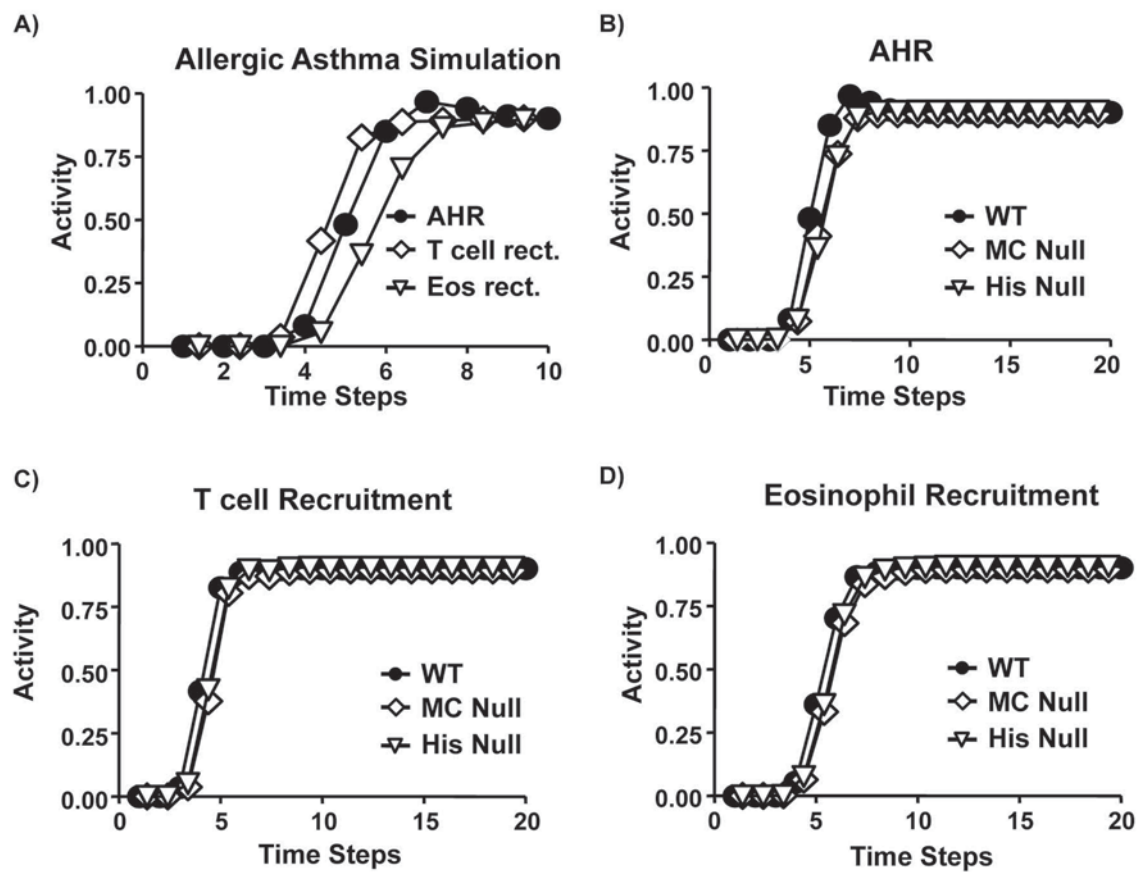
Supplementary Figure 9. Role of NKT cells in recruitment of T cells to the lung. Simulation of allergic airway inflammation and T cell recruitment in WT mice (●) or NKT cell null mice (○). Note the absence of NKT cells affects the recruitment of T cells during allergic airway inflammation.



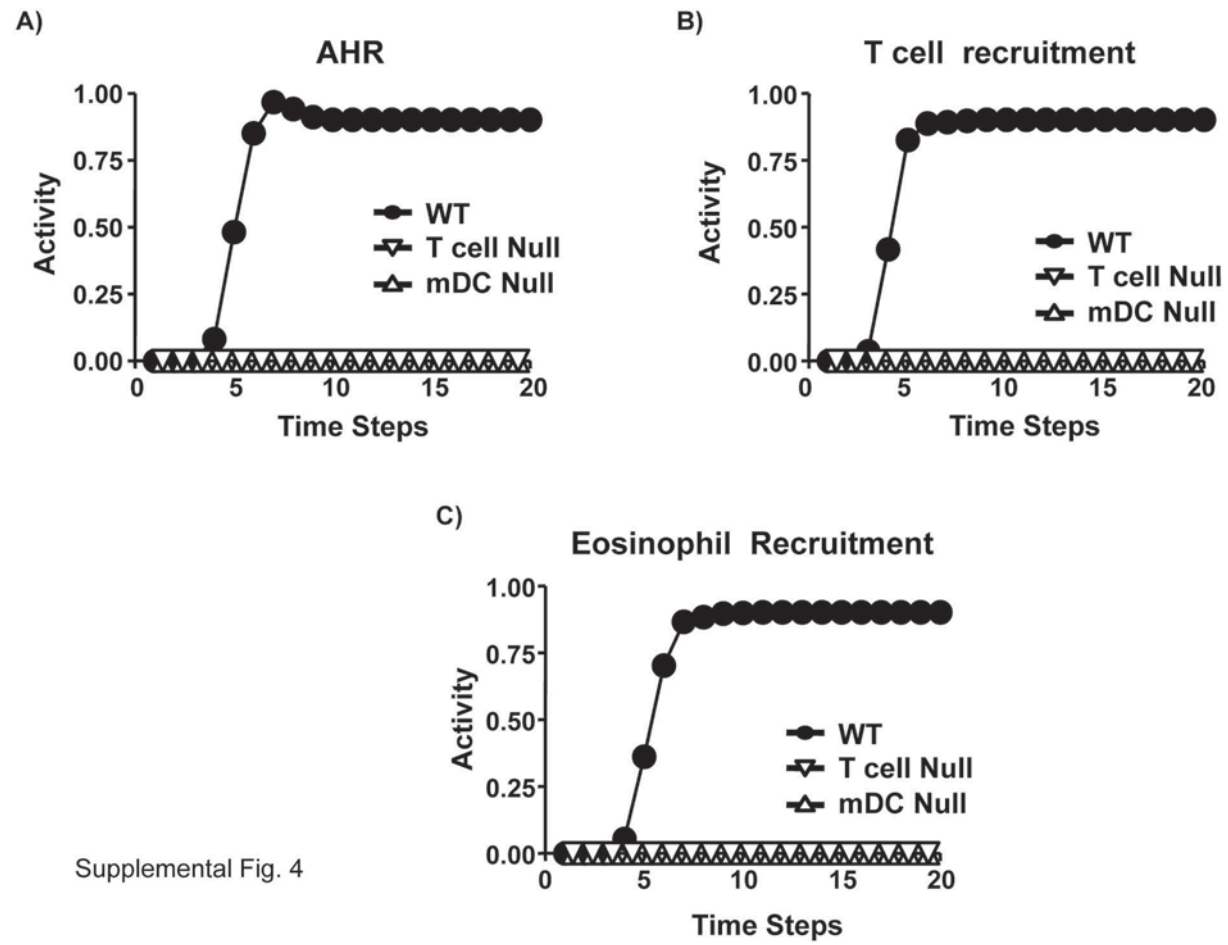
Supplemental Fig. 1



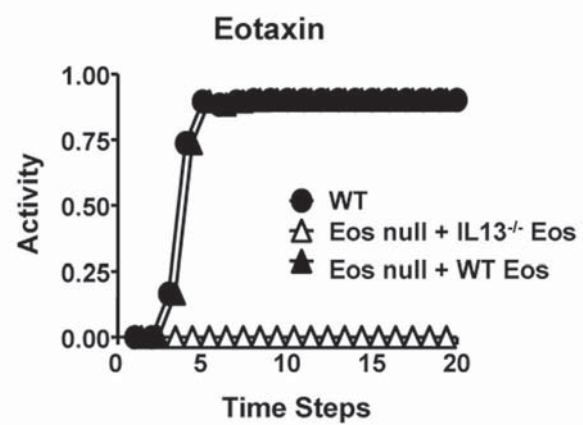
Supplemental Fig. 2



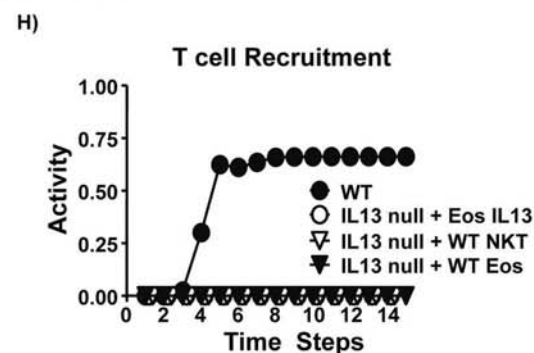
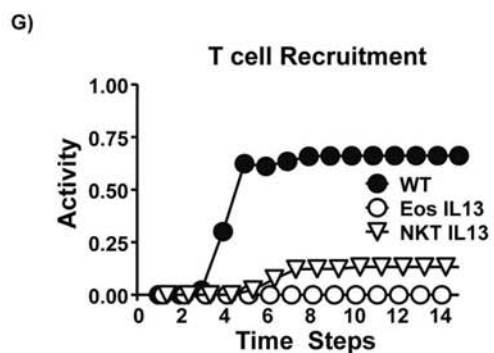
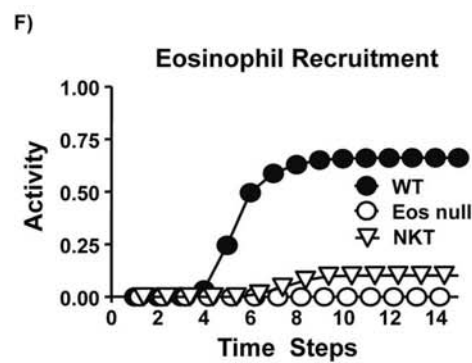
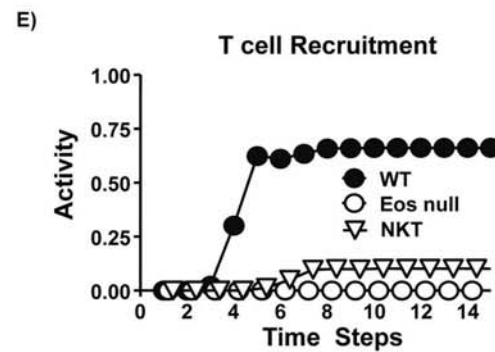
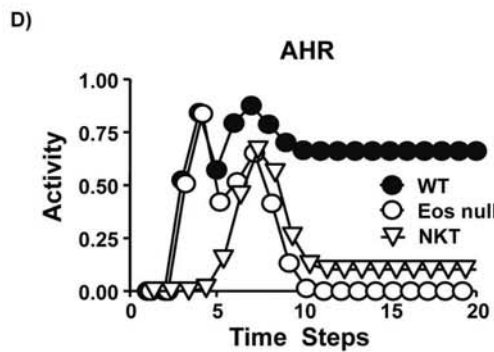
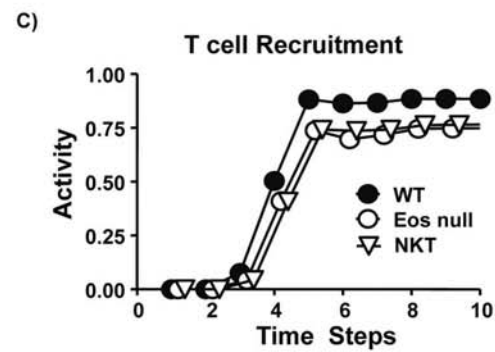
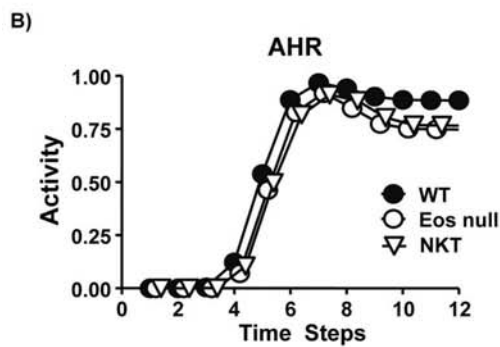
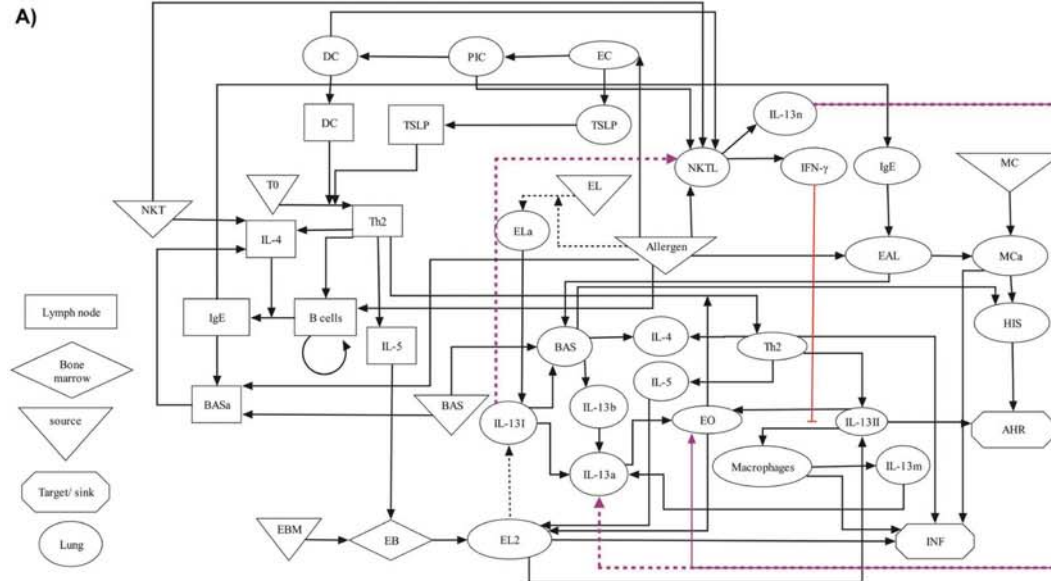
Supplemental Fig. 3

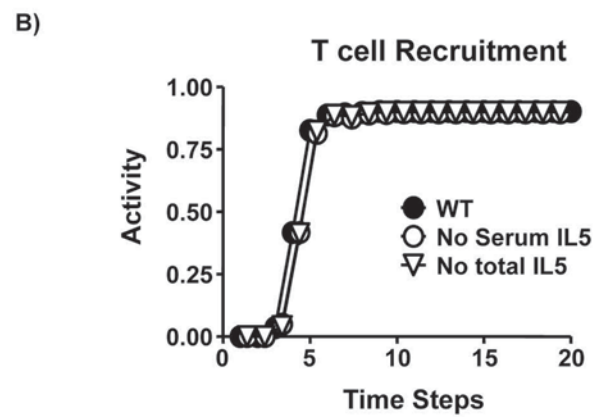
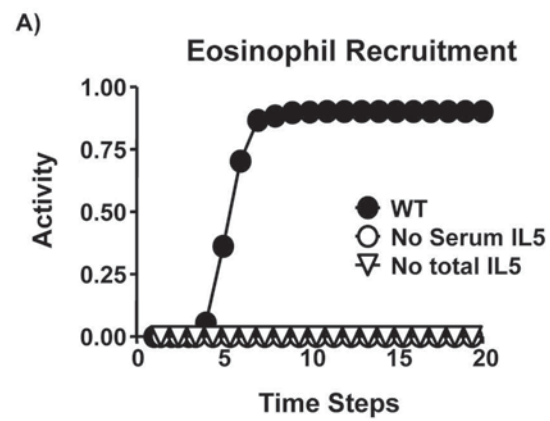


Supplemental Fig. 4

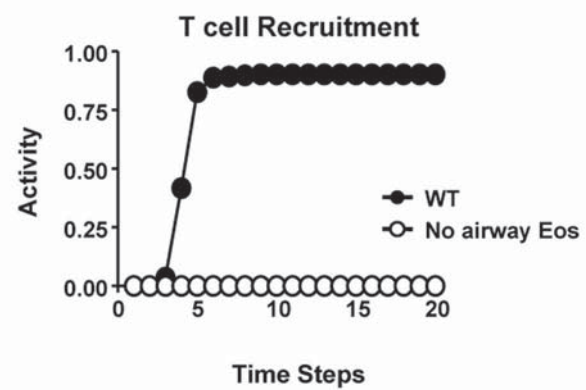


Supplemental Fig. 5

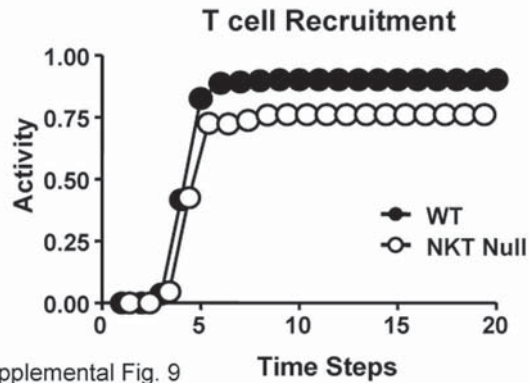




Supplemental Fig. 7



Supplemental Fig. 8



Supplemental Fig. 9