Published in final edited form as:

Trends Immunol. 2019 June ; 40(6): 538–552. doi:10.1016/j.it.2019.04.003.

Notch Signaling Orchestrates Helminth-Induced Type 2 Inflammation

Lauren M. Webb¹ and Elia D. Tait Wojno¹

¹Baker Institute for Animal Health and Department of Microbiology and Immunology, Cornell University College of Veterinary Medicine, Ithaca, New York, U.S.A.

Abstract

Infection with helminth parasites poses a significant challenge to the mammalian immune system. The Type 2 immune response to helminth infection is critical in limiting worm-induced tissue damage and expelling parasites. Conversely, aberrant Type 2 inflammation can cause debilitating allergic disease. Recent studies have revealed that key Type 2 inflammation-associated immune and epithelial cell types respond to Notch signaling, broadly regulating gene expression programs in cell development and function. Here, we discuss new advances demonstrating that Notch is active in the development, recruitment, localization, and cytokine production of immune and epithelial effector cells during Type 2 inflammation. Understanding how Notch signaling controls Type 2 inflammatory processes could inform the development of Notch pathway modulators to treat helminth infections and allergies.

The multi-faceted mechanisms of Type 2 immune activation

Multicellular eukaryotic helminth parasites afflict over a billion humans worldwide [1,2]. Soil-transmitted intestinal helminths, including *Trichuris trichiura*, *Ascaris lumbricoides* and *Ancylostoma* species, water-borne *Schistosoma* trematodes, and filarial parasites such as *Brugia malayi*, cause significant morbidity and economic loss [3]. Current anthelmintic drugs have variable efficacy, treated patients frequently become re-infected, and there are currently no effective vaccines to prevent infection in humans [4]. Thus, a better understanding of the immune mechanisms that mediate natural host protection against helminths is required to develop better treatments and vaccines to limit infection-associated pathology.

Type 2 inflammation is associated with infection with parasitic helminths. This host response can drive tissue repair of worm-induced damage and elimination or sequestration of worms and parasite products. Innate immune effector cells cooperate [5,6] to promote activation of CD4⁺ T helper 2 (Th2) cells and humoral responses [7]. Dendritic cells (DCs) educate Th2 cells in the lymph node (LN), eliciting production of antigen-specific Type 2

To whom correspondence should be addressed: Elia D. Tait Wojno. elia.taitwojno@cornell.edu.

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cytokines, including interleukins (IL)-4, 5, 9 and 13 [8]. Mammalian Th2 responses in tissues are supported by group 2 innate lymphoid cells (ILC2s) [9], basophils [10], and mast cells [5]. Type 2 cytokines produced by innate and adaptive cells also drive the polarization of **alternatively-activated macrophages** and eosinophil activation [11]; together, they facilitate wound repair and **granuloma** formation to enclose toxic helminth products [1]. Type 2 cytokines also act directly on epithelial cells in tissues, promoting increased cell turnover, mucus production, and production of anti-helminth proteins [12]. Collectively, these mechanisms are also a hallmark of debilitating inappropriate allergic inflammation in response to environmental substances such as food allergens, pollen and house dust mite (HDM) allergens that causes significant morbidity [13]. Thus, Type 2 inflammation during helminth infection and allergy has important implications for public health. Research efforts aimed at dissecting the mechanisms that control Type 2 inflammation could inform the development of new putative drugs or vaccines to manage these diseases.

While cytokines, bioactive lipids, and other factors can promote or restrain Type 2 inflammation, recent literature has highlighted the importance of the Notch receptor-ligand pathway in regulating Type 2 responses. Notch was originally identified for its functions in embryogenesis, but it also plays a role in organismal homeostasis, regulating epithelial cell differentiation and hematopoiesis; moreover, it contributes to the pathogenesis of human diseases, including hypertension, chronic obstructive pulmonary disease, and certain cancers [14]. In the past decade, Notch has been identified as a key regulator of developmental and functional gene expression programs in immune cells [15]. In this review, we discuss recent advances describing how Notch signals orchestrate Type 2 inflammation. We also highlight opportunities to therapeutically fine-tune Notch-dependent effector functions during Type 2 inflammation to treat helminth infections and allergies, and pose key questions in the field.

Notch, a key pathway for Type 2 immune mechanisms

Notch is a ubiquitous receptor-ligand signaling pathway that controls gene expression programs in many contexts, including mammalian immune activation (Box 1 and Figure 1). Catastrophic tissue damage can occur during helminth infection, for example in *Schistosoma mansoni* infection (**schistosomiasis**), in which transit of parasite eggs through tissues causes damage, fibrosis, vascular remodeling and organ dysfunction [16]. This potential for severe damage demands that host tissue-protective gene expression changes be rapid, broad-sweeping and highly organized. Notch-mediated modulation of global gene expression programs is therefore a potentially effective mechanism to elicit such changes [17]. Also, Notch-mediated cell-cell signals allow for rapid alteration of gene expression on a local scale [18], with studies employing cell lines that express luciferase reporters for Notch signaling demonstrating rapid transduction of Notch-mediated signals from the cytoplasm to the nucleus [17]. Thus, Notch can directly modulate effector cell responses in helminth-infected tissues without involving peripheral lymphoid tissues or systemic processes. Finally, as Notch signaling requires intimate cell-cell interactions [14], this mechanism could also play a role in the spatial organization of Type 2 responses in complex tissues such as the intestine.

Notch signaling intersects with other pathways, including intracellular signaling pathways and cytokine, hormone, and lipid signaling [19]; thus, Notch-responsive cells integrate a number of signals. This fact is particularly relevant in the context of murine models of helminth-induced Type 2 inflammation, in which immune effector cells in the tissue are exposed to an array of signals, including **alarmins** and cytokines such as IL-25, IL-33 and thymic stromal lymphopoietin (TSLP), released by dying and/or damaged epithelial cells [19]. Here, we discuss how Notch signaling can control the gene expression programs and functions of Type 2 immune effector and epithelial cells, as well as how the combined effects of Notch signaling on these diverse cells influence the dynamic Type 2 inflammatory environment.

ILC2s: At the forefront of helminth-induced damage detection

Like other **innate lymphoid cell** (ILC) types, ILC2s are innate sentinels found at mucosal and lymphoid tissues in humans and mice [9]. While ILC2s are rare compared to Th2 cells -- their adaptive counterparts, they are potent Type 2 cytokine producers [20]. ILC2s, unlike Th2 cells, are not activated by antigens, but respond to epithelial- and tissue-derived factors, including IL-25, IL-33, and TSLP, leading to rapid Type 2 cytokine production at sites of inflammation and in the draining lymph nodes (dLN) in mouse models [21,22]. Recent studies have highlighted critical ILC2 responses that drive Type 2 inflammation and which are also regulated by the Notch pathway.

Early studies suggested that Notch could induce ILC2 differentiation *in vitro* [23], with the strength of Notch1 signals instructing ILC2 lineage specification in human thymic progenitors *in vitro* [24]. In mice, inhibition of pan-Notch signaling in bone marrow (BM) LSK cells via retroviral transduction with a genetically-encoded dominant-negative form of the adaptor molecule **Mastermind-like (MAML)** (DNMAML, [25] (Box 1)) limited ILC2 development from LSK cells following *in vivo* transfer [26]. Following intranasal papain exposure, or during infection with the rodent hookworm-like parasite *Nippostrongylus brasiliensis*, transferred DNMAML-expressing BM ILC precursors failed to generate normal ILC2 numbers in irradiated recipients, with Notch acting upstream of the transcription factor Tcf-1 to promote ILC2 development [26].

Another study demonstrated that once murine ILC2s enter the periphery, heterogeneous ILC2 subsets emerge, with tissue-resident **natural (nILC2s)** and **inflammatory (iILC2s)** subsets accumulating in the lung during pulmonary inflammation in response to *N. brasiliensis* infection [27]. Murine iILC2s preferentially respond to IL-25 rather than IL-33, while the converse may be true for nILC2s. Murine iILC2s express KLRG1 and are functionally plastic, co-expressing the typical ILC2 product IL-13, as well as IL-17 [27] -- thought to be predominantly expressed by ILC3 [9]. Recent studies have shown that Notch plays a role in regulating these heterogeneous ILC2 populations. Specifically, in a murine model of Type 2 airway inflammation driven by IL-25 treatment, co-administration of IL-25 and the Notch-inhibiting gamma-secretase inhibitor (GSI) compound E, resulted in decreased iILC2 lung accumulation *in vivo* compared to administration of IL-25 alone, indicating that Notch was required for pulmonary iILC2 accumulation [28]. Furthermore, exposure of murine nILC2s to Notch ligands *in vitro* promoted expression of *Rorc*, encoding

a master transcription factor for ILC3 differentiation, and co-production of IL-13 and IL-17; this suggested that Notch could act as a key regulator of ILC2 functional plasticity [28]. Notch-mediated ILC2 functional plasticity might be physiologically significant in the murine lung, since IL-17 production during Type 2 immunity can result in increased neutrophilia and tissue damage [29]. Indeed, during murine lung inflammation elicited by IL-25 and HDM exposure, administration of blocking anti-Notch1/Notch2 antibodies compared to isotype control antibody resulted in reduced iILC2s co-production of IL-13 and IL-17 and reduced neutrophilia in the bronchoalveolar lavage (BAL) [28].

Notch signaling can also regulate murine lung ILC2 responses indirectly by affecting cell types that suppress inflammation, namely, T regulatory (Treg) cells. For example, during murine respiratory syncytial virus (RSV) infection, treatment with an anti-delta-like ligand 4 (Dll4) antibody (to block the Notch ligand, Dll4), relative to treatment with an isotype control antibody, resulted in increased ILC2 accumulation in lungs and dLN and elevated production of IL-5, IL-13 and IL-17 from *in vitro* RSV-restimulated mouse mediastinal LN cells [30]. This phenotype was associated with decreased Treg cell accumulation in the dLN and acquisition of Th17-like properties by Treg cells [30]. Together, these studies suggested that Notch could directly and indirectly control ILC2 development, and function in homeostasis, as well as in Type 2 inflammation in mice. Whether Notch is an essential signal for ILC2 plasticity and whether Notch preferentially controls the development or recruitment of iILC2s relative to nILC2s remains unclear and warrants robust investigation. Moreover, it is worth noting that the critical Notch target genes that can influence ILC2 phenotype and function remain to be identified in both mice and humans.

Dendritic cells: The influence of Notch on Th2 priming

In mammals, DCs are professional antigen-presenting cells (APCs) that sample antigen in tissues, migrate to dLNs, and present antigen to CD4⁺ T cells, providing costimulation and other polarizing signals to drive priming of specific T cell fates [8,31]. Even before DCs were identified as key inducers of Th2 cell priming [32,33], DC expression of Notch ligands was known to influence Th2 differentiation of murine CD4⁺ T cells [34] (Fig. 2). Specifically, mouse splenic CD11c⁺ cells [35] and GM-CSF-elicited BMDCs (GMDCs) [34] were found to express a variety of Notch ligands. Seminal work showed that GMDCs upregulated the expression of Delta-like ligand genes in conditions that promoted Th1 priming (*in vitro* stimulation with the **TLR** ligand, lipopolysaccharide), but upregulated expression of Jagged genes in conditions that promoted Th2 priming (exposure to cholera toxin or prostaglandin E₂) [34].

Subsequent studies have further dissected how DC expression of Notch ligands could influence Th2 cell priming and function. For example, compared to ovalbumin (OVA)-pulsed GMDCs transduced with empty retroviral vector, OVA-pulsed GMDCs transduced with vectors driving Dll1 and Dll4 over-expression inhibited IL-4 production and promoted IFN γ production from **OVA-specific OTII mouse T cells** *in vitro* [36]. Moreover, in a mouse model of OVA-driven allergic airway disease, adoptive transfer of OVA-primed GMDCs subjected to small-interfering RNA-mediated *Jag1* knock-down, compared to transfer of GMDCs with intact *Jag1* expression, resulted in decreased allergic disease

following OVA challenge [37]. Another study showed that intranasal HDM treatment of mice was associated with increased *Jag1* expression by CD11b⁺ **conventional DCs (cDCs)** in the mediastinal LN relative to controls [38]. Furthermore, HDM-pulsed, transferred GMDCs from *Jag1/Jag2*-floxed *Cd11c*Cre mice, lacking Jag^{1/2} expression in CD11c⁺ myeloid cells, did not promote allergic airway inflammation in the BAL following HDM challenge, compared to Jag^{1/2}-sufficient GMDCs [38]. These data support the notion that Jag ligand expression by DCs can drive Th2 priming. However, the latter study also showed that whole-body *Jag1/Jag2*-floxed *Cd11c*Cre mice and mice with intact Jag^{1/2} expression in CD11c⁺ cells exhibited similar allergic responses in the BAL following HDM sensitization and challenge [38]. Along these lines, another report indicated that while *S. mansoni* egg antigens (SEA) promoted GMDC *Jag2* upregulation *in vitro*, *Jag2* siRNA knockdown in GMDCs did not affect their ability to promote Th2 differentiation *in vitro* or *in vivo* [39]. Thus, DC expression of Jag ligands likely plays a role in Th2 priming; however, it may not be essential in all settings.

Outside of questions related to the role of Notch ligand expression by DCs during T cell priming, Notch receptor expression by DC subsets certainly impacts T helper responses. Studies utilizing DC-specific inhibition of Notch signaling in *Rbpj*-floxed *Cd11c*Cre mice have demonstrated that the absence of Notch signaling in CD11c⁺ cells alone results in a selective loss of CD8α⁺ cDCs in the spleen [40]. *Notch2*-floxed *Cd11c*Cre mice also had a specific deficiency in splenic CD8α⁺ CD11b⁺ cDCs that co-expressed the adhesion molecule **Esam**, as well as intestinal CD11b⁺CD103⁺ DCs [41]. Of note, it is unclear how Esam⁺ cDCs function in Type 2 inflammation, though CD11b⁺CD103⁺ DCs can play pleiotropic roles in **Treg** and **Th17** cell polarization [41–43] and murine intestinal cDCs and CD11b⁺CD103⁺ DCs can drive Th2 cytokine production in the dLN in response to SEA challenge [44]. Together, these data suggest that Notch may promote the development of specific murine cDC subsets that direct the phenotype and function of various Th fates, including Th2 cells in the intestine. In humans, peripheral blood cDCs and **plasmacytoid DCs (pDCs)** express Notch receptors, and *in vitro*, exposure to Notch ligands causes human DCs to upregulate Notch-responsive genes such as *HES* and *HEY1*, and to alter the capacity of cDCs and pDCs to elicit cytokine production from T cells in response to TLR ligands [45]. While this latter study did not directly assess how specific Notch ligand exposure shapes the ability of human DCs to promote Th2 priming, overall, Notch signaling in DCs may play a role in the differentiation and function of cDC subsets, with the capacity to drive **Th2 priming** in specific tissues in mice and humans.

Despite the findings described above, further study is required to understand the importance of Notch for cDC subsets and pDCs in Type 2 immunity at various tissue sites. Of particular interest is whether Notch ligands or receptors play a role in the biology of **cDC2s**, critical APCs for Th2 priming during *N. brasiliensis* infection, as well as during HDM-driven allergic inflammation in mice [46,47]. In addition, it is unclear whether cDC2s and **cDC1s**, which are primary drivers of IFNγ production [48], exhibit differential expression of Notch ligands or receptors, and whether the highly immunomodulatory products of helminths might influence DC function and phenotype [49] through alteration of Notch ligand or receptor expression. To fill the gaps in our understanding of how Notch-driven processes

govern Th2 differentiation, further mouse studies are required aiming to target Notch function in cDC2s and other DC subsets *in vivo* during Type 2 inflammation. The impact of these murine studies will be enhanced with further work using human cells.

Th2 cells: Notch as a key player in adaptive Type 2 immunity

The role of Notch in Th2 cell fate and function

Outside of the DC-intrinsic roles for Notch in regulating Th2 priming, there is a critical role for T cell-intrinsic Notch in Th2 differentiation and function. The same mouse study that described a role for distinct Notch ligands in inducing Th1 vs. Th2 differentiation also established a role for CD4⁺ T cell-intrinsic Notch signaling in regulating gene expression programs required for Th2 fate [34]. Murine CD4⁺ T cells lacking the Notch transcription factor Rbpj failed to acquire IL-4-producing capacity when exposed to Jagged-expressing DCs *in vitro*, and retroviral transduction of CD4⁺ T cells with activated Notch1 resulted in increased *Gata3* expression and direct regulation of *Il4* via Rbpj relative to controls [34]. Additionally, *in vivo*, *Rbpj*-floxed *Cd4*Cre mice displayed poor IL-4 responses to SEA challenge relative to littermate controls [50].

Additional studies have now delved into the molecular mechanisms by which Notch signaling can influence Th2 differentiation. In studies employing transduction of CD4⁺ T cells with the Notch intracellular domain (NICD) or DNMA1L-floxed *Cd4*Cre T cells and measurement of *Gata3* exon expression *in vitro*, Notch signaling directly regulated the transcription of the *Gata3* exon 1a but not 1b, acting upstream of *Gata3* to promote Th2 differentiation [50,51]. Notch-independent transcript expression of exon 1b was detected at early timepoints during Th2 differentiation following TCR stimulation of murine CD4⁺ T cells *in vitro* [52], suggesting that Notch might not play a key role in *Gata3* activation in early priming. Rather, Notch might regulate *Gata3* to promote Th2 maintenance. Consistent with this idea, DNMA1L-floxed *Cd4*Cre mice exhibited dampened Type 2 effector mechanisms during infection with the murine whipworm *Trichuris muris* relative to *Cd4*Cre mice [53,54]; however, the Notch-dependent effects on Th2 responses and worm clearance could be rescued with anti-IFN γ treatment [54]. Notch can also control murine T cell survival [55], as mice treated with a Dll4 blocking antibody compared to an isotype control presented with exacerbated allergic lung inflammation in a cockroach allergen-driven model of allergy, with increased CD4⁺ T cell activation and global *Il2* expression in the lung [56]. This study also found that murine CD4⁺ T cells exposed to Th2-inducing conditions *in vitro*, and which were cultured with Dll4 ligand for 5 days, exhibited increased apoptosis after 24–48 hours of rest time, compared to cells cultured in the absence of Dll4 ligand [56]. Together, these data indicate that T cell-intrinsic Notch does not seem to play a key role in the initiation of Th2 differentiation, but rather, that it might reinforce this process by maintaining the Th2 response, possibly via modulation of *Il4* expression in Th2 cells [50,53,54], or by promoting Th2 survival [55,56] (Fig. 2). However, these possibilities remain speculative at this point and will require further investigation.

The influence of Notch on Th2 cells beyond helminth infection

Notch-dependent T cell production of Type 2 cytokines occurs in settings outside of helminth infection or allergy, including during viral and fungal infection and **graft vs. host disease (GvHD)**. For instance, following murine RSV infection, antibody-mediated Dll4 inhibition led to increased frequencies of dLN CD4⁺ T cells and IL-13⁺ lung Foxp3⁺ Tregs [30], and increased parameters of Type 2 inflammation, including airway resistance and mucus production [57], relative to controls. During RSV-elicited exacerbation of cockroach antigen-induced allergy in mice, anti-Dll4 antibody treatment compared to isotype treatment reduced these same Type 2 inflammatory readouts after challenge [58]. Furthermore, the glycosyltransferase lunatic fringe (Lfng), which promotes Dll4-mediated Notch activation, was required in CD4⁺ T cells for fulminant Type 2 inflammation in this model, as evidenced by a decrease in mucus production and IL-4 and IL-13 production in the lungs of *Lfng*-floxed *Cd4*Cre mice, compared to control mice, following allergen challenge [58]. Upon infection with the fungus *Cryptococcus neoformans*, effective fungal control in the lung depends on IFN γ ⁺ Th1 cells driving myeloid cell anti-microbial functions and Th17 cells reinforcing optimal IFN γ production [59]. In this model, mice that expressed DNMA1L only in CD4⁺ T cells had impaired Th1 and Th2 responses relative to littermate controls, with reduced macrophage and neutrophil infiltration, and delayed fungal clearance [59]. Finally, in a murine model of GvHD in which C57BL/6 CD4⁺ T cells were transferred into irradiated Balb/c hosts, transferred DNMA1L CD4⁺ T cells displayed impaired *ex vivo* production of T helper cytokines compared to transferred wild type (WT) CD4⁺ T cells [60]. Together, these data show that functional Notch signaling can regulate the ability of CD4⁺ T cells to produce Th2 cytokines in many inflammatory settings.

Notch in CD4⁺ T cell biology

How Notch contributes to the acquisition of regulatory or other T cell fates remains under study. A recent study found that Notch signaling could promote the development of **T follicular helper (Tfh)** cells that express *Ii4* during infection with *N. brasiliensis*, with *Notch1/Notch2*-floxed *Cd4*Cre mice exhibiting reduced Tfh that were *Ii4*⁺ in the dLN compared to littermate controls [61]. Notch signals could also sustain a Treg phenotype and function during murine viral infection; specifically, anti-Dll4 antibody treatment during RSV infection (compared to isotype control), led to reduced numbers of Foxp3⁺ Tregs in the mediastinal LN and increased Treg production of effector cytokines [30]. However, significant work is warranted to assess how Notch signaling impacts the development, effector function, and maintenance of Tfh and Treg cells. In addition, how Notch acts to balance effector vs. memory fates of CD4⁺ T cells in various Th contexts remains unclear. One report indicated that the *Ii4* enhancer, CNS2, was active in CD44^{hi} CD4⁺ T cells (effector or memory precursor cells) and depended on Rbpj for activation; this in turn suggested that Notch might be able to promote T cell memory [62]. Here again, further work is needed to dissect the putative role of Notch in T cell memory formation, specifically in Th2 cells during murine and human helminth infections. Thus, many exciting investigation areas remain, and which may provide a better understanding of the influence of Notch on T cell biology in Type 2 inflammation.

Mast cells and basophils: Notch in innate granulocytes

The innate granulocytes include mast cells, eosinophils, and basophils, which release large quantities of pre-formed mediators, such as cytokines and histamine, in response to immunoglobulin E (IgE), or other antibodies binding and crosslinking surface Fc receptors [5]. For this reason, the dogma for many years dictated that gene transcription was not altered to a significant degree in these cells once they matured, even during inflammation. However, studies from the last decade have shown that these three cell types can in fact respond to environmental cues from cytokines and other factors to alter gene expression [63–65], and furthermore, that Notch signaling is a key regulator of these responses.

For instance, Dll1 signaling to Notch2 has been shown to drive the upregulation of Notch-responsive genes *Hes1* and *Gata3*, facilitating mast cell differentiation from murine **granulocyte-macrophage progenitors** *in vitro* [65]. Notch can also affect murine mast cell activities during helminth infection: in *Notch2*-floxed *Mx1*Cre mice with Notch2-deficiency in mast cells, mast cells localized to the lamina propria rather than the epithelium during *Strongyloides venezuelensis* infection, a phenotype associated with impaired parasite clearance [66]. In addition, co-culture of murine BM-derived mast cells with Dll1, Dll4, Jag1 or Jag2-expressing cells led to enhanced IL-4, IL-6, and IL-13 production *in vitro* after crosslinking of the high-affinity IgE receptor, FcεR1α, compared to culture of crosslinked mast cells alone [67]. While much remains unexplored in this area, particularly related to the role of Notch in mast cell subset biology in various tissues, these data highlight a role for Notch in mast cell differentiation and function (Fig. 3).

Recent studies have shown that Notch signaling can also affect basophil gene expression, effector function and ability to promote Type 2 inflammation. *In vitro*, murine BM-derived basophils that were subjected to Notch inhibition using a chemical GSI, displayed decreased proliferation, increased cell death, and decreased *Il4*, *Il6* and *Il13* expression in response to a calcium ionophore, compared to basophils treated with vehicle alone [68]. Moreover, in a mouse model of airway allergy elicited by transfer of BM-derived basophils into OVA-sensitized mice, *Rbpj*^{-/-}, compared to WT, basophils drove reduced mucus production and airway hyperactivity in response to methacholine [69]. In the context of murine helminth infection *in vivo*, mice that expressed DNAML in basophils alone (DNAML-floxed *Mcpt8*Cre mice) had reduced goblet cell hyperplasia and decreased clearance of the helminth *T. muris* compared to littermate control mice [10]. While loss of basophil-intrinsic Notch signaling did not alter infection-induced changes in splenic or intestinal basophil population size, it was associated with altered basophil localization within the cecum. Notch signaling-deficient basophils were less likely than Notch signaling-sufficient basophils to localize to the cecal lamina propria and near the base of crypts; this was also associated with impaired accumulation of Gata3-expressing CD4⁺ Th2 cells and a reduction in intestinal *Il4* relative to controls [10]. Together, these findings suggest that Notch signaling is a key driver of effective mast cell and basophil localization in tissues associated with helminth expulsion.

At least in the case of basophils, the effects of Notch inhibition on Type 2 immune responses in the intestine have been specifically studied in murine basophils [10]. Such approaches offer distinct advantages to the researcher in dissecting how cell-specific effects of Notch

signaling can impact broad Type 2 inflammatory responses and disease pathogenesis. Follow-up studies that employ basophil-specific deletion of Notch signaling components may thus allow for further examination of the importance of Notch-dependent basophil function in supporting Th2 responses in tissues and/or the dLN.

Data linking Notch signaling to eosinophil responses are not well developed. Human peripheral eosinophils express Notch machinery components [70], and Notch can promote GM-CSF-driven eosinophil chemokinesis, as evidenced from *in vitro* studies of human cells employing transwell or endothelial monolayer transmigration systems, and from *in vivo* studies in mice using GSI treatment during airway eosinophilia-associated OVA airway challenge [70,71]. Of note, this latter study did not target Notch inhibition *in vivo* to eosinophils alone.

Regardless, there is now strong evidence that Notch is an important regulator of effective granulocyte localization within tissues, demonstrating that proper positioning is important for optimal cell function and Type 2 immunity (Fig. 3). Collectively, these findings highlight a role for Notch in fine-tuning the transcriptional profile and localization of rare innate effectors to direct their function.

Macrophages: Does Notch have a role in alternative activation?

Given the importance of Notch signaling in regulating other myeloid populations in Type 2 inflammation, it seems likely that **alternatively-activated macrophages** are also influenced by Notch. Alternatively-activated macrophages are key in wound repair and the formation of granulomas during helminth infections [75,76]. Some studies have suggested that Notch could play a role in granuloma formation, as *Schistosoma japonicum* egg (SEA) antigen stimulation promoted alternative-activation associated with relative *Notch1* and *Jag1* upregulation in a murine cell line [77]. *In vivo*, in response to **chitin administration**, mice carrying a myeloid-cell deletion of *Rbpj* in *Lyz2*-expressing cells exhibited reduced peritoneal eosinophil and macrophage infiltration and reduced peritoneal cell expression of gene transcripts encoding Arginase1 and the mannose receptor (alternative activation-associated molecules), relative to control mice [78]. While these studies suggest that Notch may promote alternatively-activated macrophage polarization, there are a number of caveats, as the genetic approach used to target macrophages in the latter study might not adequately target all macrophages and might have off-macrophage effects [79]. Furthermore, these experiments have not been replicated in helminth infection models. Finally, there are also data that suggest that Notch signals can inhibit alternative-activation, as human monocyte-derived macrophages polarized *in vitro* with IL-4 in the presence of Dll4 have displayed reduced expression of alternatively-activated molecules such as CD206 and increased apoptosis relative to macrophages cultured in the absence of recombinant Dll4 [80]. Thus, a specific role for Notch in macrophage function during Type 2 immunity remains unclear.

Epithelial cells: Key effectors downstream of Type 2 immunity

Epithelial cells are critical players during Type 2 inflammation - they are often the first to experience helminth parasite-induced damage or allergen exposure, releasing alarmins

IL-25, IL-33 and TSLP [19,81] that transduce damage signals to the immune system. Downstream of Type 2 immune activation, intestinal epithelial cell (IEC) responses are key to expulsion of gastrointestinal helminths [81]. Specifically, essential modifications to IEC biology are initiated in response to Type 2 inflammation-associated cytokines and are termed the “weep and sweep” response; well-characterized in the mouse model of helminth infection with *T. muris*, these inflammatory changes include increased IEC turnover, differentiation of **goblet cells**, mucus production, and secretion of anti-helminth molecules such as Relm β , by goblet cells [82–85].

Epithelial cells express all receptors and ligands involved in Notch signaling [86], and Notch and Wnt are critical signaling pathways that function antagonistically to control IEC development in the crypt [87]. In mice, Notch signaling inhibition, broadly or specifically in the epithelium, results in a loss of “stemness” and proliferative capacity of intestinal crypt cells and a skew towards development of secretory cells such as goblet and **Paneth cells**, leading to goblet cell hyperplasia and increased mucin production [86–88]. For example, mice treated with anti-Notch1/Notch2 blocking antibodies compared to control mice demonstrated a loss of stem cell markers (Lgr5) in the crypt base, reduced stem cell proliferation, and an increase in expression of markers associated with secretory fate, such as lysozyme, observed using immunofluorescence microscopy [87]. Such changes can reduce the absorptive capacity of the intestine, leading to diarrhea followed by weight loss [89]. In another study, compared to control mice, animals with a global knockout or epithelial-specific deletion of Hes1 (a Notch-dependent transcriptional repressor active in the intestinal epithelium), exhibited intestinal microbial dysbiosis and reduced expression of genes encoding antimicrobial peptides such as serum amyloid A and Reg3 family members [90].

Based on the role of Notch in IEC lineage specification and epithelial function, Notch is almost certainly involved in regulating IEC responses during helminth infection. Recent studies in mice have shown that a specific IEC type, the **tuft cell**, is the primary producer of IL-25 [91–93]. While little is known about the role of Notch in tuft cell function, IEC-specific knockdown of the tuft cell-associated transcription factor Dclk in *Dclk1*-floxed *Villin*Cre mice led to dysregulation of tuft cell development and altered IEC expression of Notch1 protein [94]. Studies by our laboratory and others have shown that mice in which Notch signaling was inhibited by DNMAML, and under the control of cell-specific Cre in basophils or CD4⁺ T cells, had reduced goblet cell hyperplasia during *T. muris* infection compared to control mice [10,53,69]. These findings suggest that immune cell-intrinsic Notch signaling may help to promote production of IL-4 and IL-13 or other factors that act on IECs to elicit goblet cell responses. We hypothesize that such production of Type 2 cytokines may be required to override IEC-intrinsic Notch-dependent suppression of secretory cell differentiation [86–88], allowing for the emergence of goblet cell hyperplasia and mucus production to drive intestinal helminth expulsion [95,96]. While IL-33 can decrease Notch ligand expression in the mucosa in a murine experimental model of dextran-sulfate sodium-dependent colitis, attenuating Notch signals and allowing for goblet cell hyperplasia [97], how immune cell-derived Type 2 cytokines function in such processes during helminth infection remains unclear. Future studies will be needed to determine how

IEC subtypes that mediate helminth sensing and clearance (such as tuft and goblet cells), balance distinct roles for Notch in the intestine during homeostasis and worm infection.

Harnessing the influence of Notch to control Type 2 function: A viable option?

Given the role that Notch plays in a large variety of developmental and functional pathways, it is not surprising that Notch is a potential drug target in many conditions and diseases. However, to date, there have been limited attempts to harness Notch signaling to alter the course of Type 2 inflammation. Recent studies have highlighted opportunities to block Notch to prevent the development of allergy, asthma or fibrosis, in particular. During *S. japonicum* infection, treatment of mice with the GSI N-[N-[3,5-difluorophenacetyl]-L-alanyl]-S-phenylglycine t-butyl ester (DAPT) relative to treatment with vehicle control was associated with a reduction in liver granuloma formation, suggesting that Notch inhibition could mitigate hepatic fibrotic responses [77]. While decreasing granuloma formation may be desirable in one sense to help maintain hepatic function, conversely, the granuloma is also highly protective against egg-induced damage during schistosome infection [16,98]. In a mouse model of OVA-dependent allergic rhinitis, mice treated with DAPT at the time of OVA challenge had reduced allergic symptoms and decreased concentrations of serum IgE and Type 2 cytokines relative to control mice [99]. While these and other studies provide some proof-of-concept that Notch signaling could be inhibited to prevent or treat fibrotic or allergic disease, further work is needed to elucidate how Notch functions in Type 2 immunity before serious preclinical studies can commence. In addition, since Notch also functions in the development and function of Th1 and Treg cells [30,54,100], on-target, yet undesirable effects of any Notch-specific therapies must be considered.

In the context of helminth infection, it could be envisaged that therapies would potentiate Notch function in order to increase Type 2 immune efficacy or perhaps block Notch in IECs to promote protective epithelial responses; however, such studies have not yet been attempted. Nevertheless, activation of Notch using small molecule agonists and recombinant ligands has certainly been possible *in vivo* in rodent models in other disease states. In a murine model of acute myeloid leukemia (AML) in which human AML cells were injected subcutaneously, mice with activated Notch demonstrated tumor suppression relative to control mice [101]. However, given that over-activation of Notch is often tumorigenic, any such approach should be developed with caution. Thus, future research has the potential to generate many new opportunities to target Notch, by limiting or promoting Type 2 immunity. However, significant work using mouse models and *ex vivo* analyses of human cells in patients with ongoing Type 2 inflammation will also be required to increase our basic understanding of Notch function in Type 2 inflammation (Box 2).

Concluding remarks: Notching it up to Type 2 immunity

Recent advances have provided new and exciting insights into the function of Notch signaling in Type 2 inflammation in helminth infection and allergy. Notch has effects on a multitude of cellular players in the Type 2 immune cascade including epithelial cells, innate immune cells and adaptive CD4⁺ T cells. Leveraging this unique biology, there is a growing

possibility that the Notch signaling pathway could offer new avenues to harness Type 2 effector cells therapeutically in helminth infections and allergies. In particular, future studies investigating how Notch regulates immune and epithelial function in inflamed tissues in mice and humans may provide new insights on how tissue architecture and cellular positioning might be manipulated to rapidly and dynamically regulate Type 2 inflammatory responses. While numerous outstanding questions remain (see Outstanding Questions), studies that can ultimately translate important findings from mouse models to humans are needed to fully realize the potential of Notch pathway targeting in the treatment of helminth infections and allergies.

Acknowledgements

This work was supported by the NIH NIAID (K22 AI116729, R01 AI132708 and R01 AI130379), the Cornell University Research in Animal Health Grants Program and Cornell University start-up funds (to E.D.T.W). The content of this manuscript is solely the responsibility of the authors and does not necessarily represent the official views of the NIH. The authors thank all members of the Tait Wojno lab for their constructive contributions to discussions around this work.

Glossary

Alarmin

signal released by dead or dying cells, in response to tissue damage (e.g. by pathogen challenge).

Alternatively-activated macrophage

phenotype in Type 2 inflammation promoting wound-healing and granuloma formation.

Conventional DCs (cDCs)

express the transcription factor Zbtb46 and feature high CD11c expression.

cDC1s

express Batf3 and the surface markers CD103/CD8 α ; they prime Th1 and CD8⁺ T cells.

cDC2s

depend on Irf4, have the surface marker profile CD11b⁺CD103⁻CD8 α ⁻, and prime Th2 cells.

Chitin administration

murine model inducing Type 2 inflammation via delivery of the immunoactivatory polysaccharide chitin.

Esam

surface adhesion marker.

Experimental autoimmune encephalomyelitis (EAE)

murine model for the human demyelinating disease multiple sclerosis.

Goblet cell

secretory epithelial cells producing mucus.

Graft-versus-host-disease (GvHD)

immune-mediated disease following allogeneic bone marrow transplant with donor immune cells, attacking host tissues.

Granulocyte-macrophage progenitor

hematopoietic precursor for macrophages, DCs, monocytes, basophils, eosinophils and mast cells.

Granuloma

tissue lesion that walls off pathogens or their products; composed of leukocyte infiltrates, collagen and fibrotic tissue.

Inflammatory ILC2s (iILC2s)

IL-25-responsive subset that can develop into nILC2s.

Innate lymphoid cells (ILCs)

rare innate lymphocytes lacking antigen-specific receptors, dividing rapidly and producing cytokines upon stimulation.

Mastermind-like (MAML)

Mastermind-like protein family members act as co-activators for Notch-regulated transcription.

Natural ILC2s (nILC2s)

subset found in mucosal tissues in the steady-state, expanding during Type 2 inflammation and expressing ST2.

OVA-specific OTII mouse T cells

murine CD4⁺ T cells that express a transgenic T cell receptor specific for peptide from chicken egg ovalbumin.

Paneth cells

secretory epithelial cells producing antimicrobial compounds; they help maintain the intestinal barrier.

Plasmacytoid DCs (pDCs)

highly specialized DCs that rapidly produce Type I IFNs.

RBPJ

transcriptional repressor in the absence of Notch; transcriptional activator of target genes upon Notch activation.

Schistosomiasis

fibrotic disease caused by infection with *Schistosoma* trematodes, water-borne helminths that reside within host vasculature.

Stemness

ability of a stem cell to develop into different types of fully-differentiated cells.

T follicular helper cells (Tfh)

CD4⁺ T cells in germinal centers that support B cell selection, class switching, and affinity maturation.

Th17

CD4⁺ T cells express the transcription factor Ror γ t; they produce IL-17 and IL-22 and act against extracellular bacteria and fungi.

Th2 priming

polarization of naïve CD4⁺ T cells into Th2 cells.

TLR

pattern-recognition receptors recognizing pathogen-associated molecular patterns.

Treg

CD4⁺ T cells that suppress immune responses.

Tuft cells

chemosensory IECs producing IL-25 in response to helminths.

Type 2 inflammation

inflammation type activated during helminth parasite infection and allergy.

Weep and sweep

epithelial cell response facilitating parasite clearance, featuring increased mucus production, cell turnover and mucosal barrier permeability.

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Outstanding questions

- What are the key Notch receptors and ligands that impact immune cell function in Type 2 inflammation?
- In what tissue(s) do Type 2 immune effectors encounter Notch ligands?
- Is the timing of Notch signaling in the course of the Type 2 inflammatory response important for altering the outcome of the signal for a specific cell type?
- How do Type 2 inflammation-associated immune cells and IECs integrate Notch signals with other signals from cytokines and lipid mediators to regulate gene expression?
- Does Notch function in the development of adaptive memory responses and in innate immune cells during Type 2 inflammation?
- Is the Notch pathway involved in the development, recruitment or function of eosinophils and alternatively-activated macrophages?
- How do helminths, their products and allergens modulate the function of the Notch pathway in Type 2 inflammation?
- Are Notch receptor-ligand interactions important mediators of cellular positioning in the tissue that promotes fulminant Type 2 inflammation?

Highlights

- Notch contributes to the development and function of key Type 2 effector cell types, including ILC2s, mast cells, basophils, Th2 cells and IECs
- Notch is required to maintain Th2 and other Th fates in T cells
- Notch is a driver of epithelial cell fate in the intestine, with the intestinal site strongly influenced by Notch inhibition
- Targeted blockade of Notch signaling in various Type 2 effector cell types, including basophils and CD4⁺ T cells, leads to the abrogation of effective Type 2 immunity and impaired worm clearance during helminth infection
- Therapeutic manipulation of Notch signaling-dependent cellular responses is a potential avenue for the treatment of diseases associated with Type 2 inflammation, including helminth infection and allergy

Box 1. The Notch signaling pathway

The Notch signaling pathway is a critical mechanism of cell-cell communication that is highly conserved, from *Drosophila* to worms and all mammals [102]. It is active in many cellular processes, including embryogenesis, cell lineage specification, apoptosis, and immune activation. There are 4 receptors and a similar number of ligands that participate in sensitive and dynamic receptor-ligand interactions, influencing downstream gene expression changes at a single cell level [102]. The Notch signaling machinery is expressed by a multitude of cell types, immune as well as non-immune.

Notch signaling requires accessory enzymes for activation, and is modulated by post-translational modifications [18]. It involves rapid conversion of a cell surface receptor into an intracellular signaling molecule that promotes changes in gene transcription. Ligation of a Notch receptor (Notch1–4) by a Notch ligand (Jagged 1 or 2, or Delta-like ligands (Dll) 1, 3 or 4) leads to cleavage of the intracellular domain of the receptor (NICD) by γ secretase enzymes, allowing the translocation of the NICD to the nucleus [18]. The NICD then forms a complex with accessory proteins and coactivators, together with Mastermind-like protein (**MAML**), and the transcription factor **RBPJ** (also called CSL). This complex binds to Notch target sites across the genome, rapidly altering gene transcription [15] (Figure 1).

Box 2. Notch receptor ligand-interactions: immune regulation in time and space

A major gap in knowledge in the study of Notch in the immune system is the lack of understanding of where and when immune cells encounter critical Notch ligands and ligand sources. A recent study in a mouse model of GvHD identified LN stromal cells, particularly fibroblasts, that had expressed or were expressing Ccl19 – a key source of induction of Notch ligands in the priming of alloreactive T cells [103]. The authors suggested that chemokine expression attracted T cells to specific stromal cell niches, where they encountered activating signals, including Notch ligands, over a sustained period of time [103]. In support of this idea, deletion of Delta ligands in Ccl19⁺ stromal cells inhibited splenic Esam⁺ cDC2 development in the steady-state, and resulted in defective Tfh and germinal center formation following immunization with *S. mansoni* eggs [104]. These data indicated that LN fibroblastic stromal cells may be important sources of Notch ligands shaping DC and T cell responses, by orchestrating the spatial organization of cells within the LN.

While innate Type 2 effector cell types other than DCs may not spend considerable time in LNs, Notch might also control spatial organization of innate cells across tissues or within a tissue such as the intestine, promoting their function. Accordingly, basophil-intrinsic Notch signaling has been shown to be required for normal basophil localization in the intestinal crypt architecture associated with optimal Type 2 inflammatory and Th2 responses in the murine intestine following *T. muris* infection [10]. In this context, it is possible that basophils may encounter instructive ligand-bearing cells in the periphery enabling them to migrate and localize into tissues. Perhaps more likely, however, basophils might encounter ligand-bearing cells in inflamed tissues, with Notch signaling acting either to retain these cells and ensure their function at specific locations, or to provide a signal that promotes function, only once the basophils reach a specific location within the tissue. Candidate ligand-bearing cell types in this setting could include vascular endothelial cells, fibroblastic stromal cells and epithelial cells, although this clearly remains to be tested [103,105,106]. Ultimately, across cell types, it remains to be determined how Notch signaling organizes cellular responses in both space and time in tissues and LNs during helminth infections to promote a balanced inflammatory response that is effective in protecting the host, yet avoids unnecessary tissue damage.

Figure 1. Notch receptor activation drives rapid transcription of Notch target genes.

Ligation of a mammalian Notch receptor (Notch 1–4) by a Notch ligand (Delta/Jagged) on the cell surface (various cell types) leads to intracellular cleavage of the Notch intracellular domain (NICD) by a γ secretase enzyme. The NICD then translocates to the nucleus, where it forms a transcription-activating complex composed of the transcription factor Rbpj (also known as CSL in humans), various accessory proteins and co-activators (CoA) and Mastermind-like (MAML) proteins. This complex binds to Notch target sites across the genome to induce transcription of Notch target genes.

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Figure 2. The role of the Notch pathway in human and mouse Th2 cell development and function.

Notch signaling plays a role in T helper type 2 (Th2) cell polarization and maintenance by affecting a variety of processes during Type 2 inflammation. Conventional dendritic cells (cDCs) in mucosal tissue are exposed to antigens during Type 2 inflammation, in this case, helminth infection in the intestine. This includes Type 2-associated antigens (Type 2-Ag), including helminth Ag, and those that induce Type 1 responses (Type 1-Ag), such as those from bacteria or viral species. Type 2-Ag and Type 1-Ag drive upregulation of Jagged and Delta ligands on cDCs, respectively. cDCs traffic to the draining lymph node (dLN) and interact with naïve CD4⁺ T cells. Jagged upregulation on cDCs may support a Th2 cell fate by activating *Gata3* and *Il4* expression downstream of Notch. Delta ligands expressed by cDCs may inhibit Th2 priming and induce a Th1 cell fate by inhibiting *Il4* expression and driving *Ilfng* induction. Notch ligands are also expressed in the dLN by non-hematopoietic cells, including structural cell populations such as stromal fibroblasts. Exposure to Notch ligands on these cells may drive expression of functional genes and induce migration to inflamed tissue. Further exposure to Notch ligands in the periphery or inflamed tissue (such as the intestine) may also help to maintain a Th2 cell fate by promoting continued *Gata3* and *Il4* expression [54]. The exact location and ligand-bearing cell(s) in this interaction are not known. Notch ligands can regulate T cell survival and apoptosis, while also influencing the development and function of Tregs (not depicted), which have the potential to limit Type 2 activation to resolve inflammation [30].

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Figure 3. Notch can drive the differentiation and function of Type 2 innate effector cells in mice.

In the murine bone marrow (BM), Notch has a key role in steady-state hematopoiesis and is required for the effective development of group 2 innate lymphoid cells (ILC2s) from common-lymphoid progenitors (CLPs) and mast cell development from granulocyte-macrophage precursors (GMPs). In the murine lung, the infiltration of ILC2s during *N. brasiliensis* infection requires Notch. Notch signaling also drives recruitment of IL-25-responsive KLRG1⁺ (not depicted) inflammatory ILC2s (iILC2s) during allergic inflammation in the lung. Activation of Notch in natural, lung resident ILC2s (nILC2s) *in vitro* can induce functional plasticity, normally characteristic of iILC2s, including *Rorc* expression and co-production of IL-13/IL-17 [28]. Eosinophil accumulation in allergic inflammation may also depend on Notch signaling. In the intestine, Notch is a driver of effective granulocyte localization, key to helminth clearance. Notch signaling in mast cells helps to position them within the small intestinal epithelium. Loss of functional Notch signaling in basophils reduces the proportion of basophils in the lamina propria (LP). This diminishes the likelihood that they are in close proximity to CD4⁺ T cells at this site, associated with a reduction in the frequencies of Gata3⁺ Th2 cells in the cecum during *T. muris* infection [10]. *In vitro* studies of mast cells and basophils also suggest that Notch can facilitate optimal production of effector cytokines, including IL-4, IL-6 and IL-13 [10,67,68]. The role of Notch for the development and function of alternatively-activated macrophages remains to be determined (not depicted).