
Notch signaling during
T helper 2 cell-mediated inflammation in allergic asthma
Irma Tindemans

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Notch Signaling During T Helper 2 Cell-Mediated Inflammation in Allergic Asthma

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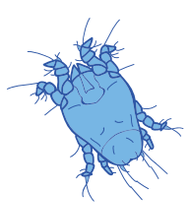

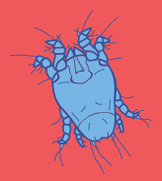

Chapter 1

Introduction

The central role of T helper 2 cells in allergic asthma

Asthma is a common, heterogeneous chronic disease of the conducting airways which is typically characterized by episodes of bronchoconstriction, airway hyperreactivity, mucus overproduction and airway inflammation. Asthma patients experience symptoms such as wheezing, shortness of breath and chest tightness. Asthma exacerbations are often induced by rather diverse stimuli including allergens (e.g. house dust mite (HDM), fungal spores, animal dander or tree or plant pollen), respiratory infections, irritants, exercise and change in weather. Worldwide, over 300 million people suffer from asthma. The majority of asthma patients responds well to standard treatment with inhaled corticosteroids, β2-adrenergic receptor agonists and oral leukotriene inhibitors[1, 2]. However, a subgroup of patients does not achieve disease control with these agents. Therefore, it is necessary to establish additional therapeutic approaches to treat asthma patients.

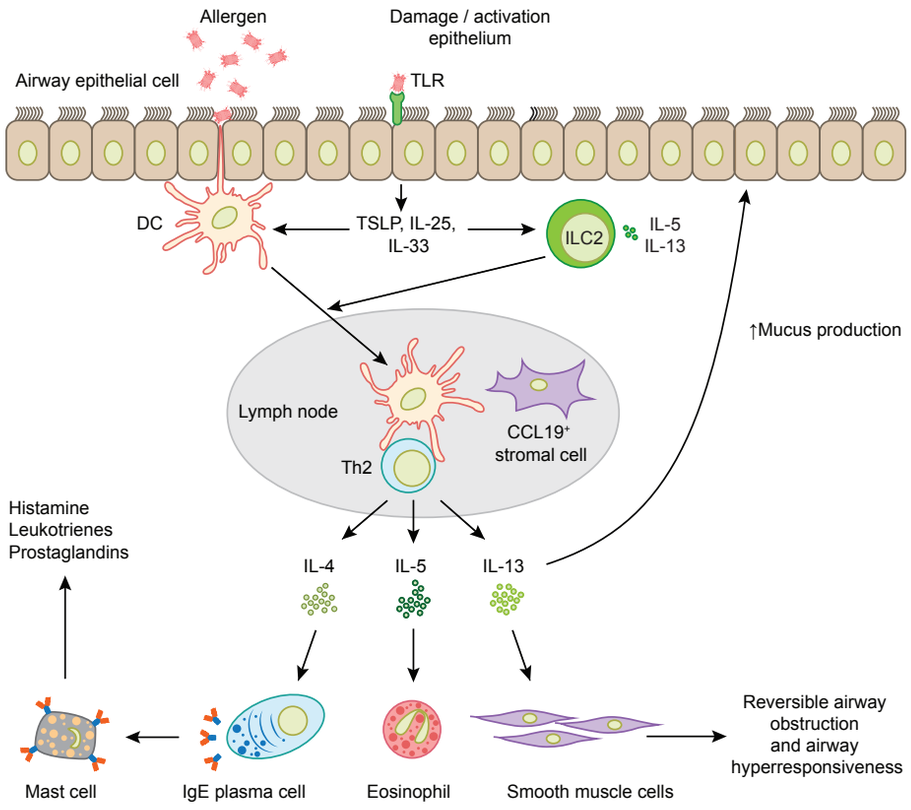

Figure 1. The central role of Th2 cells in allergic asthma

DCs can become activated by allergenic stimuli as well as epithelial-derived cytokines that also activate ILC2. Once activated, DCs migrate to draining lymph nodes which is supported by CCL19 produced by reticular stromal cells and IL-13 produced by ILC2. Antigenic stimulation of naïve T cells by DCs, together with cytokine signals induces Th2 cell differentiation. Th2 cells are potent producers of cytokines that induce IgE class switching of B cells, which induces activation of mast cells (IL-4), maturation and activation of eosinophils (IL-5) and smooth muscle hyperreactivity and goblet cell hyperplasia (IL-13).

Allergic asthma is the most common type of asthma which typically presents as eosinophilic inflammation[3-5]. Allergic asthma is generally thought to be induced by lung resident DCs that continuously sample the airway lumen for the presence of allergens. In addition to allergenic stimuli, DCs can become activated by epithelial-derived cytokines including thymic stromal lymphopoietin (TSLP), interleukin (IL)-25, IL-33 and granulocyte-macrophage colony stimulating factor (GM-CSF)[6] (**Figure 1**). Once activated, DCs mature and are attracted to draining lymph nodes by Chemokine (C-C motif) ligand 19 (CCL19), produced by reticular stromal cells, which binds to C-C chemokine receptor type 7 (CCR7) expressed by mature DCs and naïve T cells[7]. Upon antigenic stimulation by DCs, T helper 2 (Th2) cell differentiation is initiated. After subsequent allergen exposure, allergen-specific Th2 cells migrate to the lungs. Th2 cells are potent producers of the cytokines IL-4, IL-5 and IL-13 that explain many hallmarks off allergic asthma. IL-4 induces Th2 cell differentiation as well as IgE class switching of B cells, development of mast cells and mucous metaplasia[8]. IL-5 is required for the growth, maturation and activation of eosinophils[9]. IL-13 causes smooth muscle hyperreactivity and goblet cell hyperplasia[10] (**Figure 1**). In addition to Th2 cells, type 2 innate lymphoid cells (ILC2) contribute to allergic asthma by producing IL-5 and IL-13 in response to environmental signals including TSLP, IL-25 and IL-33[11, 12]. IL-13, produced by ILC2, was shown to induce migration of activated lung dendritic cells into the draining lymph node as well as production of the Th2 cell-attracting chemokine CCL17 by DCs in an asthma model induced by the protease-allergen papain[13, 14]. Moreover, it was suggested that ILC2 can crosstalk with T cells since ILC2 can, like DCs, also express major histocompatibility complex class II (MHCII), OX40L, CD80 and CD86. In addition, IL-2, derived from activated T cells can in combination with IL-33 lead to ILC2 stimulation[15].

T helper cell differentiation

Activated migratory DCs mature and enter tissue draining lymph nodes where they activate naïve $CD4^+$ T cells[16]. T cell activation requires three signals; (1) T cell receptor (TCR) stimulation with antigenic peptides in the context of MHCII, (2) co-stimulation via CD28-CD80/CD86 engagement and (3) cytokine signals[17, 18]. Upon activation, T cell acquire various cell surface receptors including CD69, CD25 (IL-2 receptor alpha chain) and CD44. In addition, T cells downregulate expression of the L-selectin CD62L and start secreting IL-2. Activated T cells proliferate in the lymph nodes for a maximum time of 1 week after which they migrate to the tissue of interest[19]. To migrate from the draining lymph nodes to the lungs, Th2 cells require expression of CCR4 which binds to its ligands CCL17 and CCL22 which are overexpressed in inflamed airways, with DCs being their major source[20]. Other chemokine receptors that have been implicated in the homing of Th2 cells to the lungs are CCR5, CXCR3, CCR6 and CCR8[21]. Egress of Th2 cells from the draining lymph nodes is mediated by Sphingosine-1-phosphate receptor 1 (S1PR1) which binds to sphingosine 1-phosphate (S1P)[22].

In 1986 it was observed that $CD4^+$ T cells can be divided into two subsets based on their cytokine production; Th1 cells and Th2 cells[23]. Today, we know at least six T cell lineages: Th1, Th2, Th9, Th17, T follicular helper (Tfh) and T regulatory (Treg) cells, characterized by their unique cytokine production profile, which is required to provide host protection against specific pathogens (**Figure 2**)[24-26]. For each T cell subset, key transcription factors have been identified. Additionally, T cell subsets are characterized by the expression of distinct members of the signaling transducer and activator (STAT) family. Moreover, depending on $CD4^+$ T cell activation, polarization and differentiation, the chemokine receptor profile expressed by T helper cells is altered, which in part is maintained in memory T cells after the inflammatory response is resolved[21]. Therefore, the differential expression of chemokine receptors can be helpful in identifying distinct T helper subtypes (**Figure 2**).

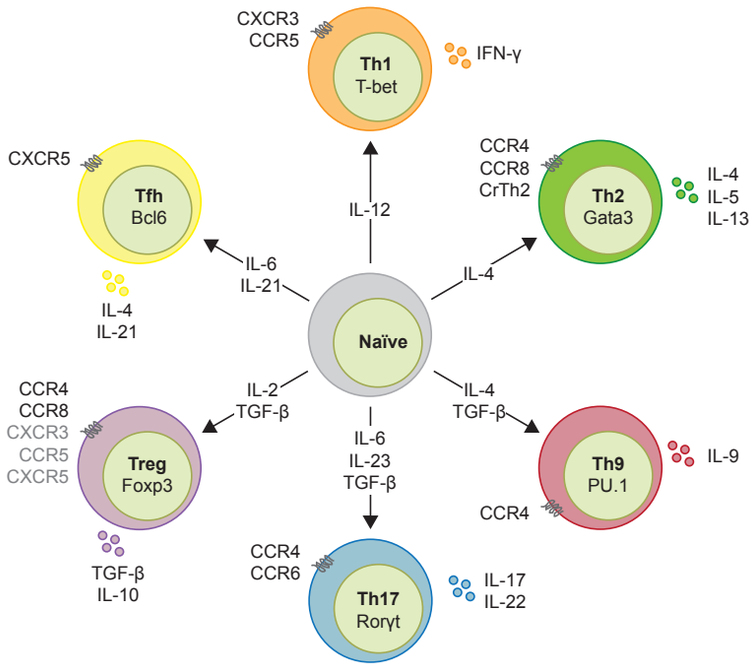

Figure 2. T helper cell differentiation

Once activated via TCR triggering by DCs, naïve T helper cells differentiate into various T helper cell lineages depending on the cytokine signals. Each lineage is defined by a critical transcription factor, expression of chemokine receptors and secretion of specific cytokines.

Th1

Th1 cells are associated with the elimination of intracellular pathogens. Th1 cells also help B cells in IgG class-switching which is required for pathogen opsonization[27]. Th1 polarization is driven by IL-12 signaling via STAT4 and induction of the key Th1 transcriptional regulator T-box-containing protein (T-bet), encoded by *Tbx21* expression. Th1 cells produce interferon gamma (IFN-γ) and Tumor Necrosis Factor alpha (TNF-α) which attract neutrophils and activate macrophages[28, 29].

Th2

Th2 cells have an important role in controlling helminth infections, eliminating extracellular microbes and for B cell help in humoral immunity[30]. Biased Th2 responses, on the other hand, can lead to allergies and asthma. Gata3 has been widely accepted as the key regulator of Th2 cell differentiation[31, 32]. Th2 differentiation is driven by IL-4 via STAT6 signaling and leads to the production of IL-4, IL-5 and IL-13. However, this raises the paradox that IL-4 is required to generate the cell type that is its major producer. The origin of the first IL-4 required for Th2 cell induction remains unclear. Several cell types including basophils, Tfh cells, NKT cells and type 2 innate lymphocytes (ILC2) are capable of producing IL-4[33-40]. However, Th2 cell responses can still be generated when only T cells can make IL-4, arguing against an essential role for an external source of IL-4[41, 42].

Th9

Th9 cells are closely related to the Th2 lineage and provide protection against helminth infections[43]. Like Th2 cells, Th9 cells require IL-4 signaling via STAT6 to differentiate but in addition require the presence of transforming growth factor beta (TGF-β)[44]. The transcription factors PU.1 and Interferon-regulatory factor 4 (IRF4) are required for Th9 cell differentiation and induce IL-9 production[45, 46].

Th17

Th17 cells provide protection against bacteria and fungi at mucosal surfaces but are also the main drivers of auto-immune diseases[25]. Th17 cells play a role in moderate to severe asthma[47]. Th17 cells are induced in response to IL-6, IL-23, and TGFβ[48-51]. Via STAT3, IL-6 induces expression of retinoic acid receptor-related orphan nuclear receptor gamma (Rorγt), leading to production of Th17 cytokines IL-17 and IL-22[52, 53].

T regulatory cells

Tregs can be classified into thymic-derived naturally occurring Tregs and inducible Tregs, both of which suppress immune responses and maintain peripheral tolerance[54-57]. Differentiation of Tregs requires high concentrations of TGF-β, with the absence of proinflammatory cytokines[58]. Cell-cell contact and IL-10 secretion is required for suppressor function, mediated through STAT5-induced activation of the lineage-specific transcription factor forkhead box P3 (Foxp3)[59, 60].

T follicular helper cells

Tfhs are involved in providing help for B cell class-switching for immunoglobulin production and germinal center formation[61]. Tfhs are characterized by expression of the transcription factor BCL-6[62-64] and by CXCR5 by which Tfhs are attracted towards CXCL13 present in the B cell zone[65]. Tfh differentiation requires activation of the inducible costimulator (ICOS) and IL-21, IL-6 and STAT3 signaling[66]. Recent studies have indicated that Tfhs, depending on the stimulus, can differentiate into effector cells[67]. In this context, it was shown that Tfhs are crucial for Th2 mediated inflammation and intranasal sensitization with HDM induces differentiation and expansion of IL-4 producing Tfh cells in the draining lymph nodes[34, 68], indicating that Tfhs can be precursors of effector Th2 cells. Apart from Th2 cells, more similarities between Tfh cells and other T helper subsets have been described. Depending on the inflammatory environment, Tfh cells can acquire low to intermediate levels of T-bet, Gata3, or Rorγt which results in a variety of Tfh cell subsets that can express low levels of specific cytokines capable of influencing B cell class-switching[67, 69]. Another Tfh subset that has recently been described are Foxp3$^+$ T follicular regulatory (Tfr) cells which act on multiple levels as regulators of the germinal center reaction[70].

The role of lung dendritic cell subsets during T helper cell differentiation

During steady-state, at least three DC subsets can be identified in the lungs; 2 types of conventional DCs (cDC1 and cDC2) and plasmacytoid DCs (pDCs)[71]. Depending on cytokine signals – all three DC subsets depend on the cytokine FMS-like tyrosine kinase 3 ligand (Flt3L) – and transcription factors, all of these subsets arise from hematopoietic stem cell (HSC) derived DC precursors and are characterized by expression of CD11c and MHCII[72]. Different functions were described for the various DC subsets during T cell activation, as well as during inflammatory responses. cDC1s depend on IFN-regulatory factor 8 (IRF8), ID2, Basic Leucine Zipper ATF-Like Transcription Factor 3 (BATF3), and Nuclear-factor interleukin-3 related protein (Nfil3) and are in the lungs characterized by CD103 expression. cDC1s are located underneath the epithelium of the large conducting airways and sense for the presence of antigens by protruding their dendrites into the airway lumen. cDC1s excel at cross-presenting and are required for the induction of $CD8^+$ T cells in immune responses against viruses and tumors[73-75]. In contrast, cDC1s were shown to dampen Th2 and Th17 responses[76-78].

cDC2 development requires various transcription factors including IRF4, v-rel avian reticuloendotheliosis viral oncogene homolog B (RELB), the Notch pathway nuclear effector Recombination Signal Binding Protein For Immunoglobulin Kappa J Region (RBPJκ; also known as CSL) and PU.1. Lung cDC2 typically express CD11b and are located underneath the epithelium in the lung lamina propria. cDC2s take up antigens efficiently, migrate to draining lymph nodes and are essential for both Th2 and Th17 cell priming[79-82].

pDC development depends on E2-2 and these cells are involved in anti-viral responses by producing type I interferons[83-85]. In addition, pDCs were implicated in the induction of Tregs[86-88] and have a tolerogenic role in allergic airway inflammation[89, 90].

During inflammatory responses, monocytes that migrate to the site of inflammation give rise to a fourth DC subset, the monocyte-derived DC (moDC)[91]. Like cDC2, moDCs express CD11c, MHCII and CD11b. In addition, like macrophages, moDCs express CD64 and FcεRI on the cell surface, are poor at migrating but are involved in locally amplifying inflammation[80].

To conclude, Th2 inflammation seems to be dependent on T cell activation and maintenance by cDC2 and moDCs, while cDC1s and pDCs have a more tolerogenic role. The capacity of DCs to induce T cell subset differentiation largely depends on the nature of DC activation, which determines the expression of co-stimulatory molecules and cytokines by DCs[92]. DCs can for example be activated by epithelial cell-derived cytokines including IL-33, IL-25, TSLP and GM-CSF as well as by antigenic triggering via pattern-recognition receptors. Co-stimulatory molecules expressed by DCs that are involved in Th2 cell differentiation include CD40, OX40L and the Notch ligand Jagged[93-95].

The Notch signaling pathway

The Notch signaling pathway is highly conserved and was first identified 100 years ago in fruit flies[96]. The Notch pathway mediates cell-cell contact-dependent signaling which regulates cell proliferation, apoptosis and a broad array of cell fate decisions and differentiation processes in neuronal, cardiac, endocrine and immune development and adult tissue homeostasis. Vertebrates carry four Notch receptors (Notch1–4) that are bound by five membrane-bound Notch ligands (Delta-like ligand (DLL) 1, 3, and 4 and Jagged 1 and 2). Notch receptors are transmembrane proteins composed of an extracellular (NECD), transmembrane (TM), and intracellular (NICD) domain. Newly generated Notch receptors are cleaved and glycosylated in the endoplasmic reticulum and Golgi apparatus, generating a heterodimer composed of NECD noncovalently attached to the TM-NICD inserted in the membrane. The capacity of different Notch ligands to trigger Notch receptor signaling is dependent on the glycosylation status of NECD by Fringe proteins. Fringe is a glycosyltransferase that adds N-acetylglucosamine to O-fucose residues present on the receptor[97, 98]. When Notch receptors carry these extra sugar moieties, Jagged-mediated Notch signaling is inhibited while the NECD preferentially signals via DLL[99]. Initiation of Notch signaling starts with ligand-receptor interaction between neighboring cells which leads to two consecutive proteolytic cleavages of the receptor. Notch receptors are first cleaved by metalloproteases from the A disintegrin and metalloproteinases (ADAM) family, which cleave the NECD external to the transmembrane domain. The released NECD is endocytosed by the ligand-expressing cell, which is

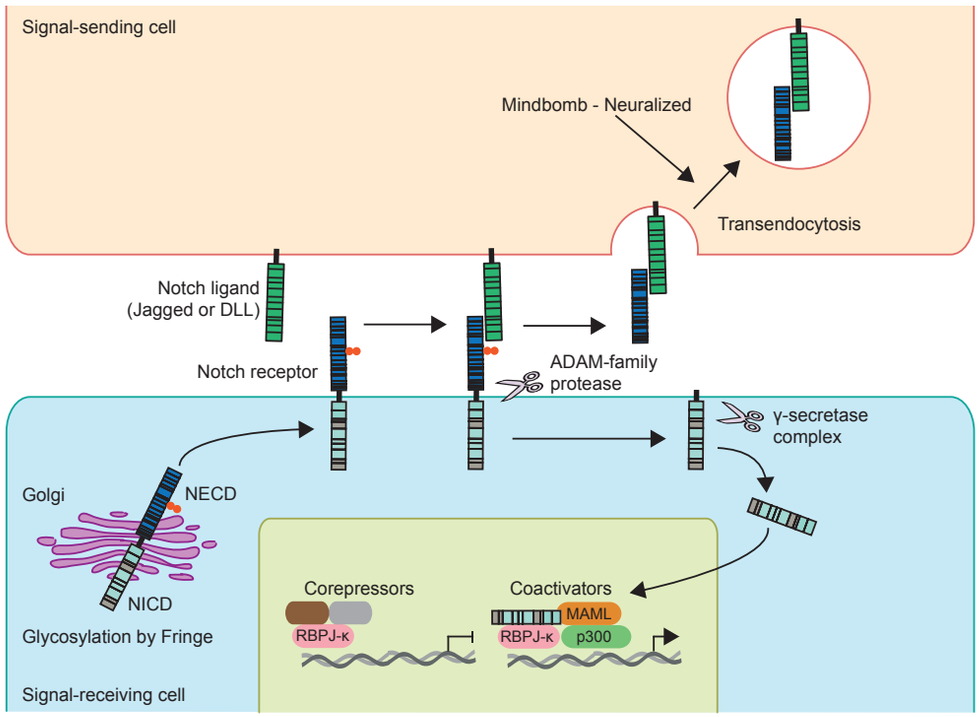

Figure 3. The Notch signaling pathway

After glycosylation by Fringe, initiation of Notch signaling starts with ligand-receptor interaction between neighboring cells, which leads to two consecutive proteolytic cleavages of the receptor. First the Notch extracellular domain (NECD) is cleaved by ADAM family metalloproteases, after which the released NECD is endocytosed by the ligand-expressing cell. This process is mediated by mindbomb and neuralized family E3-ubiquitin ligases, both of which are also required for the expression and function of Notch ligands. The second cleavage of the transmembrane domain is mediated by γ-secretase after which the Notch intracellular domain (NICD) is liberated. NICD subsequently translocates to the nucleus and heterodimerizes with the transcription factor RBPJκ which leads to recruitment of coactivators including MAML which together form a transcriptional activator complex in order to induce transcription of downstream target genes. Abbreviations are as follows: ADAM, A disintegrin and metalloproteinases; MAML, Mastermind-like; RBPJκ, Recombination-signal-binding protein for immunoglobulin Jκ region.

mediated by mono-ubiquitinylation of the cytoplasmic tail of the ligands by mindbomb and neuralized family E3-ubiquitin ligases. The second cleavage of the transmembrane domain is mediated by activity of a γ-secretase complex after which NICD is liberated. NICD subsequently translocates to the nucleus and heterodimerizes with the DNA binding transcription factor RBPJκ. NICD binding to RBPJκ leads to recruitment of other coactivators including proteins of the Mastermind-like family (MAML) to form a transcriptional activator complex in order to induce transcription of downstream target genes (**Figure 3**)[100]. Notch target genes include members of the Hairy enhancer of split (Hes) or Hairy related (Hey or Hrt) genes[101, 102], as well as many others, dependent on the tissue. Studies using genome-wide expression and chromatin immunoprecipitation (ChIP) arrays found a large number of genes that can be directly regulated by Notch[103, 104]. Moreover, there is emerging data suggesting that Notch can crosstalk to or cooperate with other signaling pathways (including mammalian target of rapamycin (mTOR), protein kinase PKCθ, NF-κB, hypoxia, glucose uptake, IL-2R or TGF-βR) and thereby broaden the spectrum of target genes that are influenced by Notch signaling[105-115].

Notch signaling during T cell development and differentiation

Notch signaling drives and regulates a wide range of developmental stages of various cell types (**Figure 4**)[116]. The importance of Notch signaling during T cell development is well studied. A Notch1 signal in bone marrow progenitors is required for cells to commit to the T cell lineage and to inhibit development of other cell types in the thymus such as myeloid cells and B cells[117-123]. For this, DLL4 expression by thymic epithelial cells is crucial[124-126]. Notch1 is especially required to restrict developing αβ T cells to the T cell lineage during early developmental stages up to the double-negative 3 (DN3) stage[127]. In this $CD44^-$ $CD25^+$ DN3 stage, first the TCRβ gene locus is rearranged and functionality of the TCRb chain is tested by cell surface expression of the pre-TCR, a process called β -selection. After β-selection, DN3-large and DN4 thymocytes proliferate before becoming $CD4^+CD8^+$ double positive (DP) cells, which subsequently undergo TCRα rearrangement to obtain a completely assembled TCR. Since thymocytes immediately downregulate Notch1 expression after successfully passing β-selection, double-positive ($CD4^+CD8^+$) thymocytes have very low levels of Notch signaling[128].

An accumulating number of studies suggest that the Notch signaling pathway is essential for $CD4^+$ T cell differentiation. Notch signaling is able to induce Th2 cell differentiation by direct activation of (1) a 3' enhancer of the *Il4* gene, and (2) an upstream promoter of *Gata3*[39, 93, 129, 130].

Moreover, Notch signaling is essential for Th2 cell-mediated responses *in vivo*[93, 130-134]. The role of Notch signaling in Th1 cell differentiation remains poorly understood. The signature Th1 genes *Ifng* and *Tbx21* were identified as direct Notch targets[135, 136]. However, while some research groups found that Notch1 and Notch2 are required for Th1 cell function, others demonstrated that Th1 cell function was unaffected when components of the Notch signaling were deleted[129-131, 135-137]. Notch signaling cooperates with TGF-β to induce Th9 cell differentiation and IL-9 expression via Jagged2 ligation[138]. Also, Th17 cell differentiation is decreased when Notch signaling is blocked and the *Rorc*, *Il17* and *Il23r* gene promoters are identified as direct Notch targets[139-143]. In addition, the key Treg transcription factor Foxp3 is a direct Notch target differentiation and Treg function requires Notch signaling in T cells[105, 144-146]. Lastly, Notch signaling is required for the differentiation of Tfh cells[147, 148]. In summary, Notch signaling is essential for the differentiation of multiple T helper cell subsets.It was suggested that Notch ligands

Delta-like ligand (DLL) and Jagged instruct Th1 and Th2 cell differentiation, respectively[93]. However, since the identification of the importance of Notch signaling in multiple T helper cell subsets, this bipotential instructional model will not be sufficient to fully explain the function of Notch signaling in Th cell differentiation. In contrast to this model, it was hypothesized that Notch signaling can act as a general amplifier of Th cell responses rather than an instructive director of specific T helper cell programs. This hypothesis was based on the observation that Notch signaling simultaneously induced Th1, Th2 and Th17 gene transcription, also under polarizing conditions that were described to favor only one of the differentiation outcomes[135]. Moreover, Notch signaling was shown to increase antigen sensitivity of $CD4^+$ T cells via promoting co-stimulatory signals in T cells and required for optimal T cell expansion, CD25 and IL-2 induction and to promote survival by enhancing anti-apoptotic signals and glucose uptake[107-110, 149, 150]. Therefore, it is currently unclear whether Notch acts as a bipotential instructor or as an unbiased amplifier during T helper cell differentiation.

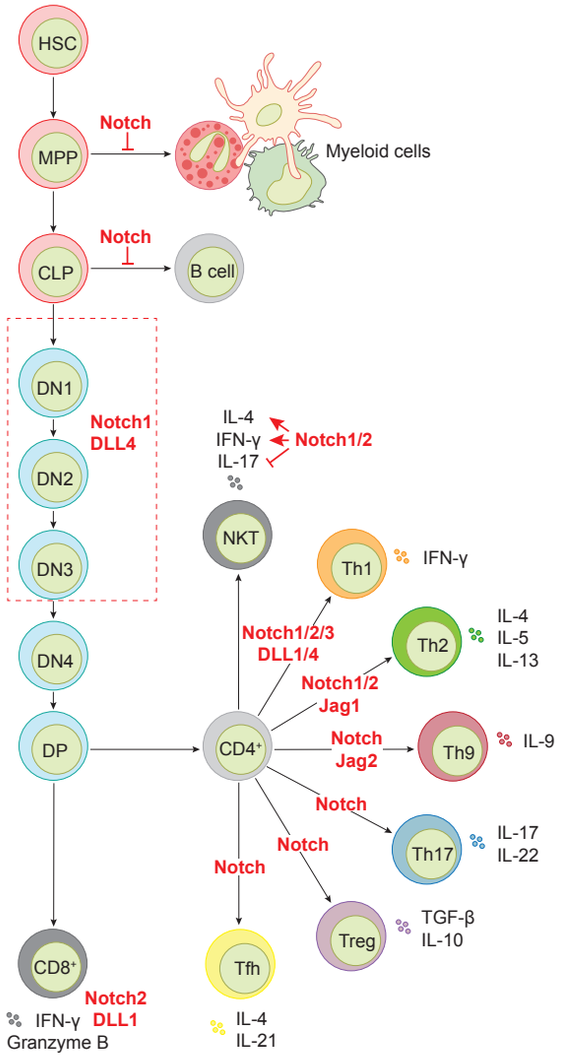

Figure 4. The function of Notch signaling during T cell development, differentiation and function

Scheme of T cell development and differentiation, showing the main lineage decisions during T cell development and T helper subset differentiation. Notch receptors or ligands that are required (if known) during these processes are indicated in red. Abbreviations are as follows: CLP, common lymphoid progenitor; CMP, common myeloid progenitor; DLL, Delta-like ligand; DN, double-negative; DP, double-positive; HSC, hematopoietic stem cell; Jag, Jagged; MPP, multipotent progenitor; NK, Natural killer.

Next to its well described role in $CD4^+$ T cells, Notch is also required for the differentiation and function of other T cell subsets including $CD8^+$ T cells and NKT cells. $CD8^+$ T cells are activated by MHC class I expressing cells and exert their cytotoxic function by secreting IFN-γ, by lysis of target cells with perforins and granzymes and by induction target cell apoptosis through FAS-FAS ligand binding. DLL1 and Notch2 have the capacity to induce Granzyme B production

and to improve cytotoxic activity of CD8⁺ T cells[151, 152]. NKT cells contribute to host immunity by rapidly producing cytokines including IL-4, IFN-γ and IL-17 upon antigen recognition[33, 153]. It was shown in mice with a conditional deletion for Notch1 and Notch2 or RBPJκ that Notch signaling coordinates NKT cell differentiation and function by positively regulating IFN-γ and IL-4 expression, and impairing IL-17 secretion[39, 154].

Collectively, an increasing number of studies showed a role for Notch in the development and function of a range of T cell subsets. Still, there is many contradicting data. Therefore, more *in vivo* loss-of-function experiments would help to exactly elucidate the role of Notch signaling in T cell development, differentiation and function.

Notch signaling during other cell-fate decisions

Next to the well-established role for Notch signaling during T cell development, Notch directs development of many other cell types including ILCs, B cells, DCs and lung structural cells (**Figure 5**). Like T cells, ILCs develop from common lymphoid progenitors (CLPs) and can be subdivided in three major subclasses based on their cytokine expression[155]. In contrast to T cells, ILCs lack antigen-specific receptors. ILC2 are dependent on RORα and Gata3 and secrete IL-5 and IL-13 during inflammation. *In vitro* studies have suggested a role for Notch signaling in ILC2 differentiation via DLL1[156-158]. This has however not yet been confirmed *in vivo*. Natural killer (NK) cells belong to the group 1 ILCs and have cytotoxic activity and functions[155]. While the role of Notch signaling in ILC1 remains unclear, NK cells do need Notch signals to develop since Notch1 and the ligands Jagged2, DLL1 and DLL4 were shown to promote NK cell differentiation and to increase IFN-γ production and cytolytic activity by NK cells[159-165]. Notch signaling has also been implicated in the differentiation and function of ILC3. The development of lymphoid tissue-inducer (LTi) cells (a type of group 3 ILC that is essential for the development and generation of secondary lymphoid organs) is dependent on RORγt[155]. Notch1 and Notch2 are required for the generation of fetal α4β7⁺ LTi cell progenitors before upregulation of RORγt. However, Notch signaling has to be subsequently downregulated again to allow the expression of RORγt and the final maturation of LTi cells[166]. IL-22 producing NKp46⁺ ILC3 are, like LTi cells, dependent on RORγt[155]. The differentiation of NKp46⁻ ILC3 into NKp46⁺ ILC3 was shown to depend on DLL1 signals[167-169]. In addition, conditional inactivation of RBPJ led to a reduction in NKp46⁺ ILCs numbers in the lamina propria of the intestine but not in Peyer's patches[170].

B cells originate from B cell progenitors in the bone marrow, after which they migrate to secondary lymphoid organs where they further mature after which they become either mature follicular B cells or marginal zone cells. In this context, the development of marginal zone B cells is dependent on Notch2 signaling via DLL1 ligation[148, 171, 172].

Although Notch signaling inhibits the differentiation of hematopoietic stem cells to myeloid progenitor cells or erythroid cells[173-176], Notch is required for the differentiation of mature myeloid cells. Notch2, but not Notch1 is specifically required for the development of a subset of cDC2 that is required for immune responses to bacterial pathogens[177-181]. In addition, Notch signaling via DLL1 is required for optimal DC maturation and activation since RBPJκ deletion in DCs led to decreased dendrite outgrowth and reduced expression of MHCII and CXCR4 in response to lipopolysaccharide (LPS)[182]. Furthermore, deletion of ADAM10 or Notch1 in DCs led to a decrease in type 2 inflammation in mice models for allergic asthma[183].

During macrophage activation, Notch signaling was shown to induce transcription of proinflammatory cytokines including IL-1β, IL-6, TNF-α and IL-12[184-186]. Lastly, *in vitro* studies have implied functions for Notch in the differentiation of granulocytes[187] and Notch signaling is required for *in vitro* survival, differentiation and cytokine production by basophils[188].

In addition to its role in immune cell differentiation, Notch also drives lung organogenesis and alveologenesis[189]. Basal cells are progenitors of airway epithelial cells that can differentiate into secretory and ciliated cells. In this context, jagged1 expression on basal cells was shown to enhance their differentiation into secretory cells[190]. While some studies describe that Notch signaling induces goblet cell differentiation[191], others found an inhibitory role for Notch in the differentiation of secretory cells into goblet cells[192].

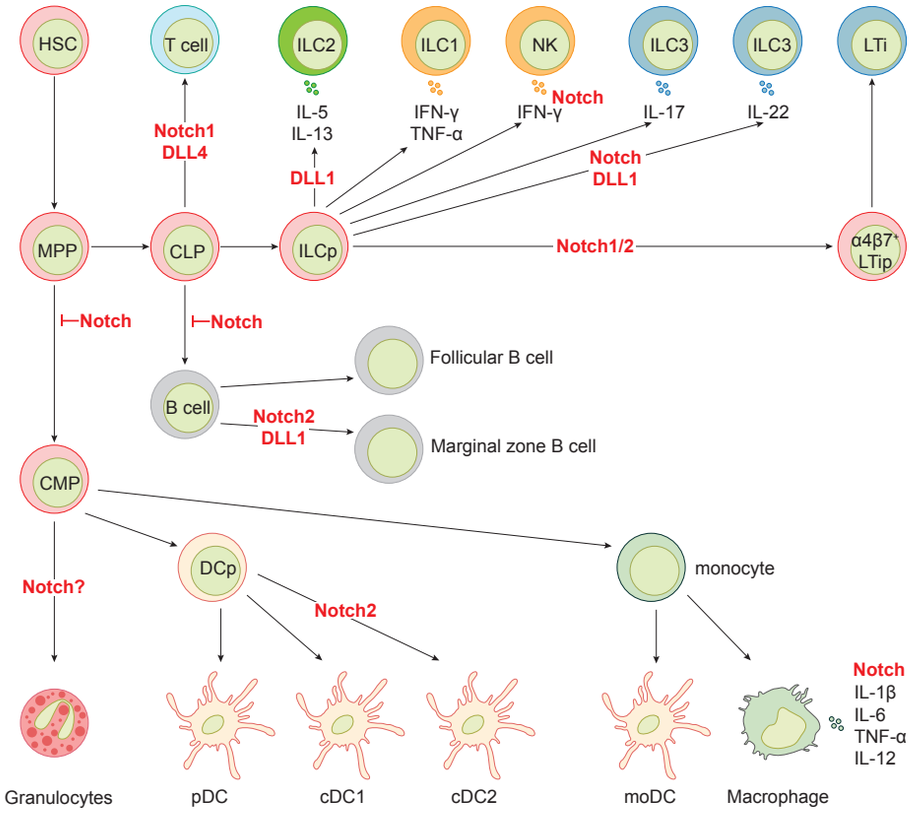

Figure 5. The role of Notch signaling during lymphoid and myeloid cell development and function

Overview of hematopoiesis, showing the main steps during lymphoid and myeloid cell development. Notch receptors or ligands that are required (if known) during these processes are indicated in red. Abbreviations are as follows: cDC, Conventional dendritic cell; CLP, common lymphoid progenitor; CMP, common myeloid progenitor; DLL, Delta-like ligand; HSC, hematopoietic stem cell; ILC, Innate lymphoid cell; Jag, Jagged; LTi, Lymphoid tissue-inducer; moDC, Monocyte-derived dendritic cell; MPP, multipotent progenitor; NK, Natural killer; p, progenitor; pDC, Plasmacytoid dendritic cell.

Taken together, Notch drives and regulates developmental programs and functions of a large range of cell types (**Figure 5**). Therefore, it is not surprising that mutations in Notch genes can result in diseases such as T cell acute lymphoblastic leukemia (T-ALL). Aberrant expression of NICD in bone marrow progenitors led to an induction of T-ALL in mice[193]. Moreover, in samples from patients with T-ALL tumors, constitutive active mutations were found in the *Notch1* gene[194, 195]. Although it is unclear how Notch signaling induces T-ALL in detail, data suggests that Notch induces T-ALL via interfering with several signaling pathways that are required for cell proliferation, growth metabolism and survival including c-Myc, mTOR, NF-κB and NFAT[196]. In addition, since Notch drives differentiation of $CD4^+$ T cell subsets, Notch signaling is thought to contribute to many immune-mediated diseases including auto-immune diseases and allergies[197]. Therefore Notch signaling might serve as a therapeutic target in those diseases. Most studies investigating Notch therapies have used γ-secretase inhibitors (GSI). However, interpretation of these findings is complicated, since GSI are not limited to Notch signaling and for example also target HLA-A2 expression and cadherins[198]. Synthetic, cell-permeable stabilized peptides that specifically target the Notch transactivation complex[199-201], as well as specific antibodies targeting Notch receptors[202-204] or Notch ligands[205, 206] have been designed. Therefore, it is crucial to elucidate what the exact role is of Notch signaling in T cell-mediated diseases and whether cell-permeable stabilized peptides or antibodies that block Notch signaling are beneficial for patients with autoimmune disease or allergies such as allergic asthma.

Aims and outline of this thesis

Notch signaling is essential for the development as well as the subset differentiation of $CD4^+$ T cells. In this thesis, we aimed to investigate what the role is of the Notch signaling pathway in Th2 cell-mediated inflammation in allergic asthma. Notch signaling has the capacity to initiate Th2 cell differentiation by direct activation of an upstream promoter of *Gata3*[129, 130], which is the key transcription factor that controls Th2 differentiation. In **chapter 2**, we will further elaborate on how Gata3 controls differentiation and function of T cells and ILCs.

Several research groups have found that Notch ligands Jagged and DLL instruct Th2 and Th1 cell differentiation, respectively[93, 207]. Jagged1, but not Jagged2, expressed on the cell surface of antigen presenting cells stimulated Th2 effector generation[93, 208-210] while surface DLL expression was shown to promote generation of Th1 cells[152, 211-213]. In addition, the expression of Jagged ligands on DCs was linked to Th2-associated stimuli while DLL ligands were upregulated in response to Th1 cell promoting stimuli[93, 205, 208, 211, 214-230 200]. In **chapter 3** we investigate the role of Notch signaling and Jagged ligands in allergic asthma. To this end, we exposed mice lacking Jagged ligands on DCs or RBPJκ specifically in T cells to a HDM-mediated model for allergic airway inflammation (AAI). We used HDM since HDM is the cause of chronic allergic sensitization in ~50% to ~85% of the asthmatic patients. HDM derives its allergenic nature from mite-derived fecal proteins which contain Toll-like receptor ligands and crude extracts of entire Dermatophagoides organisms. Inhalation of mite fecal pellets activates both epithelial cells and DCs of the lung, leading to Th2 priming in the absence of adjuvant addition[231-233].

The finding that canonical Notch signaling is required for the induction of AAI, suggested that blocking the Notch signaling pathway can serve as a potential therapeutic target in allergic asthma. Interestingly, others have shown that administration of γ-secretase inhibitors (GSI) during only the challenge in asthma models was sufficient to decrease Th2 cytokine production[132, 133]. Effects of GSI are not limited to Notch signaling and can induce unwanted side-effects including gastrointestinal toxicity[234]. Therefore, we aimed to investigate whether cell-permeable stabilized peptides that more specifically block Notch signaling can be used to alleviate AAI. In **chapter 4**, we treated mice that were exposed to our acute HDM-driven model for AAI with the synthetic, cell-permeable stabilized peptide SAHM1[199-201] to target protein-protein interfaces in the Notch transactivation complex. In addition, we investigated whether blocking Notch signaling is essential during the sensitization or during the challenge phase.

In **chapter 5**, we further investigated the role of the Notch receptors in Th2 cell inflammation. Therefore we exposed mice lacking either Notch1 or Notch2 or both receptors on T cells to acute and chronic HDM-driven models for AAI. We specifically questioned which Notch receptor (Notch1 or Notch2) is required for the induction of AAI and whether the lack of these receptors can be overcome by enforced expression of Gata3. In addition, we investigated whether Notch signaling is required for the priming of T cells or during the challenge phase. Finally, in this chapter we aimed to identify the role of Notch signaling in Th2 cells and investigated whether Notch is required for proliferation, differentiation or migration of Th2 cells.

We found in chapter 3 that Jagged expression on dendritic cells is dispensable for Th2 cell mediated inflammation in AAI. In **chapter 6** we therefore investigated the role and function of the expression of the Jagged1 and jagged2 Notch ligands on B cells, follicular reticular cells and T cells.

A subgroup of asthma patients is unable to control their disease using corticosteroids. Therefore, it is necessary to obtain knowledge about the immunological differences between steroid-controlled and uncontrolled asthmatic patients to develop additional therapeutic approaches to treat uncontrolled asthma patients. Because of the evidence that Notch signaling is required during Th2 inflammation in AAI in mice, we questioned whether Notch expression is altered on Th2 cells in allergic asthma patients. Therefore, in **chapter 7,** we compared Notch protein expression and gene expression profiles in T helper subsets from steroid-controlled and uncontrolled asthmatic patients with healthy individuals. We measured this using flow cytometry and genome-wide RNA sequencing and investigated whether our RNA and protein expression profiles correlated with clinical parameters or with circulating immune cells including eosinophils and neutrophils.

The role of Notch signaling in the differentiation of T helper cells are described in **chapter 8**. Implications of our work and potential future directions in the field of asthma research are described in **chapter 9**.

Chapter 2

GATA-3 function in innate and adaptive immunity

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Summary

The zinc-finger transcription factor GATA-3 has received much attention as a master regulator of T helper 2 (Th2) cell differentiation, during which it controls IL-4, IL-5 and IL-13 expression. More recently, GATA-3 was shown to contribute to type 2 immunity through regulation of group 2 innate lymphoid cell (ILC2) development and function. Furthermore, during thymopoiesis GATA-3 represses B cell potential in early T cell precursors, activates TCR signaling in pre-T cells and promotes the $CD4^+$ T cell lineage after positive selection. GATA-3 also functions outside the thymus in hematopoietic stem cells, regulatory T cells, $CD8^+$ T cells, thymic NK cells and ILC precursors. Here we discuss the varied functions of GATA-3 in innate and adaptive immune cells, with emphasis on its activity in T cells and ILCs, and examine the mechanistic basis for the dose-dependent, developmental stage- and cell-lineage-specific activity of this transcription factor.

Introduction

Shortly after its identification in 1990, the zinc-finger transcription factor GATA-3 was found to be required for both early T cell development in the thymus and for differentiation of naïve $CD4^+$ T cells into committed T helper type 2 (Th2) cells[31, 32, 235, 236]. The molecular function of GATA-3 has been most extensively studied in the context of transcriptional regulation of genes encoding the Th2 signature cytokines interleukin-4 (IL-4), IL-5 and IL-13, which are tightly clustered to form the Th2 cytokine locus. Within this locus, GATA-3 has a complex role: not only does it bind to the *Il5* and *Il13* promoter regions, but also to Th2-specific DNAse I hypersensitive sites (DHS) that engage chromatin remodeling machinery allowing GATA-3 to orchestrate a three-dimensional topography of type II cytokine transcription[237]. GATA-3 can repress expression of other genes, for example *Ifng* encoding the Th1 signature cytokine interferon-γ (IFN-γ), although the mechanism for this repression remains less clearly understood.

The paradigm of GATA-3 as a central mediator of type II inflammation was recently extended by the finding that GATA-3 is also essential for group 2 innate lymphoid cell (ILC2) development and Th2 cell cytokine production[238-241]. ILC2 are innate non-T and non-B lymphoid cells that produce large amounts of IL-5 and IL-13 upon activation by epithelial-derived pro-inflammatory cytokines[242-244]. However, it has become clear that GATA-3 function is not limited to innate and adaptive lymphocytes that mediate type II immunity. GATA-3 is also required in several mature T cell populations as well as in developmental cell-fate decisions during lymphoid development. For example, in addition to controlling Th2 cell differentiation, GATA-3 controls survival and proliferation of $CD8^+$ T cells and is essential for regulatory T (Treg) cell function[245-247]. Beyond the T cell lineage, GATA-3 is also involved in hematopoietic stem cell (HSC) self-renewal and maintenance[248-250] and repression of B cell commitment in lymphoid precursors[251, 252]. GATA-3 is not needed for classical NK cell development and function, but is important for several specialized subsets of NK cells[253]. In an analogous fashion to its critical role in early T lymphopoiesis, recent evidence shows that GATA-3 functions not only in mature ILC2 but also in ILC precursor cells[241, 254-256] that give rise to various ILC populations (reviewed in REF 257, 258). Thus GATA-3 is essential in the differentiation and function of multiple innate and adaptive lymphocytes.

Genome-wide analyses have identified a large number of GATA-3-binding sites in both active and silent genes in thymocytes and various mature T cell subsets[259-261], suggesting that GATA-3 can both activate and/or repress gene expression. In contrast to the detailed knowledge of GATA-3 function in transcriptional regulation of Th2 cytokine genes, little is known about mechanisms of GATA-3-dependent gene regulation in developing T cells and ILCs. As GATA-3 has crucial roles in a broad variety of cell types, it is logical to assume that GATA-3 function is context-dependent. As such, the regulatory output of GATA-3 will be dictated by its distinct protein-protein interactions in a given cell type. In this review, we highlight recent reports describing functional roles for GATA-3 in several hematopoietic cell types and discuss how genome-wide identification of binding sites support a model in which GATA-3 is recruited to distinct subsets of its potential binding sites, in a dose-dependent, developmental stage-specific and cell-lineage specific fashion.

GATA-3 and its family members

In mammals the GATA family of transcription factors consists of six members, GATA-1 to GATA-6. GATA proteins contain two N-terminal transactivation domains and two characteristic Cys_4 DNA-binding zinc finger domains, each of which is followed by a conserved basic region. The zinc finger closest to the C-terminus mediates binding to the consensus DNA sequence (A/T)-GATA-(A/G), while the N-terminal zinc finger stabilizes this binding and physically interacts with the zinc finger co-regulator protein friend of GATA (Fog)[262] (**Table 1** and discussed below).

GATA factors have pivotal roles during development, as disruption of each of the GATA genes (except GATA-5) in mice results in embryonic lethality. Most GATA factors show a tissue- and cell-restricted pattern of expression. GATA-1 and GATA-2 are primarily expressed in the hematopoietic system, while GATA-4, GATA-5, and GATA-6 are mostly expressed in the cardiac, pulmonary and digestive systems[263, 264], although GATA-6 is also expressed in peritoneal macrophages in which it is required for proliferative renewal during homeostasis and in response to inflammation[265, 266] (**Figure 1**). The broad expression of GATA-3 in multiple cell types is an exception to the rule. There is a functional overlap among GATA family members: GATA-3 can partially restore erythroid development in GATA-1-deficient embryos[267] and GATA-1, -2, -3 and -4 all have the ability to enhance IL-4 and IL-5 and to inhibit IFN-γ production in differentiated T cells[268].

GATA-1 is critically involved in the development of erythrocytes, megakaryocytes, mast cells, dendritic cells (DC), basophils and eosinophils (**Figure 1**). GATA-2 is indispensible for efficient hematopoiesis, both for the production and expansion of HSCs in the embryonic aorta-gonad-mesonephros (AGM) region and for the proliferation of HSCs in adult bone marrow[269]. During erythroid differentiation GATA-1 and GATA-2 manifest dynamic reciprocal changes in their expression profiles (see for review: REF 270). In addition, a key role for GATA-2 has been demonstrated in basophil development and mast cell generation (**Figure 1**). Surprisingly, GATA-3 overexpression in early double negative (DN1) and DN2 but not DN3 fetal thymocytes that were cultured in the absence of Notch ligands rapidly and efficiently induced mast cell specification[271]. Mast cell development usually occurs independent of GATA-3, however as GATA proteins can induce their own expression, it is likely that the ability of GATA-3 to up-regulate *Gata2* gene expression accounts for the observed reprogramming of thymocytes into mast cells.

Table 1. Interacting partners of GATA-3

Protein	Function	Cell type	Reference
CHD4	Chromodomain-helicase-DNA-binding protein 4, ATP-dependent chromatin remodeler, subunit of the repressive NuRD complex	Th2	363
CBP-p300	Histone acetyltransferease (HAT) complex – transcriptional activation	Th2	363
NuRD	Nucleosome remodeling histone deacetylase repression complex – transcriptional repression	Th2	363
Fog1	Friend of GATA-1; zinc-finger transcription factor that inhibits GATA-3 auto-activation and represses *Il5* gene transcription and Th2 differentiation	Naïve T cells	381
Rog	Repressor of GATA (also known as Zbtb32); lymphoid-specific transcription factor that is rapidly induced in activated T cells and that represses GATA-3-induced transactivation	Activated T cells	382
T-bet	T-box protein; master regulator of Th1 differentiation, essential regulator of IFN-γ expression	Th1	364
Eomes	T-box protein eomesodermin, highly homologous to T-bet and expressed in NK cells and inactivated $CD8^+$ T cells, Th1 and Th2 cells	Th1	365
Runx3	Transcription factor that represses CD4 and activates CD8 expression and promotes Th1 differentiation in naïve T cells and induces IFN-γ	Th1/Th2	383
FoxP3	Master regulator of Treg differentiation and function	Treg	245
Smad3	Intracellular signal transducer of TGF-β	Th2	384
YY1	Yin Yang 1, ubiquitously expressed zinc-finger transcription factor implicated in long-distance DNA interactions	Th2	308
Fli1	Member of the ETS transcription factor family also known as ERGB	T cells	260

In the hematopoietic system, expression of GATA-3 is confined to specific lymphocyte populations (**Figure 1**), as will be discussed below. GATA-3 is also expressed in various non-hematopoietic tissues, including adrenal glands, kidneys, central nervous system, inner ear, hair follicles, skin and mammary gland. GATA-3-deficient embryos die between embryonic day 11 and 12 and display internal bleeding, growth retardation, severe brain and spinal cord deformation and aberrations in fetal liver hematopoiesis[272]. The embryonic lethality is secondary to noradrenalin deficiency of the sympathetic nervous system, and GATA-3 mutation-induced lethality is partially averted by feeding catechol intermediates to pregnant dams[273]. Haploinsufficiency of *GATA3* in man results in an autosomal dominant developmental disorder, referred to as hypoparathyroidism-deafness-renal (HDR) dysplasia, associated with various heterozygous germline GATA-3 abnormalities, including nonsense, frameshift and missense mutations[274]. Mutations in the *GATA3* gene are commonly found in human breast cancers and low GATA-3 expression is associated with poor prognosis. GATA-3 is required for luminal epithelial cell differentiation and commitment in the mammary gland. Whereas GATA-3 expression suppresses lung metastasis, loss of GATA-3 triggers fibroblastic transformation and cell invasion (See for review: REF 275). Loss of GATA-3 is also observed in high-grade invasive bladder cancer[276].

Members of the GATA family have a highly conserved gene organization. Two distinct promoters drive lineage and tissue-specific expression and alternative first exon usage generates a series of GATA mRNA isoforms. Regulation of *Gata3* gene expression is particularly complex and is dictated by individual tissue-specific enhancers. For example, a kidney enhancer element has been identified ~113 kb 5' to the *Gata3* structural gene[277], while a cis-acting element located ~280 kb 3' to the *Gata3* structural gene regulates GATA-3 expression in the T and NK cell lineage *in vivo*[278].

The GATA-3 protein contains a classical nuclear localization signal motif and its localization between cytoplasm and nucleus is dependent on phosphorylation of critical serine residues by mitogen-activated protein kinase (MAPK) p38. MAPK is activated by T cell receptor (TCR) and IL-33R signaling in T cells and ILC2, respectively, which facilitates binding to the nuclear transporter protein importin-α resulting in nuclear carriage[250, 279, 280]. Corticosteroids, which are highly effective in suppressing allergic airway inflammation, have the capacity to suppress GATA-3 nuclear import by competing for importin-α and by inducing MAPK phosphatase-1, an inhibitor of MAPK p38[281]. Finally, it is known that the acetylation status of GATA-3 affects is transactivation ability. The GATA-3 mutant KRR-GATA-3 is hypoacetylated and shows hypomorphic functions, resulting in reduced T cell survival and altered lymphocyte homing[282].

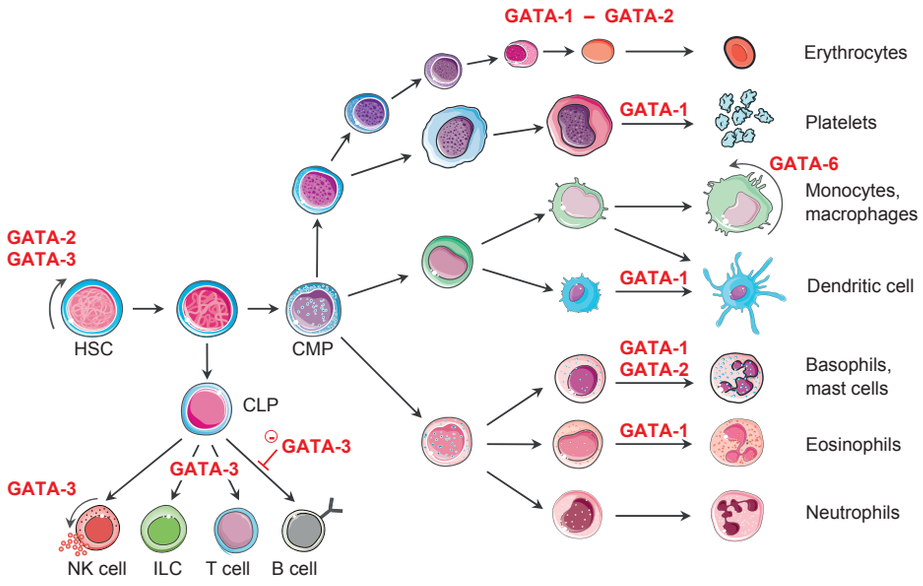

Figure 1. Function of GATA factors in hematopoietic development.
Overview of hematopoiesis, showing the main lineage commitment steps from HSC to fully maturated and functional blood cells. GATA transcription factors that are required for these processes are indicated in red: GATA-1 and GATA-2 are important for the development of the erythroid and myeloid cell lineages. In contrast, GATA-3 is only involved in the lymphoid cell lineage, whereby GATA-3 is crucial for NK cell maturation, for the development of ILCs and T cells and for the repression of B cell potential. Abbreviations: CLP, common lymphoid progenitor; CMP, common myeloid progenitor; HSC, hematopoietic stem cell; ILC, innate lymphoid cell ; NK, natural killer.

GATA-3 in Th2 cell differentiation

The role of GATA-3 in transcriptional regulation of the murine Th2 cytokine locus is well understood (**Figure 2**). This ~150 kb region contains the *Il4*, *Il5* and *Il13* genes, as well as a locus control region (LCR) that is crucial for appropriate Th2-specific cytokine expression and is located at the 5' end of the interspersed *Rad50* gene, encoding a ubiquitously expressed DNA repair protein[283]. GATA-3-mediated gene regulation and chromatin remodeling in the Th2 cytokine locus represents a model for understanding the molecular mechanisms of type II immune responses.

Initiation of Th2 differentiation and inhibition of Th1 differentiation

Multiple distinct Th cell subsets (Th1, Th2, Th9, Th17, Th22, follicular T helper (Tfh) and Treg cells), characterized by unique cytokine production and transcription factor profiles have been described (see for recent review: REF 284).

Th2 cells control helminth infections and allergic immune responses and are characterized by the expression of the proinflammatory cytokines IL-4, IL-5 and IL-13. The initiating signals that drive Th2 differentiation have been the subject of intense investigation and include IL-4 that induces phosphorylation and activation of STAT6, which in turn enhances GATA-3 expression via distal and proximal *Gata3* promoters and an upstream conserved regulatory region[285]. GATA-3 is necessary and sufficient for Th2 cytokine gene expression in T helper cells[31, 32]. Once induced, GATA-3 upregulates its own expression, either directly[286] or via the transcription factor Dec2[287]. GATA-3 is essential for the differentiation of naïve T cells to Th2 cells, as well as for the activation of already established Th2 cells[288, 289]. The induction of GATA-3 by the IL-4-STAT6 axis in differentiating Th2 cells however raises the paradox that IL-4 is essential for the generation of the cell type that is its major producer. The initial source of IL-4 that directs the Th2 response remains unclear. While a range of cell types can produce IL-4, Th2 responses can be generated when IL-4 is exclusively produced by T cells or when mice lack functional IL-4R signaling, arguing against a requisite role for an external source of IL-4[41, 42].

Other pathways have been implicated in the initial production of IL-4. TCR triggering in naïve T cells induces GATA-3 and IL-4 upregulation[290, 291] and IL-2 secretion that in turn activates STAT5a in T cells[292]. STAT5a can bind to the DNase hypersensitive sites (DHS) HSII and HSIII in the *Il4* locus (**Figure 2**), and high STAT5a activity can cooperate with GATA-3 to induce Th2 cell differentiation[293]. Several studies showed that GATA-3 and IL-4 expression can be directly regulated by Notch signaling in activated T cells[129, 130]. The role of Notch signaling in the innate and adaptive immune system has recently been reviewed[116]. Differentially expressed Notch ligands on DCs are able to instruct differentiation of naïve $CD4^+$ T helper cells: Delta-like (DLL) and Jagged ligands induce Th1 and Th2 differentiation, respectively[93]. Notch signaling induces Th2 differentiation by: (1) directly activating the upstream *Gata3* gene promoter; and (2) by regulating *Il4* gene transcription through activation of a 3' enhancer. Both of these events are dependent on a nuclear complex that contains recombination-signal-binding protein for immunoglobulin Jκ region (RBPjκ). In the absence of GATA-3, Notch no longer induces Th2 cells but instead induces Th1 cell differentiation. Mice lacking RBPjκ or the Notch1 and Notch2 receptors fail to generate robust Th2 responses to parasite antigens[129]. Therefore, it can be concluded that the Notch signaling pathway is a STAT6-independent pathway that is crucial for Th2 induction via GATA-3. Although high amounts of exogenous IL-4 can induce normal Th2

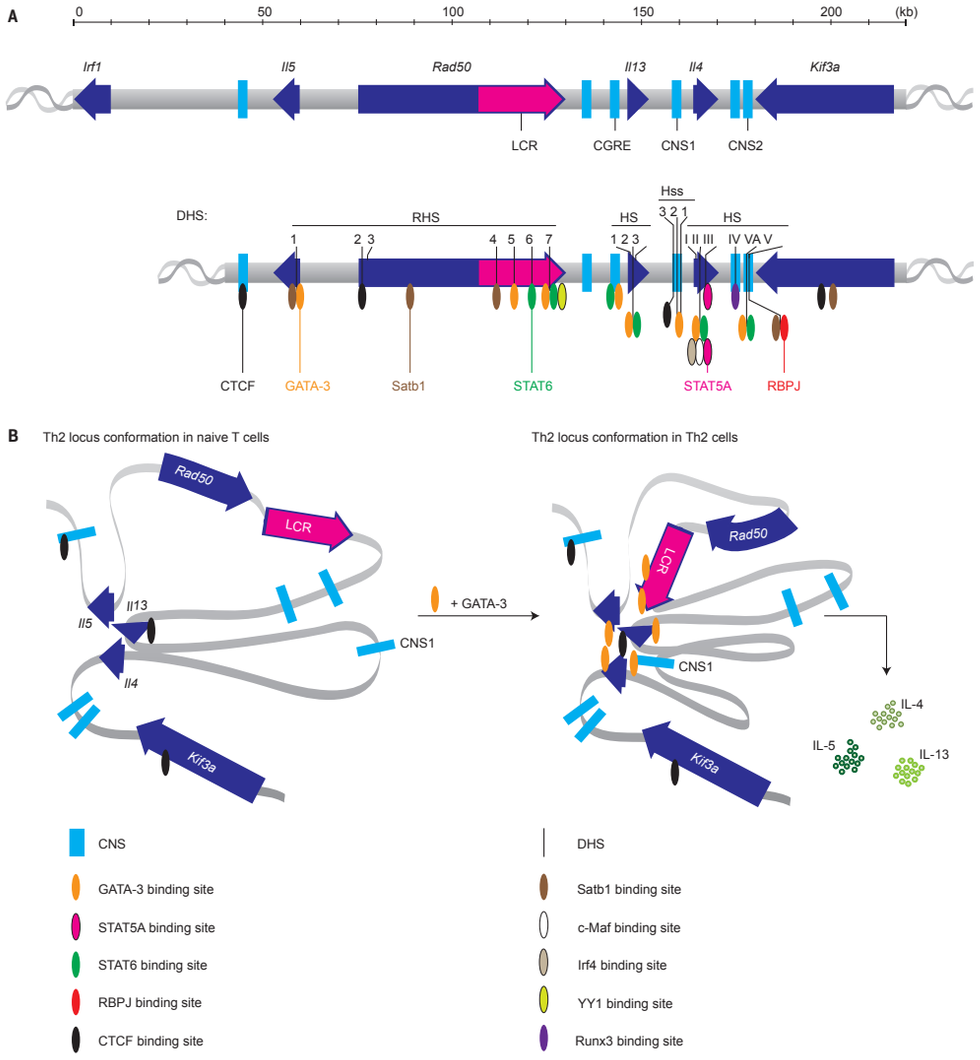

Figure 2. GATA-3-mediated regulation of the Th2 cytokine locus.

(A) GATA-3 binds to the *Il5* and *Il13* promoter regions and to the *Il13* HS1-CGRE region[302, 373, 374]. GATA-3 can also bind to a regulatory element in the first intron of the *Il4* gene[375]. GATA-3 helps establish long-range chromatin changes in the cytokine locus during Th2 cell differentiation, including the acquisition of four specific DHS sites: the HSII-intronic enhancer (IE), HSIII, HSVa and HSV, whereby HSII-IE is crucial in GATA-3 mediated activation of the *Il4* gene[298, 303, 376, 377]. HSII, controlled by GATA-3, is strongest of the known *Il4* enhancers in Th2 cells[302, 373, 374]. In the intergenic regulatory region CNS-1 located between the *Il4* and *Il13* genes, two additional Th2 cell-specific DHS sites HSS1 and HSS2 are occupied by GATA-3 *in vitro*[378]. GATA-3 also binds to RHS5 and RHS7 in the LCR located within the *Rad50* gene[379] in a STAT6-dependent manner[305]. Additional nuclear factors that have been shown to bind in the Th2 locus are indicated (see text).

(B) Schematic representation of the Th2 locus in naive T cells (*left*) and in polarized Th2 cells (*right*). In naïve T cells, the promoters for the Th2 cytokines genes are in close spatial proximity. During Th2 differentiation, activated STAT6 and Notch signaling induce GATA-3 up-regulation, leading to the participation of the LCR and CNS1 elements in this "poised" chromatin core configuration, allowing for high transcription of IL-4, IL-5 and IL-13, which is dependent on the presence of CTCF[380]. Abbreviations: CGRE, conserved GATA-3 response element; CNS1, conserved non-coding region; CTCF, CCCTC-binding factor; DHS, DNaseI hypersensitive site; Il, interleukin; IRF4, interferon regulatory factor 4; LCR, locus control region; RBPjκ, recombination-signal-binding protein for immunoglobulin Jκ region; Runx3, Runt-related transcription factor 3; Satb1, special AT-rich sequence-binding protein-1; STAT, Signal Transducer and Activator of Transcription; YY1, Yin Yang 1.

differentiation in the absence of Notch signaling[66, 129, 131], it is likely that under physiological conditions, Notch signaling and IL-4R signaling synergize to promote Th2 cell responses via the activation of GATA-3.

GATA-3 and Notch signaling are also required for efficient Th9 cell development. Th9 cells produce IL-9 and can differentiate from naïve T cells or Th2 cells under the influence of IL-4, IL-9 and TGF-β[294]. It has been shown that IL-9R expression is regulated by GATA-3[260]. Conditional deletion of Notch1 and Notch2 led to decreased IL-9 production in Th9 cultures and the Notch ligand Jagged2 - but not Delta-like 1- induced IL-9 in cells cultured with TGFβ alone[138].

Whereas Th2 cells substantially up-regulate GATA-3 levels during development, Th1 cells express very low amounts of GATA-3[31, 32, 295, 296]. During Th2 cell differentiation, GATA-3 inhibits T-bet function and IFN-γ expression. Moreover, GATA-3 suppresses Th1 cell development by down-regulating STAT4 and IL-12Rβ2 chain expression[295, 296]. This was supported by genome-wide analyses demonstrating that GATA-3-deficient Th2 cells have increased expression of STAT4 and IL-12Rβ2 mRNA[260]. Nevertheless, recent data shows that Th1 and Th2 cell differentiation is not mutually exclusive (as previously thought), as stable and functional GATA-3$^+$T-bet$^+$ T cells that produce both IL-4 and IFN-γ are generated *in vitro* and *in vivo* in parasite and lymphocytic choriomeningitis virus (LCMV) infection[297-300]. These GATA-3$^+$T-bet$^+$ T cells support both Th1 and Th2 cell-mediated immune responses but cause less immunopathology compared with committed T-bet$^+$ Th1 or GATA-3$^+$ Th2 cells, suggesting a regulatory role for these GATA-3$^+$T-bet$^+$ T cells. These studies are consistent with the notion of T helper cell flexibility and adaption depending on the characteristics of invading pathogens. Moreover, evidence is now emerging for frequent co-expression of the Th signature transcription factors GATA-3, T-bet, RORγt, Bcl6 or FoxP3 in specialized CD4$^+$ T cell subtypes, challenging the paradigm of stable T helper subsets defined by the expression of a single "master regulator"[301]. As such, T helper cell differentiation appears quite diverse and perhaps less stable compared with developmental cell-fate decisions that accompany lineage commitment, e.g. to the B or T lymphocyte lineage.

GATA-3 and the Th2 cytokine locus

GATA-3 binds directly to the *Il5* and *Il13* promoters, as well as to a binding site in the first intron of the *Il4* gene (**Figure 2**). In addition, GATA-3 plays a crucial role in establishing long-range chromatin interactions, as it binds to almost all DHS in the locus that Th2 cells acquire during their differentiation from naïve T cells, including DHS in the LCR, as well as four DHS crucial for activation of the *Il4* gene[302]. GATA-3 can induce the latter DHS in Th1 cells, which clearly demonstrates that GATA-3 is associated with chromatin remodeling activity[303]. More recently, chromatin immunoprecipitation coupled with next generation sequencing (ChIP-Seq) experiments showed that in Th2 cells genomic regions surrounding the GATA-3 binding sites in the *Il4* and *Il13* genes are associated with activating H3K4 methylation, but lack extensive repressive H3K27 trimethylation. In contrast, GATA-3 binding sites in the *Tbx21* and *Ifng* loci are associated with H3K27 trimethylation in Th2 cells[260, 304]. The finding that deletion of *Gata3* resulted in decreased H3K4 dimethylation at specific sites in the Th2 cytokine locus and decreased H3K27 trimethylation around its binding sites in the *Tbx21* and *Ifng* loci showed that GATA-3 mediates gene activation and repression by chromatin remodeling[260]. While T-bet is not expressed by Th2 cells, T-bet and GATA-3 are co-expressed in polarized human and mouse Th1 cells. Interestingly, ChIP-Seq experiments showed that many of the T-bet and GATA-3 binding sites in the *Ifng* locus in Th1 cells were coincident[259, 261], as discussed below.

Chromosome conformation capture (3C) studies show that the promoters for the Th2 cytokines genes are in close spatial proximity in various cell types, and in CD4$^+$ T cells specifically the LCR participates in this "poised" chromatin core configuration[283]. GATA-3 and STAT6 have the capacity to directly remodel the LCR[305] and are essential for the establishment or maintenance of these long-range interactions, but additional nuclear factors have been implicated in the 3D organization of the Th2 locus. These include Th2-specific transcription factors involved in *Il4* gene regulation, such as interferon regulatory factor 4 (IRF4), nuclear factor of activated T cells NFATc2 and c-Maf, but also general chromatin organizers, such as special AT-rich binding protein 1 (SATB1), Yin Yang1 (YY1), CCCTC-binding factor (CTCF) and cohesin[306-309] (**Figure 2**). Further studies are required to elucidate the exact mechanisms by which GATA-3 contributes to the formation of chromatin loops in the Th2 cytokine locus.

In addition to Th2 cells, diverse myeloid cells, including mast cells, basophils and eosinophils, are potent producers of IL-4, IL-5 and IL-13 *in vivo*. The finding that GATA-1 and GATA-2 bind HSII in the *Il4* locus in mast cells[310], indicates that in type II immunity GATA-3 function in lymphoid cells is, at least partly, substituted by GATA-1 and/or GATA-2 in myeloid cells.

GATA-3 in T cell development

As GATA-3 plays critical roles in several tissues and deletion of *Gata3* results in embryonic lethality in mice[272], analysis of GATA-3 function in immune development has been challenging. However, the use of diverse technical approaches (including blastocyst complementation, fetal liver hematopoietic stem cell reconstitution and conditional gene targeting) has provided important clues as to how GATA-3 functions during the various stages of T cell development.

One dramatic result of GATA-3 deletion is the complete absence of T cell development[236, 311, 312]. In the thymus, the T cell program is initiated by differentiation of early thymic progenitors (ETP) derived from multipotent hematopoietic precursors in the bone marrow[313]. Thymopoiesis requires several regulatory pathways for early thymocyte differentiation, including activation of the Notch1 receptor[117, 119] by its ligand delta-like 4 expressed on thymic epithelial cells[124, 125]. Notch1 triggering initiates and sustains the T cell program via activation of its transcription factor targets Tcf1 and Bcl11b[314]. GATA-3 is also up-regulated at this stage, although it is not clear whether this event is Notch-dependent. As such, the interrelationship between Notch1 and GATA-3 pathways remains unclear. Without Notch1 signals, ETPs do not develop and thymic progenitors can be diverted into the B cell pathway[120, 123, 315]. This Notch1-mediated repression of B cell development also involves GATA-3, as GATA-3-deficient pro-T cells retain a latent B cell potential despite active Notch1 triggering[252] (**Figure 3**). This is not the case with pro-T cells deficient for the Notch1 targets Tcf1 or Bcl11b[316, 317], and thus GATA-3 appears unique in its ability to "seal" Notch1-induced T cell commitment[318]. The mechanism by which GATA-3 represses the B cell program is unknown, but it is striking that repression of GATA-3 by the transcription factor early B cell factor-1 (Ebf1) is a critical component of normal B cell development[251]. In the absence of Ebf1, lymphoid progenitors exhibit increased T cell lineage potential associated with increased *Gata3* gene transcription[251]. Ebf1 ablation can divert lymphoid precursors into the ILC pathway as well[319]. As such, the relative balance between GATA-3 and Ebf1 pathways critically determines the B versus T cell or ILC cell fate decision.

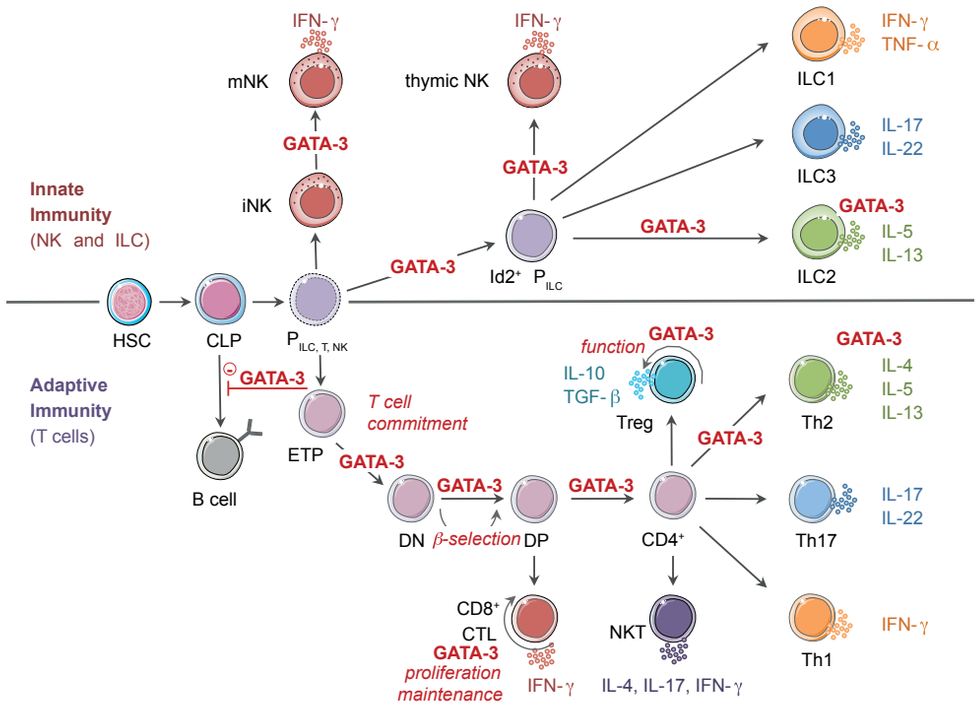

Figure 3. GATA-3 has multiple roles in ILC and T cell development and function

HSC-derived CLPs give rise to both adaptive and innate lymphoid cells. ILC development (top) mirrors T cell development (bottom). Developmental steps or cellular functions that absolutely require GATA-3 are indicated. These include the generation of a common ILC precursor, the differentiation into thymic NK cells and ILC2, as well as for NK cell maturation. GATA-3 represses B cell potential and in crucial in various stages of T cell development. In addition, GATA-3 is important for the function of $CD8^+$ T cells, Treg cells and Th2 cells, as indicated. T effector and ILC subsets are grouped according to their ability to produce different cytokines. Abbreviations: CLP, common lymphoid progenitor; CTL, cytotoxic T lymphocyte; HSC, hematopoietic stem cell; IFN, interferon; IL, interleukin; ILC, innate lymphoid cell; iNK, immature NK cell; mNK, mature NK cell; NK, natural killer cell; NKT, natural killer T cell; P, progenitor; TGF, transforming growth factor; Th, T helper cell; Treg, regulatory T cell.

Using Lck-Cre transgenic *Gata3*-floxed mice to ablate GATA-3 expression in early DN cells, it was established that GATA-3 is critical for β-selection (**Figure 3**), and thus for the generation of T cell receptor (TCRβ)-expressing DN4 thymocytes[320]. This is consistent with findings in GATA-3 reporter mice that demonstrate up-regulation of GATA-3 during pre-TCR-mediated β-selection[311]. Continued Notch1 triggering is also required for this transition[321, 322], which again suggests a close relationship between Notch1 and GATA-3 pathways at this juncture.

Using Chip-Seq technology and expression profiling in developing thymocytes, GATA-3 binding was detected at 1,500 loci with marked differences in GATA-3 occupancy between early and later stages of T cell development[260, 323]. In fact, GATA-3 binding sites did not show significant similarities between DN1 and DP cells, but rather binding was particularly enriched at "stemness" genes and "T cell identity" genes, in these respective stages[323]. These analyses suggest that GATA-3 is involved in control the expression of many genes that play a crucial role in T cell development, including key transcription factors, Notch1 and Notch 2, TCR components and the RAG enhancer. Because in DN thymocytes GATA-3 occupancy of regulatory elements in *Cd3d*, *Rag1*, *Rag2* and *Zbtb7b* loci preceded their expression, GATA-3-mediated chromatin remodeling may function to prepare loci for interactions with nuclear factors at later stages of T cell development.

Conditional ablation of GATA-3 at later stages of T cell development (at the DP stage, using CD4-Cre transgenic *Gata3*-floxed mice) demonstrates the necessity of GATA-3 for promoting $CD4^+$ lineage choice[320]. In the absence of GATA-3 differentiation of the $CD4^+$ lineage is blocked before CD4/CD8 commitment, because MHC class II-restricted GATA-3-deficient thymocytes are redirected to the $CD8^+$ T cell lineage, albeit inefficiently[324]. In this context, GATA-3 is required for the expression of the transcription factor ThPOK, which promotes $CD4^+$ lineage differentiation[325, 326]. The finding that enforced ThPOK expression does not rescue $CD4^+$ lineage differentiation of GATA-3-deficient thymocytes, indicates that GATA-3 also acts as a key $CD4^+$ lineage differentiation factor, independently of its capacity to induce ThPOK[324]. Expression of GATA-3 is induced by TCR signaling during positive selection at the DP stage and as GATA-3 enhances TCR upregulation, it is likely that GATA-3 establishes a positive feedback loop that increases TCR surface expression in developing CD4-lineage cells[327, 328]. Accordingly, GATA-3 binds strongly to the *Tcra*, *Tcrb*, *Cd3d* and *Cd3g* loci and expression of CD3δ and CD3ε mRNA is decreased in GATA-3-deleted $CD69^+$ DP cells. Although it has been suggested that Notch activity directly influences CD4/CD8 lineage commitment, it now seems that DP have very low levels of Notch signaling and that Notch does not play a direct role in lineage commitment[116, 329].

Development of $CD8^+$ SP thymocytes is not affected by lack of GATA-3. However, peripheral mature naïve $CD8^+$ T cells constitutively express GATA-3, albeit to lower levels than found in $CD4^+$ T cells, and expression is upregulated by TCR activation and cytokine signaling. GATA-3 is important in $CD8^+$ T cells as it controls proliferation by regulating c-Myc, but it is dispensable for IFN-γ production[246] (**Figure 3**). GATA-3-deficient $CD8^+$ T cells manifest defective long-term maintenance, which is attributed to lower IL-7R expression. By contrast, GATA-3 expression does not appear to be critical for the response to IL-7 in thymocytes. This is inferred from the finding that in GATA-3-deficient mice $CD8^+$ SP thymocytes develop normally, although IL-7R signaling is essential for positively selected thymocytes to express the transcription factor Runx3 to specify CD8 lineage choice and promote $CD8^+$ SP differentiation[330].

While GATA-3 is required for multiple stages of T lineage development (**Figure 3**), an additional role for GATA-3 in pre-thymic lympho-hematopoietic progenitor cells is as yet unclear. HSC express GATA-3[248], although early reports found that absence of GATA-3 did not affect the generation, maintenance or self-renewal of HSC in fetal and adult mice[261, 312, 331]. In contrast, it was recently shown that *Gata3* deletion enhances expansion of long term-multipotent HSC, consistent with a role for GATA-3 as an autonomous regulator of the balance between HSC self-renewal and differentiation[250]. Although in the absence of *Gata3* the production of functional definitive HSCs in the embryonic AGM region is impaired, this is largely independent of a cell-intrinsic role of GATA-3 and secondary to abnormalities in the developing sympathetic nervous system[249].

GATA-3 in ILC development and function

Recent work has demonstrated that GATA-3 serves as a central regulator of ILC development and function (**Figure 3**). ILCs contribute to the first-line immune defense against invading pathogens and have the capacity to promptly produce large amounts of pro-inflammatory cytokines, prior to the generation of adaptive immunity. ILCs have been categorized into three major groups, based on transcription factor dependency and cytokine production profiles, which strikingly mirror the various T helper cell subsets[257]. Group 1 ILCs (or ILC1) consist of NK cells and other IFN-γ-producing innate lymphocytes that express T-bet. ILC1 have been shown to play a major role in the defense against viruses, intracellular bacteria and protozoa[256, 332, 333]. ILC2 secrete IL-5 and IL-13 in response to stimulation with the cytokines IL-25, IL-33 or thymic stromal lymphopoietin (TSLP). ILC2 are important in the immune response against helminths, but are also associated with allergic airway inflammation and hyperreactivity and maintenance of epithelial barrier integrity during influenza infection (see for review: REF 11). Group 3 ILCs (ILC3) include several phenotypically distinct cells that express and require the transcription factor Rorγt for their development and for production of IL-17A and IL-22. ILC3 are enriched at mucosal sites and appear to regulate barrier function and epithelial cell homeostasis[258].

ILC1

Several transcription factors drive bone marrow and tissue-resident NK cell development from lymphoid precursors, including the T-box factors T-bet (encoded by *Tbx21*) and eomesodermin (Eomes), nuclear-factor interleukin-3 related (Nfil3) and GATA-3 (reviewed in REF 334). *Gata3* ablation affects the development of mature CD11b$^+$ splenic NK cells and reduces their capacity to produce IFN-γ[335]. In the thymus, GATA-3 deletion ablates the generation of IL-7Rα$^+$ NK cells[253] and more recent results show that GATA-3 is also critical for the development of a peculiar subset of CD49a$^+$Eomes$^-$NKp46$^+$NK1.1$^+$ ILC1 in the gut[256], but not in the liver[336]. This differential impact of GATA-3 deletion on diverse NK cell subsets suggests distinct developmental pathways for conventional and tissue-resident NK cells[256, 337, 338] (**Figure 3**). Consistent with this hypothesis, a bone marrow PLFZ$^+$GATA-3$^+$ ILC precursor was described that can develop into CD49a$^+$ hepatic but not conventional NK cells[254]. The signals that generate these apparently distinct ILC precursors from CLP and their capacity to promote NK cell development in the bone marrow versus tissue-specific sites remains an area of active research.

Recent work has demonstrated that other IFN-γ-producing NK1.1$^+$ cells are present in mucosal sites in humans[339] and in mice[256, 340]. These ILC1 subsets require the transcription factors T-bet, Nfil3 and GATA-3 for their generation and are phenotypically distinct from conventional NK cells that express NKp46, CD49a, IL-7Rα, CD27 but not CD11b. These ILC1 subsets seem to be an important source of IFN-γ and TNF-α in both the intestinal epithelial layer and the lamina propria under steady state conditions and during intestinal inflammation[256, 340]. The molecular mechanism through which GATA-3 contributes to ILC1 development or if its maintained expression is needed for functional attributes is unclear.

ILC2

GATA-3 plays an essential role in ILC2 development in mice[239-241, 280] and man[341] (**Figure 3**). ILC2 are generated from CLP *in vivo*[156, 157] and alterations in *Gata3* gene dosage positively correlates with ILC2 development from CLP *in vitro*[240], suggesting that GATA-3 transcriptional activity is a major determinant of ILC2 cell fate in uncommitted lymphoid precursors. Accordingly, bone marrow and lung ILC2 homeostasis in naïve mice correlates *in vivo* with *Gata3* gene copy number[240]. The transition from CLP to ILC2 is associated with up-regulation of the transcription factors inhibitor of DNA-binding 2 (Id2) and RORα, both of which are essential for ILC2 differentiation[156, 342]. Although the gene encoding Id2, which is involved in the development of all known ILC lineages[242, 343], is occupied by GATA-3 in early thymocyte precursors[323], inactivation of GATA-3 in mature ILC2 did not affect the expression of Id2 or RORα[241]. *In vitro* generation of ILC2 from CLP is dependent on Notch signaling[156, 157], although it remains to be demonstrated whether ILC2 development *in vivo* requires canonical Notch signaling. Nevertheless, certain parallels between ILC2 development and early T cell are striking: Notch1, GATA-3 and Tcf1 are critical for ILC2 and T cell development[344], with Tcf1 being a Notch1-induced target for T cell specification[316] that likely serves a similar role in ILC2 generation.

In mature ILC2, GATA-3 controls cell activation and function, including IL-5 and IL-13 cytokine secretion, cytokine responsiveness (IL-25 and IL-33 receptors) and production of amphiregulin, an epidermal growth factor family protein essential for airway epithelium integrity[238-241, 345]. The finding that ILC2 effector function directly correlates with *Gata3* gene expression suggests that GATA-3 modulation impacts pathological conditions that involve dysregulation of type II immunity. This notion gains support from genome-wide association studies showing a significant association of the *Il33* and *Il1Rl1* (encoding the IL-33R subunit T1ST2) loci with asthma in human and the increases susceptibility to allergic airway inflammation observed in mice with enforced expression of GATA-3 in T cells and ILC2[346]. In mouse models of allergic lung inflammation, ILC2 along with classical Th2 cells are major producers of IL-5 and IL-13 that orchestrate and amplify allergic inflammatory events in asthma[347]. Moreover, ILC2-derived IL-13 can promote migration of activated lung DCs that drive differentiation of naïve T cells into Th2 cells[13].

Thus, GATA-3 plays a crucial role in the induction of IL-5 and IL-13 cytokine production both in ILC2 and in Th2 cells, which synergize in type II immunity and are activated through different mechanisms and with different kinetics.

ILC3

ILC3 are a heterogeneous population and include CCR6$^+$ lymphoid tissue inducer (LTi) cells that are needed for lymphoid tissue organogenesis in lymph nodes and Peyer's patches during fetal life and for postnatal formation of intestinal lymphoid clusters[348]. Moreover, adult CD4$^+$ ILC3 that can be found in secondary lymphoid tissues and in mucosal sites produce IL-17A and IL-22 that can contribute to immune defense[349]. Another subset of ILC3 that express the NK cell receptor NKp46 is non-cytotoxic and produces IL-22 but not IFN-γ[350-352]. NKp46$^+$ ILC3 are CCR6$^-$, are found primarily in the intestinal lamina propria, and cross-talk with epithelial cells to stimulate cell proliferation and production of anti-microbial peptides that regulate communities of commensal bacteria[351, 353]. Heterogeneous CD4$^-$ NKp46$^-$ 'double negative' ILC3 have also been described and produce IL-17A, IL-22, IFN-γ and TNF-α and are involved in intestinal inflammation[354-356]. Both NKp46$^+$ and a subset of double negative ILC3 express the transcription factor T-bet, which is critical for their development[168, 354, 357].

More recently, GATA-3 was shown to be crucial for development of both LTi cells and T-bet$^+$ ILC3[241, 255]. ILC3 express abundant GATA-3 protein, albeit in lower amounts than observed in mature ILC2. Hematopoietic chimeric mice derived from GATA-3-deficient fetal liver cells failed to generate intestinal ILC3 subsets and showed defective IL-22 production and maintenance of mucosal barrier homeostasis upon infection[23]. Conditional *Gata3* ablation using Vav1-Cre generates a similar defect in ILC3 development[241]. Moreover, in the fetus GATA-3 is critical for differentiation of CD135$^+$α4β7$^+$ CLP-like cells and cell-intrinsic GATA-3 expression is essential to generate fetal liver RORγt^{hi}IL7Rα^{hi} precursor cells[255]. While the GATA-3-dependent transcriptional pathways that drive ILC3 development remain unclear, RNA-seq analyses revealed that GATA-3 ablation does not modify *Rorc, Runx1, Runx3, AhR, Id2* or *Tcf7* expression in mature ILC3[241]. This result probably reflects the context-dependent role for GATA-3 in early ILC development that is not recapitulated in mature ILC3, as previous reports demonstrate that GATA-3 expression in mature Id2$^+$ ILC3 is not essential for their homeostasis[239].

GATA-3 as a central regulator of ILC development

The observation that GATA-3 ablation severely affects development of several distinct ILC subsets (ILC2, ILC3, intestinal CD49a$^+$NK1.1$^+$ ILC1, thymic NK cells) suggests that GATA-3 could operate at an early stage of ILC differentiation and perhaps via the generation of common ILC precursors (**Figure 3**). In the fetus, a subset of α4β7$^+$ fetal liver precursor (Lin$^-$ IL-7Rα$^+$ Sca-1^{int}c-Kit^{lo}) cells have been described that fail to give rise to B and T cells but retain NK cell and ILC3 potential[356, 358]. A similar α4β7$^+$Lin-IL-7Rα$^+$ bone marrow subset exists, although it includes more mature ILC2[239, 240]. Recently, two reports identified that this α4β7$^+$ subset contains committed ILC progenitors[254, 256]. Both groups used GFP reporter mice (in either the *Zbtb16* (PLZF) or *Id2* loci) to show that putative fetal and adult BM ILC precursors could give rise to ILC1-3 subsets *in vivo* and *in vitro* at the single cell level. These ILC precursors gave rise to ILC2, ILC3 (especially NKp46$^+$ ILC3) and the peculiar CD49a$^+$NKp46$^+$ ILC1 subset that has been identified in the liver and gut. Interestingly, these ILC precursors had reduced capacity to generate conventional NK cells. As such, these ILC precursors had the developmental potential for the same ILC subsets that require GATA-3 for their development[168, 254, 255, 335, 354]. Accordingly, one group found that PLZF$^+$ α4β7$^+$ cells co-expressed GATA-3 protein, suggesting a link between GATA-3 expression in these ILC precursors and their cell fate potential[254].

As CLP are GATA-3$^-$ Id2$^-$ and PLZF$^-$[240, 254], identifying the signals that allow for the emergence of Id2$^+$PLZF$^+$GATA-3$^+$ ILC precursors from CLP will be of considerable interest. Soluble factors (such as IL-7) and cell-intrinsic transcription factors (including Notch1, Tox, Runx1) are important for the normal development of distinct ILC subsets[166, 168, 170, 359-361]. One possibility is that GATA-3 up-regulation is an early event in CLP that effectively restricts B lineage fate and thereby generates T and ILC precursors (**Figure 3**). Such 'bi-potent' precursors would then further differentiate to more restricted T lineage precursors (ETP) or ILC precursors; up-regulation of Id2 would be a dominant factor in promoting the development of the latter. This model is consistent with the existing data and would clearly distinguish the developmental pathway of conventional NK cells (GATA-3-independent) from other ILC subsets (GATA-3-dependent).

Although GATA-3 function in ILC precursors and ETP may be partly overlapping, e.g. to repress B cell potential, it is conceivable that collaboration of GATA-3 with other transcription factors, such as RBPjκ, Id2, Tcf1, Nfil3, T-bet or RORg may enforce differentiation into the distinct ILC subsets or the T cell lineage.

GATA-3 cofactors and target genes

GATA-3 and its interacting partners

As GATA factors are expressed in a variety of cell types and can act as transcriptional activators or repressors, it was expected that their functional outcome would depend on their interactions with other transcriptional regulators. Indeed, using a biotinylation tagging/proteomics approach in erythroid cells, the association of specific interacting partners were linked to activating versus repressive functions of GATA-1[362]. Likewise, it was recently shown that GATA-3 and chromodomain helicase DNA binding protein 4 (Chd4) complex form functionally distinct activating and repressive assemblies with histone acetyltransferase (HAT) and histone deacetylase (HDAC) activity, respectively[363]. Many other proteins are known to interact with GATA-3 (**Table 1**). Genome-wide identification of GATA-3 binding sites in thymocytes and various effector T cell populations using Chip-Seq technology showed that in addition to the primary 5'-(A/T)GATA(A/G)-3'motif they contained several secondary motifs, including binding sites for the Ets, Runx, AP1 and TCF11 transcription factors, or even contained only secondary motifs[260]. Therefore, GATA-3 can be recruited through physical interactions with another transcription factor, or protein interactions may stabilize binding of GATA-3 to non-canonical target sequences.

T-bet represses Th2 lineage commitment through a physical interaction with GATA-3 that is enhanced by T-bet tyrosine phosphorylation and interferes with GATA-3 binding to its target DNA[364]. However, recent genome-wide comparison of T-bet and GATA-3 binding sites revealed that many of the Th1-specific GATA-3 binding sites overlapped with T-bet binding motifs[259, 261]. As in Th1 cells, GATA-3 binds to T-box motifs and not to cognate GATA-3 sites, GATA-3 occupancy is mediated through association with T-bet and T-box motifs[261]. On the other hand, the T-box factor Eomes interacts with GATA-3 and suppresses the transcriptional activity of GATA-3 (and IL-5 expression) in memory T cells[365]. Interaction of GATA-3 and the transcription factor Runx3 actively represses production of IFN-γ in Th1 cells. These examples demonstrate how GATA-3 targets repression and can be the target of repression.

FoxP3 forms a complex with GATA-3 specifically in activated Treg cells. Under inflammatory settings, GATA-3 limits Rorγt expression and thus restrains excessive polarization and inflammatory cytokine production by Treg cells[247, 366]. GATA-3 is induced upon TCR and IL-2 stimulation and is required for the accumulation of Treg cells at inflamed sites. In Treg cells, GATA-3 specifically binds to regulatory elements of the *Foxp3* locus and thereby directly controls FoxP3 expression[245]. Conversely, FoxP3 binds to the promoter and intronic regions of the *Gata3* locus, and thus FoxP3 and GATA-3 proteins not only physically interact, but also reciprocally control each other's expression. Because FoxP3-GATA-3 complex formation is dependent on TCR stimulation, GATA-3$^+$ Treg cells are subject to immune control in response to environmental changes.

YY1 occupies regulatory elements in the Th2 locus and is required for subsequent GATA-3 binding[308]. Thus, cooperation of YY1 with GATA-3 is essential for regulation of the Th2 cytokine locus and Th2 cell differentiation. The Ets-family member Fli1 binds to ~70% of all GATA-3-bound sites in Th2 cells[260]. Upon *Gata3* deletion, Fli1 occupancy at the majority of shared GATA-3 and Fli1 binding sites is lost (including binding to the *IL13* and *Rad50* loci) indicating mutualistic binding of these two factors.

The differential complex formation provides a mechanism by which GATA-3 can be directed to a distinct subset of its potential binding sites in a cell type-specific fashion, and can help explain GATA-3 function as a transcriptional activator or repressor. Moreover, context-dependent cooperative binding of GATA-3 with different transcription factors dramatically increases the regulatory complexity. Indeed, even closely related T cell subsets (e.g. DN2 and DP thymocytes or Th1 and Th17 effector cells) exhibited different GATA-3 binding patterns, despite nearly identical amounts of total GATA-3 protein present[260, 323].

GATA-3 target genes

To identify GATA-3 target genes genome-wide, GATA-binding sites have been identified by Chip-Seq and changes in gene expression upon *Gata3* deletion were evaluated in various thymocyte and mature T cell subsets[260, 261, 323]. In these studies, >7,000 and >14,000 GATA-3 binding sites were identified genome-wide in murine and human Th2 cells, respectively. Although GATA-3 binding was enriched in gene regions just upstream of transcription start sites and 5'UTR, a majority of binding sites was >2kb distal to known gene promoters. These distal sites were frequently at conserved sequences, coinciding with DHS, and enriched for both activating H3K4 methylation marks, indicative for cis-regulatory elements[261]. Gene Ontology analysis revealed that genes harboring distal GATA-3 binding sites - but not genes bound by GATA-3 only at their promoter region - were enriched for immune response functions. Upon deletion of *Gata3* only a minority of GATA-3 bound genes (<10%) showed a significant increase or decrease in expression, but ~46% of its bound genes manifested a significantly changes in H3K4 or H3K27 histone methylation marks. Therefore, the observed epigenetic modifications are most likely regulated by GATA-3 activity and not a consequence of transcriptional activation or silencing.

In addition to GATA-3 targets in the Th2 locus, these genome-wide studies identified several other complex loci controlled by GATA-3 in Th2 cells. These include the ~145-kb cytokine cluster containing the *Il10* gene and the homologues *Il19*, *Il20* and *Il24*[367]. Consequently, *Gata3* deletion resulted in significantly decreased expression of IL-10 and IL-24. Furthermore, the large genomic regions encompassing the chemokine receptor genes (*CCR9*, *CXCR6*, *XCR1*, *CCR1*, *CCR3*, *CCR2*, *CCR5*, *CCRL2*) or the *Il1r1*, *Il1rl2*, *Il1l1* (encoding IL-33R or T1ST2), *Il18r1* and *Il18rap* genes contains many GATA-binding sites, whereby GATA-3 binds to sites in the *Il18r1* gene in both Th1, Th2, Th17 and iTreg cells[260, 261]. As described above, expression of IL-33R is crucial for ILC2 activation and enforced expression of GATA-3 in transgenic mice induced the formation of Th2 memory cells expressing high amounts of IL-33R[368].

To date, the identification of GATA-3 targets in the various mature ILC subsets and in developing ILCs is not only hampered by the low cell numbers of the individual ILC subsets and their precursors, but also by the fact that ILC precursors are still poorly defined. Nevertheless, comparison of genes expressed in Th2 cells and mature ILC2 showed that only 55 genes were positively or negatively regulated by GATA-3 both in Th2 and ILC2, including *Il5*, *Il13*, *Areg*, (encoding amphiregulin) and *Il1rl1*[241]. In contrast, 281 unique targets were regulated by GATA-3 in ILC2 (e.g. Icos, Il2ra and Kit), while 568 targets were regulated in Th2 cells (e.g. *Il4* and *Maf*). Although these may represent both direct and indirect targets of GATA-3, these findings indicate that GATA-3 has mostly unique functions in these two functionally related cell types. In a similar fashion, less than 4% of genes regulated by GATA-3 in ILC2 were also regulated by GATA-3 in ILC3.

Concluding remarks

Over two decades of research on GATA-3 biology has demonstrated that within the hematopoietic stem, GATA-3 has multiple and diverse roles that are mediated in a complex, dose-dependent, developmental stage-specific and cell-lineage specific fashion. Context-dependent activating or repressive functions of GATA-3 are provided by differential cooperative binding of GATA-3 with several different transcription factors, whereby related T cell or ILC subsets exhibit very different genome-wide GATA-3 occupancy.

Highly-sensitive approaches to examine genome-wide GATA-3 binding sites in small populations of ILC subsets and their precursors may help to elucidate the critical GATA-3-dependent developmental pathways from CLPs to individual lymphocyte precursors. Moreover, such analyses should identify crucial GATA-3 targets as well as the complex relationships between GATA-3, Notch signaling and key transcription factors such as Id2, RORα, RORγ, Tcf1 and Nfil3 in lymphocyte cell-fate decisions.

Parallels exist between ILC in the bone marrow and T cell development in the thymus, as they both require – in addition to GATA-3 - similar transcription factors (e.g. Tcf-1) and Notch signaling. Obviously, instructive signals from the micro-environment such as cytokines, Notch ligands or Wnt signaling are different between bone marrow and thymus, but also ETP and ILC precursors will have different intrinsic developmental capacities. Therefore, future experiments should show common and unique GATA-3 targets and their epigenetic configurations in ILC precursors and ETP.

In mice, GATA-3 functions in a dose-dependent fashion in both ILC2 and T cells[240, 346, 369]. Whereas human GATA-3 haploinsufficiency affects T cell function, its effects on ILC subsets are not known. Enforced expression of GATA-3 during T cell development induces DP T cell lymphoma, whereby malignant transformation involves cooperation with c-Myc and the induction of activating Notch1 mutations[370]. Likewise, recent genome-wide germline SNP analysis identified *GATA3* gene variants that influence susceptibility to Philadelphia chromosome-positive acute lymphoblastic leukemia and risk of relapse[371]. It will be important to understand how dysregulated GATA-3 influences neoplastic transformation in hematopoietic cells and non-hematopoietic lineages[275].

Although to date the role of GATA-3 in the regulation of Th2 cytokine expression is known in molecular detail, one of the unresolved questions that remains is how signals from the micro-environment cooperate to induce GATA-3 expression in activated T cells. Next to TCR, IL-4R and Notch signaling, very recent studies indicate that also nucleic acids (NA) released from dead cells and complexes with antimicrobial peptides or histones can upregulate GATA-3 expression, independently of known NA sensors[372]. GATA-3 expression is sufficient and required for development and function of Th2 cells and ILC2 that play a central role in allergic inflammation. Therefore, inhibiting GATA-3 function, e.g. by inhibition of its translocation to the nucleus[49], could be an excellent starting point for drug discovery strategies.

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Chapter 3

Notch signaling in T cells is essential for allergic airway inflammation, but expression of Notch ligands Jagged1 and Jagged2 on dendritic cells is dispensable

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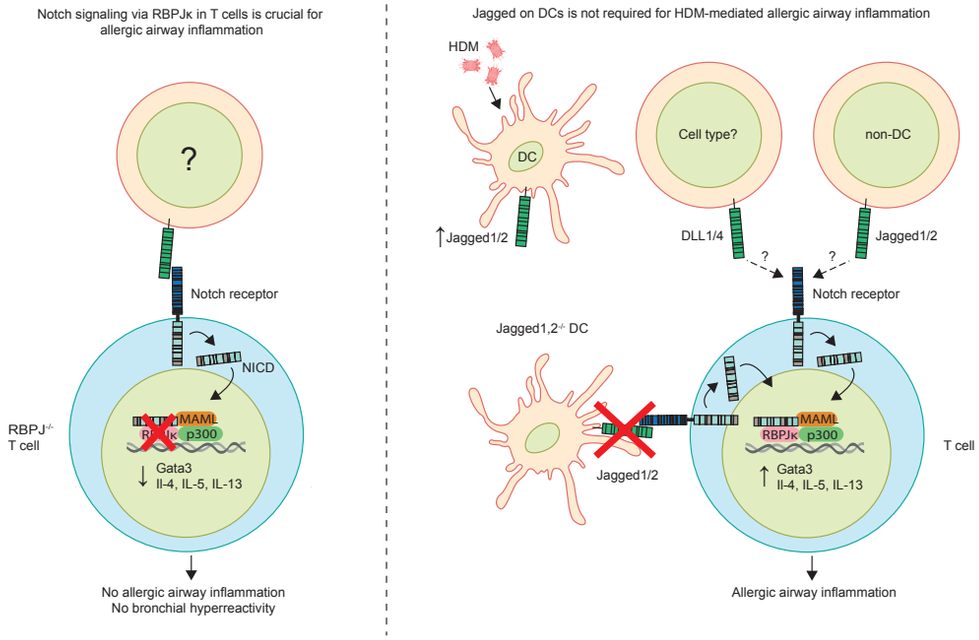
Graphical Abstract



Abstract

Background: Allergic asthma is characterized by a T helper 2 (Th2) response induced by dendritic cells (DCs) that present inhaled allergen. Although the mechanisms by which they instruct Th2 differentiation are still poorly understood, expression of the Notch ligand Jagged on DCs has been implicated in this process.

Objective: To establish whether Notch signaling, induced by DCs, is critical for house-dust mite (HDM) driven allergic airway inflammation (AAI) *in vivo*.

Methods: The induction of Notch ligand expression on DC subsets by HDM was quantified by qRT-PCR. We used a HDM-driven asthma mouse model to compare the capacity of Jagged1 and Jagged2 single and double-deficient DCs to induce AAI. In addition, we studied AAI in mice with a T cell-specific deletion of RBPJκ, a downstream effector of Notch signaling.

Results: HDM exposure promoted the expression of Jagged1, but not Jagged2, on DCs. In agreement with published findings, *in vitro* differentiated and HDM-pulsed Jagged1 and Jagged2 double-deficient DCs lacked the capacity to induce AAI. However, following *in vivo* intranasal sensitization and challenges with HDM, DC-specific Jagged1, Jagged2 single or double-deficient mice developed an eosinophilic airway inflammation and Th2 cell activation phenotype that was not different from that in control littermates. In contrast, RBPJκ-deficient mice failed to develop AAI and airway hyperreactivity.

Conclusion: Our results show that the Notch signaling pathway in T cells is crucial for the induction of Th2-mediated AAI in a HDM-driven asthma model, but that expression of Jagged1 or Jagged2 on DCs is not required.

Clinical Implications: The Notch signaling pathway in T cells is critical for development of house-dust mite driven allergic airway inflammation in mice, indicating it could be a potential therapeutic target in asthma.

Introduction

Allergic asthma is a T helper 2 (Th2) cell-mediated disease characterized by chronic airway inflammation, airway hyperreactivity and episodes of bronchoconstriction. Inflammatory dendritic cells (DCs) are necessary for induction of Th2 immunity to inhaled house dust mite (HDM) allergen in mice, as was shown in CD11c-diphtheria toxin receptor (DTR) mice in which DCs were specifically depleted by DT exposure[385]. Lung resident DCs continuously sample the airway lumen for the presence of allergens such as HDM and once activated, these cells mature and migrate to draining lymph nodes to activate naïve T cells (reviewed in REF 386). Upon antigenic stimulation by DCs, Th2 cell differentiation is initiated whereby the polarizing cytokine IL-4, which induces phosphorylation and activation of STAT6, enhances expression of the key Th2 transcriptional regulator Gata3[285]. Th2 cells are potent producers of cytokines that induce IgE synthesis (IL-4), recruit eosinophils (IL-5) and cause smooth muscle hyperreactivity and goblet cell hyperplasia (IL-13).

Therefore, the initiation of Th2 cell differentiation via the IL-4/STAT6 axis is suggestive of an autocrine loop that leads to the expansion of IL-4 producing T cells. However, the primary origin of IL-4 that induces the Th2 response remains unclear. One of the pathways that has been implicated in the initiation of Th2 cell differentiation is the Notch signaling pathway. It has been demonstrated that Notch signaling has the capacity to induce Th2 cell differentiation by (I) directly activating the upstream *Gata3* gene promoter and by (II) regulating *Il4* gene transcription through activation of a 3' enhancer[93, 129, 130]. Both of these are dependent on a nuclear complex that includes recombination-signal-binding protein for immunoglobulin Jκ region (RBPjκ) and the co-activator Mastermind-like 1 (MAML1). Notch signaling in $CD4^+$ T cells is required for physiological Th2 responses to parasite antigens, as was shown in mice deficient for RBPjκ or the Notch1 and Notch2 receptors[129] and in mice expressing dominant-negative MAML[131]. Moreover, pharmacological inhibition of γ-secretase, the enzyme that liberates the intracellular Notch domain from the plasma membrane allowing it to function as a transcription factor in the nucleus, led to decreased Th2 cytokine production after immunization with ovalbumin in an asthma model[133].

Several lines of research support that Notch ligands Delta-like ligand (DLL) and Jagged instruct Th1 and Th2 cell differentiation, respectively[207]. Surface DLL expression was shown to promote generation of Th1 cells and to reduce Th2 responses, whereas Jagged expressing antigen presenting cells stimulated Th2 effector generation[93]. Jagged1 can be upregulated on DCs by stimuli that promote Th2 cell responses. For instance, via thymic stromal lymphopoietin, produced by diesel exhaust particle-treated human bronchial epithelial cells[220], and upon stimulation with *T. brucei*-derived antigens as well as TNF, Dermatophagoides pteronyssinus group 7 allergen (Derp7) and low-dose LPS[214, 219, 387]. Jagged1 was shown to be crucial in the induction of a Th2 response in a model of airway hyperresponsiveness using ovalbumin-pulsed *in vitro* cultured granulocyte-macrophage colony-stimulating factor (GM-CSF) bone marrow-derived (bm)DCs[208]. Although evidence was provided that Jagged2 is dispensable for the induction of Th2 cells *in vivo*[209, 210], Jagged2 was shown to have the capacity to induce Th2 cell differentiation *in vitro*[209]. Correspondingly, DLL1 and DLL4 ligands are induced on DCs by stimuli that elicit Th1 responses and have the capacity to induce Th1 differentiation *in vitro*[225, 388].

In contrast to this model, it has been hypothesized that Notch signaling acts as a general amplifier of T helper cell responses rather than an instructive director of specific cell fates. This could either be by enhancing proliferation, cytokine production and anti-apoptotic signals[135, 149, 150] or by boosting antigen sensitivity via promoting co-stimulatory signals in T cells[107, 108].

Therefore, in this report we aimed to determine whether Notch signaling is critical for HDM-driven allergic airway inflammation (AAI) *in vivo*. In particular, we questioned whether Jagged1 and Jagged2 on DCs are required for the induction of polarization of naïve T cells into Th2 cells. We found that the expression of Jagged1 or Jagged2 on DCs is not required while T cells do need Notch signals, specifically to differentiate into Th2 cells.

Methods

Mice

Wild-type (WT) mice were purchased from Harlan (Zeist, the Netherlands). *Jag1*$^{fl/fl}$ mice[389], *Jag2*$^{fl/fl}$ mice[390] and Rosa26-stop-EYFP reporter (ROSA^{EYFP}) (Jackson Laboratories, Bar Harbor, ME, USA) mice were bred with CD11c-cre transgenic mice[177]. RBPJκ$^{fl/fl}$ mice[391] were crossed with CD4-cre transgenic mice[392]. All mice were bred on a C57BL/6 background in the Erasmus MC animal facility under specific-pathogen free conditions and genotyped by PCR as described[177, 389-392]. In experiments, both male and female 6-14-week-old mice were used. Mice were given ad libitum access to food and water. All experiments involving animals were approved by the Erasmus MC Animal Ethics Committee.

Single cell suspension preparation

Single cell suspensions were obtained as previously described[347]. Briefly, directly after harvest, spleen and lymph nodes were mechanically disrupted in a cell strainer. Lungs were digested using 20 µg/ml liberase (Roche Applied Science, Almere, the Netherlands) and 2 µg/ml DNase I (Sigma-Aldrich, St. Louis, MO, USA) for 30 minutes at 37°C while shaking. Erythrocytes in BM, lung and spleen were lysed with osmotic lysis buffer for 1 minute.

Bone marrow-derived dendritic cell cultures

GM-CSF bmDCs were generated as described[393]. Briefly, to prepare single cell suspensions from bone marrow (BM), femurs and tibias from mice were cleaned with 70% ethanol and mechanically disrupted in RPMI 1640 containing GlutaMAX-I (Invitrogen, Carlsbad, CA, USA) after which cells were separated from bones using a 100 µm cell strainer (BD Falcon, Bedford, MA, USA). $2 \cdot 10^6$ bone marrow (BM) cells/ml were cultured in 6-well plates in 2 ml of complete medium with 20 ng/ml GM-CSF (kindly provided by Dr. K. Thielemans, Belgium). 2 ml new medium supplemented with GM-CSF was added at day 3. At day 6, 2 ml of culture medium was discarded and replaced by 2 ml fresh medium with GM-CSF. Cells were harvested at day 10.

DC subset sorting

DCs were sorted using a FACS Aria equipped with BD FACS Diva software (BD Biosciences, San Jose, CA, USA). Cells were selected on negativity for DAPI (invitrogen). Doublets were depleted using side scatter- and forward scatter width and height and cells were further gated as indicated in the figure legends. A list of all used fluorochrome labeled antibodies can be found in **Supplementary table 1**.

Sorted DCs were collected in FCS and stimulated overnight with 10µg/ml house-dust mite (HDM) extract (Greer, Lenoir, NC, USA)(endotoxin; 1397.5 EU/vial, protein; 5.59 mg/vial), 10 ng/ml LPS (Enzo life sciences, Farmingdale, NY, USA) or a similar volume of PBS (invitrogen) after which cells were stored in RNA lysisbuffer (Qiagen, Venlo, the Netherlands) containing 2-mercaptoethanol.

To activate *in vivo* DCs from draining lymph nodes, WT mice and *Jag1*$^{fl/fl}$*Jag2*$^{fl/fl}$ CD11c-cre^{+} (*Jg1Jg2*$^{\Delta CD11c/\Delta CD11c}$) mice were treated intranasally (i.n.) with 50 µg HDM in 40 µl PBS or with 40 µl PBS under isoflurane anesthesia 72 hrs prior to sacrifice. To obtain sufficient numbers of cells, MedLN from 6 mice were pooled per sample. After sorting, cells were collected in RNA lysis buffer containing 2-mercaptoethanol.

RNA extraction and quantitative real-time PCR

RNA was extracted using RNeasy Micro Kit (Qiagen) according to manufactures' protocol. RNA from cultured DCs was synthesized into cDNA using RevertAid H Minus Reverse Transcriptase and random hexamer primers in the presence of RiboLock RNase inhibitor (Thermo scientific, Waltham, MA, USA). Amplified cDNA from sorted *ex vivo* DC RNA was prepared using Ovation PicoSL WTA System V2 (Nugen, San Carlos, CA, USA).

For qRT-PCR reactions, probes from the Universal ProbeLibrary Set (Roche Applied Science) and Taqman Universal Mastermix were used (Applied Biosystems, Foster City, CA, USA). qRT-PCR reactions were performed by an Applied Biosystems Prism 7300 Sequence Detection System (Applied Biosystems). Primers were designed using transcript sequences obtained from www.ensembl.org and were specific for *Jag1* (forward: 5'-accagaacggcaacaaaact-3', reverse: 5'-gacccatgcttgggactg-3', probe 97), *Jag2* (forward: 5'-cgtcattccctttcagttcg-3', reverse: 5'-cctcatctggagtggtgtca-3', probe 95), *Dll1* (forward: 5'-gggcttctctggcttcaac-3', reverse: 5'-taagagttgccgaggtccac-3', probe 103) and *Dll4* (forward: 5'-gaggaacgagtgtgtgattgc-3', reverse: 5'-gtcccatacaggatgcaatgt-3', probe 3). mRNA levels of genes of interest were normalized to glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) (forward: 5'-TTCACCACCATGGAGAAGGC-3', reverse: 5'-GGCATGGACTGTGGTCATGA-3', probe TGCATCCTGCACCACCAACTG). Primers were checked for specificity and efficacy using standard criteria.

Mouse studies

To induce AAI with DCs, mice were sensitized with 30.000 unsorted GM-CSF bmDCs from *Jg1Jg2*$^{\Delta CDnc/\Delta CDnc}$ or *Jg1Jg2*$^{+/+}$ mice (stimulated overnight with 5 µg/ml HDM or with an equal volume of PBS as a control) intratracheally (i.t.) while anesthetized with ketamine 75 mg/kg and medetomidine 1.0 mg/kg as previously described[80]. From day 7, mice were challenged with 10 µg HDM in 40 µl PBS for 5 consecutive days. During HDM treatments, mice were anesthetized with isoflurane. 1 day after the last challenge, mice were sacrificed and analyzed.

HDM-mediated AAI was induced as described before[394]. Briefly, mice were sensitized with 1 or 10 µg HDM (as indicated in the figures) i.n. in 40 µl or with PBS. At day 7-10, mice were i.n. exposed to 10 µg HDM (40 µl) for 5 consecutive days. During HDM/PBS treatments, mice were anesthetized with isoflurane. Mice were sacrificed and analyzed 4 days after the last challenge, or at 1 day when bronchial hyperreactivity (BHR) measurements were performed.

To study Th1 responses, mice were injected subcutaneously in the tail base with 100 µl of a mixture of 2 mg/ml OVA and Incomplete Freund's Adjuvant (IFA, BD biosciences) or PBS with IFA as a control. At day 7, mice were sacrificed and inguinal- and axillary lymph nodes were collected and restimulated *in vitro* with 100 µg/ml OVA for 72 hrs.

Mice were immunized i.p. with TNP-KLH(29) (Biosearch Technologies, Petaluma, CA, USA) day 0 (in combination with alum) and day 35 (without alum), as described[395]. For immunoglobulin analysis, blood was drawn from the tail vain at day 0 (baseline), 7, 35 and 42. Mice were sacrificed at day 42.

Bronchial hyperreactivity measurement

BHR was analyzed 24 hrs after the last challenge with HDM using flexiVent invasive measurement of dynamic resistance (SCIREQ Scientific Respiratory Equipment Inc., Montreal, Canada) as described previously[396]. Mice were anesthetized with urethane, paralyzed using d-tubocurarine and tracheotomized with an 18-gauge catheter, followed by mechanical ventilation with a flexiVent apparatus (SCIREQ). Respiratory frequency was set at 120 breaths per min with a tidal volume of 0.2 ml and a positive end-expiratory pressure of 2 ml H_2O. Increasing concentrations of metacholine (0–100 mg/ml) (Sigma-Aldrich) were administered via the jugular vein. Dynamic resistance and compliance were recorded after a standardized inhalation maneuver given every 10 s for 2 min. Baseline resistance was restored before administering the subsequent doses of metacholine.

Flow cytometry

Single cell suspensions were stained with a mixture of fluorochrome labeled antibodies in FACS buffer containing 0.25% BSA, 0.5 mM EDTA, 0.05% NaN_3 in PBS as described previously[397]. A list of all fluorochrome labeled antibodies that were used can be found in **Supplementary table 1**. For intracellular cytokine measurements, cells were restimulated with a mixture of 50 ng/ml PMA, 500 ng/ml ionomycin (both Sigma-Aldrich) and protein transport inhibitor (golgistop, BD biosciences) in complete medium. Next, cells were fixed and permeabilized with paraformaldehyde and 0.5% saponin in FACS buffer prior to intracellular staining. For staining of transcription factors, cells were fixed and permeabilized with Foxp3/Transcription Factor Staining Buffer Set (eBioscience, San Diego, CA, USA).

Data was acquired by a LSR II flow cytometer and FACS Diva software 6.1 (BD biosciences) and analyzed using Flowjo 9.8.5 (tree Star Inc., Ashland, OR, USA).

Cytokine and immunoglobulin measurements

Cytokines were quantified by commercial enzyme-linked immunoasorbent assay (ELISA) for IL-5, IL-12, TNF-α (eBioscience), IgE, IL-6 (BD biosciences) and KC (R&D, Minneapolis, MN, USA) according to manufactures' protocol.

HDM-specific IgE and IgG1 (antibodies from BD biosciences) were measured as described[398].
Levels of TNP-KLH specific IgM, IgG1 and IgG2a were determined as described[399]. To discriminate high- versus total affinity anti–TNP-KLH IgG1 Abs, plates were coated with TNP (13)-KLH and TNP (29)-KLH, respectively.

Statistical analysis

Reported values are shown as mean + SEM. For statistical analysis, a Mann-Whitney U-test was performed with GraphPad Prism software (version 5.01, La Jolla, USA). P values were considered significant when values were <0.05.

Results

Jagged1 is upregulated on *in vitro* GM-CSF bmDCs upon exposure to HDM

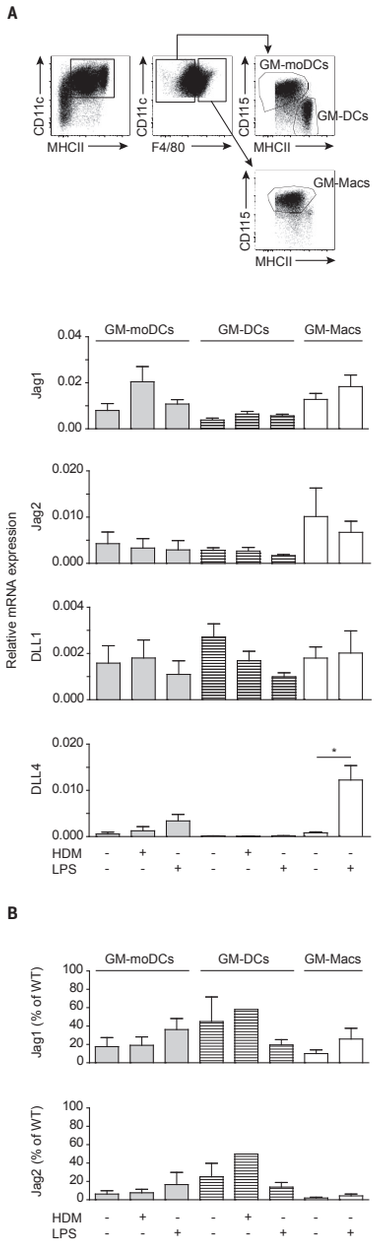
Because several research groups have shown a role for Jagged in the orchestration of T cell responses by employing GM-CSF bmDCs[93, 208-210, 387], we first investigated the expression of Notch ligands on bmDCs upon stimulation with the pro-Th2 stimulus HDM and the pro-Th1 stimulus LPS. GM-CSF bmDCs were cultured from WT mice and sorted at day 9 into CD11c$^+$MHCII^{int}F4/80$^-$CD115$^+$ GM-moDCs, CD11c$^+$MHCII^{high}F4/80$^-$CD115$^-$ GM-DCs and CD11c$^+$MHCII^{int}F4/80$^+$CD115$^+$ GM-Macs (**Figure 1A**), based on the study by Helft et al.[400]. Upon HDM stimulation, Jagged1 mRNA was upregulated on GM-moDCs and GM-DCs, whereas LPS stimulation induced upregulation of DLL4 mRNA on GM-moDCs and GM-Macs. Expression of Jagged2 and DLL1 was not altered on GM-CSF bmDCs upon stimulation (**Figure 1A**).

Thus, Jagged1 mRNA is substantially upregulated on *in vitro* GM-CSF bmDCs after HDM stimulation.

Jagged is crucial during the sensitization phase in a model that employs GM-CSF bmDCs to induce AAI

To delete *Jag1* and *Jag2* specifically in DCs, we employed *Jag1*$^{fl/fl}$ and *Jag2*$^{fl/fl}$ mice, in which the Jag loci contain loxP sites, as well as CD11c-cre transgenic mice, expressing Cre recombinase under the control of the DC-specific CD11c promoter. Efficiency of CD11c-cre mediated-deletion was confirmed in CD11c-cre transgenic ROSA^{EYFP} mice with cre-mediated excision of a loxP-flanked transcriptional STOP sequence. GM-CSF bmDCs were cultured from CD11c-cre×ROSA^{EYFP} mice and WT×ROSA^{EYFP} mice with GM-CSF. Analysis of EYFP expression by flow cytometry indicated that GM-CSF bmDC subsets manifested Cre-mediated deletion in 70-74% of the cells (**Supplementary figure 1A**).

Next, we analyzed Jagged mRNA expression in DCs from CD11c-Cre transgenic *Jag1*$^{fl/fl}$*Jag2*$^{fl/fl}$ mice (*Jg1Jg2*$^{\Delta CDnc/\Delta CDnc}$) and *Jg1Jg2*$^{+/+}$ control mice (**Figure 1B**). We found reduced expression of Jagged1 and Jagged2 compared to WT DCs in all GM-CSF bmDC subsets. Finally, recombination of *Jag1* and *Jag2* was confirmed on genomic DNA of GM-CSF bmDCs from *Jg1Jg2*$^{\Delta CDnc/\Delta CDnc}$, compared with *Jg1Jg2*$^{+/+}$ mice (**Supplementary figure 1B**).



To confirm that Jagged expression on DCs is essential for allergic airway inflammation (AAI) induction by intratrachael transfer of allergen-pulsed GM-CSF bmDC, we sensitized WT mice with HDM-pulsed total GM-CSF bmDCs from *Jg1Jg2*$^{\Delta CDnc/\Delta CDnc}$ or *Jg1Jg2*$^{+/+}$ mice and challenged the mice with HDM (**Figure 2A**). HDM stimulated *Jg1Jg2*$^{+/+}$ GM-CSF bmDCs induced AAI, as evidenced by a significant increase in numbers of eosinophils, macrophages, neutrophils, B cells, T cells and DCs in BAL, compared with mice that were sensitized with PBS treated GM-CSF bmDCs (**Figure 2B**). In contrast, GM-CSF bmDCs from *Jg1Jg2*$^{\Delta CDnc/\Delta CDnc}$ mice lacked the capacity to induce AAI (**Figure 2B**). Accordingly, IL-4$^+$, IL-5$^+$, IL-13$^+$, IFN-γ$^+$ and IL-17A$^+$ T cells in BAL (**Figure 2C**) or Gata3$^+$ Th2 cells in mediastinal lymph nodes (MedLN) (**Figure 2D and 2E**), were reduced, when mice were sensitized with *Jg1Jg2*$^{\Delta CDnc/\Delta CDnc}$ *GM-CSF* bmDCs compared with control DCs. Numbers of Rorγt$^+$ Th17 cells or Foxp3$^+$ Tregs in MedLN were not different between the two groups of mice, and T-bet expression was not detected (not shown).

The defective capacity of *Jg1Jg2*$^{\Delta CDnc/\Delta CDnc}$ GM-CSF bmDCs (**Supplementary figure 2A**) to induce Th2 polarization *in vivo* was likely not due to cell-intrinsic defects, because these DCs expressed similar levels of co-stimulatory molecules (**Supplementary figure 2B**), DLL1 and DLL4 (**Supplementary figure 2C**) and produced similar amounts of pro-inflammatory cytokines (**Supplementary figure 2D**) as control DCs did upon *in vitro* activation with a variety of stimuli. Finally, to investigate whether expression of Jagged1 and Jagged2 is perhaps also required during the challenge phase of AAI induction, *Jg1Jg2*$^{\Delta CDnc/\Delta CDnc}$ or *Jg1Jg2*$^{+/+}$ mice were sensitized with WT GM-CSF bmDCs and challenged with HDM. We found comparable AAI induction in *Jg1Jg2*$^{\Delta CDnc/\Delta CDnc}$ and *Jg1Jg2*$^{+/+}$ mice (not shown), indicating that for AAI induction Jagged expression is only required on GM-CSF bmDCs during the sensitization phase and not during HDM challenge.

Taken together, these findings confirm that expression of Jagged1 and Jagged2 is crucial during the sensitization phase in a model where GM-CSF bmDCs are used to induce HDM driven AAI.

Figure 1. Jagged1 is upregulated on bmDCs upon HDM exposure and CD11c-cre is effective in GM-CSF bmDCs

(A) Flow cytometric gating strategy for bmDC subsets from C57BL/6 mice. Live cells were analyzed for CD11c and MHCII and gated as indicated (*top*). mRNA expression of the indicated Notch ligands, quantified by qRT-PCR, in GM-moDCs, GM-DCs and GM-Macrophages and stimulated overnight in the presence or absence of HDM or LPS, as indicated (*bottom*).

(B) Quantification of relative Jagged1 and Jagged2 expression by qRT-PCR in bmDC subsets that were FACS sorted from *Jg1Jg2*$^{\Delta CD11c/\Delta CD11c}$ mice compared to WT C57BL/6 mice, which were set to 100%. Data are shown as mean + SEM of four mice per group in one experiment, except for HDM stimulated GM-DCs (n=2 in panel 1A; n=1 in panel 1B). * p < 0.05 using Mann-Whitney U-test.

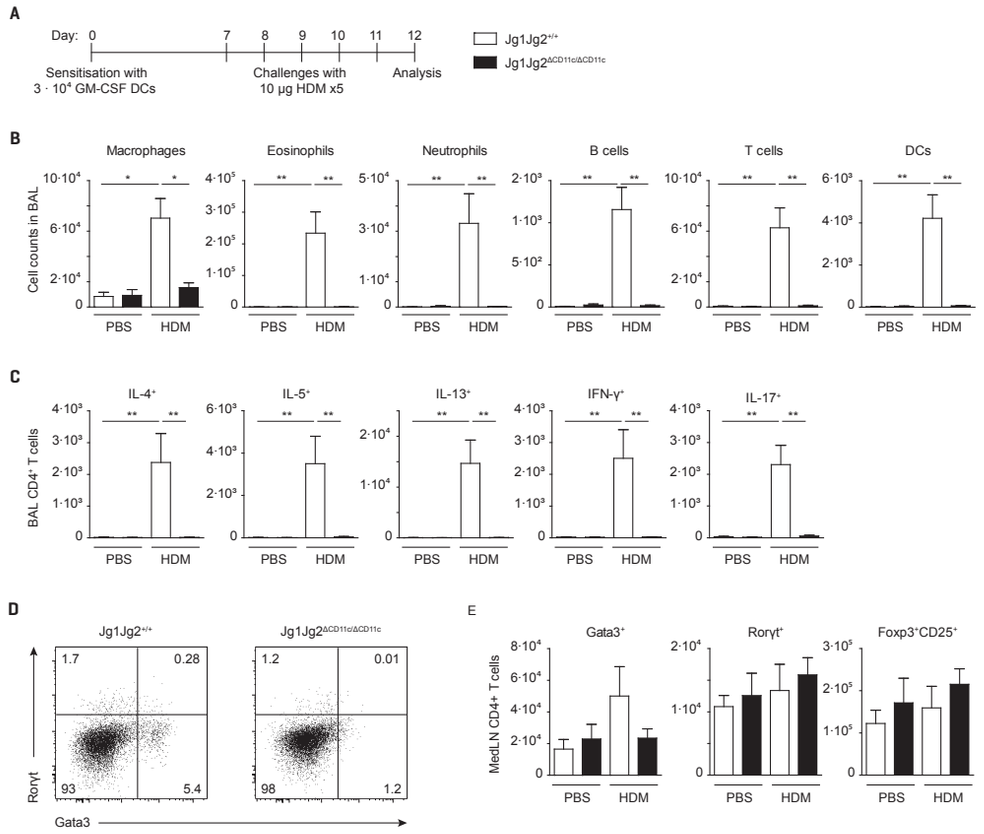


Figure 2. Jagged1 and Jagged2 are crucial during the sensitization phase when using GM-CSF bmDCs to induce AAI

(A) Sensitization and challenge scheme of HDM-driven AAI in mice, using cultured total bmDCs.

(B) Numbers of macrophages (FSC^{high}SSC^{high}autofluorescent$^+$CD11c$^+$Siglec-F$^+$), eosinophils (FSC^{int}SSC^{high}Siglec-F$^+$), neutrophils (Ly-6G$^+$), B cells (CD19$^+$), T cells (CD3$^+$), DCs (CD11c$^+$MHCII^{hi}) in BAL in mice treated with either PBS-pulsed or HDM-pulsed bmDCs from *Jg1Jg2*$^{\Delta CD11c/\Delta CD11c}$ or *Jg1Jg2*$^{+/+}$ mice.

(C) Numbers of IL-4$^+$, IL-5$^+$, IL-13$^+$, IFN-γ$^+$ and IL-17A$^+$ CD3$^+$CD4$^+$ T cells in BAL in mice treated with either PBS-pulsed or HDM-pulsed bmDCs.

(D) Flow cytometry profile of Gata3/Rorγt expression in CD3$^+$CD4$^+$ T cells in mice treated with HDM-pulsed bmDCs from *Jg1Jg2*$^{\Delta CD11c/\Delta CD11c}$ or *Jg1Jg2*$^{+/+}$ mice. (E) Total numbers of Gata3$^+$, Rorγt$^+$ and Foxp3$^+$CD25$^+$ CD3$^+$CD4$^+$ T cells in MedLN from mice treated with PBS-pulsed or HDM-pulsed bmDCs from *Jg1Jg2*$^{\Delta CD11c/\Delta CD11c}$ or *Jg1Jg2*$^{+/+}$ mice, as indicated.

Data are shown as mean + SEM of four mice (PBS) or six mice (HDM) per group, in one experiment. * p < 0.05, ** < 0.01, using Mann-Whitney U-test.

Jagged1 is highly upregulated on *in vivo* migratory CD11b⁺ cDCs upon HDM exposure

To analyze the role of Jagged expression in a more physiological HDM-driven airway inflammation model, we first aimed to establish which *in vivo* DC subsets expresses crucial Notch ligands during HDM exposure. In this context, CD11b⁺ conventional DCs (cDCs) were shown to be the main DC subset involved in the induction of Th2 cells in draining lymph nodes, whereas monocyte-derived DCs (moDCs) play a crucial role during the challenge phase[80]. We sorted resident moDCs, migratory moDCs, resident CD11b⁺ cDCs and migratory CD11b⁺ cDCs from MedLN from WT mice intranasally (i.n.) treated with HDM or PBS for 72 hrs. In migratory CD11b⁺ cDCs, both Jagged1 and DLL4 were expressed at baseline and significantly upregulated upon exposure to HDM, whereas Jagged2 and DLL1 were not detected (**Figure 3A**). Resident moDCs, migratory moDCs and resident CD11b⁺ cDCs expressed very low levels of Jagged1 mRNA and expression of other Notch ligands was not detected (not shown).

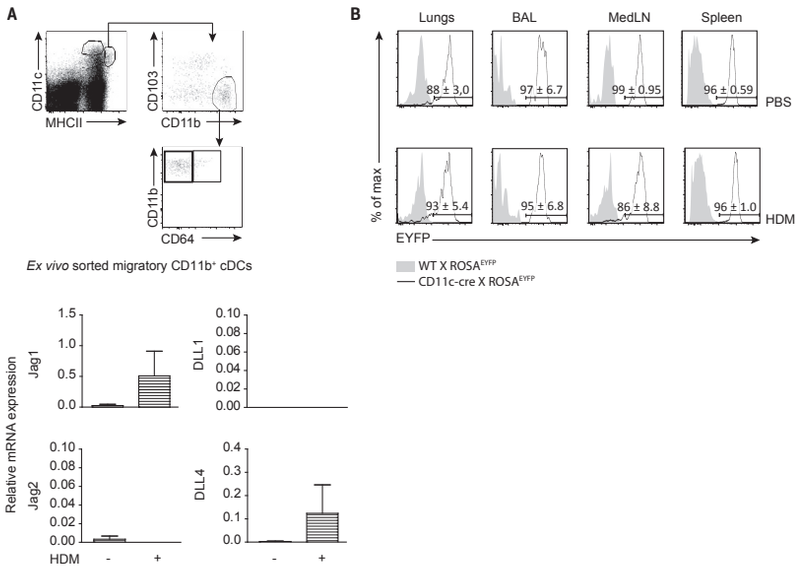


Figure 3. Jagged1 is upregulated on migratory CD11b⁺ cDCs upon HDM exposure and CD11c-cre is effective in *in vivo* DCs
(A) Gating strategy of *ex vivo* sorted DC subsets from C57BL/6 mice that were i.n. treated with 50 µg HDM or PBS (*top*). mRNA expression of the indicated Notch ligands, as determined by qRT-PCR, in DAPI⁻MHCII^hiCD11b⁺CD103⁻CD64⁻ (migratory) DCs from MedLN after 72 hrs of *in vivo* stimulation (*bottom*). 6 mice were pooled per sample. Data are shown as mean + SEM of three samples per group, in one experiment. (B) EYFP expression in CD11c⁺MHCII^hi DCs in the indicated tissues from WT×ROSA^EYFP and CD11c-cre×ROSA^EYFP mice after 72 hrs of *in vivo* stimulation with 50 µg HDM or PBS. Data are shown as histogram overlays of EYFP expression in the indicated mice. Samples were concatenated, data are shown as mean + SD of four mice (CD11c-cre X ROSA^EYFP) or 2-3 mice (WT X ROSA^EYFP) per group, in one experiment.

Jag1 and *Jag2* are effectively deleted in DCs from *Jg1Jg2^ΔCD11c/ΔCD11c* mice

To check the efficacy of CD11c-cre-mediated *in vivo* gene deletion, we analyzed DCs from CD11c-cre×ROSA^EYFP mice and control mice. EYFP was expressed in 88-97% of CD11c⁺MHCII^high DCs in lungs, bronchoalveolar lavage (BAL), MedLN and spleen and was unaltered when mice were challenged with 50 mg of HDM 72 prior to analysis (**Figure 3B;** see **Supplementary Table 2** for a detailed analysis of EYFP expression in DC subsets and other immune cells). In accordance with the EYFP data, Jagged1 and Jagged2 mRNA expression was not detected in migratory CD11b⁺ cDCs sorted from MLN from *Jg1Jg2^ΔCD11c/ΔCD11c* mice (data not shown).

Together, these data show that Jagged1, but not Jagged2, is substantially upregulated on migratory CD11b⁺ cDCs upon stimulation with HDM. In addition, DCs from *Jg1Jg2^ΔCD11c/ΔCD11c* mice show almost complete *in vivo* deletion of both Jagged1 and Jagged2.

Mice lacking Jagged expression on DCs develop AAI similar to WT animals

Next, we used an acute AAI model by sensitizing and challenging *Jg1Jg2^ΔCD11c/ΔCD11c* and *Jg1Jg2^+/+* mice with HDM. Four days after the last challenge, mice were analyzed (**Figure 4A**). Surprisingly, following HDM exposure both *Jg1Jg2^ΔCD11c/ΔCD11c* and *Jg1Jg2^+/+* mice developed similar AAI inflammation characterized by increased macrophages, eosinophils, neutrophils, B cells and T cells in BAL, compared to PBS-sensitized mice (**Figure 4B**). *Jg1Jg2^ΔCD11c/ΔCD11c* and *Jg1Jg2^+/+* mice showed similar increases in IL-4, IL-5, IL-13 and IL-9 expressing CD4⁺ T cells and also the numbers of IFN-γ or IL-17A T helper cells were similar (**Figure 4C and 4D**). Accordingly, restimulated MedLN cells from *Jg1Jg2^ΔCD11c/ΔCD11c* and *Jg1Jg2^+/+* mice showed no differences in production of HDM-induced IL-5 (**Figure 4E**). In addition, numbers of Gata3⁺ T cells were higher in HDM-treated *Jg1Jg2^ΔCD11c/ΔCD11c* mice, compared with *Jg1Jg2^+/+* control mice. In these experiments the numbers of Rorγt⁺ and Foxp3⁺ T cells were not different between the two groups. (**Figure 4F**). T-bet⁺ T cells were not detected (not shown). Whereas total serum IgE levels were higher in *Jg1Jg2^ΔCD11c/ΔCD11c* mice, compared with *Jg1Jg2^+/+* mice, HDM specific IgE and IgG1 in serum were similar in the two HDM-treated mouse groups (**Figure 4G**). When we analyzed single gene conditional knockouts, we found, as expected, that *Jg1^ΔCD11c/ΔCD11c* and *Jg2^ΔCD11c/ΔCD11c* mice developed AAI similar to WT littermates upon HDM exposure (**Figure 4H**).

To verify that the DC migration and responsiveness was comparable between *Jg1Jg2^ΔCD11c/ΔCD11c* and *Jg1Jg2^+/+* mice, the DC response to HDM was analyzed 24 hr after intranasal administration of either PBS, 10 µg HDM or 50 µg HDM (**Supplementary figure 3A**). We did not detect differences in the numbers of cells of individual DC subsets (**Supplementary figure 3B and 3C**) nor in the expression of co-stimulatory molecules on total DCs (**Supplementary figure 3D**) or separate DC subsets (not shown) in the MedLN or lungs between *Jg1Jg2^ΔCD11c/ΔCD11c* and *Jg1Jg2^+/+* mice. We noticed a small but significant increase in DLL4 expression on DCs in the MedLN of *Jg1Jg2^ΔCD11c/ΔCD11c* mice, compared with *Jg1Jg2^+/+* mice.

Taken together, our analysis demonstrates that in the HDM-driven asthma model, there is no evidence for a role for Jagged1 or Jagged2 expression on DCs.

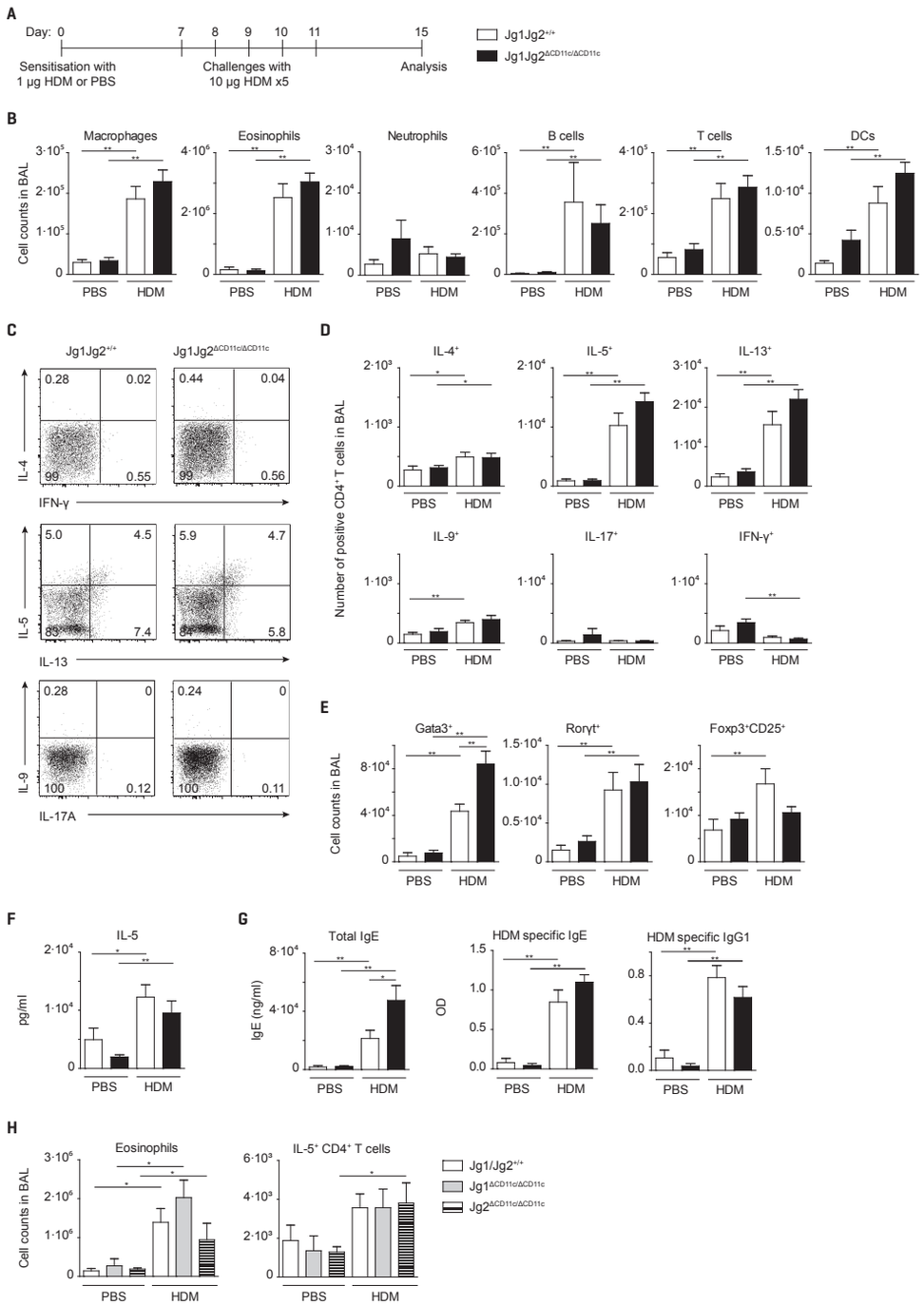


Figure 4. Jagged1 and Jagged2 expression on DCs is dispensable for the development of AAI *in vivo*

Conditional Jagged1 and Jagged2 knockout mice develop normal Th1 responses *in vivo*

Although Th2 responses still developed in the HDM model in mice with Jagged-deficient DCs, it remained possible that these mice had a shift in the Th1/Th2 balance. However, when we analyzed *in vitro* recall responses to OVA, there was no difference in T cell activation, Th1 cells or Th2 cells (**Supplementary figure 4A-4D**), or IL-4$^+$ and IFN-γ$^+$ T cells (not shown) between *in vitro* OVA-restimulated lymph node cells from *Jg1Jg2*$^{\Delta CD11c/\Delta CD11c}$ and *Jg1Jg2*$^{+/+}$ mice. Likewise, no differences were found in T cell-dependent B cell responses, because total or high affine TNP-KLH-specific IgM, Th2-driven IgG1 and Th1-driven IgG2c levels were similar in *Jg1Jg2*$^{\Delta CD11c/\Delta CD11c}$ and *Jg1Jg2*$^{+/+}$ mice (**Supplementary figure 4F and 4G**). Therefore, the absence of Jagged expression on DCs does not affect the Th1/Th2 balance *in vivo*.

Canonical Notch signaling via RBPJκ in CD4$^+$ T cells is required for the development of AAI

To establish whether Notch signaling in T cells is critical for the induction of Th2 differentiation, mice with T cell-specific conditional deletion of the downstream transcription factor RBPJκ[93] were studied. We exposed CD4-Cre transgenic *RBPJκ*$^{fl/fl}$ mice (termed *RBPJκ*$^{\Delta CD4/\Delta CD4}$) and non CD4-Cre expressing *RBPJκ*$^{fl/fl}$ littermates (termed *RBPJκ*$^{+/+}$) to our HDM-driven AAI model (**Figure 5A**). Strikingly, in the absence of RBPJκ in T cells, mice displayed a significant decrease in the number of macrophages, eosinophils, neutrophils, B cells, T cells and DCs in BAL compared to WT littermates (**Figure 5B**). Also, the numbers and percentages of IL-4$^+$, IL-5$^+$ and IL-13$^+$ T cells were lower in *RBPJκ*$^{\Delta CD4/\Delta CD4}$ than in *RBPJκ*$^{+/+}$ mice, whereas we found similar numbers and increased percentages of IFN-γ$^+$ and IL-17A$^+$ T cells in BAL, MedLN and lungs (**Figure 5C and 5D and data not shown**). Moreover, the ratio of cytokine-producing T cells, shifted from a predominant Th2-phenotype to a more equal Th1/Th2/Th17 phenotype in the absence of RBPJκ in T cells (**Figure 5E**). In addition, induction of Gata3 was particularly impaired in *RBPJκ*$^{\Delta CD4/\Delta CD4}$ mice in CD4$^+$ cells in BAL, MedLN and lungs (**Figure 5F, 5G and data not shown**), Furthermore, serum IgE levels (**Figure 5H**) and airway resistance to methacholine were significantly lower in *RBPJκ*$^{\Delta CD4/\Delta CD4}$ mice, compared with *RBPJκ*$^{+/+}$ mice (**Figure 5I**).

In summary, these results demonstrate that canonical RBPJκ-mediated Notch signaling in CD4$^+$ T cells is crucial for the induction of AAI and airway hyperreactivity *in vivo*.

Figure 4. Jagged1 and Jagged2 expression on DCs is dispensable for the development of AAI *in vivo*

(A) Scheme of HDM-mediated AAI induction in mice.
(B) Total numbers of the indicated cell populations in BAL from PBS- or HDM-treated *Jg1Jg2*$^{\Delta CD11c/\Delta CD11c}$ or *Jg1Jg2*$^{+/+}$ mice.
(C) Intracellular flow cytrometric analysis of cytokine production by CD3$^+$CD4$^+$ T cells in BAL from the indicated mice and (D) quantification of the total numbers of cytokine-positive CD3$^+$CD4$^+$ T cells in BAL.
(E) Quantification of IL-5 production *in vitro* by MedLN cells that were restimulated with 15 µg/ml HDM for 7 days, as quantified by ELISA.
(F) Numbers of Gata3$^+$, Rorγt$^+$ and Foxp3$^+$CD25$^+$ CD3$^+$CD4$^+$ T cells in BAL from PBS- or HDM-treated *Jg1Jg2*$^{\Delta CD11c/\Delta CD11c}$ or *Jg1Jg2*$^{+/+}$ mice.
(G) Total IgE levels and levels of HDM-specific IgE and IgG1 in serum of the indicated mice.
(H) Cell counts of eosinophils and IL-5$^+$ CD3$^+$CD4$^+$ T cells in BAL from PBS- or HDM-treated in *Jg1*$^{\Delta CD11c}$ and *Jg2*$^{\Delta CD11c}$ mice with WT littermates.
Data are shown as mean + SEM of 6-7 mice per group and are representative of three independent experiments. * p < 0.05, ** < 0.01, using Mann-Whitney U-test.

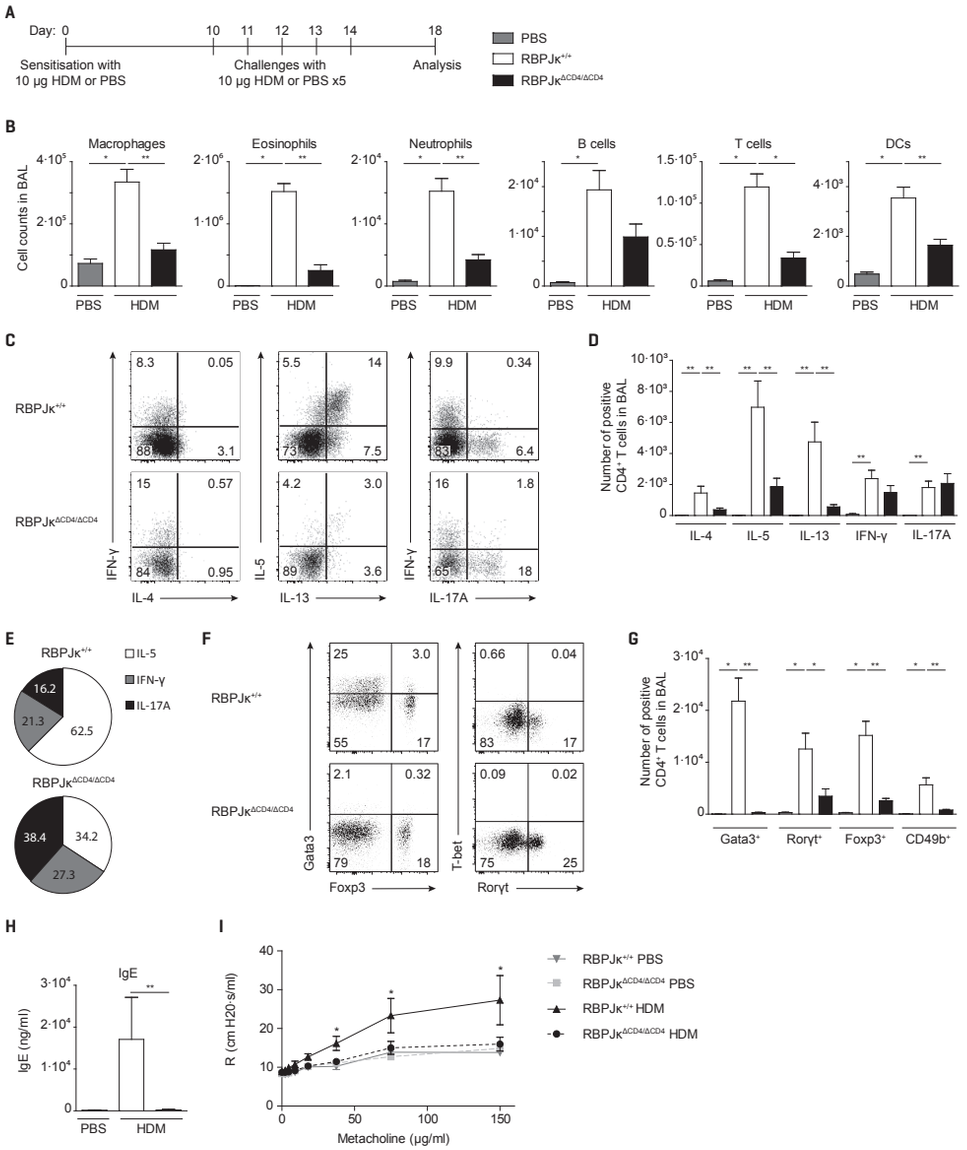


Figure 5. Notch signaling in CD4⁺ T cells is crucial for the induction of AAI

(A) Scheme of HDM-mediated AAI induction in *RBPJκ*$^{\Delta CD4/\Delta CD4}$ and *RBPJκ*$^{+/+}$ mice.

(B) Total numbers of the indicated cell populations in BAL from PBS- or HDM-treated mice.

(C-E) Intracellular flow cytrometric analysis of cytokine production by CD3⁺CD4⁺ T cells in BAL from the indicated mice and

(D) quantification of the total numbers of cytokine-positive CD3⁺CD4⁺ T cells in BAL and

(E) distribution of Th1, Th2 and Th17 cells, as signified by their key cytokines IL-5, IFN-γ and IL-17A, in BAL of the indicated mice.

(F) Flow cytometry profile of transcription factor expression in CD3⁺CD4⁺ T cells in mice treated with HDM.

(G) Quantification of Gata3⁺, Rorγt⁺, Foxp3⁺ CD4⁺ T cells and CD49b⁺ CD4⁺ NKT cells in BAL.

(H) Total IgE levels in serum, as determined by ELISA.

(I) Airway resistance, measured directly after administration of increasing doses of methacholine, using Flexivent, in the indicated mouse groups.

Data are shown as mean + SEM of 4-6 mice per group (B-I) and are representative of 6 independent experiments (B-H). * p<0.05, **p <0.01, using Mann-Whitney U-test.

Discussion

To induce a Th2 response, Notch signaling in T cells is crucial. This was shown earlier in mouse models using parasite antigens[129, 131] and in asthma models using OVA[133]. In line with these reports, we found that mice with T cell-specific RBPJκ-deficiency did not mount a Th2 response in a HDM-induced mouse AAI model. However, the role of the Notch ligands Jagged1 and Jagged2 in Th2 induction remains more elusive. Here we show that upon HDM exposure, Jagged1 is specifically upregulated on migratory CD11b⁺ cDC in medLN, but expression of Jagged1 and Jagged2 on DCs is dispensable for the induction of HDM induced AAI *in vivo*.

Whereas we found a substantial increase of Jagged1 expression upon HDM stimulation *in vivo* and *in vitro*, Jagged2 expression was low and remained unaltered. In addition, Jagged1 was shown to be crucial in the induction of a Th2 response in a AAI model using OVA-pulsed *in vitro* cultured GM-CSF bmDCs[208], whereas Jagged2 is not required for Th2 induction *in vivo*[209, 210]. Therefore, we hypothesized that Jagged1, but not Jagged2, would be critical in the induction of AAI *in vivo*. However, in our physiological model using HDM to sensitize and challenge mice, we found that expression of Jagged1 and Jagged2 on DCs was dispensable. Nevertheless, our data based on transfer of *in vitro* HDM-activated GM-CSF bmDCs confirmed earlier literature showing that Jagged-deficient GM-CSF bmDCs are incapable of inducing AAI *in vivo*[208] in an OVA-based model. Thus, the requirement for Jagged expression on GM-CSF bmDCs for their capacity to induce AAI does not appear to be dependent on the nature of the allergen (HDM or OVA), but is likely related to the use of GM-CSF bmDCs to sensitize the mice. In particular, it was recently shown that GM-CSF bmDCs comprise a heterogeneous cell population consisting of both conventional DC-like cells and monocyte-derived macrophages[400]. These findings indicate that data obtained employing *in vivo* transfers of GM-CSF bmDCs should be interpreted with care.

While there is no doubt that Notch is required to induce proper effector T cell responses, it is currently under debate whether Notch ligands have an instructive role in T helper cell differentiation or whether Notch signaling acts as an amplifier of T helper cell responses[207]. The results obtained after instillation of Jagged deficient DCs would appear to support a general role for Notch in promoting T helper cell responses. In contrast, in RBPJκ deficient mice treated with HDM, we clearly observed a selective defect in Th2 cell responses, while numbers of Th1 and Th17 cells were similar to those in wild type mice, arguing for a role for Notch as a Th2 instructive signal. We speculate that Notch can perform both roles, enhance general T cell activation and function as a more specific promoter of Th2 responses, depending on the repertoire of signals mobilized. Thus, when HDM treated DCs are used to prime the response, the repertoire of additional T cell activating signals may be limited. In that case, T cell activation would become more dependent on activation of Notch. When, on the other hand, HDM is inhaled, many cell types (innate lymphocytes, epithelial cells, tissue resident myeloid cells) will contribute to the generation of activating signals that may override the requirement for Notch in priming of the T cells. In this latter scenario, only the Th2-promoting function of Notch would then be critical.

It has previously been suggested that Notch ligands DLL and Jagged instruct Th1 and Th2 responses, respectively[93]. However, we found that mice with a conditional deletion for Jagged1 and Jagged2 in DCs developed Th2 responses to HDM to a similar extent as their WT littermates. These findings indicate either (I) a critical role for other Jagged-expressing cells, implying an instructive role for Notch signaling or (II) redundancy between various Notch ligands (Jagged1, Jagged2, DLL1 and DLL4) on DCs during the induction of Th2 responses, which would argue for a role for Notch as an unbiased amplifier.

One explanation for the induction of a Th2 response in the absence of Jagged1 and Jagged2 on DCs could be that there is a redundancy of other Jagged expressing cells. It is not likely that Jagged expression on alveolar macrophages is required for Th2 priming. Firstly, although macrophages can take up HDM, they have been reported to lack the capacity to induce T cell proliferation[80]. Secondly, our finding of >94% EYFP expression in alveolar macrophages from CD11c-cre×ROSA^{EYFP} mice (**Supplementary figure 2B**), would indicate that in the *Jg1Jg2*$^{\Delta CD11c/\Delta CD11c}$ also these cells are Jagged-deficient. Another candidate would be B cells which have been implicated in the induction of Th2 mediated AAI[34, 401, 402]. Also, B cells are important in the development and maintenance of T follicular helper (Tfh) cells[403], which play an important role in AAI by secreting IL-4 and IL-2[134, 68, 404, 405]. However, in FACS-sorted activated and non-activated B cells from HDM treated- and control mice Jagged1 was not detected and levels of Jagged2 were very low (I.T., unpublished findings), inconsistent with a role for Jagged expression on B cells in Th2 cell induction.

We found that upon stimulation with HDM, DLL4 was increased on migratory CD11b^{+} cDCs *in vivo* (**Figure 1B**). In the absence of Jagged1 and Jagged2 on DCs, DLL4 expression was increased (**Supplementary figure 4D**), raising the possibility that DLL4 compensates for the absence of Jagged1 and Jagged2. DLL4 signaling was originally thought to be associated with the induction of Th1 responses[93, 225]. Indeed, DLL4 is upregulated on DCs in response to Th1 stimuli, including bacterial LPS, respiratory syncytial virus (RSV) and dengue virus[223, 225, 229]. Later studies however showed that it is also induced by certain Th2 stimuli, including cockroach allergen, low doses LPS and RSV-mediated allergic asthma exacerbations[214, 217, 406]. Furthermore, a regulatory role for DLL4 was demonstrated in Th2 responses to cockroach allergen[217], and when DLL4-pretreated bmDCs stimulated with OVA were adoptively transferred to induce AAI[218]. On the other hand, Th2 responses were decreased when DLL4 was neutralized *in vivo* in a mouse model for RSV-mediated allergic asthma exacerbations[406]. It is therefore unclear if DLL4 compensates for the absence of Jagged molecules on DC or if DLL4 has a regulatory role in this setting. Further studies targeting both Jagged1 and DLL4 Notch ligands are required to resolve this question.

In summary, we showed that Notch signaling is crucial for the induction of HDM-mediated eosinophilia, Th2 responses and airway hyperreactivity *in vivo*, indicating that Notch on T cells could be a potential therapeutic target in allergic asthma patients. In addition, our data indicate that there is redundancy, either between various Jagged-expressing cells or between Jagged and Delta-like ligands on DCs. Therefore, further studies are required to identify which cells and which ligands provide the Notch signals that are essential for Th2-induction in allergic asthma.

Acknowledgements

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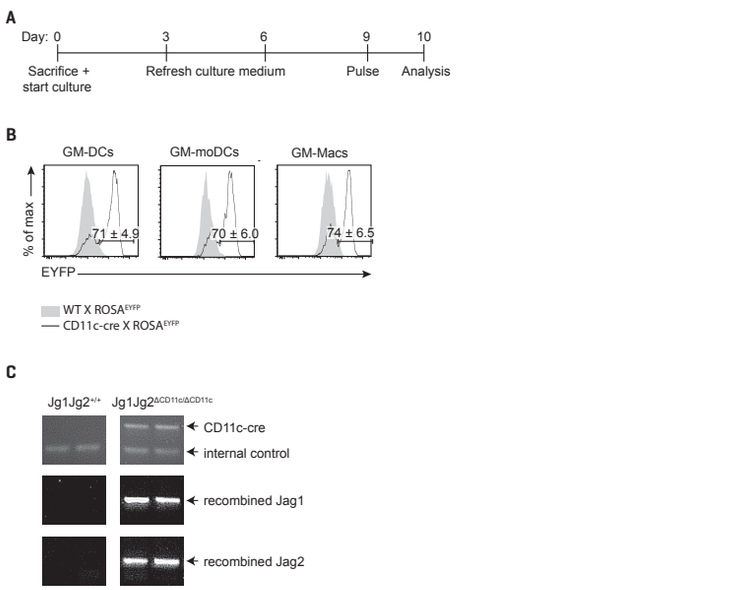
Supplementary figures and tables

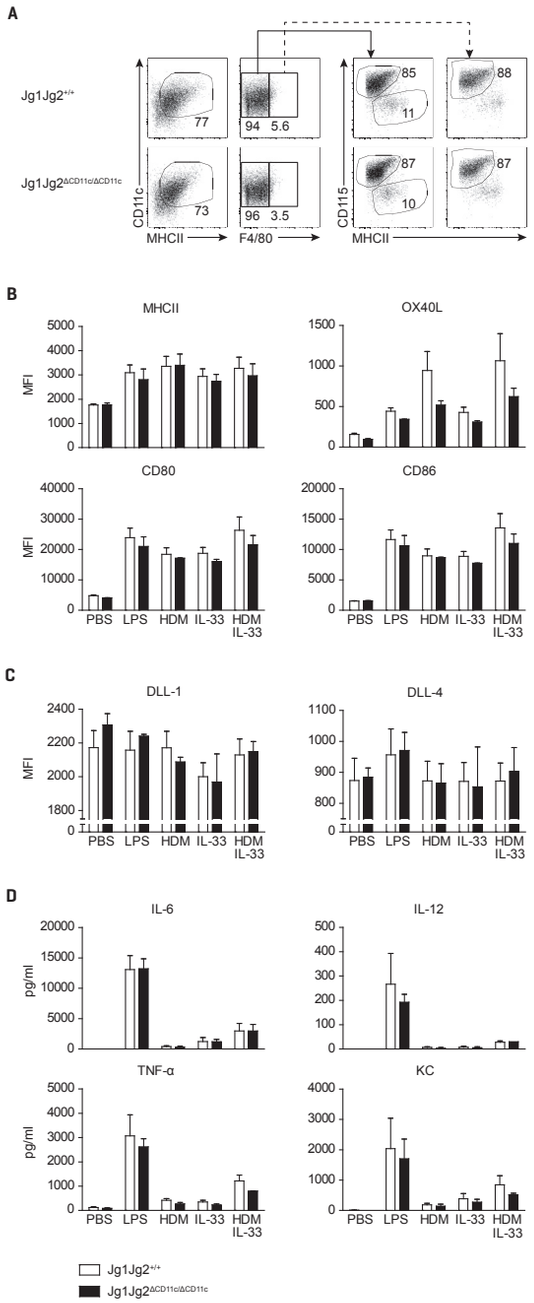


Supplementary figure 1. Generation of Jagged deficient bmDCs

(A) Scheme of bmDCs culture and stimulation.

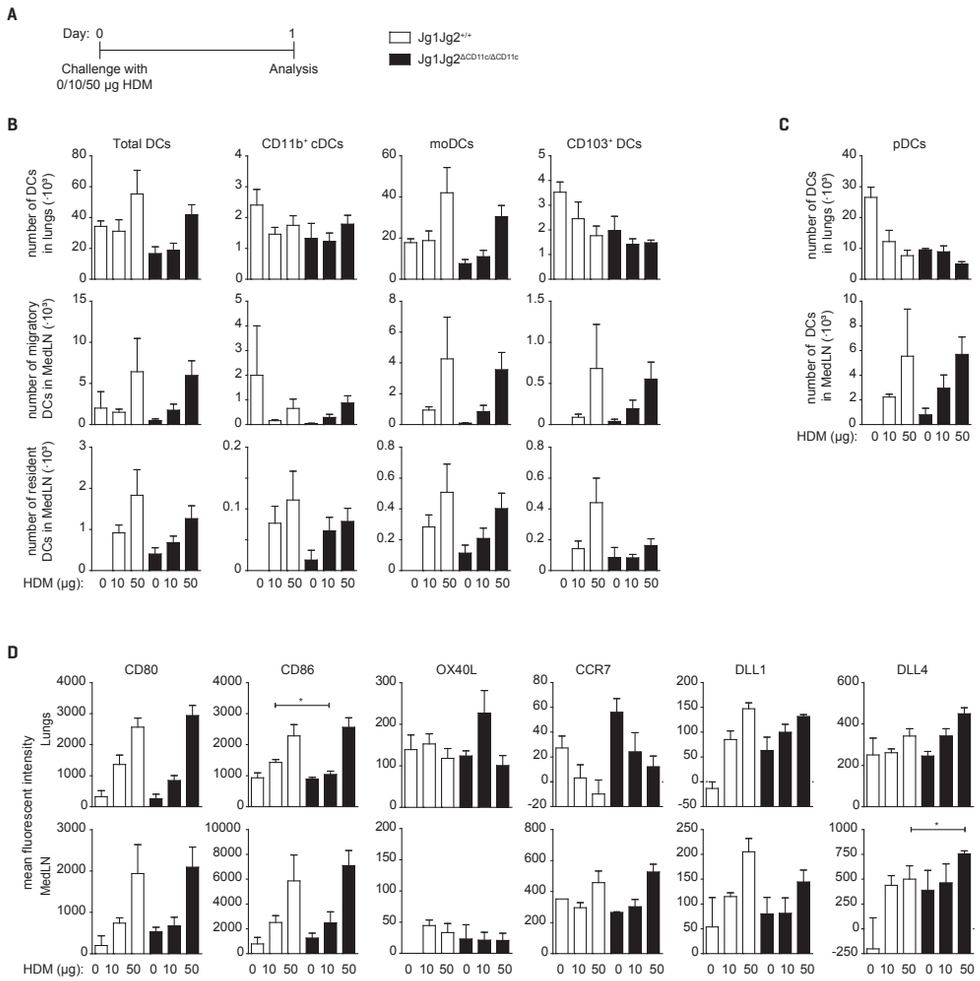
(B) EYFP expression in bmDC subsets from WT×ROSA^{EYFP} and from CD11c-cre×ROSA^{EYFP} mice, stimulated with 10 µg/ml HDM. Data are shown as mean + SD of 2-4 mice per group.

(C) PCR analysis of genomic DNA from total bmDCs for the presence of the CD11c-cre transgene and for the presence of loxP-mediated deletion of *Jag1* and *Jag2* in GM-CSF bmDCs (shown for 2 mice per genotype) from the indicated mice.



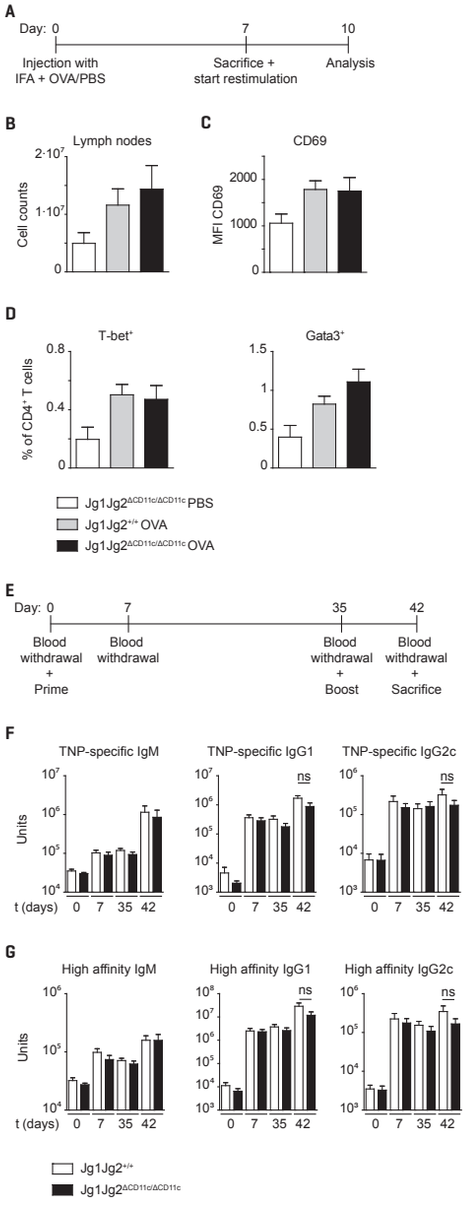
Supplementary figure 2. BmDCs from *Jg1Jg2$^{\Delta CD11c/\Delta CD11c}$* and *Jg1Jg2$^{+/+}$* mice are similar in expression of co-stimulatory molecules, DLL ligands and pro-inflammatory cytokines

(A) Flow cytometric gating strategy for bmDC subsets from *Jg1Jg2$^{\Delta CD11c/\Delta CD11c}$* and *Jg1Jg2$^{+/+}$* mice. Live cells were analyzed for CD11c, MHCII and F4/80. Gated cells were analyzed for expression of CD115 and MHCII.
(B, C) Mean fluorescent intensity (MFI) values of the indicated co-stimulatory molecules (B) and Notch ligands (C) on CD11c^{+}MHCII^{+} bmDCs, stimulated with PBS, 10 ng/ml LPS, 10 µg/ml HDM, 0.1 µg/ml IL-33 or 1 µg/ml HDM and 0.1 µg/ml IL-33.
(D) Protein concentrations of IL-6, IL-12, TNF-α and KC (CXCL1), determined in bmDC culture supernatants, measured by ELISA.
Data are shown as mean values + SEM of 3-4 mice per group. No significant differences were found between *Jg1Jg2$^{\Delta CD11c/\Delta CD11c}$* and *Jg1Jg2$^{+/+}$* bmDCs, using Mann-Whitney U-tests.



Supplementary figure 3. DCs from *Jg1Jg2$^{+/+}$* and *Jg1Jg2$^{\Delta CD11c/\Delta CD11c}$* mice are similar in numbers and expression of co-stimulatory factors in an innate response to HDM

(A) Scheme of innate DC activation: *Jg1Jg2$^{+/+}$* and *Jg1Jg2$^{\Delta CD11c/\Delta CD11c}$* mice were i.n. challenged with the indicated amounts of HDM and analyzed one day later by flow cytometry.
(B) Numbers of total DCs, CD11b^{+} cDCs (CD11b^{+}CD103^{-}CD64^{-}FcεRI^{-}), moDCs (CD11b^{+}CD103^{-}CD64^{+}FcεRI^{+}) and CD103^{+} DCs (CD11b^{-}CD103^{+}CD64^{-}FcεRI^{-}) in lungs (CD11c^{+}MHCII^{+}), migratory DCs (CD11c^{+}MHCII^{hi}) in MedLN and resident DCs (CD11c^{+}MHCII^{+}) in MedLN, upon stimulation with the indicated amounts of HDM.
(C) Numbers of plasmacytoid DCs (pDCs; SSC^{low}FSC^{low}CD11c^{int}MHCII^{int}CD11b^{-}Ly6C^{hi}) in lungs and MedLN, upon stimulation with the indicated amounts of HDM.
(D) Expression levels of CD80, CD86, OX40L, CCR7, DLL1 and DLL4 gated on total DCs (CD11c^{+}MHCII^{+}) in lungs and MedLN, expressed as MFI values determined by flow cytometry.
Data are shown as mean values + SEM of 3-6 mice per group. Statistical evaluations were performed, whereby side-by-side comparisons were done between *Jg1Jg2$^{+/+}$* and *Jg1Jg2$^{\Delta CD11c/\Delta CD11c}$* mice. Differences were not significant unless indicated; * $p < 0.05$, using Mann-Whitney U-test.



Supplementary figure 4. Jagged1 and Jagged2 molecules on DCs are not required for the development of a Th1 response *in vivo*

(A) *In vivo* immunization scheme and *in vitro* restimulation of inguinal lymph node cells.

(B-D) Total cells in inguinal lymph nodes (B), MFI of CD69 expression on $CD3^+CD4^+$ T cells (C) and proportions of $Gata3^+$ and $T\text{-}bet^+$ $CD3^+CD4^+$ T cells (D) in $Jg1Jg2^{\Delta CD11c/\Delta CD11c}$ and $Jg1Jg2^{+/+}$ mice.

(E) Scheme of TNP-KLH immunization and analysis.

(F,G) Total TNP-KLH-specific IgM, IgG1 and IgG2c and high-affine TNP-KLH-specific IgM, IgG1 and IgG2c in the serum of $Jg1Jg2^{\Delta CD11c/\Delta CD11c}$ and $Jg1Jg2^{+/+}$ mice at different time points, as measured by ELISA.

Data are shown as mean + SEM of 4-8 mice per group. ns, not significant, using Mann-Whitney U-test.

IFA, incomplete Freund's adjuvant; TNP-KLH, tri-nitrophenol keyhole limpet hemagglutinin.

Supplementary Table 1. Antibodies used for flow cytometry

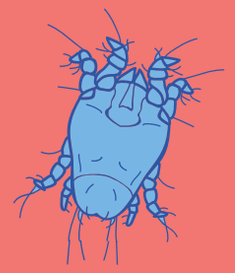
Target	Conjugate	Company	Clone
120G8	FITC	Own production	
B220	Biotin	BD biosciences	RA3-6B2
B220	PE	eBioscience	RA3-6B2
B220	PE-Cy7	eBioscience	RA3-6B2
CCR7	PE	eBioscience	4B12
CD3	APC-ef780	eBioscience	17A2
CD3	PE	eBioscience	145-2c11
CD3	PE-CF594	BD biosciences	145-2C11
CD4	AF700	eBioscience	GK1.5
CD4	APC-H7	BD biosciences	GK1,5
CD4	BV605	BD biosciences	RM4-5
CD4	PerCP-Cy5.5	eBioscience	RM4-5
CD8a	FITC	eBioscience	Ly-2
CD8a	PE-Cy7	eBioscience	53-6.7
CD8a	PerCP	BD biosciences	53-6.7
CD11b	ef450	eBioscience	M1/70
CD11b	PerCP-Cy5.5	BD biosciences	M1/70
CD11c	APC-ef780	eBioscience	N418
CD11c	BV786	BD biosciences	HL3
CD11c	PE-Cy7	eBioscience	N418
CD11c	PE-Texas Red	Invitrogen	N418
CD19	AF700	eBioscience	eBio1D3
CD19	APC-ef780	eBioscience	1D3
CD24	Biotin	BD biosciences	M1/69
CD24	PE	BD biosciences	M1/69
CD25	PE-Cy7	eBioscience	PC61.5
CD25	PerCP-Cy5.5	eBioscience	PC61.5
CD49b	AF647	BD biosciences	HMa2
CD64	AF647	BD biosciences	X54-5/7.1
CD64	BV711	biolegend	X54-5/7.1
CD69	PE	eBioscience	H1.2F3
CD80	PerCP-Cy5.5	BD biosciences	16-10A1
CD86	PE-Cy7	BD biosciences	GL1
CD103	ef450	eBioscience	2E7
CD103	PE	eBioscience	2E7
CD115	PE	eBioscience	AFS98
DLL1	AF488	eBioscience	HMD1-5
DLL4	APC	Biolegend	HMD4-1
F4/80	APC-ef780	eBioscience	BM8
F4/80	Biotin	eBioscience	BM8
F4/80	FITC	eBioscience	BM8

FcεRI-α	Biotin	eBioscience	MAR-1
Foxp3	AF488	eBioscience	FJK-16s
Foxp3	PE-Cy7	eBioscience	FJK-16s
Gata3	ef660	eBioscience	TWAJ-14
IFN-γ	BV650	BD biosciences	XMG1.2
IFN-γ	PE-Cy7	eBioscience	XMG1.2
IL-4	PE	BD biosciences	11B11
IL-4	BV711	BD biosciences	11B11
IL-5	APC	BD biosciences	TRFK-5
IL-5	Biotin	BD biosciences	TRFK4
IL-9	PE	BD biosciences	D93D2C12
IL-13	ef450	eBioscience	eBio13A
IL-13	ef660	eBioscience	eBio13A
IL-17A	AF700	BD biosciences	TC11-18H10.1
Live/Dead	Amcyan	Invitrogen	
Ly-6C	BV605	BD biosciences	AL-21
Ly-6G	PE	BD biosciences	1A8
Ly-6G	PE-Cy7	BD biosciences	1A8
MHC class II	AF700	eBioscience	M5/114.15.3
MHC class II	APC	eBioscience	M5/114.15.2
MHC class II	APC-Cy7	Biolegend	M5/114.15.2
MHC class II	BV650	BD biosciences	M5/114.15.2
OX40L	PE	eBioscience	RM134L
Rorγt	PE	BD biosciences	Q31-378
Siglec-F	PE	BD biosciences	E50-2440
Siglec-F	PE-CF594	BD biosciences	E50-2440
Streptavidin	APC-ef780	eBioscience	
Streptavidin	BV650	BD biosciences	
Streptavidin	PE-Cy7	eBioscience	
T-bet	BV421	BD biosciences	04-46

Supplementary Table 2. CD11c-cre activity in *in vivo* DCs

Cell type	Lungs	BAL	MedLN	Spleen
CD11b$^+$ moDCs[1]	89,88 ± 4,54[2]	97,48 ± 3,36	87,85 ± 9,08	82,85 ± 11,71
CD103$^+$/CD8$^+$ DCs	95,70 ± 3,03	99,50 ± 1,00	74,28 ± 2,00	83,78 ± 2,29
Resident CD11b$^+$ cDCs	[3]		67,85 ± 12,18	
Resident moDCs[1]			68,63 ± 10,40	
Resident CD103$^+$ DCs			39,20 ± 9,53	
CD8$^+$ moDCs				84,88 ± 4,77
CD11b$^-$CD4$^+$ DCs				43,03 ± 9,99
CD11b$^-$CD4$^+$ moDCs[1]				56,45 ± 12,71
pDCs	35,78 ± 4,94		57,05 ± 7,36	11,98 ± 2,38
(Alveolar) macrophages	93,70 ± 1,15	98,28 ± 0,43	9,86 ± 4,17	74,23 ± 9,27
Interstitial macrophages	13,05 ± 0,79			
B cells	5,03 ± 1,39	18,15 ± 9,75		5,75 ± 1,63
T cells[4]	11,89 ± 4,45	16,07 ± 7,32		12,18 ± 4,34
NK cells	10,40 ± 1,18	10,38 ± 7,47		11,47 ± 1,35

1) moDCs were characterized as CD64$^+$FcεRI$^+$CD4$^+$ in the spleen and CD64$^+$FcεRI$^+$ in other organs.
2) Proportions of EYFP$^+$ cells in DC subsets and other immune cells in lungs, BAL, medLN and spleen from CD11c-cre×ROSA^{EYFP} mice, stimulated with 50 µg HDM 72 hrs prior to sacrifice. Proportions of EYFP$^+$ basophils, eosinophils and granulocytes were <10%. Shown is cells in HDM stimulated mice. Percentages of EYFP positivity were similar in PBS treated mice. Data are shown as mean + SD of 2-4 mice per group.
3) Grey, Not determined.
4) EYFP expression in CD3$^+$ T cells was comparable to CD4$^+$ and CD8$^+$ T cells in all organs.



Chapter 4

The Notch pathway inhibitor stapled α-helical peptide derived from mastermind-like 1 (SAHM1) abrogates the hallmarks of allergic asthma

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Abstract

Background: The Notch signaling pathway has been implicated in the pathogenesis of allergic airway inflammation (AAI). Targeting the active Notch transactivation complex by using the cell-permeable, hydrocarbon-stapled synthetic peptide stapled α-helical peptide derived from mastermind-like 1 (SAHM1) resulted in genome-wide suppression of Notch-activated genes in leukemic cells and other models. However, the efficacy of SAHM1 in allergic asthma models has remained unexplored.

Objective: We aimed to investigate the therapeutic efficacy of SAHM1 in a house dust mite (HDM)-driven asthma model**.**

Methods: Topical therapeutic intervention with SAHM1 or a control peptide was performed during sensitization, challenge or both with HDM in mice. Airway inflammation was assessed by using multicolor flow cytometry, and bronchial hyperreactivity (BHR) was studied. Additionally, SAHM1 therapy was investigated in mice with established AAI and in a model in which we neutralized IFN-γ during HDM challenge to support the Th2 response and exacerbate asthma.

Results: SAHM1 treatment during the challenge phase led to a marked reduction of eosinophil and T cell numbers in bronchoalveolar lavage, compared with those in diluent-treated or control peptide-treated mice. Likewise, T cell cytokine content and bronchial hyperreactivity were reduced. SAHM1 treatment dampened Th2-inflammation during ongoing HDM challenge and enhanced recovery after following established asthma. Additionally, in the presence of anti-IFN-γ antibodies, SAHM1 downregulated expression of the key Th2 transcription factor Gata3 and intracellular IL-4 in bronchoalveolar lavage T cells, but expression of the Th17 transcription factor retinoic-actid-related orphan receptor gt or intracellular IL-17 was not affected. SAHM1 therapy also reduced serum IgE levels.

Conclusions: Therapeutic intervention of Notch signaling by SAHM1 inhibits AAI in mice and is therefore an interesting new topical treatment opportunity in asthmatic patients.

Clinical Implications: We show that targeting the Notch transactivation complex with SAHM1, a synthetic cell-permeable peptide, inhibits allergic airway inflammation in mice, indicating that SAHM1 is an interesting novel treatment opportunity in asthma.

Introduction

Allergic asthma is characterized by bronchial hyperreactivity (BHR), eosinophilic airway inflammation and increased IgE levels[407, 408]. The hallmarks of asthma are the direct consequences of enhanced activation of T helper 2 (Th2) cells producing the cytokines IL-4, IL-5 and IL-13, expression of which is controlled by the key Th2 transcription factor (TF) Gata3[31, 32, 409]. Central to the initiation of the differentiation of naïve T cells into the Th2 direction is IL-4, which induces Gata3 through IL-4 receptor (IL-4R) and signal transducer and activator of transcription 6 (STAT6).

Intriguingly, the induction of Gata3 by the IL-4/Stat6 axis in differentiating Th2 cells raises the paradox that IL-4 is required for the generation of the cell type that is its major producer. However, in this context it was demonstrated that antigen presenting dendritic cells (DCs) use the Notch signaling pathway to instruct T cell differentiation, independently of IL-4[93]. In this model, expression of Jagged Notch ligands on DCs constitutes an instructive signal for Th2 differentiation, whereby Notch signaling regulates Gata3 gene transcription from an upstream promoter, as well as *Il4* gene transcription in parallel with Gata3[129, 130]. Conversely, expression of the Notch Delta-like ligands on DCs, which is induced by stimulation with microbial products, promotes Th1 cell differentiation[225, 388, 410]. Supporting a critical role for Notch signaling in Th2 differentiation, we recently found that house dust mite (HDM)-driven allergic airway inflammation (AAI), Th2 activation and BHR were diminished in mice lacking the canonical Notch signaling mediator recombination signal-binding protein for IgJκ region (RBPj) in T cells[108]. However, in this HDM-driven asthma model, expression of the Jagged Notch ligands on DCs was dispensable.

Notch signaling also sensitizes T cells to exogenous cytokines[135], potentiates T cell receptor and CD28 signaling, and stimulates metabolic reprogramming and IL-2 secretion during priming of naïve T cells[108]. Moreover, Notch is required to maintain Th1 and Th2 programs, controls memory Th cell survival by regulating glucose uptake[109, 135], and acts as a general amplifier of T cells[108]. RBPj in T cells affected the ability of Th17 cells to adequately respond to IL-23[141].

In addition to its role in T cell differentiation, Notch is also important during lung organogenesis, alveologenesis, and differentiation[189, 190]. In addition, Notch signaling has been implicated in other immune cells and is also involved, for example, in DC differentiation and maturation[411].

Ligand binding to the Notch heterodimeric cell-surface receptor initiates its intramolecular cleavage mediated by a γ-secretase complex, resulting in the release of the Notch intracellular domain (NICD), which thereby translocates from the cytosol into the nucleus[116, 207]. There, NICD forms a transactivation complex with mastermind-like (MAML) proteins and RBPj, resulting in the activation of target genes. Binding of RBPj to DNA in the absence of NICD prevents target gene transcription by recruiting co-repressors. Interaction of NICD with RBPj removes co-repressors and recruits co-activators, including MAML, which in turn recruit DNA modification enzymes and induce Notch target gene transcription.

Interestingly, blocking Notch signaling by means of intranasal administration of γ-secretase inhibitors (GSIs) reduced allergic lung inflammation in a mouse asthma model[133]. Because GSIs are associated with severe, on-target gastrointestinal toxicity, other Notch inhibitors are being developed. For example, it has been demonstrated that therapeutic antibodies blocking Notch signaling prevent immune activation[200, 201] and activation of AKT downstream of Notch can be inhibited by the phosphatidylinositol 3-kinase inhibitor PI-103[412]. Assembly of the NICD-MAML-RBPj nuclear complex can be prevented by the synthetic, cell-permeable inhibitor stapled α-helical peptide derived from mastermind-like 1 (SAHM1)[199, 413]. SAHM1 proved to be effective in a murine model of T cell acute lymphoblastic leukemia caused by inappropriate Notch activation and outside the cancer field[199, 414, 415]. Importantly, this inhibitor can be more specific for Notch than the commonly used GSIs, which also affect cleavage of many other substrates of this enzyme complex[199, 416], or might preferentially affect certain tissues because of pharmacologic differences.

Given the prominent role of Notch signaling in type 2 immunity, we investigated the capacity of the SAHM1 peptide to mitigate pathology in eosinophilic lung inflammation in an HDM-driven asthma model. We found SAHM1 therapy to be beneficial because it reduced all hallmarks of asthma, including eosinophilic airway inflammation, Th2 differentiation and BHR.

Methods

Mice

C57bl/6 mice (Envigo, Zeist, The Netherlands) and OTII (C57bl/6; Erasmus MC, Rotterdam, The Netherlands) mice were kept under specific pathogen free conditions, provided with water and food ad libitum, and were used at the age of 6 to 11 weeks. All experiments were approved by the Erasmus MC Animal Ethics Committee.

House dust mite allergic airway inflammation model and therapy

Isoflurane-anesthetized mice were sensitized intratracheally (i.t.) with 10 μg of HDM (Greer Laboratories, Lenoir, NC) extract in 80 μL of PBS, or PBS only was used to induce AAI on day 0[346]. Ten days later, anesthetized mice were challenged with 10 μg of HDM in 50μL of PBS intranasally (i.n.) for 5 consecutive days. In the first set of experiments, treatment with HDM or control PBS was admixed with diluent (dimethylsulfoxide (DMSO)); 0.3, 1.0 or 3 mg SAHM1; or D1 (stock solution, 25 mg/L in DMSO; see the Methods section in this article's Online Repository at www.jacionline.org for supplementary data). The treatment preparation was made just before use. Four days after the last challenge, mice were killed and bronchoalveolar lavage (BAL) was performed by flushing the lungs 3 times with 1 ml PBS containing EDTA (Sigma-Aldrich, St Louis, Mo). The Lungs were snap-frozen in liquid nitrogen and stored at -80°C until further processing for histological analysis. In some mice, lung function was measured after increasing doses of nebulized methacholine (for details, see the Methods section in this article's Online Repository). Innate immune responses were studied with HDM or IL-33 (for details, see the Methods section in this article's Online Repository at www.jacionline.org).

Flow cytometric analysis and immunohistochemistry

BAL fluid cells were collected for cellular differentiation by using flow cytometry, as previously described[346]. For details of flow cytometry, see **Figure E1** in this article's Online Repository at www.jacionline.org. Immunohistochemical staining was performed in a half-automatic stainer (Sequenza, Milan, Italy) as previously described[417]. Sections were stained with rat anti-Siglec-F (clone E50-2440; BD Biosciences, San Jose, Calif).

Statistical analysis

Reported values are shown as means + SEMs. Statistical analyses were performed with SPSS software (SPSS, Chicago, Ill) by using the Kruskal Wallis test followed by a Mann-Whitney U-test. Resulting p values of less than 0.05, 0.01 and 0.001 are indicated and considered significant. Test results that did not reach significance ($p > 0.05$) are not indicated.

Results

SAHM1 abrogates eosinophilic airway inflammation

To evaluate the efficacy of SAHM1, we used an acute airway inflammation model, sensitizing and challenging C57bl/6 mice with HDM, and mice were analyzed 4 days after the last challenge (**Figure 1A**). In this model mice had AAI characterized by increased eosinophil, neutrophil, macrophage, DC, T cell, B cell and type 2 innate lymphocyte cell (ILC2) counts in BAL, compared with those in PBS-sensitized mice (**Figure 1B and 1C**). Treatment with an optimal dose range of 0.3 to 3 mg of SAHM1 during HDM sensitization and challenge abrogated eosinophilic airway inflammation in lung tissue and BAL fluid (**Figure 1A-C**).

Next, we used intracellular flow cytometry to analyze cytokine-producing T cells in BAL fluid and observed that although the proportions of Th2 cytokine-positive cells were not affected, SAHM1 treatment reduced significantly the total numbers of CD4 T cells positive for IL-5, IL-13, IL-17 and IFN-γ (see **Figure E2** in this article's Online Repository at www.jacionline.org). Thus SAHM1 treatment during both sensitization and challenge reduced Th2 cell differentiation and eosinophilic airway inflammation.

SAHM1 treatment is effective during challenge

Because Notch signaling has been implicated in both the induction and in maintenance of Th2 differentiation[93, 135], we compared the effects of SAHM1 (1mg) treatment administered during either sensitization or challenge (**Figure 1D**). We found that SAHM1 treatment effectively reduced eosinophilic and T cell accumulation in BAL fluid only when given during the challenge phase (**Figure 1E**). SAHM1 treatment during sensitization only did not show any effects. IL-4 is not only important for the initiation of Th2 differentiation, but also induces serum IgE (see the Results section in this article's Online Repository), another hallmark of asthma. We observed that the levels of total serum IgE were reduced to control levels when mice were treated with SAHM1 during challenge, showing the effectiveness of SAHM1 treatment (**Figure 1F**).

No effects of SAHM1 treatment were observed in an innate response to a single dose of 100 mg HDM regarding BAL differentiation, DC subset cell numbers and CD86 expression (see **Figures E3-E5** in this article's Online Repository at www.jacionline.org), indicating that in this context Notch signaling is not important for DC activation. Next, when we investigated IL-33-driven alveolar inflammation (see **Figure E6** in this article's Online Repository at www.jacionline.org), we noticed that SAHM1 treatment did not impair the IL-33-driven eosinophilic inflammation. Therefore SAHM1 does not have a direct effect on recruitment and accumulation of eosinophils.

Next, we adoptively transferred OVA-specific (OTII) T cells in mice that received SAHM1 therapy or D1 or diluent. No difference in OTII cell division was seen in the presence of SAHM1 therapy during this primary response. OVA restimulation of the adoptively transferred OVA-specific OTII T cells obtained from lung draining mediastinal lymph nodes (MedLNs) indicates a reduced level of IFN-γ (see **Figure E7** in this article's Online Repository at www.jacionline.org).

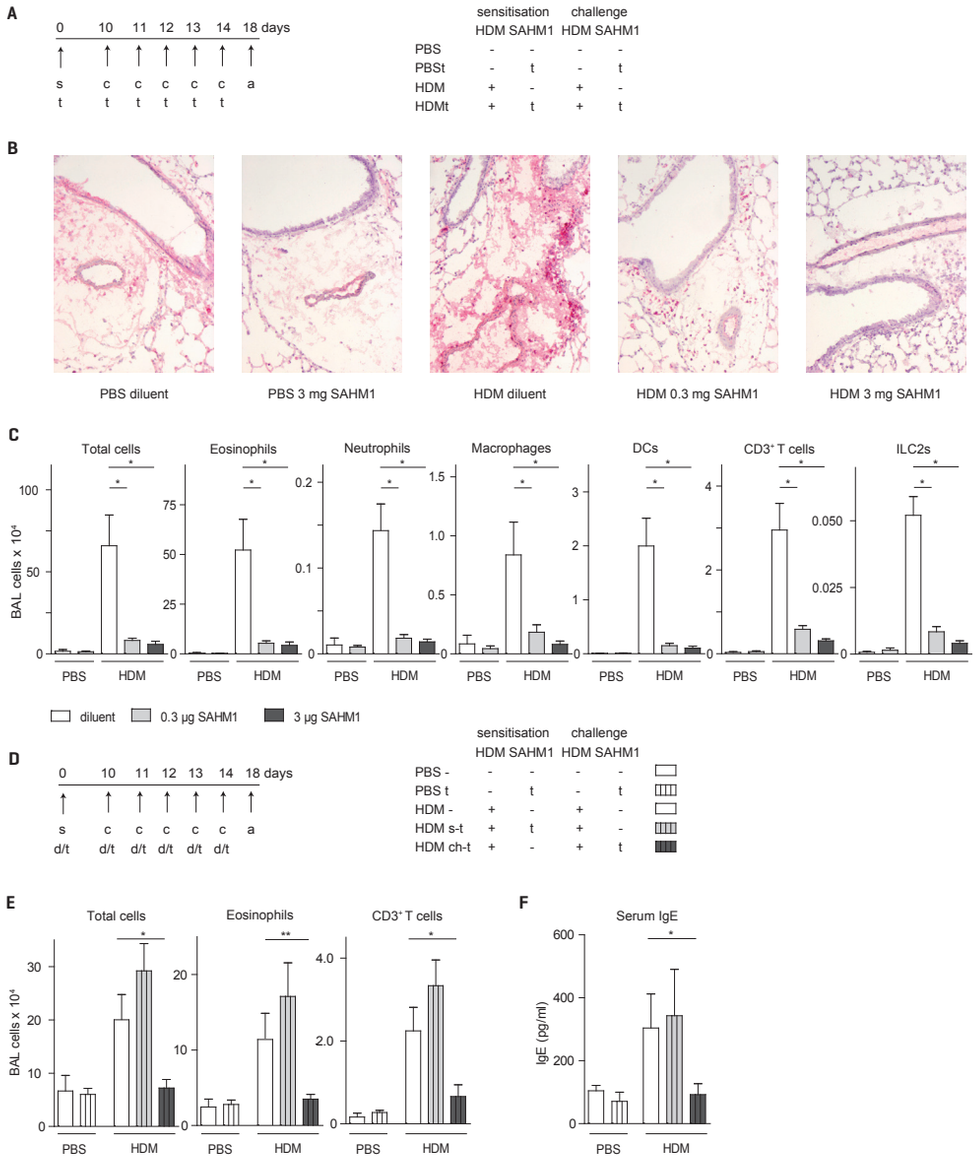

Figure 1. SAHM1 abrogates eosinophilic airway inflammation.

(A) Experimental HDM-driven asthma design, showing intratracheal sensitization (s) and challenge (c) of 10 µg of HDM admixed with diluent, 0.3 and 3 mg SAHM1 (t), or PBS admixed with diluent or 3 mg SAHM1 as a control. Arrows indicated PBS or HDM treatment and SAHM1 or diluent therapy (↑). Analyses (a) were performed 4 days after the last challenge. (B) Immunohistochemical Siglec-F staining (red) to identify eosinophils in indicated representative lung samples. (C) Quantification of flow cytometric analyses of the indicated populations of BAL cells. The results shown represent one of 2 independent experiments with 3 to 6 animals per group and are expressed as means + SEMs. *p<0.05, **p<0.01. (D) Experimental HDM-driven asthma design, showing SAHM1 treatment either during sensitization (s) or challenge (c) with 10 µg of HDM admixed with diluent or 1 mg SAHM1 treatment (t), including PBS exposures admixed with diluent or 1 mg SAHM1 as controls. Analyses (a) were performed 4 days after the last challenge. (E) Quantification of flow cytometric analyses of the indicated populations of BAL fluid cells. (F) Total serum IgE levels were measured by means of ELISA. Results are from one experiment with 3 to 6 animals per group and shown as means + SEMs. *p<0.05, **p<0.01.

SAHM1, but not the mutant control D1, abrogates eosinophilic airway inflammation

Next, we investigated whether a control mutant peptide affected airway inflammation when given during HDM challenge (**Figure 2A**). In contrast to the active SAHM1 inhibitor, the mutant peptide D1[199] did not abrogate eosinophilic airway inflammation (**Figure 2B**). When we investigated intracellular cytokine content of $CD4^+$ T cells using flow cytometry, we observed a significant reduction in total numbers of Th2 cells producing IL-5 or IL-13 in BAL fluid when HDM SAHM1-treated mice were compared with HDM D1-treated mice (**Figure 2C and 2D**).

SAHM1 targets the nuclear complex downstream of the Notch signaling pathway by preventing the binding of MAML in the RBPj complex, which is important for transcription of Notch target genes, which include Gata3, which has an RBPJ-binding site in its upstream promoter[129, 130]. Intracellular flow cytometric analyses were performed to investigate the effect of SAHM1 on key T cell subset TFs, which showed that SAHM1 treatment induced a significant reduction in the numbers of $Gata3^+$, retinoic acid-related orphan receptor γt $(Ror\gamma t)^+$ and forkhead box P3 $(Foxp3)^+$ T cells in BAL fluid (**Figure 3**). For Rorγt and Foxp3, the reduction was explained mostly by the lower number of T cells found in SAHM1-treated animals. In contrast, numbers of $Gata3^+$ $CD4^+$ T cells were also reduced as a proportion of the total T cells, suggesting that inhibition of Notch specifically affected Gata3 expression, independent from diminishing T cell numbers. And therefore both the proportions (**Figure 3A)** and absolute numbers of $Gata3^+$ T cells (**Figure 3B**) were reduced markedly. In agreement with the limited involvement of Th1 cells in HDM-driven airway inflammation in mice, the key Th1 TF T box-containing protein was not induced and not affected by SAHM1 treatment. Taken together, these findings demonstrate that SAHM1 is able to reduce the numbers of key inflammatory cells in mice with AAI and particularly the Th2 inflammatory response.

SAHM1 abrogates airway BHR

BHR is another hallmark of asthma that we investigated in our HDM-driven asthma model, in which PBS control mice and HDM-exposed mice were treated only during allergen challenge with either 1 mg of SAHM1 or control diluent (**Figure 4A**). No differences were observed between the 4 groups of mice in baseline airway responsiveness, as measured based on lung resistance. BHR to increasing doses of methacholine was significantly increased in HDM-challenged mice treated with diluent, compared with diluent-treated PBS control mice (**Figure 4B**). Importantly, treatment with SAHM1 was potent enough to abrogate BHR in HDM-challenged mice.

SAHM1 improves recovery from AAI

Because asthmatic patients present with clinical symptoms only after airway hypersensitivity has already developed fully, we investigated whether SAHM1 treatment would improve airway inflammation in mice with already established asthma. To this end, we investigated 5 different groups of mice (**Figure 5A**). Two control groups consisted of PBS/diluent or PBS/SAHM1 controls through sensitization and challenge only. Three other groups were sensitized with HDM, followed on day 10 to 14 and thereafter by 5 additional HDM challenges. These HDM-exposed mice were then challenged again on 5 successive days (days 15-19) with either HDM in the presence of diluent (group 3) or PBS with either diluent or SAHM1 (group 4 and 5, respectively; **Figure 5A**). This setup allowed us to investigate wether SAHM1 enhances the recovery after establishment of asthma.

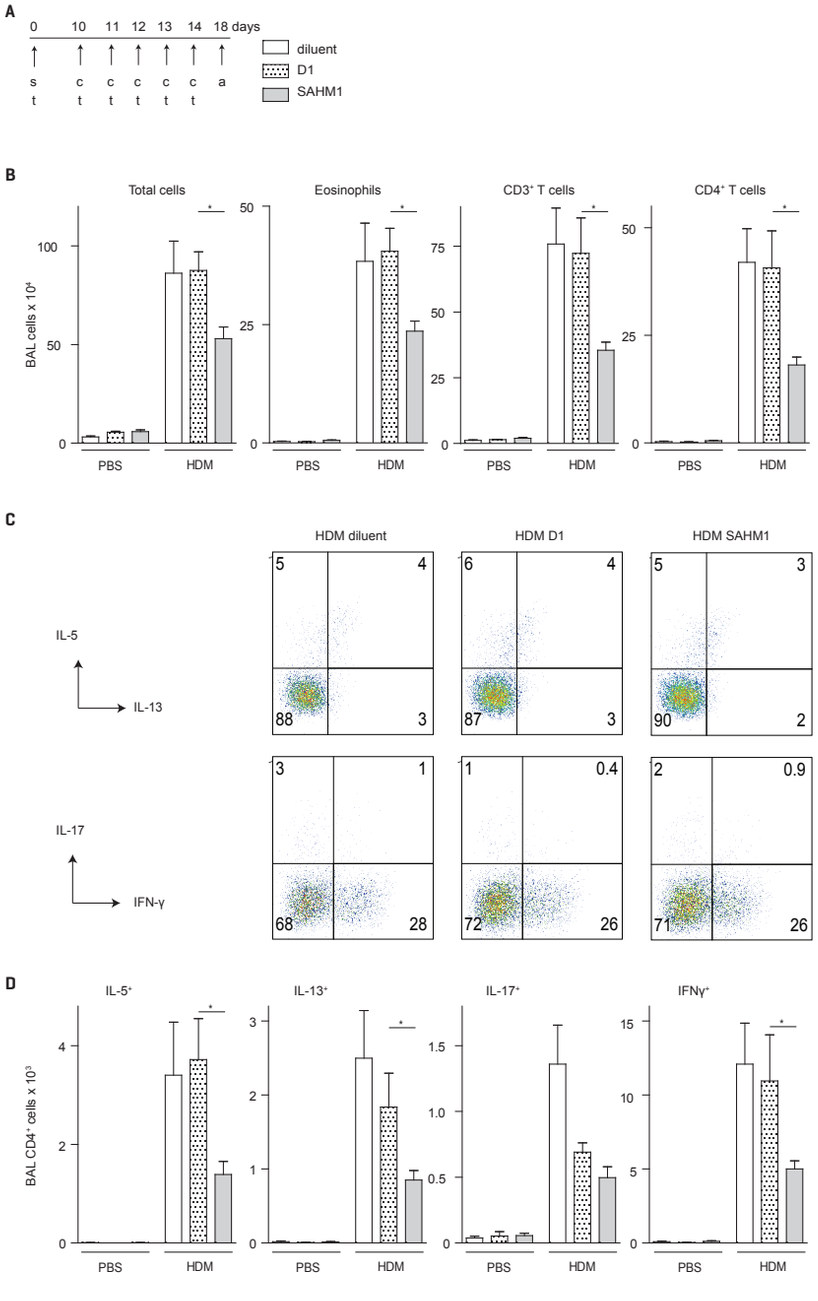

Figure 2. SAHM1, but not the control peptide D1, abrogates eosinophilic airway inflammation.

(A) Experimental HDM-driven asthma design, showing intratracheal sensitization (s) and challenge (c) with 10 µg of HDM admixed with diluents, 3 mg of D1 control peptide, or 3mg of SAHM1. PBS admixed with diluents, 3mg of D1, or 3 mg of SAHM1 were included as controls. Analysis (a) were performed 4 days after the last challenge. (B) Quantification of flow cytometric analyses of the indicated populations of BAL fluid cells. (C) Cytokine expression profiles of gated BAL CD3⁺CD4⁺ T cells upon 4 hrs of phorbol 12-myristate 13-acetate/ionomycin stimulation. (D) Quantification of flow cytometric analyses of cytokine positive CD4⁺ T cells. Results are from one experiment with 3 to 6 animals per group and shown as as means + SEMs. *p<0.05, **p<0.01.

When analyzed at day 23, the PBS controls showed no signs of allergic inflammation in BAL fluid. The group that was HDM-sensitized and then HDM exposed from days 10 to 19 exhibited significantly increased numbers of total cells, eosinophils and T cells, indicating an allergic response (**Figure 5B**). Importantly, when SAHM1 treatment was started 5 days after HDM challenges, the SAHM1-treated mice (HDM PBS SAHM1, **Figure 5**) still showed a strong reduction in eosinophil and T cell numbers, compared with the mice receiving diluent (HDM PBS diluent, **Figure 5**). This showed that SAHM1 treatment was able to enhance recovery from AAI. Moreover, SAHM1 treatment reduced the numbers of Th2 cytokine-producing T cells, which reached significance for IL-5 and IL-13 (**Figure 5A**), as well as the numbers of neutrophils and DCs, in the BAL fluid (data not shown). Therefore SAHM1 treatment improves the recovery of already established AAI in an HDM-driven asthma mouse model.

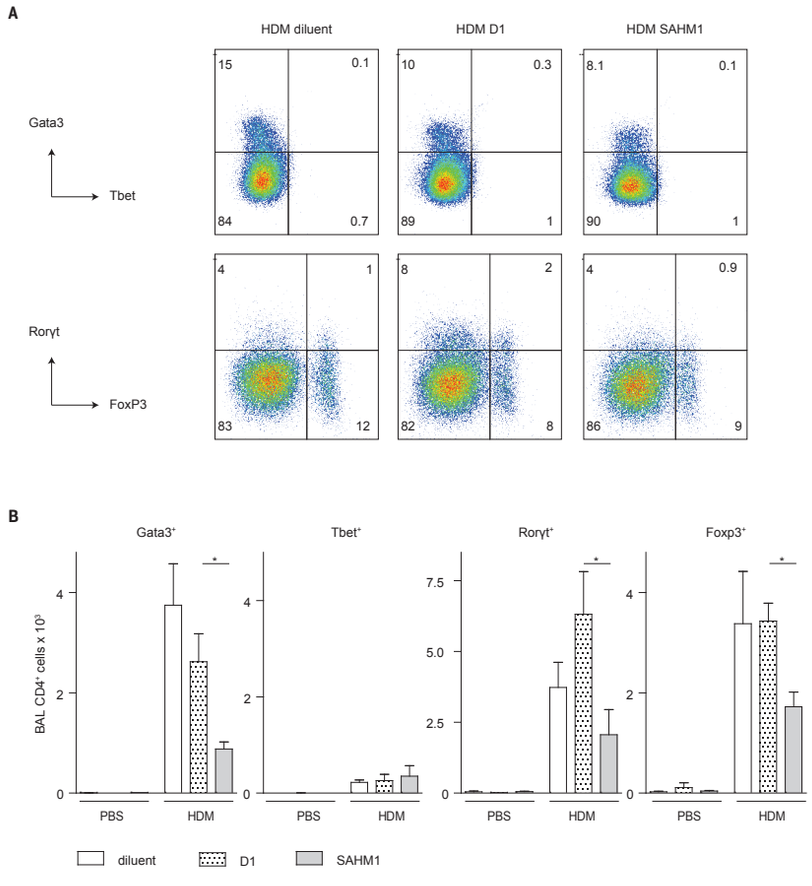

Figure 3. SAHM1, but not D1, reduces key T cell subset TF expression.

(A) TF expression profiles of gated BAL fluid CD3⁺CD4⁺ T cells. (B) Quantification of intracellular flow cytometric analyses of the indicated populations of BAL fluid T cells. Results are from one experiment with 3 (PBS) or 6 (HDM-exposed) mice per group and shown as as means + SEMs. *p<0.05, **p<0.01.

SAHM1 treatment suppresses α-IFN-γ-induced asthma exacerbation

A previous study showed that inhibition of Notch resulted in loss of Th2 responses to a helminth parasite, but that Th2 responses could be restored by blocking IFN-γ[135]. This result suggested the possibility that a main function of Notch is to counteract the inhibitory activity of IFN-γ towards development of Th2 type responses. The imbalance between Th1- and Th2-type cytokines in favor of Th2 cytokines is a major cause of allergic diseases in human patients. If the primary function of Notch is indeed to counteract the inhibitory function of IFN-γ on Th2 responses, a prediction would be that neutralization of IFN-γ would also obviate the ability of Notch inhibition to mitigate the development of allergic asthma.

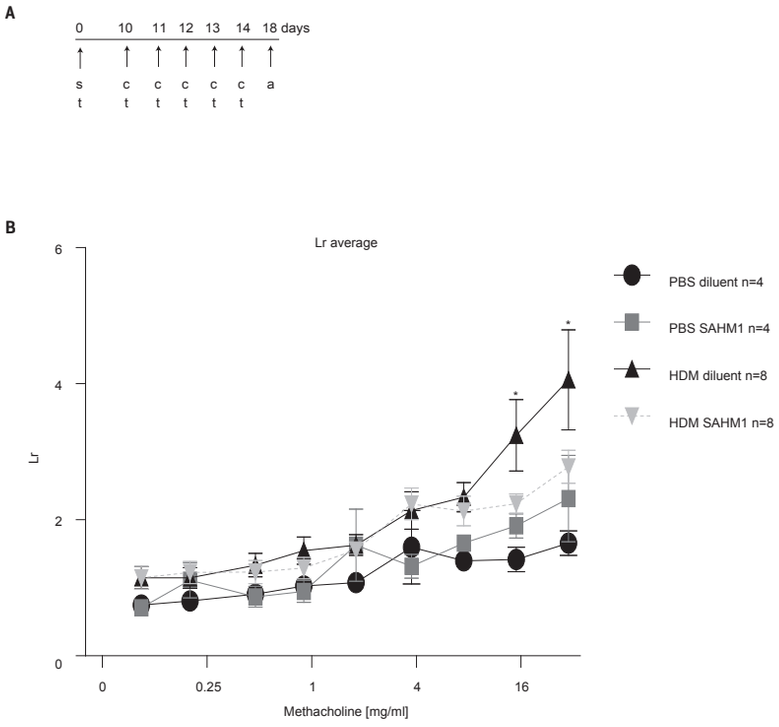

Figure 4. SAHM1 treatment abrogates airway BHR.

(A) Experimental HDM-driven asthma design, showing intratracheal sensitization (s) and challenge (c) with of 10 µg HDM admixed with either diluent or 1 mg SAHM1. PBS admixed with either diluent or 1 mg of SAHM1 served as controls. Analyses (a) were performed 4 days after the last challenge in the indicated mouse groups. (B) Bronchial hyperresponsiveness measurement (Lr, lung resistance) with Buxco in the indicated mouse groups. Results are from one experiment with 4 (PBS) or 8 (HDM-exposed) mice per group and expressed as as means + SEMs. *p<0.05, **p<0.01.

Therefore we tested whether SAHM1 treatment could suppress development of eosinophilic airway inflammation when mice are injected with neutralizing antibodies to IFN-γ-induced exacerbation. To this end, we investigated eosinophilic airway inflammation in HDM-exposed mice in the presence of α-IFN-γ or control rat immunoglobulin (Ig) treated with either diluent or SAHM1 (**Figure 6A**). On the basis of previous reports showing an increased Th2 response when Th1 activity is suppressed by binding of free IFN-γ[135, 418], we expected that the presence of α-IFN-γ during the challenge phase would exacerbate the AAI. Indeed, eosinophilic airway inflammation was more severe in the presence of α-IFN-γ , as evidenced by increased induction of focal dense infiltrates (**Figure 6B**). Importantly, the inflammation was less severe when mice were treated with SAHM1 (**Figure 6B**). Likewise, numbers of eosinophils, neutrophils, macrophages and T cells were significantly increased by treatment with

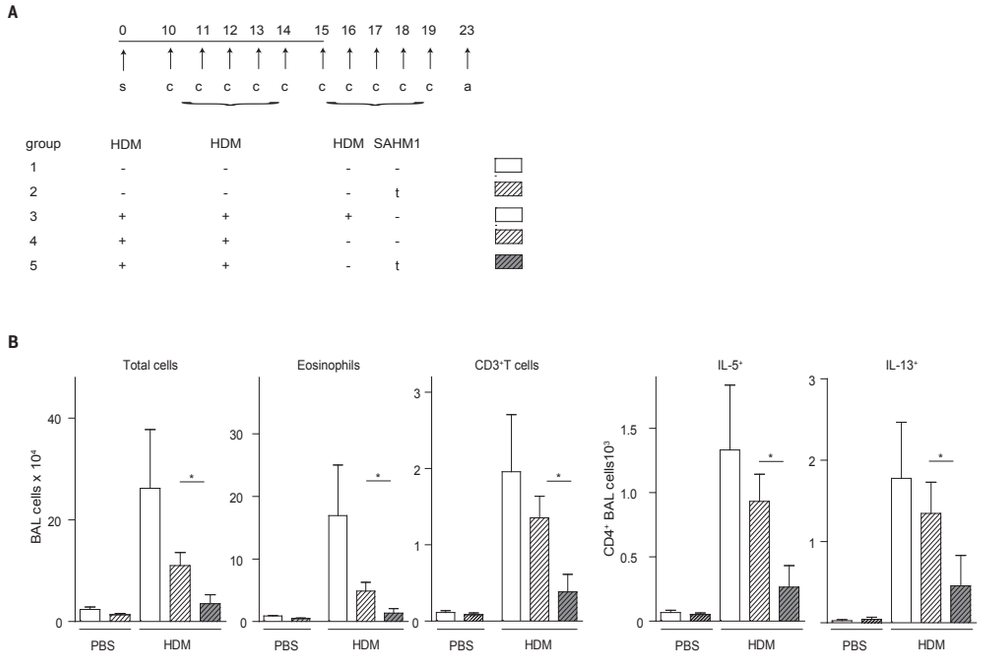

Figure 5. SAHM1 improves recovery from AAI

(A) Experimental HDM-driven asthma design, showing intratracheal sensitization (s) and challenge (c) with HDM during the first interval (day 10-14) to induce asthma. During the second interval (day 15-19), HDM (ongoing inflammation) or PBS (resolution phase) was given with either 1 mg of SAHM1 treatment (t) or diluent as a control. Analyzes (a) were performed four days after the last challenge in the indicated mouse groups. (B) Quantification of flow cytometric analyses of the indicated populations of BAL fluid cells during ongoing inflammation and resolution. Results are from one experiment with 3 to 6 animals per group and are shown as as means + SEMs. *p<0.05.

α-IFN-γ (**Figure 6C**). Asthma exacerbation by α-IFN-γ during HDM challenge was associated with increased numbers of eosinophils, neutrophils, macrophages and T cells in BAL fluid, but additional treatment with SAHM1 restored these numbers to those observed in HDM and control rat Ig-exposed, diluent-treated mice (**Figure 6C**).

Intracellular flow cytometry showed that the numbers of Th2 (IL-4), Th1 (IFN-γ) and Th17 (IL-17) cells in BAL fluid were significantly increased in the presence of α-IFN-γ compared with those in mice that received control rat Ig, but only a limited effect of SAHM1 on the relative proportions of cells producing these cytokines content could be detected (**Figure 6D**). Therefore, we investigated TF expression in BAL fluid Th cells and saw that blocking IFN-γ resulted in reduced proportions of $Gata3^+CD4^+$ T cells and increased proportions of $Ror\gamma t^+CD4^+$ T cells in BAL fluid (**Figure 6E**). Importantly, in the presence of α-IFN-γ , SAHM1 treatment still reduced the proportions of Gata3-expressing $CD4^+$ T cells in BAL fluid compared with diluent treatment (**Figure 6E**). This reduction was greater than the reduction of the CD4 T cell number by SAHM1 (**Figure 6D**), suggesting that Notch inhibition specifically reduced Th2 responses in this model, even when IFN-γ was neutralized (**Figure 6E**). Quantification of absolute numbers of TF expressing $CD4^+$ T cells in BAL demonstrated that, in the presence of α-IFN-γ , SAHM1 treatment reduced $Gata3^+CD4$ T cells numbers, but did not affect numbers of $Ror\gamma t^+$ or $FoxP3^+CD4^+$ T cells (**Figure 6F**). We observed that total serum IgE levels were increased in HDM-exposed mice, compared with PBS control mice, but the effects of α-IFN-γ or SAHM1 treatment in HDM-exposed mice were limited (**Figure 6G**). SAHM1-treated HDM/α-IFN-γ -exposed mice showed some reduction of serum IgE levels compared with those in diluent-treated control mice, but this did not reach significance (**Figure 6G**).

From these experiments we conclude that SAHM1 treatment reduces AAI, even in the setting of α-IFN-γ -induced asthma exacerbation. Furthermore, these findings indicate that Notch signaling in the HDM-mediated airway inflammation model does not only function to counteract inhibitory effects of IFN-γ on Th2 differentiation.

Figure 6. SAHM1 treatment suppresses α-IFN-γ induced asthma exacerbation

(A) Experimental HDM-driven asthma design, showing intratracheal sensitization (s) and challenge (c). During challenge, HDM was admixed either with 150 mg control rat immunoglobulin or α-IFN-γ (to exacerbate asthma) and diluent or 3mg of SAHM1. Analyses (a) were performed 4 days after the last challenge in the indicated mouse groups. (B) Immunohistochemical Siglec-F staining (red) to identify eosinophils in the indicated representative lung samples. (C) Quantification of flow cytometric analyses of the indicated populations of BAL fluid. Cell numbers were significantly less for PBS (statistics not indicated in figure). (D) Total $CD4^+$ T cell numbers in BAL fluid (left) and pie charts of $CD4^+$ T cells classified as Th2 (IL-4), Th1 (IFN-γ) and Th17 (IL-17) cells (right). (E) TF expression profiles of gated BAL fluid $CD3^+CD4^+$ T cells. (F) Quantification of flow cytometric analyses of the indicated populations of TF-expressing BAL fluid T cells in the indicated experimental mouse groups. (G) Total serum IgE levels were measured by means of ELISA. Results shown are from one experiment with 3 to 6 animals per group and are shown as as means + SEMs. *p<0.05.

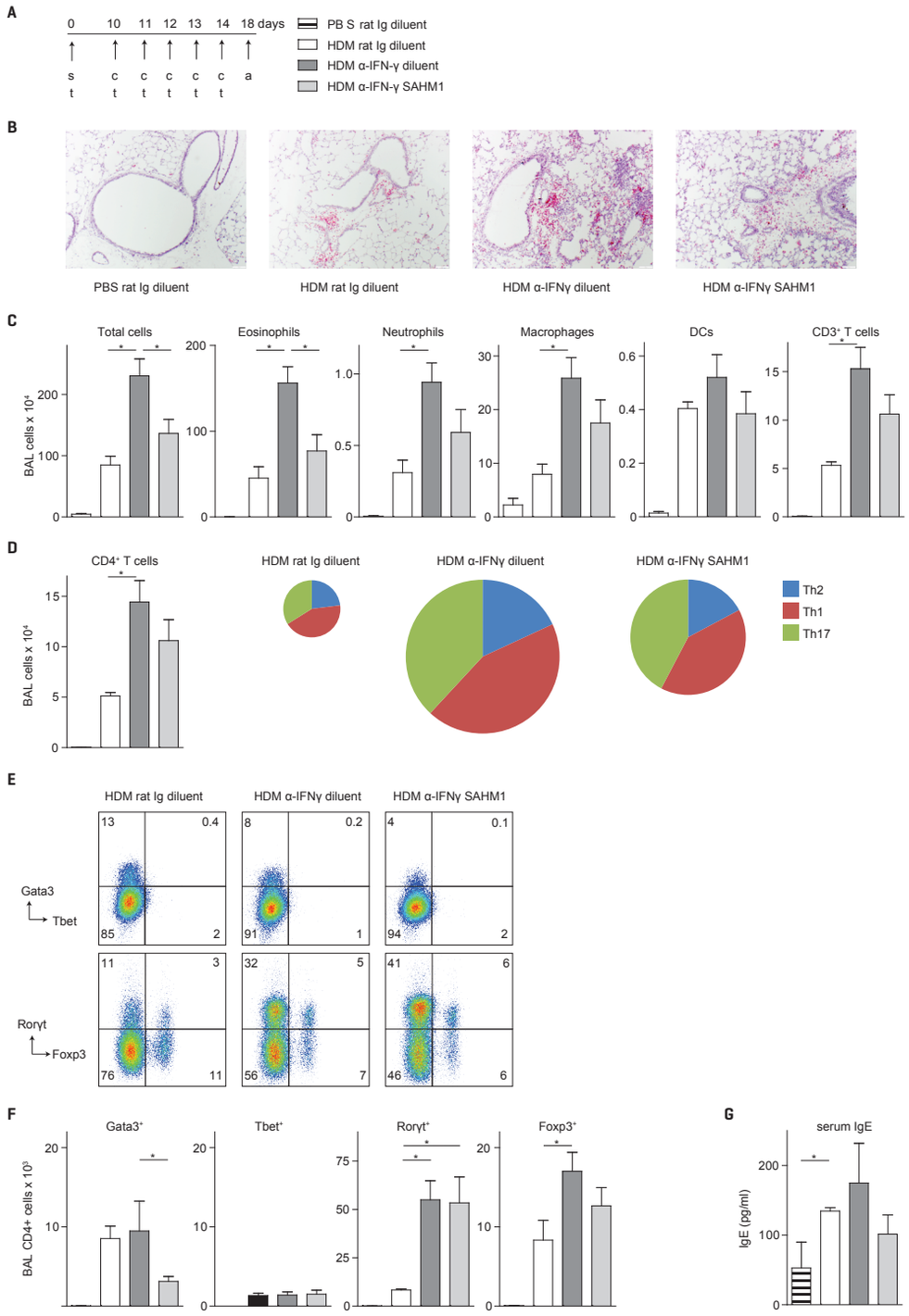

Figure 6. SAHM1 treatment suppresses α-IFN-γ induced asthma exacerbation

Discussion

The Notch signaling pathway has been implicated in asthma pathogenesis. Here we show that the cell-permeable, hydrocarbon-stapled peptide SAHM1 is effective in an HDM-driven model for AAI in mice. SAHM1 treatment reduced functional airway abnormalities: HDM-exposed mice showed all signs of allergic inflammation, including BHR to methacholine and eosinophilia. However, when they received SAHM1 therapy, they showed fewer signs of BHR and eosinophilic airway inflammation.

Notch signaling in $CD4^+$ T cells induces the formation of a nuclear complex containing NICD, RBPJ and MAML. When RBPJ is deleted in $CD4^+$ T cells, this complex cannot be formed, and the Notch pathway is blunted[93, 419]. SAHM1 binds this nuclear complex at the MAML interface and thereby functions as a competitor. Because an RBPJ-binding site is present in the Gata3 upstream promoter (exon 1a) and because we found that Gata3 expression is reduced by SAHM1, it is conceivable that this drug dampens AAI by inhibiting the expression of Gata3 from the upstream promoter. Direct therapeutic targeting of Gata3 is challenging, although recently, improvements in both early and late asthmatic responses after allergen provocation were demonstrated in patients after treatment with a DNAzyme that specifically targets *Gata3* mRNA[420]. Intriguingly, there are differences in the regulation of Il4 gene transcription (which is partly Notch dependent) and *Il5* and *Il13* gene transcription (which is Gata3 and IL-4R/signal transducer and activator of transcription 6 (STAT6) dependent)[39, 93]. This might implicate that when Notch signaling is inhibited in a strong asthma protocol, initial production of IL-4 can still induce IL-4R/STAT6-dependent transcription of Gata3 from the exon 1b promoter and thus transcription of the *Il5* and *Il13* genes.

GSIs, which act as Notch inhibitors, were used successfully in models of AAI, showing the importance of the Notch pathway in IL-4-producing $CD4^+$ T cells[208, 421, 422]. The finding that Notch inhibition is effective during both the primary and secondary immune response illustrated the ongoing need of the Notch pathway for maintenance of Th2 responses[208, 421]. Unfortunately, GSI show significant side effects and are not specific. Here we demonstrate that in HDM-driven AAI, which is an acute and short T cell-dependent asthma model, Notch signaling is important during allergen challenge, which is in agreement with earlier findings that Notch is required for maintaining the Th2 program[135]. Several features of SAHM1 are of significant interest in the context of its application as a new treatment strategy in asthmatic patients. We observed that SAHM1 is effective in dampening HDM-induced AAI and α-IFN-γ -induced exacerbation of allergic inflammation. Moreover, SAHM1 was potent in reducing inflammation in mice with established AAI and to enhance and accelerate recovery. Furthermore, these findings indicate that Notch signaling in the HDM-mediated airway inflammation model does not only function to counteract inhibitory effects of IFN-γ on Th2 differentiation. In addition, the Notch signaling pathway is not only critical for T cells, but also for other cells involved in AAI, including epithelial cells and DCs[423]. Because in endothelial cells the *Il33* gene is a Notch target[424], SAHM1 treatment can result in impaired IL-33 responses. Of note, IL-33 production by airway epithelial cells plays an important role in patients with AAI[425]. Notch is a common differentiation signal for T cell priming of $CD11b^+$ DC subsets in the spleen and intestine[178], and deletion of RBPJ resulted in a reduced capacity of DCs to activate T cells[426]. Human bronchial epithelial cells treated with diesel exhaust particles generate oxidative stress and upregulate Jagged1 and OX40 ligand in myeloid DCs through thymic stromal lymphopoietin, which resulted in myeloid DC-driven Th2 responses[220]. These findings indicate that environmental factors, including air pollution, influence Notch signaling.

Although Notch activation in epithelial cells is sufficient to induce mucous metaplasia, which is a hallmark of asthma, the lack of Notch signaling seems to be beneficial[427]. In this context Notch and STAT6 signaling operate in parallel and independent pathways to regulate mucous metaplasia[191]. Thus, pharmacological targeting of Notch signaling in epithelial cells, such as by SAHM1, can be considered in patients with airway diseases associated with mucous metaplasia[191]. It is very well possible that in our HDM-driven AAI model, topical intratracheal application of SAHM1 also beneficially targeted epithelial or endothelial cells. Neutralization of Notch2 reverses established goblet cell formation in an IL-13-induced mouse model of mucus hypersecretion[423, 428]. Taken together, these effects on the airway epithelium could be seen as a bonus of SAHM1 topical therapy in the airways targeting inflammatory cells. Nevertheless, our previous finding that HDM-driven airway inflammation is diminished in mice with conditional RBPJ deletion exclusively in the T cell lineage demonstrated the critical role of Notch signaling in T cells[419].

Our study has implications for the therapeutic use of peptidomimetic compounds in general. Although stapled peptides have been used *in vivo* and several of these are in human clinical testing, this is the first report of topical delivery. This suggests the potential to use this class of inhibitor for localized delivery in patients with asthma and other indications.

In conclusion, we demonstrate that the Notch pathway is important for an *in vivo* type 2 immune response. Importantly, targeting the NICD-MAML-RBPJ complex by SAHM1 reduced Th2 inflammation during ongoing HDM challenge and enhanced recovery after established asthma. Therefore SAHM1 might represent a novel therapeutic opportunity to abrogate both airway inflammation and BHR in patients with allergic asthma.

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Chapter 8

Notch signaling in T helper cell subsets: instructor or unbiased amplifier?

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Summary

For protection against pathogens, it is essential that naïve $CD4^+$ T cells differentiate into specific effector T helper (Th) cell subsets following activation by antigen presented by dendritic cells (DCs). Next to T cell receptor and cytokine signals, membrane-bound Notch ligands have an important role in orchestrating Th cell differentiation. Several studies provided evidence that DC activation is accompanied by surface expression of Notch ligands. Intriguingly, DCs that express the Delta-like or Jagged Notch ligands gain the capacity to instruct Th1 or Th2 cell polarization, respectively. However, in contrast to this model it has also been hypothesized that Notch signaling acts as a general amplifier of Th cell responses rather than an instructive director of specific T cell fates. In this alternative model Notch enhances proliferation, cytokine production and anti-apoptotic signals or promotes co-stimulatory signals in T cells. An instructive role for Notch ligand expressing DCs in the induction of T helper cell differentiation is further challenged by evidence for the involvement of Notch signaling in differentiation of Th9, Th17, regulatory T cells and follicular T helper cells. In this review, we will discuss the two opposing models, referred to as the 'instructive' and the 'unbiased amplifier' model. We highlight both the function of different Notch receptors on $CD4^+$ T cells and the impact of Notch ligands on antigen-presenting cells.

Introduction

Following signals from both antigen presenting cells (APCs) and the micro-environment, activated $CD4^+$ T cells are triggered to initiate secretion of specific effector cytokines. Since the original observation in 1986 that upon antigenic stimulation naïve $CD4^+$ T cells can differentiate into T helper 1 (Th1) or Th2 effector T cells depending on polarizing cytokine signals[23], various additional Th subsets have been recognized. These include Th9, Th17, Th22, follicular T helper cells (Tfh) and regulatory T cells (Tregs), each characterized by a unique cytokine production profile and a key transcription factor (see for recent review: REF 24). These Th subsets play a crucial role in appropriate immune responses during host defense, but are also involved in the pathogenesis of inflammatory diseases[25, 26].

Th1 cells mainly produce IFN-γ and TNF-α and are associated with the elimination of intracellular pathogens. Th1 development is facilitated by either IL-12 and STAT4 or by IFN-γ, STAT1 and the key Th1 transcriptional regulator T-box-containing protein (T-bet), encoded by *Tbx21*[28]. Th2 cells control helminth infections and are implicated in allergic immune responses such as allergic asthma. They are potent producers of Th2 cytokines that induce IgE synthesis (IL-4), recruit eosinophils (IL-5) and cause smooth muscle hyperreactivity and goblet cell hyperplasia (IL-13). Therefore, Th2 cells are central in the orchestration and amplification of inflammatory events in allergic asthma. The master transcription factor Gata3 is necessary and sufficient for Th2 cytokine gene expression in Th2 cells[409]. Because Th2 differentiation is driven by IL-4, this raises the paradox that IL-4 is required to generate the cell type that is its major producer. But, the origin of the first IL-4 required for Th2 cell induction remains unclear. While a range of cell types are able to produce IL-4, Th2 cell responses can still be generated when only T cells can make IL-4, arguing against an essential role for an external source of IL-4[41, 42].

An accumulating number of studies suggest that the Notch signaling pathway, which also plays a crucial role in early hematopoietic development and at multiple steps of T lineage development, is essential for Th cell differentiation (for recent review: REF 207). Currently, two opposing models have been proposed that explain how Notch ligands can influence Th subset differentiation. According to the 'instructive' model, Jagged and Delta-like ligands (DLL) on APCs induce Th2 and Th1 differentiation, respectively[93]. Alternatively, the 'unbiased amplifier' model proposes that Notch ligands are not instructive but rather function to generally amplify Th cell responses[135]. In this review, we will discuss these two contrasting hypotheses on the role of Notch signaling. We will focus both on Notch receptor expressing T cells and on Notch ligand-expressing cells.

The Notch signaling pathway

There are five Notch ligands: two Jagged (Jagged1 and Jagged2) and three Delta-like ligands (DLL1, DLL3 and DLL4), which are bound by four receptors, Notch1-4. For these ligands to be functional their ubiquitination by Mindbomb1 or Neuralized within the cell is required[500]. Details of the Notch signaling pathway are discussed in various excellent reviews[444, 459]. Briefly, following ligand-receptor binding, the Notch intracellular domain (NICD) is cleaved by a γ-secretase complex and translocates to the nucleus and binds to the transcription factor RBPJk (recombination signal binding protein for immunoglobulin Jk region; **Figure 1**). Finally, additional co-activating proteins are recruited, such as mastermind-like proteins (MAML1-3) and p300 to induce transcription of target genes. Notch signaling does not only induce Th lineage-defining transcription factors and cytokines (described below), but also general pathways critical for T cell activation, including IL-2 production, upregulation of the IL-2 receptor and glucose uptake[107-110]. Notch signaling potentiates phosphatidylinositol 3-kinase-dependent signaling downstream of the T cell receptor (TCR) and CD28 by inducing activation of Akt kinase and mammalian target of rapamycin, which enhances T cell effector functions and survival and allows them to respond to lower antigen doses[108, 111, 112]. Notch signaling can be enhanced by the protein kinase PKCθ, which is crucial for TCR and CD28 signaling and regulation of the actin cytoskeleton[113]. Moreover, upon TCR stimulation NICD interacts with other proteins in the cell in a non-canonical, RBPJκ-independent pathway that leads to NFκB activation[114, 115].

Induction of Notch ligands on APCs

Th2-promoting stimuli including helminth eggs, prostaglandin E2, cholera toxin and allergens such as house dust mite (HDM), birch pollen and cockroach allergens were shown to induce Jagged expression on APCs, as summarized in **Table 1**. Conversely, microbial Th1-inducing stimuli, e.g. dengue virus, respiratory syncytial virus (RSV), bacterial LPS and the TRL9 ligand CpG, upregulate the Notch ligands DLL1/DLL4 on APCs (**Table 1**). Other studies, however, do not show exclusive upregulation of either DLL or Jagged molecules, but rather upregulation of Notch ligands of both families upon stimulation[93, 205, 208, 214, 215, 217, 221, 222, 227, 419, 501]. Interestingly, whereas surface induction of DLL requires MyD88, this is not the case for Jagged induction[93, 210, 216, 223-225]. LPS can promote both Th1 and Th2 responses, which are MyD88-dependent and Myd88-independent, respectively, but the molecular mechanisms responsible for Jagged induction by LPS are unknown[502-504]. Together, although there is also evidence that particular stimuli can induce both Th1 and Th2 differentiation, many studies support an instructive role of DLL and Jagged expression on APCs.

The role of Notch ligands in Th2 and Th1 differentiation and function

Th2 cells

Notch signaling can initiate Th2 cell differentiation by direct activation of (i) a 3' enhancer of the *Il4* gene, and (ii) an upstream promoter of *Gata3*[39, 93, 129, 130]. Several studies using mice expressing a dominant negative (DN) MAML transgene have demonstrated that Notch signaling is essential for Th2 cell differentiation and function[130, 131]. When γ-secretase inhibitors (GSI) were used to block Notch signaling in OVA-induced asthma or food allergy models, Th2 cytokine production by T cells was inhibited while IFN-γ production was increased[132-134]. Moreover, upon gene ablation of Notch1/Notch2 or RBPJk, IL-4 production was abrogated and functional responses against parasitical pathogens were reduced[93]. At the same time, IFN-γ expression was unaffected, supporting an instructive role for Notch signaling. In line with an instructive model, DLL4 was demonstrated to have a regulatory role in Th2 responses to cockroach allergen, OVA, RSV or Schistosoma Mansoni egg antigen (**Table 1**) and in an experimental autoimmune encephalomyelitis (EAE) model[212]. A protective Th1 response to RSV in the lungs was converted into an allergic Th2 response by DLL4-neutralization *in vivo*[216].

However, defective Th2 responses against the intestinal helminth *Trichuris muris* in DN-MAML transgenic mice were restored when mice received anti-IFN-γ antibodies, indicating that Notch functions to optimize rather than to initiate the Th2 response[135]. Moreover, decreased Th2 responses were found when DLL4 was blocked in a mouse model for RSV-mediated allergic asthma exacerbations[406]. Finally, we very recently found that whereas mice with RBPJk-deficient T cells failed to develop HDM-driven allergic airway inflammation (AAI) and airway hyperreactivity (AHR), mice with a DC-specific conditional deficiency of both Jagged1 and Jagged2 developed normal AAI following *in vivo* HDM-exposure (**Chapter 3**). Although most studies using bmDCs would support an instructive role for Jagged in the induction of Th2 cell differentiation and function (**Table 1**), our studies indicate that induction of Th2 responses in HDM-driven AAI is dependent on Jagged expression on other cell types than DCs or alternatively on cooperation between Jagged and DLL on DCs.

Taken together, although several lines of evidence indicate that DCs use the Notch pathway to instruct Th cell fates, Notch may also act as an unbiased amplifier of Th cell differentiation.

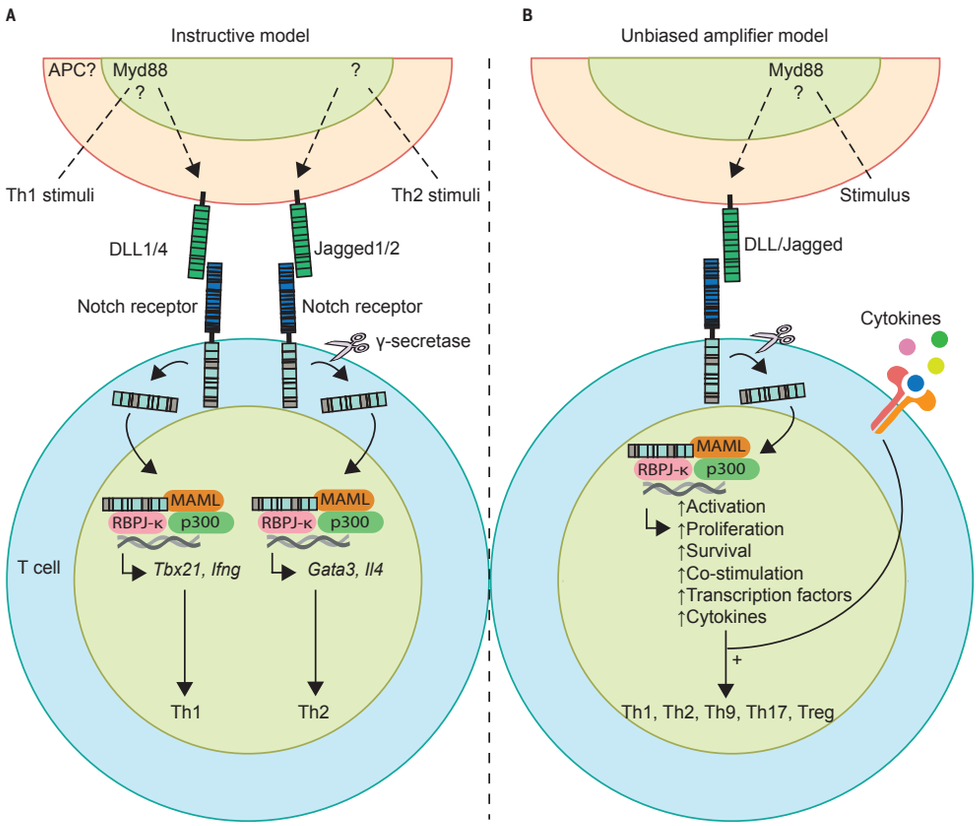

Figure 1: Schematic overview of the two models describing the role of Notch signaling in Th cell differentiation.
(A) According to the instructive model, Th1-stimuli and Th2-stimuli induce DLL and Jagged ligand expression on APCs, respectively. Upon receptor-ligand binding, Th1 differentiation is induced by Notch intracellular domain (NICD) binding and activating transcription of the Th1 transcription factor gene *Tbx21* and signature cytokine *Ifng*. For Th2 differentiation, Notch induces transcription of *Gata3* and *Il4*.
(B) Notch ligands act as an unbiased amplifier, thereby sensitizing cells to the environment to ensure that activated $CD4^+$ T cells overcome a Th cell commitment threshold. Notch induces activation, proliferation, enhance anti-apoptotic signals and is simultaneously recruited to Th1, Th2 and Th17 genes. So, in this hypothesis Notch acts as an enabler of differentiation, whereby the outcome depends on signals of the environment, such as cytokines.

Table 1: Evidence supporting instructive roles for Jagged and DLL in Th2 and Th1 cell differentiation, respectively.

1) Abbreviations used: AAI, Allergic airway inflammation; AHR, Airway hyperreactivity; APE, Aqueous birch pollen extract; bmDC, Bone marrow-derived DC; CFA, Complete Freund's Adjuvant; CNS, Central nervous system; DLL, Delta-like ligand; EAE, Experimental autoimmune encephalomyelitis; GP, Viral glycoprotein peptide; Jag, Jagged; mDCs, Myeloid immature DCs; Mib, Mindbomb; moDCs, monocyte-derived DCs; RSV, Respiratory syncytial virus; TMEV, Theiler's murine encephalomyelitis virus.

2) ↑, increased; ↓, decreased; 0, unaffected, L, Low expression; -, Not determined;

Instruction of Th2 differentiation							
APC	**Stimulant**	**Notch Ligand**				**Additional findings**	**Reference**
		Jag1	**Jag2**	**DLL1**	**DLL4**		
GM-CSF bmDCs [1)]	PGE2 or Cholera toxin	0 [2)]	↑	0	0	Jag1-expressing APC induce Th2 cytokines in $CD4^+$ T cells	93
GM-CSF bmDCs	Endotoxin$^+$ OVA	↑	0	-	↑	Jag1-FC (but not siRNA-Jag1) enhances AHR, eosinophilia and Th2 cytokine production	208, 214
GM-CSF bmDCs	Cholera toxin CpG	- -	↑ 0	- -	0 ↑	c-kit deficient DCs lack Jag2 (but not DLL4) and induce reduced Th2 inflammation and AAI	215
GM-CSF bmDCs	LPS$^+$ OVA/GP peptide	-	-	-	-	DCs lacking Mib1 show impaired Th2 but not Th1 differentiation *in vitro*	222
GM-CSF bmDCs	CpG, RSV	-	-	-	↑	Blocking of DLL4 induces increased Th2 cytokine secretion and AHR	216
GM-CSF bmDCs	Cockroach allergen	↑	0	↑	↑↑	DLL4 suppresses Th2 cytokines and blocking of DLL4 induces increased Th2 cytokine secretion and AHR	217
GM-CSF bmDCs	OVA OVA$^+$ DLL4	0 ↑	0 0	0 ↑	↑ ↑	DCs, pretreated with DLL4, induce reduced AHR and AAI	218
GM-CSF bmDCs	Derp7 LPS	↑↑ ↑	- -	- -	- -	Derp7 induces IL-4 secretion by $CD4^+$ T cells	219
Human $BDCA1^+$mDCs	-	-	-	-	-	Jag1 expression correlated with IL-4 expressing T cells	228
Human $BDCA1^+$mDCs	Diesel-exhaust particles (DEP)	↑	0	-	L	In a DC-$CD4^+$ T cell co-culture anti-Jag1 decreases the IL-5/IFN-γ ratio	220
Human GM-CSF moDCs	APE APE/PGE2$^+$LPS	0 0	↑ ↑	↑ ↓	0 ↓	In a DC-$CD4^+$ T cell co-culture, APE increases IL-5 and IL-10 secretion	221

Instruction of Th1 differentiation							
APC	**Stimulant**	**Notch Ligand**				**Additional findings**	**Reference**
		Jag1	**Jag2**	**DLL1**	**DLL4**		
$CD11c^+$ GM-CSF bmDCs	RSV	0	0	-	↑	MyD88-/- DCs have reduced DLL4 and cannot induce IFN-γ in $CD4^+$ T cells	223
GM-CSF bmDCs	P. acnes, CpG	-	-	-	↑	DLL4 promotes Th1 development by inhibition of IL-4 production in T cells	224
GM-CSF bmDCs	TMEV	-	-	-	↑	Blocking of DLL4 induces decreased Th1 cytokines in demyelinating disease	211
Splenic DCs ($CD11c^+CD8^-$)	LPS	L	L	L	↑	DLL4 expressing APCs induce IFN-γ (but not IL-4) in $CD4^+$ T cells *in vitro*	225
$CD11c^+$ DCs, $CD19^+$ B cells	MOG35-55 peptide in CFA	↑ 0	- -	↑ ↑	- -	DLL1-Fc increases Th1 cells, anti-Delta1 antibodies decrease Th1 cells; anti-Jag1 antibodies worsened EAE	205, 226
Unknown	MOG(35-55)/CFA	-	-	-	-	DLL4-blockade decreases IFN-γ and TNF-α, promotes IL-4 production by T cells and decreases CNS inflammation	212, 213
Human GM-CSF moDCs	LPS	↑	L	0	↑	Expression of DLL4 correlated with IFN-γ inducing capabilities of DCs	227
Human $BDCA1^+$ mDCs	R-848	↓	L	L	↑	Jag1 expression negatively correlated with IFN-γ expressing T cells	228
Human monocytes, macrophages, GM-CSF moDCs	Dengue-virus	 L L L	 L L L	 ↑ ↑ ↑	 0 ↑ ↑	DLL1 induces IFN-γ but not IL-4 production by $CD4^+$ T cells *in vitro*	229
Human $CD1c^+$ DCs and pDCs	R-848	-	-	-	↑	DLL4-blockade decreases IFN-γ and IL-17 expressing $CD4^+$ T cells *in vitro*	230

Th1 cells

The signature Th1 genes *Ifng* and *Tbx21* were identified as direct Notch targets[135, 136]. Mice in which T cells were Notch1/Notch2 double-deficient showed impaired IFN-γ secretion by Th1 cells during *in vivo* Leishmania major parasite infection, but reports employing DN-MAML transgenic or conditional RBPJκ knockout mice, demonstrated that Th1 cell function was unaffected[129-131, 137, 419]. Therefore, these findings suggest that signals that regulate Th1 differentiation involve RBPJκ-independent functions of Notch. Studies using GSI showed that Th1 differentiation was impaired in an *in vivo* EAE model[135, 136]. By contrast, an increase in Th1 differentiation (and a concomitant decrease in Th2 cytokine production) was seen in an OVA-driven AAI model[133]. The interpretation of these apparently conflicting findings remains complicated, because effects of GSI are not limited to Notch signaling and e.g. also involve HLA-A2 expression and cadherins[198].

The capacity of DLL1/DLL4 to induce Th1 cell differentiation is supported by many *in vitro* and *in vivo* experiments, as outlined in **Table 1**. For example, anti-DLL4 antibodies reduced IFN-γ and TNF-α secretion by T cells *in vivo*[211-213]. DLL1-blockade decreased Th1 cell numbers in an allograft model[152]. Conversely, Jagged1-Fc had no effect and anti-Jagged1 antibodies worsened EAE disease[205, 226]. Gene ablation of Jagged1 or Mindbomb1, which is critical for expression of functional Notch ligands, did not affect Th1 differentiation *in vitro*[208, 222].

In conclusion, although most studies would support an instructive role for DLL1/DLL4 in Th1 induction, the role of Notch signaling in Th1 cell differentiation remains incompletely understood.

Other T helper cell subsets

Given the increasing complexity of T cell subset biology, it is not unexpected that the bipotential instructional model is not sufficient to fully explain the function of Notch signaling in Th cell differentiation. For example, Notch signaling cooperates with TGF-β to induce Th9 cell differentiation and IL-9 expression via Jagged2 ligation[138]. The *Rorc*, *Il17* and *Il23r* gene promoters are direct Notch targets and, accordingly, Th17 cell differentiation is impaired when Notch signaling is blocked[139-143]. Hereby, DLL1, DLL3 and DLL4 ligands were found to be essential[212, 213, 230, 406, 505], but a role for Jagged1 remains controversial[506-508]. Remarkably, addition of DLL3 enhanced Th17 differentiation *in vitro*[509], although it was shown that DLL3 cannot activate Notch in adjacent cells, but inhibits signaling when expressed in the same cell as the Notch receptor[510]. Differentiation and function of Tregs requires Notch signaling in T cells[105, 144-146], whereby both DLL and Jagged ligands can promote Treg expansion[511-518]. Although the key Treg transcription factor Foxp3 is a direct Notch target[519], the role of Notch in Tregs seems rather complex, because targeting of DLL4 or Treg-specific components of the Notch pathway was associated with an increase of Tregs in *in vivo* autoimmune models[212, 520, 521]. Moreover, hepatocytes and plasmacytoid DCs can induce IL-10 production in T cells via Jagged1 and DLL4, respectively[515, 522, 523]. Finally, the finding that the absence of Notch receptors on T cells or DLL4 on lymph node stromal cells, resulted in a deficiency of Tfh cells[147, 148], implicates Notch signaling in Tfh cell differentiation.

"Instructive" versus "unbiased amplifier" model

As summarized in **Table 1**, considerable evidence supports an "instructive model" whereby pathogens direct Th1 and Th2 differentiation via upregulation of DLL or Jagged ligands on DCs, respectively (**Figure 1**). This implies that different Notch ligands induce distinct cellular responses in T cells, largely by the same signaling components. Although it has been speculated that different ligands might induce qualitatively different signals, e.g. RBPJk-dependent or independent, or signals that differ in strength or kinetics[524], the molecular mechanisms involved are currently unknown.

It has been shown that DLL4 induces a stronger Notch signal than DLL1 or Jagged1[516]. Also, the ability of ligands to induce Notch signaling is dependent on the glycosylation status of the extracellular domain of Notch: Notch receptors carrying N-acetylglucosamine preferentially signal via Delta ligands, while Jagged binding is inhibited[99]. Absence or overexpression of Fringe glycosyltransferase proteins alters Th1 and Th2 differentiation[406, 422]. Another possibility would be that different ligands preferentially activate different Notch receptors, which may each have unique downstream nuclear targets to induce distinct cellular programs. Indeed, it has been reported that whereas Notch1 and Notch2 activate Th2 differentiation, Notch3 promotes Th1 differentiation and IFN-γ production[129, 388]. The expression of all these Notch receptors is induced on T cells upon TCR stimulation[137, 151, 461]. Because different NICDs have different target gene preferences[525], distinct ligand-receptor combinations may produce quantitatively or qualitatively distinct signals[526]. However, this is not supported by the findings that both Th1 and Th2 differentiation is affected in T cells that are Notch1/Notch2 double-deficient[129, 137] and that retroviral expression of Notch1 as well as Notch3 was associated with increased Th1 responses[136, 388]. This issue is further complicated by the observation that individual Notch receptors are upregulated with different kinetics[527]. It is therefore conceivable that they have distinct functions depending on the phase of the response.

Several studies are in apparent conflict with the "instructive model". For example, DLL ligands were reported to promote Th2 responses or Jagged ligands were implicated in Th1 induction[406, 528]. Neither Jagged1 or DLL1 could instruct Th2 or Th1 cytokine differentiation *in vitro* in the absence of polarizing cytokines[150]. Importantly, Bailis *et al*. showed that Notch signaling simultaneously induced Th1, Th2 and Th17 gene transcription, also under polarizing conditions that were described to favor only one of the differentiation outcomes[135]. In addition, Notch signaling via DLL4 was shown to boost antigen sensitivity of $CD4^+$ T cells via promoting co-stimulatory signals in T cells[108]. Together, this would suggest that Notch acts as a co-stimulating factor that orchestrates multiple Th cell programs by sensitizing cells to exogenous cytokines, thereby ensuring that activated $CD4^+$ T cells overcome a Th cell commitment threshold. In support of a role for Notch as an unbiased amplifier (**Figure 1**), Notch signaling was shown to be required for optimal T cell expansion, CD25 and IL-2 induction *in vitro* of both Th1 and Th2 cells[107, 108, 110, 150]. Finally, Notch signals promote survival by enhancing anti-apoptotic signals and glucose uptake[109, 149].

It is conceivable that minor differences in experimental design or conditions form the basis of the discrepant results that support one of the two opposing models for Notch function in Th differentiation. Many studies on Notch ligands on APCs have employed GM-CSF cultured bmDCs (**Table 1**),

which were recently shown to contain not only DCs, but also monocyte-derived macrophages[400]. In our own studies, we found that Jagged expression was required for the induction of a Th2 response in the lung when *in vitro* HDM-pulsed bmDCs were used for allergen sensitization, but not when mice were *in vivo* sensitized by endogenous airway DCs (**Chapter 3**). Moreover, studies are complicated by the finding that Notch ligands are not only induced on DCs, but also on macrophages, B and T cells or lymph node stromal cells[148, 205, 460, 461]. Stimulation via CD46 and CD3 was shown to upregulate Jagged1 on human T cells[462], suggesting that T cells can provide Notch signals to each other. However, it is of note that normally several mechanisms, including lateral inhibition, are used to regulate Notch activity when similar cell types express both ligand and receptor. By lateral inhibition signal-sending cells actively repress their Notch signaling pathway[529] which would hamper concerted Notch-mediated differentiation and polarization of adjacent T helper cells. Finally, Notch receptors can become activated independent of ligand binding[471]. Indeed, spontaneous Notch cleavage has been observed upon TCR triggering[107, 110, 114]. Ligand-independent Notch signaling would also be supported by the recent identification of a PKCθ-dependent mechanism that enhances Notch activation[113]. More experiments targeting Notch ligands in various cells types are required to determine how the Notch signaling pathway is activated in T cell subsets *in vivo*.

Another concern is that some gain-of-function approaches, involving overexpression of Notch receptors or ligands, may be associated with strong or prolonged, less physiological Notch signals. In this context, it is interesting that variable Notch signal strength allows induction of distinct responses by the same signaling pathway[530, 531], paralleling previous experiments demonstrating Th1 or Th2 cells are induced by strong or weak TCR signals, respectively[532, 533]. Therefore, in studies on the effects of Notch ligands on Th differentiation, it may be critical to use a range of antigen doses. Finally, since it has recently been shown that Th2 inflammation also crucially involves IL-4-producing Tfh cells[34, 68], findings of impaired *in vivo* Th2 cell differentiation may point at Tfh rather than Th2 defects and should therefore be interpreted with care.

Conclusions and future directions

Given the increasing number of characterized Th subsets, it is unlikely that Notch signaling simply acts as a bimodal molecular switch for the induction of either Th1 and Th2 differentiation, based on DLL and Jagged expression on DCs, respectively. Nevertheless, many studies described above support the notion that individual Notch ligands have differential effects on T helper cell differentiation, which cannot be explained by the unbiased amplifier model. The two models, however, may not necessarily be mutually exclusive. Effects of Notch signaling could be quite different during induction and during maintenance of Th subset differentiation. Moreover, the finding that there is quite some plasticity between Th subsets[24] and that Th2 differentiation may involve a Tfh phase has further complicated the role of Notch signaling in Th differentiation. We also conclude that the elucidation of the role of Notch ligands on particular cell types requires comprehensive *in vivo* studies, using cell-specific knockout of individual Notch ligands or combinations.

Since Notch signaling is involved in the differentiation of basically all Th subsets, it could serve as a potential therapeutic target, for example by inhibiting Th2 responses in allergies or Th1/Th17 responses in autoimmune diseases. However, because effects of GSI are not limited to Notch signaling, it will be valuable to develop more specific compounds targeting Notch signaling components. Indeed synthetic, cell-permeable stabilized peptides that target a critical protein-protein interface in the Notch transactivation complex[199-201], as well as specific antibodies that target Notch receptors[202-204] or Notch ligands[205, 206] have been designed. Promising results were obtained with Notch pathway blocking antibodies in cancer patients[534] and future studies should explore whether these antibodies are beneficial for allergic or autoimmune patients.

Interestingly, GSI administration during only the challenge in asthma models was sufficient to decrease Th2 cytokine production[132, 133]. These findings imply that Notch signaling is not likely critical to initiate IL-4 production in activated T cells and thus the initial source of IL-4, for example in AAI, remains unclear. While several cells including basophils, Tfh cells, NKT cells and ILC2 are capable of producing IL-4[33-40], mice deficient for NKT cells, ILC2 or basophils are still capable of inducing Th2 responses[385, 535, 536], suggesting that IL-4 production by Tfh cells could be crucial for Th2 cell induction. Nevertheless, the finding that in animal models allergic disease symptoms are reduced by GSI administration during challenge only, indicates that Notch signaling is important in maintaining rather than inducing Th2 cell responses. This makes Notch signaling an interesting target for development of therapeutic strategies in allergic asthma.

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Chapter 9

Conclusions and future directions

In this thesis we showed that Notch signaling is required for Th2 cell-mediated allergic airway inflammation (AAI). We demonstrated this in mice lacking either RBPJκ or both Notch1 and Notch2 specifically in T cells and by using the synthetic, cell-permeable stabilized peptide SAHM1 to target protein-protein interfaces in the Notch transactivation complex (**Chapter 3-5**). In addition, we found that AAI, induced by HDM, develops independently of the Notch ligand Jagged on DCs, alveolar macrophages, fibroblastic reticular cells, B cells and T cells (**Chapter 3 and 6**). Our experiments further provide evidence that Notch signaling was not required for the initiation of Th2 cell differentiation or proliferation during the sensitization phase, but was needed to promote or maintain Th2 cell-mediated inflammation upon repeated exposure to house-dust mite (HDM). Transcriptional as well as flow cytometric analyses of Th2 cells from WT and Notch-deficient mice revealed that Notch signaling has a function in lymphocyte adhesion and responsiveness to cytokines (**Chapter 5**). Lastly, we characterized Th2 cells from asthmatic patients and healthy subjects in detail. We found that frequencies of Notch1 and Notch2 positive memory T cells were increased in peripheral blood from asthma patients with low asthma control. This correlated with the expression of the prostaglandin DP2 receptor CRTH2 on Th2 cells (**Chapter 7**). The data described in this thesis do not only indicate that targeting of the Notch signaling pathway is a promising therapeutic approach for asthma patients, but also provide new questions for future research on the function of Notch signaling during Th2 cell-mediated inflammation in allergic asthma.

Outstanding questions

- What ligand on which cell type is required for Notch-mediated Th2 cell inflammation in an HDM-mediated model for allergic airway inflammation?
- What distinct cellular responses are mediated by the different Notch ligands and how do different Notch ligands and Notch receptors induce distinct cellular responses in T cells, largely by the same signaling components?
- Via what mechanism does Notch signaling support migration of Th2 cells from the lymph nodes to the lungs?
- In which phase of HDM-mediated allergic airway inflammation is Notch signaling required most?
- Via which mechanisms does Notch signaling enhance differentiation and inflammation of other (non-Th2) T helper cell subsets?
- Can the Notch inhibitor SAHM1 reduce airway inflammation and alleviate symptoms in patients with allergic asthma?

What ligand on which cell type is required for Notch-mediated Th2 cell inflammation in an HDM-mediated model for allergic airway inflammation?

A noticeable amount of evidence supports an instructive model whereby allergens direct differentiation of Th1 and Th2 cells via upregulation of DLL or Jagged ligands on APCs, respectively (**Chapter 8**). Nevertheless, we did not find any evidence for a role for the Notch ligand Jagged on DCs, alveolar macrophages, fibroblastic reticular cells, B cells and T cells (**Chapter 3 and 6**), although we found considerable Jagged expression on DCs and lymph node fibroblastic reticular cells. This suggests redundancy of Notch ligand expression on other cell types or redundancy with other Notch ligands. Given the increasing number of characterized Th subsets and the increasing number of studies that show plasticity of these T helper subsets[455], it seems unlikely that Notch signaling just acts as a bimodal instructor of either Th1 and Th2 differentiation based on the presented ligand. Our findings that Notch signaling has a function during a later phase in the Th2 cell response and that Notch mediates cytokine responsiveness of Th2 cells, add to the hypothesis that Notch acts as a unbiased amplifier of T helper cell responses in which Notch together with environmental triggers steers T helper cell differentiation. Finding which ligands on which cell types are involved in the Th2 response will be crucial to understand the mechanism of Notch-driven Th2 cell inflammation.

What distinct cellular responses are mediated by the different Notch ligands and how do different Notch ligands and Notch receptors induce distinct cellular responses in T cells, largely by the same signaling components?

The Notch signaling pathway is a relatively simple pathway containing a limited amount of signaling components. As described in **chapter 8**, little is known about how the 5 different Notch ligands in combination with the 4 different Notch receptors induce distinct cellular responses in T cells, largely by the same signaling components. In addition, it is unclear how the distribution of expression of different Notch ligands and receptors on different cell types and tissue compartments contributes to the type of interactions and cellular responses. The instructive model implies that different Notch ligands induce distinct cellular responses in T cells while the unbiased amplifier simply suggests that all ligands can induce T helper cell differentiation. Evidence suggests that different ligands might induce qualitatively different signals or signals that differ in strength or kinetics[524]. Also, ligands might preferentially bind to Notch receptors depending on the glycosylation status of the extracellular domain of the Notch receptor[99]. In addition, different Notch intracellular domains (NICDs) might have different target gene preferences[525]. It is therefore not unlikely that distinct ligand-receptor combinations can produce quantitatively or qualitatively distinct signals[526] and could therefore have distinct functions. A better understanding of the molecular mechanisms involved in the different ligand-receptor interactions in various T cell subsets will contribute to the understanding of the function of the different ligands during Notch signaling-mediated T helper cell differentiation and activation.

Via what mechanism does Notch signaling support migration of Th2 cells from the lymph nodes to the lungs and in which phase of HDM-mediated allergic airway inflammation is Notch signaling required most?

Previous *in vitro* experiments have shown that Notch signaling can directly induce transcription of the *Il4* and *Gata3* genes[93, 129, 130]. In contrast, our experiments indicate that there is no crucial role for Notch signaling during the induction of proliferation or cytokine production of Th2 cells, although Gata3 expression was reduced in the absence of Notch signaling. Moreover, Notch signaling was necessary during the challenge phase of AAI and mediated lymph node egress of Th2 cells, adhesion and cytokine responsiveness (**Chapter 4 and 5**). Together, these data provide a new perspective on the role of Notch signaling in T helper differentiation. Our findings suggest that Notch signaling does not induce Th2 cell differentiation independent of STAT6, but instead steers Th2 cell differentiation together with cytokines and mediates lymph node egress of Th2 cells. Investigating which factors (cytokines or Notch signaling) induce Th2 cell differentiation requires a range of complex experiments that comprises experiments that block Notch signaling together with IL-4 during the priming of Th2 cells. In addition, it would be helpful to measure a range of time points after one or multiple allergen challenges to find at what time point Notch signaling is most required for which processes during the Th2 cell lifespan. This is complex, since it is well possible that the function of Notch signaling during Th2 cell differentiation differs per type of model and stimulus and whether experiments are performed *in vitro* or *in vivo*.

Via which mechanisms does Notch signaling enhance differentiation and inflammation of other (non-Th2) T helper cell subsets?

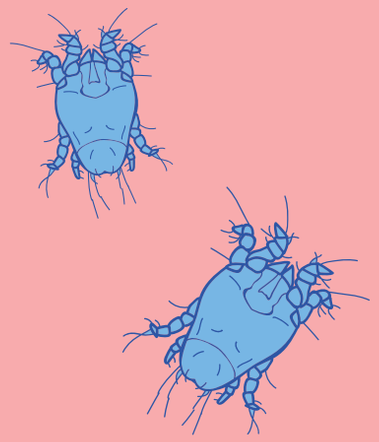
Notch signaling was not only described to induce differentiation of Th2 cells, but also for the differentiation of multiple T helper cell subsets (as described in detail in **Chapters 1 and 8**). These include Th1 cells, since the Th1 genes *Ifng* and *Tbx21* were identified as direct Notch targets[135, 136]. Moreover, Notch signaling cooperates with TGF-β to induce Th9 cell differentiation and IL-9 expression[138] and the promoter regions of the Th17 cell-associated Rorc, Il17 and Il23r genes are identified as direct Notch targets[139-143]. Also, the key Treg transcription factor Foxp3 is a direct Notch target. Lastly, Notch signaling is required for the differentiation of Tfh cells[147, 148]. To our knowledge, it has not been established if Notch signaling is actually required for the early transcriptional program during initiation of differentiation of Th1 cells, Th9 cells, Th17 cells, Tregs and Tfh cells. Since we found that Notch signaling is mainly required during a later phase of the Th2 cell response, it is possible that the Notch function is similar during immune responses mediated by other T helper cell subsets. Since Notch signaling could be a potential therapeutic target in many $CD4^+$ T cell-driven diseases, it would be helpful to better understand the mechanisms involved in function or control of Notch signaling in these cells.

Can the Notch inhibitor SAHM1 reduce airway inflammation and alleviate symptoms in patients with allergic asthma?

Assembly of the NICD-MAML-RBPJκ nuclear complex can effectively be prevented by the synthetic Notch inhibitor SAHM1. We found that treating mice intranasally with the Notch inhibitor SAHM1 during only the challenge phase of our HDM-driven model for AAI is sufficient to abolish Th2 cell inflammation, eosinophilia and bronchial hyperreactivity (**Chapter 4**). Importantly, our finding that inhibiting Notch signaling can reduce already established AAI is a strong indicator that patients with allergic asthma could benefit from an inhaled Notch inhibitor such as SAHM1. We found increased proportions of $Notch1^+$ and $Notch2^+$ memory $CD4^+$ T cells and Th2 cells in peripheral blood from asthma patients (**Chapter 7**). Herein, we did not find differences between allergic and non-allergic asthma patients. Since the percentage of Notch positive T cells was low and highly variable between patients it would be helpful to obtain information about Notch expression on $CD4^+$ T cells in lungs or lymph nodes, which may be higher than in peripheral blood $CD4^+$ T cells. In addition, when SAHM1 or other inhibitors of the Notch pathway would be tested as a therapeutic agent in asthma patients, the therapeutic effects in patients with high and low Notch expression should be compared. Such an analysis would reveal whether or not only patients with high Notch expression on $CD4^+$ T cells would benefit from Notch inhibitors. In addition, since Notch signaling is a general pathway and not specific for T cells, the effects of SAHM1 on other cell types should be evaluated.

Altogether, we showed that Notch signaling is crucial during Th2 cell-mediated AAI in an HDM-driven model for AAI and we found increased proportions of $Notch1^+$ and $Notch2^+$ cells in the memory $CD4^+$ T cell and Th2 cell populations from asthmatic patients. Therefore, we conclude that the Notch signaling pathway is a promising therapeutic target for patients with asthma.

References



1. Bateman ED, Boushey HA, Bousquet J, Busse WW, Clark TJ, Pauwels RA, et al. Can guideline-defined asthma control be achieved? The Gaining Optimal Asthma ControL study. Am J Respir Crit Care Med 2004; 170:836-44.
2. Fanta CH. Asthma. N Engl J Med 2009; 360:1002-14.
3. Dolan CM, Fraher KE, Bleecker ER, Borish L, Chipps B, Hayden ML, et al. Design and baseline characteristics of the epidemiology and natural history of asthma: Outcomes and Treatment Regimens (TENOR) study: a large cohort of patients with severe or difficult-to-treat asthma. Ann Allergy Asthma Immunol 2004; 92:32-9.
4. Haselkorn T, Borish L, Miller DP, Weiss ST, Wong DA. High prevalence of skin test positivity in severe or difficult-to-treat asthma. J Asthma 2006; 43:745-52.
5. Craig TJ. Aeroallergen sensitization in asthma: prevalence and correlation with severity. Allergy Asthma Proc 2010; 31:96-102.
6. Hammad H, Lambrecht BN. Barrier Epithelial Cells and the Control of Type 2 Immunity. Immunity 2015; 43:29-40.
7. Forster R, Davalos-Misslitz AC, Rot A. CCR7 and its ligands: balancing immunity and tolerance. Nat Rev Immunol 2008; 8:362-71.
8. Barnes PJ. The cytokine network in asthma and chronic obstructive pulmonary disease. J Clin Invest 2008; 118:3546-56.
9. Stirling RG, van Rensen EL, Barnes PJ, Chung KF. Interleukin-5 induces CD34(+) eosinophil progenitor mobilization and eosinophil CCR3 expression in asthma. Am J Respir Crit Care Med 2001; 164:1403-9.
10. Wills-Karp M. Interleukin-13 in asthma pathogenesis. Immunol Rev 2004; 202:175-90.
11. Li BW, Hendriks RW. Group 2 innate lymphoid cells in lung inflammation. Immunology 2013; 140:281-7.
12. Halim TY, Krauss RH, Sun AC, Takei F. Lung natural helper cells are a critical source of Th2 cell-type cytokines in protease allergen-induced airway inflammation. Immunity 2012; 36:451-63.
13. Halim TY, Steer CA, Matha L, Gold MJ, Martinez-Gonzalez I, McNagny KM, et al. Group 2 innate lymphoid cells are critical for the initiation of adaptive T helper 2 cell-mediated allergic lung inflammation. Immunity 2014; 40:425-35.
14. Halim TY, Hwang YY, Scanlon ST, Zaghouani H, Garbi N, Fallon PG, et al. Group 2 innate lymphoid cells license dendritic cells to potentiate memory TH2 cell responses. Nat Immunol 2016; 17:57-64.
15. Halim TY. Group 2 innate lymphoid cells in disease. Int Immunol 2016; 28:13-22.
16. Tacke F, Ginhoux F, Jakubzick C, van Rooijen N, Merad M, Randolph GJ. Immature monocytes acquire antigens from other cells in the bone marrow and present them to T cells after maturing in the periphery. J Exp Med 2006; 203:583-97.
17. Catron DM, Itano AA, Pape KA, Mueller DL, Jenkins MK. Visualizing the first 50 hr of the primary immune response to a soluble antigen. Immunity 2004; 21:341-7.
18. Mempel TR, Henrickson SE, Von Andrian UH. T-cell priming by dendritic cells in lymph nodes occurs in three distinct phases. Nature 2004; 427:154-9.
19. Ley K. The second touch hypothesis: T cell activation, homing and polarization. F1000Res 2014; 3:37.
20. Crapster-Pregont M, Yeo J, Sanchez RL, Kuperman DA. Dendritic cells and alveolar macrophages mediate IL-13-induced airway inflammation and chemokine production. J Allergy Clin Immunol 2012; 129:1621-7 e3.
21. Islam SA, Luster AD. T cell homing to epithelial barriers in allergic disease. Nat Med 2012; 18:705-15.
22. Rosen H, Gonzalez-Cabrera PJ, Sanna MG, Brown S. Sphingosine 1-phosphate receptor signaling. Annu Rev Biochem 2009; 78:743-68.
23. Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, Coffman RL. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. J Immunol 1986; 136:2348-57.
24. DuPage M, Bluestone JA. Harnessing the plasticity of CD4(+) T cells to treat immune-mediated disease. Nat Rev Immunol 2016; 16:149-63.
25. Hirahara K, Nakayama T. $CD4^+$ T-cell subsets in inflammatory diseases: beyond the Th1/Th2 paradigm. Int Immunol 2016; 28:163-71.
26. Li P, Spolski R, Liao W, Leonard WJ. Complex interactions of transcription factors in mediating cytokine biology in T cells. Immunol Rev 2014; 261:141-56.
27. Smith KM, Pottage L, Thomas ER, Leishman AJ, Doig TN, Xu D, et al. Th1 and Th2 $CD4^+$ T cells provide help for B cell clonal expansion and antibody synthesis in a similar manner *in vivo*. J Immunol 2000; 165:3136-44.
28. Lazarevic V, Glimcher LH, Lord GM. T-bet: a bridge between innate and adaptive immunity. Nat Rev Immunol 2013; 13:777-89.
29. Caza T, Landas S. Functional and Phenotypic Plasticity of CD4(+) T Cell Subsets. Biomed Res Int 2015; 2015:521957.
30. Zeng WP. 'All things considered': transcriptional regulation of T helper type 2 cell differentiation from precursor to effector activation. Immunology 2013; 140:31-8.
31. Zheng W, Flavell RA. The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells. Cell 1997; 89:587-96.
32. Zhang DH, Cohn L, Ray P, Bottomly K, Ray A. Transcription factor GATA-3 is differentially expressed in murine Th1 and Th2 cells and controls Th2-specific expression of the interleukin-5 gene. J Biol Chem 1997; 272:21597-603.

33. Akbari O, Stock P, Meyer E, Kronenberg M, Sidobre S, Nakayama T, et al. Essential role of NKT cells producing IL-4 and IL-13 in the development of allergen-induced airway hyperreactivity. Nat Med 2003; 9:582-8.
34. Ballesteros-Tato A, Randall TD, Lund FE, Spolski R, Leonard WJ, Leon B. T Follicular Helper Cell Plasticity Shapes Pathogenic T Helper 2 Cell-Mediated Immunity to Inhaled House Dust Mite. Immunity 2016; 44:259-73.
35. Min B, Prout M, Hu-Li J, Zhu J, Jankovic D, Morgan ES, et al. Basophils produce IL-4 and accumulate in tissues after infection with a Th2-inducing parasite. J Exp Med 2004; 200:507-17.
36. Noval Rivas M, Burton OT, Oettgen HC, Chatila T. IL-4 production by group 2 innate lymphoid cells promotes food allergy by blocking regulatory T-cell function. J Allergy Clin Immunol 2016; 138:801-11 e9.
37. Pelly VS, Kannan Y, Coomes SM, Entwistle LJ, Ruckerl D, Seddon B, et al. IL-4-producing ILC2s are required for the differentiation of TH2 cells following Heligmosomoides polygyrus infection. Mucosal Immunol 2016; 9:1407-17.
38. Seder RA, Paul WE, Dvorak AM, Sharkis SJ, Kagey-Sobotka A, Niv Y, et al. Mouse splenic and bone marrow cell populations that express high-affinity Fc epsilon receptors and produce interleukin 4 are highly enriched in basophils. Proc Natl Acad Sci U S A 1991; 88:2835-9.
39. Tanaka S, Tsukada J, Suzuki W, Hayashi K, Tanigaki K, Tsuji M, et al. The interleukin-4 enhancer CNS-2 is regulated by Notch signals and controls initial expression in NKT cells and memory-type CD4 T cells. Immunity 2006; 24:689-701.
40. Yoshimoto T, Paul WE. CD4pos, NK1.1pos T cells promptly produce interleukin 4 in response to *in vivo* challenge with anti-CD3. J Exp Med 1994; 179:1285-95.
41. Jankovic D, Kullberg MC, Noben-Trauth N, Caspar P, Paul WE, Sher A. Single cell analysis reveals that IL-4 receptor/Stat6 signaling is not required for the *in vivo* or *in vitro* development of CD4$^+$ lymphocytes with a Th2 cytokine profile. J Immunol 2000; 164:3047-55.
42. Schmitz J, Thiel A, Kuhn R, Rajewsky K, Muller W, Assenmacher M, et al. Induction of interleukin 4 (IL-4) expression in T helper (Th) cells is not dependent on IL-4 from non-Th cells. J Exp Med 1994; 179:1349-53.
43. Schmitt E, Bopp T. Discovery and initial characterization of Th9 cells: the early years. Semin Immunopathol 2017; 39:5-10.
44. Veldhoen M, Uyttenhove C, van Snick J, Helmby H, Westendorf A, Buer J, et al. Transforming growth factor-beta 'reprograms' the differentiation of T helper 2 cells and promotes an interleukin 9-producing subset. Nat Immunol 2008; 9:1341-6.
45. Chang HC, Sehra S, Goswami R, Yao W, Yu Q, Stritesky GL, et al. The transcription factor PU.1 is required for the development of IL-9-producing T cells and allergic inflammation. Nat Immunol 2010; 11:527-34.
46. Staudt V, Bothur E, Klein M, Lingnau K, Reuter S, Grebe N, et al. Interferon-regulatory factor 4 is essential for the developmental program of T helper 9 cells. Immunity 2010; 33:192-202.
47. Newcomb DC, Peebles RS, Jr. Th17-mediated inflammation in asthma. Curr Opin Immunol 2013; 25:755-60.
48. Bettelli E, Carrier Y, Gao W, Korn T, Strom TB, Oukka M, et al. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. Nature 2006; 441:235-8.
49. Harrington LE, Hatton RD, Mangan PR, Turner H, Murphy TL, Murphy KM, et al. Interleukin 17-producing CD4$^+$ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. Nat Immunol 2005; 6:1123-32.
50. Park H, Li Z, Yang XO, Chang SH, Nurieva R, Wang YH, et al. A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. Nat Immunol 2005; 6:1133-41.
51. Gaffen SL, Jain R, Garg AV, Cua DJ. The IL-23-IL-17 immune axis: from mechanisms to therapeutic testing. Nat Rev Immunol 2014; 14:585-600.
52. Ivanov, II, McKenzie BS, Zhou L, Tadokoro CE, Lepelley A, Lafaille JJ, et al. The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17$^+$ T helper cells. Cell 2006; 126:1121-33.
53. Mathur AN, Chang HC, Zisoulis DG, Stritesky GL, Yu Q, O'Malley JT, et al. Stat3 and Stat4 direct development of IL-17-secreting Th cells. J Immunol 2007; 178:4901-7.
54. Josefowicz SZ, Rudensky A. Control of regulatory T cell lineage commitment and maintenance. Immunity 2009; 30:616-25.
55. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. J Immunol 1995; 155:1151-64.
56. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. Science 2003; 299:1057-61.
57. Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4$^+$CD25$^+$ regulatory T cells. Nat Immunol 2003; 4:330-6.
58. Gu AD, Wang Y, Lin L, Zhang SS, Wan YY. Requirements of transcription factor Smad-dependent and -independent TGF-beta signaling to control discrete T-cell functions. Proc Natl Acad Sci U S A 2012; 109:905-10.
59. Hara M, Kingsley CI, Niimi M, Read S, Turvey SE, Bushell AR, et al. IL-10 is required for regulatory T cells to mediate tolerance to alloantigens *in vivo*. J Immunol 2001; 166:3789-96.
60. Annacker O, Pimenta-Araujo R, Burlen-Defranoux O, Barbosa TC, Cumano A, Bandeira A. CD25$^+$ CD4$^+$ T cells regulate the expansion of peripheral CD4 T cells through the production of IL-10. J Immunol 2001; 166:3008-18.
61. He J, Tsai LM, Leong YA, Hu X, Ma CS, Chevalier N, et al. Circulating precursor CCR7(lo)PD-1(hi) CXCR5(+) CD4(+) T cells indicate Tfh cell activity and promote antibody responses upon antigen reexposure. Immunity 2013; 39:770-81.
62. Lee SK, Rigby RJ, Zotos D, Tsai LM, Kawamoto S, Marshall JL, et al. B cell priming for extrafollicular antibody responses requires Bcl-6 expression by T cells. J Exp Med 2011; 208:1377-88.
63. Liu X, Yan X, Zhong B, Nurieva RI, Wang A, Wang X, et al. Bcl6 expression specifies the T follicular helper cell program *in vivo*. J Exp Med 2012; 209:1841-52, S1-24.
64. Hatzi K, Nance JP, Kroenke MA, Bothwell M, Haddad EK, Melnick A, et al. BCL6 orchestrates Tfh cell differentiation via multiple distinct mechanisms. J Exp Med 2015; 212:539-53.
65. Moser B. CXCR5, the Defining Marker for Follicular B Helper T (TFH) Cells. Front Immunol 2015; 6:296.
66. Nurieva RI, Chung Y, Hwang D, Yang XO, Kang HS, Ma L, et al. Generation of T follicular helper cells is mediated by interleukin-21 but independent of T helper 1, 2, or 17 cell lineages. Immunity 2008; 29:138-49.
67. Luthje K, Kallies A, Shimohakamada Y, Belz GT, Light A, Tarlinton DM, et al. The development and fate of follicular helper T cells defined by an IL-21 reporter mouse. Nat Immunol 2012; 13:491-8.
68. Coquet JM, Schuijs MJ, Smyth MJ, Deswarte K, Beyaert R, Braun H, et al. Interleukin-21-Producing CD4(+) T Cells Promote Type 2 Immunity to House Dust Mites. Immunity 2015; 43:318-30.
69. Hale JS, Ahmed R. Memory T follicular helper CD4 T cells. Front Immunol 2015; 6:16.
70. Maceiras AR, Fonseca VR, Agua-Doce A, Graca L. T follicular regulatory cells in mice and men. Immunology 2017.
71. Sichien D, Lambrecht BN, Guilliams M, Scott CL. Development of conventional dendritic cells: from common bone marrow progenitors to multiple subsets in peripheral tissues. Mucosal Immunol 2017; 10:831-44.
72. Guilliams M, Ginhoux F, Jakubzick C, Naik SH, Onai N, Schraml BU, et al. Dendritic cells, monocytes and macrophages: a unified nomenclature based on ontogeny. Nat Rev Immunol 2014; 14:571-8.
73. Hildner K, Edelson BT, Purtha WE, Diamond M, Matsushita H, Kohyama M, et al. Batf3 deficiency reveals a critical role for CD8alpha$^+$ dendritic cells in cytotoxic T cell immunity. Science 2008; 322:1097-100.
74. GeurtsvanKessel CH, Willart MA, van Rijt LS, Muskens F, Kool M, Baas C, et al. Clearance of influenza virus from the lung depends on migratory langerin$^+$CD11b- but not plasmacytoid dendritic cells. J Exp Med 2008; 205:1621-34.
75. Fuertes MB, Kacha AK, Kline J, Woo SR, Kranz DM, Murphy KM, et al. Host type I IFN signals are required for antitumor CD8$^+$ T cell responses through CD8{alpha}$^+$ dendritic cells. J Exp Med 2011; 208:2005-16.
76. Bernatchez E, Gold MJ, Langlois A, Lemay AM, Brassard J, Flamand N, et al. Pulmonary CD103 expression regulates airway inflammation in asthma. Am J Physiol Lung Cell Mol Physiol 2015; 308:L816-26.
77. Engler DB, Reuter S, van Wijck Y, Urban S, Kyburz A, Maxeiner J, et al. Effective treatment of allergic airway inflammation with Helicobacter pylori immunomodulators requires BATF3-dependent dendritic cells and IL-10. Proc Natl Acad Sci U S A 2014; 111:11810-5.
78. Zelante T, Wong AY, Ping TJ, Chen J, Sumatoh HR, Vigano E, et al. CD103(+) Dendritic Cells Control Th17 Cell Function in the Lung. Cell Rep 2015; 12:1789-801.
79. Lambrecht BN, Hammad H. Lung dendritic cells in respiratory viral infection and asthma: from protection to immunopathology. Annu Rev Immunol 2012; 30:243-70.
80. Plantinga M, Guilliams M, Vanheerswynghels M, Deswarte K, Branco-Madeira F, Toussaint W, et al. Conventional and monocyte-derived CD11b(+) dendritic cells initiate and maintain T helper 2 cell-mediated immunity to house dust mite allergen. Immunity 2013; 38:322-35.
81. Persson EK, Uronen-Hansson H, Semmrich M, Rivollier A, Hagerbrand K, Marsal J, et al. IRF4 transcription-factor-dependent CD103(+)CD11b(+) dendritic cells drive mucosal T helper 17 cell differentiation. Immunity 2013; 38:958-69.
82. Schlitzer A, McGovern N, Teo P, Zelante T, Atarashi K, Low D, et al. IRF4 transcription factor-dependent CD11b$^+$ dendritic cells in human and mouse control mucosal IL-17 cytokine responses. Immunity 2013; 38:970-83.
83. Dalod M, Hamilton T, Salomon R, Salazar-Mather TP, Henry SC, Hamilton JD, et al. Dendritic cell responses to early murine cytomegalovirus infection: subset functional specialization and differential regulation by interferon alpha/beta. J Exp Med 2003; 197:885-98.
84. Grayson MH, Ramos MS, Rohlfing MM, Kitchens R, Wang HD, Gould A, et al. Controls for lung dendritic cell maturation and migration during respiratory viral infection. J Immunol 2007; 179:1438-48.
85. Smit JJ, Rudd BD, Lukacs NW. Plasmacytoid dendritic cells inhibit pulmonary immunopathology and promote clearance of respiratory syncytial virus. J Exp Med 2006; 203:1153-9.
86. Lombardi V, Speak AO, Kerzerho J, Szely N, Akbari O. CD8alpha(+)beta(-) and CD8alpha(+)beta(+) plasmacytoid dendritic cells induce Foxp3(+) regulatory T cells and prevent the induction of airway hyper-reactivity. Mucosal Immunol 2012; 5:432-43.
87. Lewkowich IP, Herman NS, Schleifer KW, Dance MP, Chen BL, Dienger KM, et al. CD4$^+$CD25$^+$ T cells protect against experimentally induced asthma and alter pulmonary dendritic cell phenotype and function. J Exp Med 2005; 202:1549-61.

88. Oriss TB, Ostroukhova M, Seguin-Devaux C, Dixon-McCarthy B, Stolz DB, Watkins SC, et al. Dynamics of dendritic cell phenotype and interactions with CD4$^+$ T cells in airway inflammation and tolerance. J Immunol 2005; 174:854-63.
89. Kool M, van Nimwegen M, Willart MA, Muskens F, Boon L, Smit JJ, et al. An anti-inflammatory role for plasmacytoid dendritic cells in allergic airway inflammation. J Immunol 2009; 183:1074-82.
90. Takagi H, Fukaya T, Eizumi K, Sato Y, Sato K, Shibazaki A, et al. Plasmacytoid dendritic cells are crucial for the initiation of inflammation and T cell immunity *in vivo*. Immunity 2011; 35:958-71.
91. Jakubzick C, Gautier EL, Gibbings SL, Sojka DK, Schlitzer A, Johnson TE, et al. Minimal differentiation of classical monocytes as they survey steady-state tissues and transport antigen to lymph nodes. Immunity 2013; 39:599-610.
92. Vroman H, van den Blink B, Kool M. Mode of dendritic cell activation: the decisive hand in Th2/Th17 cell differentiation. Implications in asthma severity? Immunobiology 2015; 220:254-61.
93. Amsen D, Blander JM, Lee GR, Tanigaki K, Honjo T, Flavell RA. Instruction of distinct CD4 T helper cell fates by different notch ligands on antigen-presenting cells. Cell 2004; 117:515-26.
94. MacDonald AS, Straw AD, Dalton NM, Pearce EJ. Cutting edge: Th2 response induction by dendritic cells: a role for CD40. J Immunol 2002; 168:537-40.
95. Jenkins SJ, Perona-Wright G, Worsley AG, Ishii N, MacDonald AS. Dendritic cell expression of OX40 ligand acts as a costimulatory, not polarizing, signal for optimal Th2 priming and memory induction *in vivo*. J Immunol 2007; 179:3515-23.
96. Morgan TH. The theory of the gene. Am Nat 1917; 51:513-44.
97. Stanley P. Regulation of Notch signaling by glycosylation. Curr Opin Struct Biol 2007; 17:530-5.
98. Stanley P, Guidos CJ. Regulation of Notch signaling during T- and B-cell development by O-fucose glycans. Immunol Rev 2009; 230:201-15.
99. Haines N, Irvine KD. Glycosylation regulates Notch signalling. Nat Rev Mol Cell Biol 2003; 4:786-97.
100. Gordon WR, Arnett KL, Blacklow SC. The molecular logic of Notch signaling--a structural and biochemical perspective. J Cell Sci 2008; 121:3109-19.
101. Kageyama R, Ohtsuka T, Kobayashi T. The Hes gene family: repressors and oscillators that orchestrate embryogenesis. Development 2007; 134:1243-51.
102. Iso T, Kedes L, Hamamori Y. HES and HERP families: multiple effectors of the Notch signaling pathway. J Cell Physiol 2003; 194:237-55.
103. Palomero T, Lim WK, Odom DT, Sulis ML, Real PJ, Margolin A, et al. NOTCH1 directly regulates c-MYC and activates a feed-forward-loop transcriptional network promoting leukemic cell growth. Proc Natl Acad Sci U S A 2006; 103:18261-6.
104. Weng AP, Millholland JM, Yashiro-Ohtani Y, Arcangeli ML, Lau A, Wai C, et al. c-Myc is an important direct target of Notch1 in T-cell acute lymphoblastic leukemia/lymphoma. Genes Dev 2006; 20:2096-109.
105. Samon JB, Champhekar A, Minter LM, Telfer JC, Miele L, Fauq A, et al. Notch1 and TGFbeta1 cooperatively regulate Foxp3 expression and the maintenance of peripheral regulatory T cells. Blood 2008; 112:1813-21.
106. Poellinger L, Lendahl U. Modulating Notch signaling by pathway-intrinsic and pathway-extrinsic mechanisms. Curr Opin Genet Dev 2008; 18:449-54.
107. Adler SH, Chiffoleau E, Xu L, Dalton NM, Burg JM, Wells AD, et al. Notch signaling augments T cell responsiveness by enhancing CD25 expression. J Immunol 2003; 171:2896-903.
108. Laky K, Evans S, Perez-Diez A, Fowlkes BJ. Notch signaling regulates antigen sensitivity of naïve CD4$^+$ T cells by tuning co-stimulation. Immunity 2015; 42:80-94.
109. Maekawa Y, Ishifune C, Tsukumo S, Hozumi K, Yagita H, Yasutomo K. Notch controls the survival of memory CD4$^+$ T cells by regulating glucose uptake. Nat Med 2015; 21:55-61.
110. Palaga T, Miele L, Golde TE, Osborne BA. TCR-mediated Notch signaling regulates proliferation and IFN-gamma production in peripheral T cells. J Immunol 2003; 171:3019-24.
111. Perumalsamy LR, Nagala M, Sarin A. Notch-activated signaling cascade interacts with mitochondrial remodeling proteins to regulate cell survival. Proc Natl Acad Sci U S A 2010; 107:6882-7.
112. Sade H, Krishna S, Sarin A. The anti-apoptotic effect of Notch-1 requires p56lck-dependent, Akt/PKB-mediated signaling in T cells. J Biol Chem 2004; 279:2937-44.
113. Britton GJ, Ambler R, Clark DJ, Hill EV, Tunbridge HM, McNally KE, et al. PKCtheta links proximal T cell and Notch signaling through localized regulation of the actin cytoskeleton. Elife 2017; 6.
114. Dongre A, Surampudi L, Lawlor RG, Fauq AH, Miele L, Golde TE, et al. Non-Canonical Notch Signaling Drives Activation and Differentiation of Peripheral CD4[+] T Cells. Front Immunol 2014; 5:54.
115. Shin HM, Tilahun ME, Cho OH, Chandiran K, Kuksin CA, Keerthivasan S, et al. NOTCH1 Can Initiate NF-kappaB Activation via Cytosolic Interactions with Components of the T Cell Signalosome. Front Immunol 2014; 5:249.
116. Radtke F, MacDonald HR, Tacchini-Cottier F. Regulation of innate and adaptive immunity by Notch. Nat Rev Immunol 2013; 13:427-37.
117. Radtke F, Wilson A, Stark G, Bauer M, van Meerwijk J, MacDonald HR, et al. Deficient T cell fate specification in mice with an induced inactivation of Notch1. Immunity 1999; 10:547-58.
118. Han H, Tanigaki K, Yamamoto N, Kuroda K, Yoshimoto M, Nakahata T, et al. Inducible gene knockout of transcription factor recombination signal binding protein-J reveals its essential role in T versus B lineage decision. Int Immunol 2002; 14:637-45.
119. Pui JC, Allman D, Xu L, DeRocco S, Karnell FG, Bakkour S, et al. Notch1 expression in early lymphopoiesis influences B versus T lineage determination. Immunity 1999; 11:299-308.
120. Wilson A, MacDonald HR, Radtke F. Notch 1-deficient common lymphoid precursors adopt a B cell fate in the thymus. J Exp Med 2001; 194:1003-12.
121. Bell JJ, Bhandoola A. The earliest thymic progenitors for T cells possess myeloid lineage potential. Nature 2008; 452:764-7.
122. Wada H, Masuda K, Satoh R, Kakugawa K, Ikawa T, Katsura Y, et al. Adult T-cell progenitors retain myeloid potential. Nature 2008; 452:768-72.
123. Feyerabend TB, Terszowski G, Tietz A, Blum C, Luche H, Gossler A, et al. Deletion of Notch1 converts pro-T cells to dendritic cells and promotes thymic B cells by cell-extrinsic and cell-intrinsic mechanisms. Immunity 2009; 30:67-79.
124. Hozumi K, Mailhos C, Negishi N, Hirano K, Yahata T, Ando K, et al. Delta-like 4 is indispensable in thymic environment specific for T cell development. J Exp Med 2008; 205:2507-13.
125. Koch U, Fiorini E, Benedito R, Besseyrias V, Schuster-Gossler K, Pierres M, et al. Delta-like 4 is the essential, nonredundant ligand for Notch1 during thymic T cell lineage commitment. J Exp Med 2008; 205:2515-23.
126. Mohtashami M, Shah DK, Nakase H, Kianizad K, Petrie HT, Zuniga-Pflucker JC. Direct comparison of Dll1- and Dll4-mediated Notch activation levels shows differential lymphomyeloid lineage commitment outcomes. J Immunol 2010; 185:867-76.
127. Wendorff AA, Koch U, Wunderlich FT, Wirth S, Dubey C, Bruning JC, et al. Hes1 is a critical but context-dependent mediator of canonical Notch signaling in lymphocyte development and transformation. Immunity 2010; 33:671-84.128. Yashiro-Ohtani Y, He Y, Ohtani T, Jones ME, Shestova O, Xu L, et al. Pre-TCR signaling inactivates Notch1 transcription by antagonizing E2A. Genes Dev 2009; 23:1665-76.
129. Amsen D, Antov A, Jankovic D, Sher A, Radtke F, Souabni A, et al. Direct regulation of Gata3 expression determines the T helper differentiation potential of Notch. Immunity 2007; 27:89-99.
130. Fang TC, Yashiro-Ohtani Y, Del Bianco C, Knoblock DM, Blacklow SC, Pear WS. Notch directly regulates Gata3 expression during T helper 2 cell differentiation. Immunity 2007; 27:100-10.
131. Tu L, Fang TC, Artis D, Shestova O, Pross SE, Maillard I, et al. Notch signaling is an important regulator of type 2 immunity. J Exp Med 2005; 202:1037-42.
132. Zhou M, Cui ZL, Guo XJ, Ren LP, Yang M, Fan ZW, et al. Blockade of Notch Signalling by gamma-Secretase Inhibitor in Lung T Cells of Asthmatic Mice Affects T Cell Differentiation and Pulmonary Inflammation. Inflammation 2015; 38:1281-8.
133. Kang JH, Kim BS, Uhm TG, Lee SH, Lee GR, Park CS, et al. Gamma-secretase inhibitor reduces allergic pulmonary inflammation by modulating Th1 and Th2 responses. Am J Respir Crit Care Med 2009; 179:875-82.
134. Jiang S, Han S, Chen J, Li X, Che H. Inhibition effect of blunting Notch signaling on food allergy through improving TH1/TH2 balance in mice. Ann Allergy Asthma Immunol 2017; 118:94-102.
135. Bailis W, Yashiro-Ohtani Y, Fang TC, Hatton RD, Weaver CT, Artis D, et al. Notch simultaneously orchestrates multiple helper T cell programs independently of cytokine signals. Immunity 2013; 39:148-59.
136. Minter LM, Turley DM, Das P, Shin HM, Joshi I, Lawlor RG, et al. Inhibitors of gamma-secretase block *in vivo* and *in vitro* T helper type 1 polarization by preventing Notch upregulation of *Tbx21*. Nat Immunol 2005; 6:680-8.
137. Auderset F, Schuster S, Coutaz M, Koch U, Desgranges F, Merck E, et al. Redundant Notch1 and Notch2 signaling is necessary for IFN-gamma secretion by T helper 1 cells during infection with Leishmania major. PLoS Pathog 2012; 8:e1002560.
138. Elyaman W, Bassil R, Bradshaw EM, Orent W, Lahoud Y, Zhu B, et al. Notch receptors and Smad3 signaling cooperate in the induction of interleukin-9-producing T cells. Immunity 2012; 36:623-34.
139. Mukherjee S, Schaller MA, Neupane R, Kunkel SL, Lukacs NW. Regulation of T cell activation by Notch ligand, DLL4, promotes IL-17 production and Rorc activation. J Immunol 2009; 182:7381-8.
140. Keerthivasan S, Suleiman R, Lawlor R, Roderick J, Bates T, Minter L, et al. Notch signaling regulates mouse and human Th17 differentiation. J Immunol 2011; 187:692-701.
141. Meyer Zu Horste G, Wu C, Wang C, Cong L, Pawlak M, Lee Y, et al. RBPJ Controls Development of Pathogenic Th17 Cells by Regulating IL-23 Receptor Expression. Cell Rep 2016; 16:392-404.
142. Coutaz M, Hurrell BP, Auderset F, Wang H, Siegert S, Eberl G, et al. Notch regulates Th17 differentiation and controls trafficking of IL-17 and metabolic regulators within Th17 cells in a context-dependent manner. Sci Rep 2016; 6:39117.
143. Zhang W, Zhang X, Sheng A, Weng C, Zhu T, Zhao W, et al. gamma-Secretase Inhibitor Alleviates Acute Airway Inflammation of Allergic Asthma in Mice by Downregulating Th17 Cell Differentiation. Mediators Inflamm 2015; 2015:258168.

144. Anastasi E, Campese AF, Bellavia D, Bulotta A, Balestri A, Pascucci M, et al. Expression of activated Notch3 in transgenic mice enhances generation of T regulatory cells and protects against experimental autoimmune diabetes. J Immunol 2003; 171:4504-11.
145. Barbarulo A, Grazioli P, Campese AF, Bellavia D, Di Mario G, Pelullo M, et al. Notch3 and canonical NF-kappaB signaling pathways cooperatively regulate Foxp3 transcription. J Immunol 2011; 186:6199-206.
146. Fu T, Zhang P, Feng L, Ji G, Wang XH, Zheng MH, et al. Accelerated acute allograft rejection accompanied by enhanced T-cell proliferation and attenuated Treg function in RBP-J deficient mice. Mol Immunol 2011; 48:751-9.
147. Auderset F, Schuster S, Fasnacht N, Coutaz M, Charmoy M, Koch U, et al. Notch signaling regulates follicular helper T cell differentiation. J Immunol 2013; 191:2344-50.
148. Fasnacht N, Huang HY, Koch U, Favre S, Auderset F, Chai Q, et al. Specific fibroblastic niches in secondary lymphoid organs orchestrate distinct Notch-regulated immune responses. J Exp Med 2014; 211:2265-79.
149. Helbig C, Gentek R, Backer RA, de Souza Y, Derks IA, Eldering E, et al. Notch controls the magnitude of T helper cell responses by promoting cellular longevity. Proc Natl Acad Sci U S A 2012; 109:9041-6.
150. Ong CT, Sedy JR, Murphy KM, Kopan R. Notch and presenilin regulate cellular expansion and cytokine secretion but cannot instruct Th1/Th2 fate acquisition. PLoS One 2008; 3:e2823.
151. Maekawa Y, Minato Y, Ishifune C, Kurihara T, Kitamura A, Kojima H, et al. Notch2 integrates signaling by the transcription factors RBP-J and CREB1 to promote T cell cytotoxicity. Nat Immunol 2008; 9:1140-7.
152. Riella LV, Ueno T, Batal I, De Serres SA, Bassil R, Elyaman W, et al. Blockade of Notch ligand delta1 promotes allograft survival by inhibiting alloreactive Th1 cells and cytotoxic T cell generation. J Immunol 2011; 187:4629-38.
153. Coquet JM, Chakravarti S, Kyparissoudis K, McNab FW, Pitt LA, McKenzie BS, et al. Diverse cytokine production by NKT cell subsets and identification of an IL-17-producing CD4-NK1.1- NKT cell population. Proc Natl Acad Sci U S A 2008; 105:11287-92.
154. Oh SJ, Ahn S, Jin YH, Ishifune C, Kim JH, Yasutomo K, et al. Notch 1 and Notch 2 synergistically regulate the differentiation and function of invariant NKT cells. J Leukoc Biol 2015; 98:781-9.
155. Klose CS, Artis D. Innate lymphoid cells as regulators of immunity, inflammation and tissue homeostasis. Nat Immunol 2016; 17:765-74.
156. Wong SH, Walker JA, Jolin HE, Drynan LF, Hams E, Camelo A, et al. Transcription factor RORalpha is critical for nuocyte development. Nat Immunol 2012; 13:229-36.
157. Yang Q, Saenz SA, Zlotoff DA, Artis D, Bhandoola A. Cutting edge: Natural helper cells derive from lymphoid progenitors. J Immunol 2011; 187:5505-9.
158. Gentek R, Munneke JM, Helbig C, Blom B, Hazenberg MD, Spits H, et al. Modulation of Signal Strength Switches Notch from an Inducer of T Cells to an Inducer of ILC2. Front Immunol 2013; 4:334.
159. Bachanova V, McCullar V, Lenvik T, Wangen R, Peterson KA, Ankarlo DE, et al. Activated notch supports development of cytokine producing NK cells which are hyporesponsive and fail to acquire NK cell effector functions. Biol Blood Marrow Transplant 2009; 15:183-94.
160. Beck RC, Padival M, Yeh D, Ralston J, Cooke KR, Lowe JB. The Notch ligands Jagged2, Delta1, and Delta4 induce differentiation and expansion of functional human NK cells from CD34$^+$ cord blood hematopoietic progenitor cells. Biol Blood Marrow Transplant 2009; 15:1026-37.
161. Benne C, Lelievre JD, Balbo M, Henry A, Sakano S, Levy Y. Notch increases T/NK potential of human hematopoietic progenitors and inhibits B cell differentiation at a pro-B stage. Stem Cells 2009; 27:1676-85.
162. DeHart SL, Heikens MJ, Tsai S. Jagged2 promotes the development of natural killer cells and the establishment of functional natural killer cell lines. Blood 2005; 105:3521-7.
163. Felices M, Ankarlo DE, Lenvik TR, Nelson HH, Blazar BR, Verneris MR, et al. Notch signaling at later stages of NK cell development enhances KIR expression and functional maturation. J Immunol 2014; 193:3344-54.
164. Haraguchi K, Suzuki T, Koyama N, Kumano K, Nakahara F, Matsumoto A, et al. Notch activation induces the generation of functional NK cells from human cord blood CD34-positive cells devoid of IL-15. J Immunol 2009; 182:6168-78.
165. Manaster I, Gazit R, Goldman-Wohl D, Ster-γinossar N, Mizrahi S, Yagel S, et al. Notch activation enhances IFN-gamma secretion by human peripheral blood and decidual NK cells. J Reprod Immunol 2010; 84:1-7.
166. Cherrier M, Sawa S, Eberl G. Notch, Id2, and RORgammat sequentially orchestrate the fetal development of lymphoid tissue inducer cells. J Exp Med 2012; 209:729-40.
167. Chea S, Perchet T, Petit M, Verrier T, Guy-Grand D, Banchi EG, et al. Notch signaling in group 3 innate lymphoid cells modulates their plasticity. Sci Signal 2016; 9:ra45.
168. Rankin LC, Groom JR, Chopin M, Herold MJ, Walker JA, Mielke LA, et al. The transcription factor T-bet is essential for the development of NKp46$^+$ innate lymphocytes via the Notch pathway. Nat Immunol 2013; 14:389-95.
169. Viant C, Rankin LC, Girard-Madoux MJ, Seillet C, Shi W, Smyth MJ, et al. Transforming growth factor-beta and Notch ligands act as opposing environmental cues in regulating the plasticity of type 3 innate lymphoid cells. Sci Signal 2016; 9:ra46.
170. Lee JS, Cella M, McDonald KG, Garlanda C, Kennedy GD, Nukaya M, et al. AHR drives the development of gut ILC22 cells and postnatal lymphoid tissues via pathways dependent on and independent of Notch. Nat Immunol 2012; 13:144-51.
171. Saito T, Chiba S, Ichikawa M, Kunisato A, Asai T, Shimizu K, et al. Notch2 is preferentially expressed in mature B cells and indispensable for marginal zone B lineage development. Immunity 2003; 18:675-85.
172. Hozumi K, Negishi N, Suzuki D, Abe N, Sotomaru Y, Tamaoki N, et al. Delta-like 1 is necessary for the generation of marginal zone B cells but not T cells *in vivo*. Nat Immunol 2004; 5:638-44.
173. Bigas A, Martin DI, Milner LA. Notch1 and Notch2 inhibit myeloid differentiation in response to different cytokines. Mol Cell Biol 1998; 18:2324-33.
174. Milner LA, Bigas A, Kopan R, Brashem-Stein C, Bernstein ID, Martin DI. Inhibition of granulocytic differentiation by mNotch1. Proc Natl Acad Sci U S A 1996; 93:13014-9.
175. Kumano K, Chiba S, Shimizu K, Yamagata T, Hosoya N, Saito T, et al. Notch1 inhibits differentiation of hematopoietic cells by sustaining GATA-2 expression. Blood 2001; 98:3283-9.
176. Lam LT, Ronchini C, Norton J, Capobianco AJ, Bresnick EH. Suppression of erythroid but not megakaryocytic differentiation of human K562 erythroleukemic cells by notch-1. J Biol Chem 2000; 275:19676-84.
177. Caton ML, Smith-Raska MR, Reizis B. Notch-RBP-J signaling controls the homeostasis of CD8- dendritic cells in the spleen. J Exp Med 2007; 204:1653-64.
178. Lewis KL, Caton ML, Bogunovic M, Greter M, Grajkowska LT, Ng D, et al. Notch2 receptor signaling controls functional differentiation of dendritic cells in the spleen and intestine. Immunity 2011; 35:780-91.
179. Ferrero I, Held W, Wilson A, Tacchini-Cottier F, Radtke F, MacDonald HR. Mouse CD11c[+] B220[+] Gr1[+] plasmacytoid dendritic cells develop independently of the T-cell lineage. Blood 2002; 100:2852-7.
180. Radtke F, Ferrero I, Wilson A, Lees R, Aguet M, MacDonald HR. Notch1 deficiency dissociates the intrathymic development of dendritic cells and T cells. J Exp Med 2000; 191:1085-94.
181. Satpathy AT, Briseno CG, Lee JS, Ng D, Manieri NA, Kc W, et al. Notch2-dependent classical dendritic cells orchestrate intestinal immunity to attaching-and-effacing bacterial pathogens. Nat Immunol 2013; 14:937-48.
182. Wang YC, Hu XB, He F, Feng F, Wang L, Li W, et al. Lipopolysaccharide-induced maturation of bone marrow-derived dendritic cells is regulated by notch signaling through the up-regulation of CXCR4. J Biol Chem 2009; 284:15993-6003.
183. Damle SR, Martin RK, Cockburn CL, Lownik JC, Carlyon JA, Smith AD, et al. ADAM10 and Notch1 on murine dendritic cells control the development of type 2 immunity and IgE production. Allergy 2017.
184. Monsalve E, Ruiz-Garcia A, Baladron V, Ruiz-Hidalgo MJ, Sanchez-Solana B, Rivero S, et al. Notch1 upregulates LPS-induced macrophage activation by increasing NF-kappaB activity. Eur J Immunol 2009; 39:2556-70.
185. Hu X, Chung AY, Wu I, Foldi J, Chen J, Ji JD, et al. Integrated regulation of Toll-like receptor responses by Notch and interferon-gamma pathways. Immunity 2008; 29:691-703.
186. Xu H, Zhu J, Smith S, Foldi J, Zhao B, Chung AY, et al. Notch-RBP-J signaling regulates the transcription factor IRF8 to promote inflammatory macrophage polarization. Nat Immunol 2012; 13:642-50.
187. Schroeder T, Just U. Notch signalling via RBP-J promotes myeloid differentiation. EMBO J 2000; 19:2558-68.
188. Qu SY, Lin JJ, Zhang J, Song LQ, Yang XM, Wu CG. Notch signaling pathway regulates the growth and the expression of inflammatory cytokines in mouse basophils. Cell Immunol 2017; 318:29-34.
189. Tsao PN, Matsuoka C, Wei SC, Sato A, Sato S, Hasegawa K, et al. Epithelial Notch signaling regulates lung alveolar morphogenesis and airway epithelial integrity. Proc Natl Acad Sci U S A 2016; 113:8242-7.
190. Gomi K, Staudt MR, Salit J, Kaner RJ, Heldrich J, Rogalski AM, et al. JAG1-Mediated Notch Signaling Regulates Secretory Cell Differentiation of the Human Airway Epithelium. Stem Cell Rev 2016; 12:454-63.
191. Guseh JS, Bores SA, Stanger BZ, Zhou Q, Anderson WJ, Melton DA, et al. Notch signaling promotes airway mucous metaplasia and inhibits alveolar development. Development 2009; 136:1751-9.
192. Tsao PN, Wei SC, Wu MF, Huang MT, Lin HY, Lee MC, et al. Notch signaling prevents mucous metaplasia in mouse conducting airways during postnatal development. Development 2011; 138:3533-43.
193. Pear WS, Aster JC, Scott ML, Hasserjian RP, Soffer B, Sklar J, et al. Exclusive development of T cell neoplasms in mice transplanted with bone marrow expressing activated Notch alleles. J Exp Med 1996; 183:2283-91.
194. Weng AP, Ferrando AA, Lee W, Morris JPt, Silverman LB, Sanchez-Irizarry C, et al. Activating mutations of NOTCH1 in human T cell acute lymphoblastic leukemia. Science 2004; 306:269-71.
195. Ellisen LW, Bird J, West DC, Soreng AL, Reynolds TC, Smith SD, et al. TAN-1, the human homolog of the Drosophila notch gene, is broken by chromosomal translocations in T lymphoblastic neoplasms. Cell 1991; 66:649-61.
196. Radtke F, Fasnacht N, Macdonald HR. Notch signaling in the immune system. Immunity 2010; 32:14-27.
197. Zong D, Ouyang R, Li J, Chen Y, Chen P. Notch signaling in lung diseases: focus on Notch1 and Notch3. Ther Adv Respir Dis 2016.

198. Beel AJ, Sanders CR. Substrate specificity of gamma-secretase and other intramembrane proteases. Cell Mol Life Sci 2008; 65:1311-34.
199. Moellering RE, Cornejo M, Davis TN, Del Bianco C, Aster JC, Blacklow SC, et al. Direct inhibition of the NOTCH transcription factor complex. Nature 2009; 462:182-8.
200. Lafkas D, Shelton A, Chiu C, de Leon Boenig G, Chen Y, Stawicki SS, et al. Therapeutic antibodies reveal Notch control of transdifferentiation in the adult lung. Nature 2015; 528:127-31.
201. Tran IT, Sandy AR, Carulli AJ, Ebens C, Chung J, Shan GT, et al. Blockade of individual Notch ligands and receptors controls graft-versus-host disease. J Clin Invest 2013; 123:1590-604.
202. Wu Y, Cain-Hom C, Choy L, Hagenbeek TJ, de Leon GP, Chen Y, et al. Therapeutic antibody targeting of individual Notch receptors. Nature 2010; 464:1052-7.
203. Aste-Amezaga M, Zhang N, Lineberger JE, Arnold BA, Toner TJ, Gu M, et al. Characterization of Notch1 antibodies that inhibit signaling of both normal and mutated Notch1 receptors. PLoS One 2010; 5:e9094.
204. Li K, Li Y, Wu W, Gordon WR, Chang DW, Lu M, et al. Modulation of Notch signaling by antibodies specific for the extracellular negative regulatory region of NOTCH3. J Biol Chem 2008; 283:8046-54.
205. Elyaman W, Bradshaw EM, Wang Y, Oukka M, Kivisakk P, Chiba S, et al. JAGGED1 and delta1 differentially regulate the outcome of experimental autoimmune encephalomyelitis. J Immunol 2007; 179:5990-8.
206. Hoey T, Yen WC, Axelrod F, Basi J, Donigian L, Dylla S, et al. DLL4 blockade inhibits tumor growth and reduces tumor-initiating cell frequency. Cell Stem Cell 2009; 5:168-77.
207. Amsen D, Helbig C, Backer RA. Notch in T Cell Differentiation: All Things Considered. Trends Immunol 2015; 36:802-14.
208. Okamoto M, Matsuda H, Joetham A, Lucas JJ, Domenico J, Yasutomo K, et al. Jagged1 on dendritic cells and Notch on CD4$^+$ T cells initiate lung allergic responsiveness by inducing IL-4 production. J Immunol 2009; 183:2995-3003.
209. Worsley AG, LeibundGut-Landmann S, Slack E, Phng LK, Gerhardt H, Reis e Sousa C, et al. Dendritic cell expression of the Notch ligand jagged2 is not essential for Th2 response induction *in vivo*. Eur J Immunol 2008; 38:1043-9.
210. Krawczyk CM, Sun J, Pearce EJ. Th2 differentiation is unaffected by Jagged2 expression on dendritic cells. J Immunol 2008; 180:7931-7.
211. Takeichi N, Yanagisawa S, Kaneyama T, Yagita H, Jin YH, Kim BS, et al. Ameliorating effects of anti-Dll4 mAb on Theiler's murine encephalomyelitis virus-induced demyelinating disease. Int Immunol 2010; 22:729-38.
212. Bassil R, Zhu B, Lahoud Y, Riella LV, Yagita H, Elyaman W, et al. Notch ligand delta-like 4 blockade alleviates experimental autoimmune encephalomyelitis by promoting regulatory T cell development. J Immunol 2011; 187:2322-8.
213. Eixarch H, Mansilla MJ, Costa C, Kunkel SL, Montalban X, Godessart N, et al. Inhibition of delta-like ligand 4 decreases Th1/Th17 response in a mouse model of multiple sclerosis. Neurosci Lett 2013; 541:161-6.
214. Okamoto M, Takeda K, Lucas JJ, Joetham A, Yasutomo K, Gelfand EW. Low-dose lipopolysaccharide affects lung allergic responses by regulating Jagged1 expression on antigen-pulsed dendritic cells. Int Arch Allergy Immunol 2012; 157:65-72.
215. Krishnamoorthy N, Oriss TB, Paglia M, Fei M, Yarlagadda M, Vanhaesebroeck B, et al. Activation of c-Kit in dendritic cells regulates T helper cell differentiation and allergic asthma. Nat Med 2008; 14:565-73.
216. Schaller MA, Neupane R, Rudd BD, Kunkel SL, Kallal LE, Lincoln P, et al. Notch ligand Delta-like 4 regulates disease pathogenesis during respiratory viral infections by modulating Th2 cytokines. J Exp Med 2007; 204:2925-34.
217. Jang S, Schaller M, Berlin AA, Lukacs NW. Notch ligand delta-like 4 regulates development and pathogenesis of allergic airway responses by modulating IL-2 production and Th2 immunity. J Immunol 2010; 185:5835-44.
218. Huang HM, Hsiao G, Fan CK, Lin CL, Leu SJ, Chiang BL, et al. Notch ligand delta-like 4-pretreated dendritic cells alleviate allergic airway responses by enhancing IL-10 production. PLoS One 2013; 8:e63613.
219. Tsai JJ, Wang HC, Chiu CL, Liao EC. The effect of Dermatophagoides pteronyssinus group 7 allergen (Der p 7) on dendritic cells and its role in T cell polarization. Immunobiology 2016.
220. Bleck B, Tse DB, Gordon T, Ahsan MR, Reibman J. Diesel exhaust particle-treated human bronchial epithelial cells upregulate Jagged-1 and OX40 ligand in myeloid dendritic cells via thymic stromal lymphopoietin. J Immunol 2010; 185:6636-45.
221. Gilles S, Beck I, Lange S, Ring J, Behrendt H, Traidl-Hoffmann C. Non-allergenic factors from pollen modulate T helper cell instructing notch ligands on dendritic cells. World Allergy Organ J 2015; 8:2.
222. Jeong HW, Kim JH, Kim JY, Ha SJ, Kong YY. Mind bomb-1 in dendritic cells is specifically required for Notch-mediated T helper type 2 differentiation. PLoS One 2012; 7:e36359.
223. Rudd BD, Schaller MA, Smit JJ, Kunkel SL, Neupane R, Kelley L, et al. MyD88-mediated instructive signals in dendritic cells regulate pulmonary immune responses during respiratory virus infection. J Immunol 2007; 178:5820-7.
224. Sun J, Krawczyk CJ, Pearce EJ. Suppression of Th2 cell development by Notch ligands Delta1 and Delta4. J Immunol 2008; 180:1655-61.
225. Skokos D, Nussenzweig MC. CD8- DCs induce IL-12-independent Th1 differentiation through Delta 4 Notch-like ligand in response to bacterial LPS. J Exp Med 2007; 204:1525-31.
226. Jiao Z, Wang W, Xu H, Wang S, Guo M, Chen Y, et al. Engagement of activated Notch signalling in collagen II-specific T helper type 1 (Th1)- and Th17-type expansion involving Notch3 and Delta-like1. Clin Exp Immunol 2011; 164:66-71.
227. Wakui M, Nakano K, Matsushita S. Notch ligand mRNA levels of human APCs predict Th1/Th2-promoting activities. Biochem Biophys Res Commun 2007; 358:596-601.
228. Liotta F, Frosali F, Querci V, Mantei A, Fili L, Maggi L, et al. Human immature myeloid dendritic cells trigger a TH2-polarizing program via Jagged-1/Notch interaction. J Allergy Clin Immunol 2008; 121:1000-5 e8.
229. Li Y, Wu S, Pu J, Huang X, Zhang P. Dengue virus up-regulates expression of notch ligands Dll1 and Dll4 through interferon-beta signalling pathway. Immunology 2015; 144:127-38.
230. Meng L, Bai Z, He S, Mochizuki K, Liu Y, Purushe J, et al. The Notch Ligand DLL4 Defines a Capability of Human Dendritic Cells in Regulating Th1 and Th17 Differentiation. J Immunol 2016; 196:1070-80.
231. Schuijs MJ, Willart MA, Vergote K, Gras D, Deswarte K, Ege MJ, et al. Farm dust and endotoxin protect against allergy through A20 induction in lung epithelial cells. Science 2015; 349:1106-10.
232. Gregory LG, Lloyd CM. Orchestrating house dust mite-associated allergy in the lung. Trends Immunol 2011; 32:402-11.
233. Thomas WR, Hales BJ, Smith WA. House dust mite allergens in asthma and allergy. Trends Mol Med 2010; 16:321-8.
234. Milano J, McKay J, Dagenais C, Foster-Brown L, Pognan F, Gadient R, et al. Modulation of notch processing by gamma-secretase inhibitors causes intestinal goblet cell metaplasia and induction of genes known to specify gut secretory lineage differentiation. Toxicol Sci 2004; 82:341-58.
235. Yamamoto M, Ko LJ, Leonard MW, Beug H, Orkin SH, Engel JD. Activity and tissue-specific expression of the transcription factor NF-E1 multigene family. Genes Dev 1990; 4:1650-62.
236. Ting CN, Olson MC, Barton KP, Leiden JM. Transcription factor GATA-3 is required for development of the T-cell lineage. Nature 1996; 384:474-8.
237. Lee GR, Kim ST, Spilianakis CG, Fields PE, Flavell RA. T helper cell differentiation: regulation by cis elements and epigenetics. Immunity 2006; 24:369-79.
238. Liang HE, Reinhardt RL, Bando JK, Sullivan BM, Ho IC, Locksley RM. Divergent expression patterns of IL-4 and IL-13 define unique functions in allergic immunity. Nat Immunol 2012; 13:58-66.
239. Hoyler T, Klose CS, Souabni A, Turqueti-Neves A, Pfeifer D, Rawlins EL, et al. The transcription factor GATA-3 controls cell fate and maintenance of type 2 innate lymphoid cells. Immunity 2012; 37:634-48.
240. Klein Wolterink RG, Serafini N, van Nimwegen M, Vosshenrich CA, de Bruijn MJ, Fonseca Pereira D, et al. Essential, dose-dependent role for the transcription factor Gata3 in the development of IL-5$^+$ and IL-13$^+$ type 2 innate lymphoid cells. Proc Natl Acad Sci U S A 2013; 110:10240-5.
241. Yagi R, Zhong C, Northrup DL, Yu F, Bouladoux N, Spencer S, et al. The Transcription Factor GATA3 Is Critical for the Development of All IL-7Ralpha-Expressing Innate Lymphoid Cells. Immunity 2014; 40:378-88.
242. Moro K, Yamada T, Tanabe M, Takeuchi T, Ikawa T, Kawamoto H, et al. Innate production of T(H)2 cytokines by adipose tissue-associated c-Kit(+)Sca-1(+) lymphoid cells. Nature 2010; 463:540-4.
243. Saenz SA, Siracusa MC, Perrigoue JG, Spencer SP, Urban JF, Jr., Tocker JE, et al. IL25 elicits a multipotent progenitor cell population that promotes T(H)2 cytokine responses. Nature 2010; 464:1362-6.
244. Neill DR, Wong SH, Bellosi A, Flynn RJ, Daly M, Langford TK, et al. Nuocytes represent a new innate effector leukocyte that mediates type-2 immunity. Nature 2010; 464:1367-70.
245. Wang Y, Su MA, Wan YY. An essential role of the transcription factor GATA-3 for the function of regulatory T cells. Immunity 2011; 35:337-48.
246. Wang Y, Misumi I, Gu AD, Curtis TA, Su L, Whitmire JK, et al. GATA-3 controls the maintenance and proliferation of T cells downstream of TCR and cytokine signaling. Nat Immunol 2013; 14:714-22.
247. Rudra D, deRoos P, Chaudhry A, Niec RE, Arvey A, Samstein RM, et al. Transcription factor Foxp3 and its protein partners form a complex regulatory network. Nat Immunol 2012; 13:1010-9.
248. Ku CJ, Hosoya T, Maillard I, Engel JD. GATA-3 regulates hematopoietic stem cell maintenance and cell-cycle entry. Blood 2012; 119:2242-51.
249. Fitch SR, Kimber GM, Wilson NK, Parker A, Mirshekar-Syahkal B, Gottgens B, et al. Signaling from the sympathetic nervous system regulates hematopoietic stem cell emergence during embryogenesis. Cell Stem Cell 2012; 11:554-66.
250. Frelin C, Herrington R, Janmohamed S, Barbara M, Tran G, Paige CJ, et al. GATA-3 regulates the self-renewal of long-term hematopoietic stem cells. Nat Immunol 2013; 14:1037-44.
251. Banerjee A, Northrup D, Boukarabila H, Jacobsen SE, Allman D. Transcriptional repression of Gata3 is essential for early B cell commitment. Immunity 2013; 38:930-42.
252. Garcia-Ojeda ME, Klein Wolterink RG, Lemaitre F, Richard-Le Goff O, Hasan M, Hendriks RW, et al. GATA-3 promotes T-cell specification by repressing B-cell potential in pro-T cells in mice. Blood 2013; 121:1749-59.

253. Vosshenrich CA, Garcia-Ojeda ME, Samson-Villeger SI, Pasqualetto V, Enault L, Richard-Le Goff O, et al. A thymic pathway of mouse natural killer cell development characterized by expression of GATA-3 and CD127. Nat Immunol 2006; 7:1217-24.
254. Constantinides MG, McDonald BD, Verhoef PA, Bendelac A. A committed precursor to innate lymphoid cells. Nature 2014.
255. Serafini N, Klein Wolterink RG, Satoh-Takayama N, Xu W, Vosshenrich CA, Hendriks RW, et al. Gata3 drives development of RORgammat+ group 3 innate lymphoid cells. J Exp Med 2014; 211:199-208.
256. Klose CS, Flach M, Mohle L, Rogell L, Hoyler T, Ebert K, et al. Differentiation of Type 1 ILCs from a Common Progenitor to All Helper-like Innate Lymphoid Cell Lineages. Cell 2014; 157:340-56.
257. Spits H, Artis D, Colonna M, Diefenbach A, Di Santo JP, Eberl G, et al. Innate lymphoid cells--a proposal for uniform nomenclature. Nat Rev Immunol 2013; 13:145-9.
258. Walker JA, Barlow JL, McKenzie AN. Innate lymphoid cells--how did we miss them? Nat Rev Immunol 2013; 13:75-87.
259. Jenner RG, Townsend MJ, Jackson I, Sun K, Bouwman RD, Young RA, et al. The transcription factors T-bet and GATA-3 control alternative pathways of T-cell differentiation through a shared set of target genes. Proc Natl Acad Sci U S A 2009; 106:17876-81.
260. Wei G, Abraham BJ, Yagi R, Jothi R, Cui K, Sharma S, et al. Genome-wide analyses of transcription factor GATA3-mediated gene regulation in distinct T cell types. Immunity 2011; 35:299-311.
261. Kanhere A, Hertweck A, Bhatia U, Gokmen MR, Perucha E, Jackson I, et al. T-bet and GATA3 orchestrate Th1 and Th2 differentiation through lineage-specific targeting of distal regulatory elements. Nat Commun 2012; 3:1268.
262. Fox AH, Kowalski K, King GF, Mackay JP, Crossley M. Key residues characteristic of GATA N-fingers are recognized by FOG. J Biol Chem 1998; 273:33595-603.
263. Bresnick EH, Katsumura KR, Lee HY, Johnson KD, Perkins AS. Master regulatory GATA transcription factors: mechanistic principles and emerging links to hematologic malignancies. Nucleic Acids Res 2012; 40:5819-31.
264. Chlon TM, Crispino JD. Combinatorial regulation of tissue specification by GATA and FOG factors. Development 2012; 139:3905-16.
265. Rosas M, Davies LC, Giles PJ, Liao CT, Kharfan B, Stone TC, et al. The Transcription Factor Gata6 Links Tissue Macrophage Phenotype and Proliferative Renewal. Science 2014.
266. Okabe Y, Medzhitov R. Tissue-specific signals control reversible program of localization and functional polarization of macrophages. Cell 2014; 157:832-44.
267. Tsai FY, Browne CP, Orkin SH. Knock-in mutation of transcription factor GATA-3 into the GATA-1 locus: partial rescue of GATA-1 loss of function in erythroid cells. Dev Biol 1998; 196:218-27.
268. Ranganath S, Murphy KM. Structure and specificity of GATA proteins in Th2 development. Mol Cell Biol 2001; 21:2716-25.
269. Ling KW, Ottersbach K, van Hamburg JP, Oziemlak A, Tsai FY, Orkin SH, et al. GATA-2 plays two functionally distinct roles during the ontogeny of hematopoietic stem cells. J Exp Med 2004; 200:871-82.
270. Vicente C, Conchillo A, Garcia-Sanchez MA, Odero MD. The role of the GATA2 transcription factor in normal and malignant hematopoiesis. Crit Rev Oncol Hematol 2012; 82:1-17.
271. Taghon T, Yui MA, Rothenberg EV. Mast cell lineage diversion of T lineage precursors by the essential T cell transcription factor GATA-3. Nat Immunol 2007; 8:845-55.
272. Pandolfi PP, Roth ME, Karis A, Leonard MW, Dzierzak E, Grosveld FG, et al. Targeted disruption of the GATA3 gene causes severe abnormalities in the nervous system and in fetal liver haematopoiesis. Nat Genet 1995; 11:40-4.
273. Lim KC, Lakshmanan G, Crawford SE, Gu Y, Grosveld F, Engel JD. Gata3 loss leads to embryonic lethality due to noradrenaline deficiency of the sympathetic nervous system. Nat Genet 2000; 25:209-12.
274. Ali A, Christie PT, Grigorieva IV, Harding B, Van Esch H, Ahmed SF, et al. Functional characterization of GATA3 mutations causing the hypoparathyroidism-deafness-renal (HDR) dysplasia syndrome: insight into mechanisms of DNA binding by the GATA3 transcription factor. Hum Mol Genet 2007; 16:265-75.
275. Chou J, Provot S, Werb Z. GATA3 in development and cancer differentiation: cells GATA have it! J Cell Physiol 2010; 222:42-9.
276. Higgins JP, Kaygusuz G, Wang L, Montgomery K, Mason V, Zhu SX, et al. Placental S100 (S100P) and GATA3: markers for transitional epithelium and urothelial carcinoma discovered by complementary DNA microarray. Am J Surg Pathol 2007; 31:673-80.
277. Hasegawa SL, Moriguchi T, Rao A, Kuroha T, Engel JD, Lim KC. Dosage-dependent rescue of definitive nephrogenesis by a distant Gata3 enhancer. Dev Biol 2007; 301:568-77.
278. Hosoya-Ohmura S, Lin YH, Herrmann M, Kuroha T, Rao A, Moriguchi T, et al. An NK and T cell enhancer lies 280 kilobase pairs 3' to the gata3 structural gene. Mol Cell Biol 2011; 31:1894-904.
279. Maneechotesuwan K, Xin Y, Ito K, Jazrawi E, Lee KY, Usmani OS, et al. Regulation of Th2 cytokine genes by p38 MAPK-mediated phosphorylation of GATA-3. J Immunol 2007; 178:2491-8.
280. Furusawa J, Moro K, Motomura Y, Okamoto K, Zhu J, Takayanagi H, et al. Critical role of p38 and GATA3 in natural helper cell function. J Immunol 2013; 191:1818-26.
281. Maneechotesuwan K, Yao X, Ito K, Jazrawi E, Usmani OS, Adcock IM, et al. Suppression of GATA-3 nuclear import and phosphorylation: a novel mechanism of corticosteroid action in allergic disease. PLoS Med 2009; 6:e1000076.
282. Yamagata T, Mitani K, Oda H, Suzuki T, Honda H, Asai T, et al. Acetylation of GATA-3 affects T-cell survival and homing to secondary lymphoid organs. EMBO J 2000; 19:4676-87.
283. Spilianakis CG, Flavell RA. Long-range intrachromosomal interactions in the T helper type 2 cytokine locus. Nat Immunol 2004; 5:1017-27.
284. Vahedi G, A CP, Hand TW, Laurence A, Kanno Y, O'Shea JJ, et al. Helper T-cell identity and evolution of differential transcriptomes and epigenomes. Immunol Rev 2013; 252:24-40.
285. Scheinman EJ, Avni O. Transcriptional regulation of GATA3 in T helper cells by the integrated activities of transcription factors downstream of the interleukin-4 receptor and T cell receptor. J Biol Chem 2009; 284:3037-48.
286. Ouyang W, Lohning M, Gao Z, Assenmacher M, Ranganath S, Radbruch A, et al. Stat6-independent GATA-3 autoactivation directs IL-4-independent Th2 development and commitment. Immunity 2000; 12:27-37.
287. Yang XO, Angkasekwinai P, Zhu J, Peng J, Liu Z, Nurieva R, et al. Requirement for the basic helix-loop-helix transcription factor Dec2 in initial TH2 lineage commitment. Nat Immunol 2009; 10:1260-6.
288. Pai SY, Truitt ML, Ho IC. GATA-3 deficiency abrogates the development and maintenance of T helper type 2 cells. Proc Natl Acad Sci U S A 2004; 101:1993-8.
289. Zhu J, Min B, Hu-Li J, Watson CJ, Grinberg A, Wang Q, et al. Conditional deletion of Gata3 shows its essential function in T(H)1-T(H)2 responses. Nat Immunol 2004; 5:1157-65.
290. Grogan JL, Mohrs M, Harmon B, Lacy DA, Sedat JW, Locksley RM. Early transcription and silencing of cytokine genes underlie polarization of T helper cell subsets. Immunity 2001; 14:205-15.
291. Yamane H, Zhu J, Paul WE. Independent roles for IL-2 and GATA-3 in stimulating naïve CD4+ T cells to generate a Th2-inducing cytokine environment. J Exp Med 2005; 202:793-804.
292. Lin JX, Leonard WJ. The role of Stat5a and Stat5b in signaling by IL-2 family cytokines. Oncogene 2000; 19:2566-76.
293. Cote-Sierra J, Foucras G, Guo L, Chiodetti L, Young HA, Hu-Li J, et al. Interleukin 2 plays a central role in Th2 differentiation. Proc Natl Acad Sci U S A 2004; 101:3880-5.
294. Kaplan MH. Th9 cells: differentiation and disease. Immunol Rev 2013; 252:104-15.
295. Ouyang W, Ranganath SH, Weindel K, Bhattacharya D, Murphy TL, Sha WC, et al. Inhibition of Th1 development mediated by GATA-3 through an IL-4-independent mechanism. Immunity 1998; 9:745-55.
296. Usui T, Preiss JC, Kanno Y, Yao ZJ, Bream JH, O'Shea JJ, et al. T-bet regulates Th1 responses through essential effects on GATA-3 function rather than on *IFNG* gene acetylation and transcription. J Exp Med 2006; 203:755-66.
297. Fang M, Xie H, Dougan SK, Ploegh H, van Oudenaarden A. Stochastic cytokine expression induces mixed T helper cell States. PLoS Biol 2013; 11:e1001618.
298. Antebi YE, Reich-Zeliger S, Hart Y, Mayo A, Eizenberg I, Rimer J, et al. Mapping differentiation under mixed culture conditions reveals a tunable continuum of T cell fates. PLoS Biol 2013; 11:e1001616.
299. Peine M, Rausch S, Helmstetter C, Frohlich A, Hegazy AN, Kuhl AA, et al. Stable T-bet(+)GATA-3(+) Th1/Th2 hybrid cells arise *in vivo*, can develop directly from naïve precursors, and limit immunopathologic inflammation. PLoS Biol 2013; 11:e1001633.
300. Hegazy AN, Peine M, Helmstetter C, Panse I, Frohlich A, Bergthaler A, et al. Interferons direct Th2 cell reprogramming to generate a stable GATA-3(+)T-bet(+) cell subset with combined Th2 and Th1 cell functions. Immunity 2010; 32:116-28.
301. Oestreich KJ, Weinmann AS. Master regulators or lineage-specifying? Changing views on CD4+ T cell transcription factors. Nat Rev Immunol 2012; 12:799-804.
302. Tanaka S, Motomura Y, Suzuki Y, Yagi R, Inoue H, Miyatake S, et al. The enhancer HS2 critically regulates GATA-3-mediated *Il4* transcription in T(H)2 cells. Nat Immunol 2011; 12:77-85.
303. Lee HJ, Takemoto N, Kurata H, Kamogawa Y, Miyatake S, O'Garra A, et al. GATA-3 induces T helper cell type 2 (Th2) cytokine expression and chromatin remodeling in committed Th1 cells. J Exp Med 2000; 192:105-15.
304. Chang S, Aune TM. Dynamic changes in histone-methylation 'marks' across the locus encoding interferon-gamma during the differentiation of T helper type 2 cells. Nat Immunol 2007; 8:723-31.
305. Lee DU, Rao A. Molecular analysis of a locus control region in the T helper 2 cytokine gene cluster: a target for STAT6 but not GATA3. Proc Natl Acad Sci U S A 2004; 101:16010-5.
306. Barski A, Cuddapah S, Cui K, Roh TY, Schones DE, Wang Z, et al. High-resolution profiling of histone methylations in the human genome. Cell 2007; 129:823-37.
307. Parelho V, Hadjur S, Spivakov M, Leleu M, Sauer S, Gregson HC, et al. Cohesins functionally associate with CTCF on mammalian chromosome arms. Cell 2008; 132:422-33.

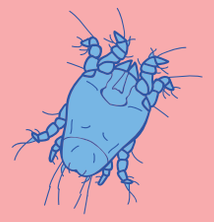
308. Hwang SS, Kim YU, Lee S, Jang SW, Kim MK, Koh BH, et al. Transcription factor YY1 is essential for regulation of the Th2 cytokine locus and for Th2 cell differentiation. Proc Natl Acad Sci U S A 2013; 110:276-81.
309. Cai S, Lee CC, Kohwi-Shigematsu T. SATB1 packages densely looped, transcriptionally active chromatin for coordinated expression of cytokine genes. Nat Genet 2006; 38:1278-88.
310. Kwan M, Powell DR, Nachman TY, Brown MA. An intron GATA-binding site regulates chromatin accessibility and is essential for IL-4 gene expression in mast cells. Eur J Immunol 2005; 35:1267-74.
311. Hendriks RW, Nawijn MC, Engel JD, van Doorninck H, Grosveld F, Karis A. Expression of the transcription factor GATA-3 is required for the development of the earliest T cell progenitors and correlates with stages of cellular proliferation in the thymus. Eur J Immunol 1999; 29:1912-8.
312. Hosoya T, Kuroha T, Moriguchi T, Cummings D, Maillard I, Lim KC, et al. GATA-3 is required for early T lineage progenitor development. J Exp Med 2009; 206:2987-3000.
313. Rothenberg EV, Taghon T. Molecular genetics of T cell development. Annu Rev Immunol 2005; 23:601-49.
314. Chi AW, Bell JJ, Zlotoff DA, Bhandoola A. Untangling the T branch of the hematopoiesis tree. Curr Opin Immunol 2009; 21:121-6.
315. Sambandam A, Maillard I, Zediak VP, Xu L, Gerstein RM, Aster JC, et al. Notch signaling controls the generation and differentiation of early T lineage progenitors. Nat Immunol 2005; 6:663-70.
316. Weber BN, Chi AW, Chavez A, Yashiro-Ohtani Y, Yang Q, Shestova O, et al. A critical role for TCF-1 in T-lineage specification and differentiation. Nature 2011; 476:63-8.
317. Li L, Leid M, Rothenberg EV. An early T cell lineage commitment checkpoint dependent on the transcription factor Bcl11b. Science 2010; 329:89-93.
318. Rothenberg EV. GATA-3 locks the door to the B-cell option. Blood 2013; 121:1673-4.
319. Nechanitzky R, Akbas D, Scherer S, Gyory I, Hoyler T, Ramamoorthy S, et al. Transcription factor EBF1 is essential for the maintenance of B cell identity and prevention of alternative fates in committed cells. Nat Immunol 2013; 14:867-75.
320. Pai SY, Truitt ML, Ting CN, Leiden JM, Glimcher LH, Ho IC. Critical roles for transcription factor GATA-3 in thymocyte development. Immunity 2003; 19:863-75.
321. Bender TP, Kremer CS, Kraus M, Buch T, Rajewsky K. Critical functions for c-Myb at three checkpoints during thymocyte development. Nat Immunol 2004; 5:721-9.
322. Wolfer A, Wilson A, Nemir M, MacDonald HR, Radtke F. Inactivation of Notch1 impairs VDJbeta rearrangement and allows pre-TCR-independent survival of early alpha beta Lineage Thymocytes. Immunity 2002; 16:869-79.
323. Zhang JA, Mortazavi A, Williams BA, Wold BJ, Rothenberg EV. Dynamic transformations of genome-wide epigenetic marking and transcriptional control establish T cell identity. Cell 2012; 149:467-82.
324. Wang L, Wildt KF, Zhu J, Zhang X, Feigenbaum L, Tessarollo L, et al. Distinct functions for the transcription factors GATA-3 and ThPOK during intrathymic differentiation of CD4(+) T cells. Nat Immunol 2008; 9:1122-30.
325. He X, He X, Dave VP, Zhang Y, Hua X, Nicolas E, et al. The zinc finger transcription factor Th-POK regulates CD4 versus CD8 T-cell lineage commitment. Nature 2005; 433:826-33.
326. Sun G, Liu X, Mercado P, Jenkinson SR, Kypriotou M, Feigenbaum L, et al. The zinc finger protein cKrox directs CD4 lineage differentiation during intrathymic T cell positive selection. Nat Immunol 2005; 6:373-81.
327. Hernandez-Hoyos G, Anderson MK, Wang C, Rothenberg EV, Alberola-Ila J. GATA-3 expression is controlled by TCR signals and regulates CD4/CD8 differentiation. Immunity 2003; 19:83-94.
328. Ling KW, van Hamburg JP, de Bruijn MJ, Kurek D, Dingjan GM, Hendriks RW. GATA3 controls the expression of CD5 and the T cell receptor during CD4 T cell lineage development. Eur J Immunol 2007; 37:1043-52.
329. Laky K, Fowlkes BJ. Notch signaling in CD4 and CD8 T cell development. Curr Opin Immunol 2008; 20:197-202.
330. Park JH, Adoro S, Guinter T, Erman B, Alag AS, Catalfamo M, et al. Signaling by intrathymic cytokines, not T cell antigen receptors, specifies CD8 lineage choice and promotes the differentiation of cytotoxic-lineage T cells. Nat Immunol 2010; 11:257-64.
331. Buza-Vidas N, Duarte S, Luc S, Bouriez-Jones T, Woll PS, Jacobsen SE. GATA3 is redundant for maintenance and self-renewal of hematopoietic stem cells. Blood 2011; 118:1291-3.
332. Orr MT, Lanier LL. Natural killer cell education and tolerance. Cell 2010; 142:847-56.
333. Kupz A, Scott TA, Belz GT, Andrews DM, Greyer M, Lew AM, et al. Contribution of Thy1+ NK cells to protective IFN-gamma production during Salmonella typhimurium infections. Proc Natl Acad Sci U S A 2013; 110:2252-7.
334. Vosshenrich CA, Di Santo JP. Developmental programming of natural killer and innate lymphoid cells. Curr Opin Immunol 2013.
335. Samson SI, Richard O, Tavian M, Ranson T, Vosshenrich CA, Colucci F, et al. GATA-3 promotes maturation, IFN-gamma production, and liver-specific homing of NK cells. Immunity 2003; 19:701-11.
336. Sojka DK, Plougastel-Douglas B, Yang L, Pak-Wittel MA, Artyomov MN, Ivanova Y, et al. Tissue-resident natural killer (NK) cells are cell lineages distinct from thymic and conventional splenic NK cells. Elife 2014; 3:e01659.
337. Daussy C, Faure F, Mayol K, Viel S, Gasteiger G, Charrier E, et al. T-bet and Eomes instruct the development of two distinct natural killer cell lineages in the liver and in the bone marrow. J Exp Med 2014; 211:563-77.
338. Di Santo JP, Vosshenrich CA. Bone marrow versus thymic pathways of natural killer cell development. Immunol Rev 2006; 214:35-46.
339. Bernink JH, Peters CP, Munneke M, te Velde AA, Meijer SL, Weijer K, et al. Human type 1 innate lymphoid cells accumulate in inflamed mucosal tissues. Nat Immunol 2013; 14:221-9.
340. Fuchs A, Vermi W, Lee JS, Lonardi S, Gilfillan S, Newberry RD, et al. Intraepithelial type 1 innate lymphoid cells are a unique subset of IL-12- and IL-15-responsive IFN-gamma-producing cells. Immunity 2013; 38:769-81.
341. Mjosberg J, Bernink J, Golebski K, Karrich JJ, Peters CP, Blom B, et al. The Transcription Factor GATA3 Is Essential for the Function of Human Type 2 Innate Lymphoid Cells. Immunity 2012; 37:649-59.
342. Halim TY, MacLaren A, Romanish MT, Gold MJ, McNagny KM, Takei F. Retinoic-acid-receptor-related orphan nuclear receptor alpha is required for natural helper cell development and allergic inflammation. Immunity 2012; 37:463-74.
343. Yokota Y, Mansouri A, Mori S, Sugawara S, Adachi S, Nishikawa S, et al. Development of peripheral lymphoid organs and natural killer cells depends on the helix-loop-helix inhibitor Id2. Nature 1999; 397:702-6.
344. Yang Q, Monticelli LA, Saenz SA, Chi AW, Sonnenberg GF, Tang J, et al. T cell factor 1 is required for group 2 innate lymphoid cell generation. Immunity 2013; 38:694-704.
345. Monticelli LA, Sonnenberg GF, Abt MC, Alenghat T, Ziegler CG, Doering TA, et al. Innate lymphoid cells promote lung-tissue homeostasis after infection with influenza virus. Nat Immunol 2011; 12:1045-54.
346. KleinJan A, Klein Wolterink RG, Levani Y, de Bruijn MJ, Hoogsteden HC, van Nimwegen M, et al. Enforced expression of Gata3 in T cells and group 2 innate lymphoid cells increases susceptibility to allergic airway inflammation in mice. J Immunol 2014; 192:1385-94.
347. Klein Wolterink RG, Kleinjan A, van Nimwegen M, Bergen I, de Bruijn M, Levani Y, et al. Pulmonary innate lymphoid cells are major producers of IL-5 and IL-13 in murine models of allergic asthma. Eur J Immunol 2012; 42:1106-16.
348. van de Pavert SA, Ferreira M, Domingues RG, Ribeiro H, Molenaar R, Moreira-Santos L, et al. Maternal retinoids control type 3 innate lymphoid cells and set the offspring immunity. Nature 2014; 508:123-7.
349. Takatori H, Kanno Y, Watford WT, Tato CM, Weiss G, Ivanov, II, et al. Lymphoid tissue inducer-like cells are an innate source of IL-17 and IL-22. J Exp Med 2009; 206:35-41.
350. Cella M, Fuchs A, Vermi W, Facchetti F, Otero K, Lennerz JK, et al. A human natural killer cell subset provides an innate source of IL-22 for mucosal immunity. Nature 2009; 457:722-5.
351. Satoh-Takayama N, Vosshenrich CA, Lesjean-Pottier S, Sawa S, Lochner M, Rattis F, et al. Microbial flora drives interleukin 22 production in intestinal NKp46+ cells that provide innate mucosal immune defense. Immunity 2008; 29:958-70.
352. Sanos SL, Bui VL, Mortha A, Oberle K, Heners C, Johner C, et al. RORgammat and commensal microflora are required for the differentiation of mucosal interleukin 22-producing NKp46+ cells. Nat Immunol 2009; 10:83-91.
353. Sonnenberg GF, Monticelli LA, Alenghat T, Fung TC, Hutnick NA, Kunisawa J, et al. Innate lymphoid cells promote anatomical containment of lymphoid-resident commensal bacteria. Science 2012; 336:1321-5.
354. Klose CS, Kiss EA, Schwierzeck V, Ebert K, Hoyler T, d'Hargues Y, et al. A T-bet gradient controls the fate and function of CCR6-RORgammat+ innate lymphoid cells. Nature 2013; 494:261-5.
355. Buonocore S, Ahern PP, Uhlig HH, Ivanov, II, Littman DR, Maloy KJ, et al. Innate lymphoid cells drive interleukin-23-dependent innate intestinal pathology. Nature 2010; 464:1371-5.
356. Sawa S, Cherrier M, Lochner M, Satoh-Takayama N, Fehling HJ, Langa F, et al. Lineage relationship analysis of RORgammat+ innate lymphoid cells. Science 2010; 330:665-9.
357. Sciume G, Hirahara K, Takahashi H, Laurence A, Villarino AV, Singleton KL, et al. Distinct requirements for T-bet in gut innate lymphoid cells. J Exp Med 2012; 209:2331-8.
358. Yoshida H, Kawamoto H, Santee SM, Hashi H, Honda K, Nishikawa S, et al. Expression of alpha(4)beta(7) integrin defines a distinct pathway of lymphoid progenitors committed to T cells, fetal intestinal lymphotoxin producer, NK, and dendritic cells. J Immunol 2001; 167:2511-21.
359. Eberl G, Marmon S, Sunshine MJ, Rennert PD, Choi Y, Littman DR. An essential function for the nuclear receptor RORgamma(t) in the generation of fetal lymphoid tissue inducer cells. Nat Immunol 2004; 5:64-73.
360. Aliahmad P, de la Torre B, Kaye J. Shared dependence on the DNA-binding factor TOX for the development of lymphoid tissue-inducer cell and NK cell lineages. Nat Immunol 2010; 11:945-52.
361. Tachibana M, Tenno M, Tezuka C, Sugiyama M, Yoshida H, Taniuchi I. Runx1/Cbfbeta2 complexes are required for lymphoid tissue inducer cell differentiation at two developmental stages. J Immunol 2011; 186:1450-7.
362. Rodriguez P, Bonte E, Krijgsveld J, Kolodziej KE, Guyot B, Heck AJ, et al. GATA-1 forms distinct activating and repressive complexes in erythroid cells. EMBO J 2005; 24:2354-66.

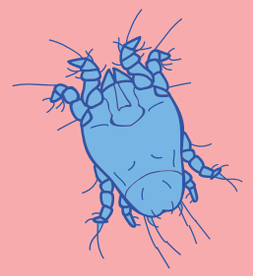
363. Hosokawa H, Tanaka T, Suzuki Y, Iwamura C, Ohkubo S, Endoh K, et al. Functionally distinct Gata3/Chd4 complexes coordinately establish T helper 2 (Th2) cell identity. Proc Natl Acad Sci U S A 2013; 110:4691-6.
364. Hwang ES, Szabo SJ, Schwartzberg PL, Glimcher LH. T helper cell fate specified by kinase-mediated interaction of T-bet with GATA-3. Science 2005; 307:430-3.
365. Endo Y, Iwamura C, Kuwahara M, Suzuki A, Sugaya K, Tumes DJ, et al. Eomesodermin controls interleukin-5 production in memory T helper 2 cells through inhibition of activity of the transcription factor GATA3. Immunity 2011; 35:733-45.
366. Wohlfert EA, Grainger JR, Bouladoux N, Konkel JE, Oldenhove G, Ribeiro CH, et al. GATA3 controls Foxp3(+) regulatory T cell fate during inflammation in mice. J Clin Invest 2011; 121:4503-15.
367. Jones EA, Flavell RA. Distal enhancer elements transcribe intergenic RNA in the IL-10 family gene cluster. J Immunol 2005; 175:7437-46.
368. Nawijn MC, Dingjan GM, Ferreira R, Lambrecht BN, Karis A, Grosveld F, et al. Enforced expression of GATA-3 in transgenic mice inhibits Th1 differentiation and induces the formation of a T1/ST2-expressing Th2-committed T cell compartment in vivo. J Immunol 2001; 167:724-32.
369. Nawijn MC, Ferreira R, Dingjan GM, Kahre O, Drabek D, Karis A, et al. Enforced expression of GATA-3 during T cell development inhibits maturation of CD8 single-positive cells and induces thymic lymphoma in transgenic mice. J Immunol 2001; 167:715-23.
370. van Hamburg JP, de Bruijn MJ, Dingjan GM, Beverloo HB, Diepstraten H, Ling KW, et al. Cooperation of Gata3, c-Myc and Notch in malignant transformation of double positive thymocytes. Mol Immunol 2008; 45:3085-95.
371. Perez-Andreu V, Roberts KG, Harvey RC, Yang W, Cheng C, Pei D, et al. Inherited GATA3 variants are associated with Ph-like childhood acute lymphoblastic leukemia and risk of relapse. Nat Genet 2013; 45:1494-8.
372. Imanishi T, Ishihara C, Badr Mel S, Hashimoto-Tane A, Kimura Y, Kawai T, et al. Nucleic acid sensing by T cells initiates Th2 cell differentiation. Nat Commun 2014; 5:3566.
373. Kishikawa H, Sun J, Choi A, Miaw SC, Ho IC. The cell type-specific expression of the murine IL-13 gene is regulated by GATA-3. J Immunol 2001; 167:4414-20.
374. Zhang DH, Yang L, Ray A. Differential responsiveness of the IL-5 and IL-4 genes to transcription factor GATA-3. J Immunol 1998; 161:3817-21.
375. Tykocinski LO, Hajkova P, Chang HD, Stamm T, Sozeri O, Lohning M, et al. A critical control element for interleukin-4 memory expression in T helper lymphocytes. J Biol Chem 2005; 280:28177-85.
376. Agarwal S, Rao A. Modulation of chromatin structure regulates cytokine gene expression during T cell differentiation. Immunity 1998; 9:765-75.
377. Yamashita M, Ukai-Tadenuma M, Miyamoto T, Sugaya K, Hosokawa H, Hasegawa A, et al. Essential role of GATA3 for the maintenance of type 2 helper T (Th2) cytokine production and chromatin remodeling at the Th2 cytokine gene loci. J Biol Chem 2004; 279:26983-90.
378. Takemoto N, Kamogawa Y, Jun Lee H, Kurata H, Arai KI, O'Garra A, et al. Cutting edge: chromatin remodeling at the IL-4/IL-13 intergenic regulatory region for Th2-specific cytokine gene cluster. J Immunol 2000; 165:6687-91.
379. Lee GR, Fields PE, Griffin TJ, Flavell RA. Regulation of the Th2 cytokine locus by a locus control region. Immunity 2003; 19:145-53.
380. Ribeiro de Almeida C, Heath H, Krpic S, Dingjan GM, van Hamburg JP, Bergen I, et al. Critical role for the transcription regulator CCCTC-binding factor in the control of Th2 cytokine expression. J Immunol 2009; 182:999-1010.
381. Zhou M, Ouyang W, Gong Q, Katz SG, White JM, Orkin SH, et al. Friend of GATA-1 represses GATA-3-dependent activity in CD4+ T cells. J Exp Med 2001; 194:1461-71.
382. Miaw SC, Choi A, Yu E, Kishikawa H, Ho IC. ROG, repressor of GATA, regulates the expression of cytokine genes. Immunity 2000; 12:323-33.
383. Yagi R, Junttila IS, Wei G, Urban JF, Jr., Zhao K, Paul WE, et al. The transcription factor GATA3 actively represses RUNX3 protein-regulated production of interferon-gamma. Immunity 2010; 32:507-17.
384. Blokzijl A, ten Dijke P, Ibanez CF. Physical and functional interaction between GATA-3 and Smad3 allows TGF-beta regulation of GATA target genes. Curr Biol 2002; 12:35-45.
385. Hammad H, Plantinga M, Deswarte K, Pouliot P, Willart MA, Kool M, et al. Inflammatory dendritic cells--not basophils--are necessary and sufficient for induction of Th2 immunity to inhaled house dust mite allergen. J Exp Med 2010; 207:2097-111.
386. Lambrecht BN, Hammad H. Dendritic cell and epithelial cell interactions at the origin of murine asthma. Ann Am Thorac Soc 2014; 11 Suppl 5:S236-43.
387. Pletinckx K, Stijlemans B, Pavlovic V, Laube R, Brandl C, Kneitz S, et al. Similar inflammatory DC maturation signatures induced by TNF or Trypanosoma brucei antigens instruct default Th2-cell responses. Eur J Immunol 2011; 41:3479-94.
388. Maekawa Y, Tsukumo S, Chiba S, Hirai H, Hayashi Y, Okada H, et al. Delta1-Notch3 interactions bias the functional differentiation of activated CD4+ T cells. Immunity 2003; 19:549-59.
389. Kiernan AE, Xu J, Gridley T. The Notch ligand JAG1 is required for sensory progenitor development in the mammalian inner ear. PLoS Genet 2006; 2:e4.
390. Xu J, Krebs LT, Gridley T. Generation of mice with a conditional null allele of the Jagged2 gene. Genesis 2010; 48:390-3.
391. Tanigaki K, Han H, Yamamoto N, Tashiro K, Ikegawa M, Kuroda K, et al. Notch-RBP-J signaling is involved in cell fate determination of marginal zone B cells. Nat Immunol 2002; 3:443-50.
392. Wolfer A, Bakker T, Wilson A, Nicolas M, Ioannidis V, Littman DR, et al. Inactivation of Notch 1 in immature thymocytes does not perturb CD4 or CD8T cell development. Nat Immunol 2001; 2:235-41.
393. Kool M, Soullie T, van Nimwegen M, Willart MA, Muskens F, Jung S, et al. Alum adjuvant boosts adaptive immunity by inducing uric acid and activating inflammatory dendritic cells. J Exp Med 2008; 205:869-82.
394. Li BW, de Bruijn MJ, Tindemans I, Lukkes M, KleinJan A, Hoogsteden HC, et al. T cells are necessary for ILC2 activation in house dust mite-induced allergic airway inflammation in mice. Eur J Immunol 2016; 46:1392-403.
395. Maas A, Dingjan GM, Savelkoul HF, Kinnon C, Grosveld F, Hendriks RW. The X-linked immunodeficiency defect in the mouse is corrected by expression of human Bruton's tyrosine kinase from a yeast artificial chromosome transgene. Eur J Immunol 1997; 27:2180-7.
396. Hammad H, Kool M, Soullie T, Narumiya S, Trottein F, Hoogsteden HC, et al. Activation of the D prostanoid 1 receptor suppresses asthma by modulation of lung dendritic cell function and induction of regulatory T cells. J Exp Med 2007; 204:357-67.
397. Li BW, de Bruijn MJ, Tindemans I, Lukkes M, KleinJan A, Hoogsteden HC, et al. T cells are necessary for ILC2 activation in house dust mite-induced allergic airway inflammation in mice. Eur J Immunol 2016.
398. Post S, Heijink IH, Petersen AH, de Bruin HG, van Oosterhout AJ, Nawijn MC. Protease-activated receptor-2 activation contributes to house dust mite-induced IgE responses in mice. PLoS One 2014; 9:e91206.
399. Corneth OB, de Bruijn MJ, Rip J, Asmawidjaja PS, Kil LP, Hendriks RW. Enhanced Expression of Bruton's Tyrosine Kinase in B Cells Drives Systemic Autoimmunity by Disrupting T Cell Homeostasis. J Immunol 2016.
400. Helft J, Bottcher J, Chakravarty P, Zelenay S, Huotari J, Schraml BU, et al. GM-CSF Mouse Bone Marrow Cultures Comprise a Heterogeneous Population of CD11c(+)MHCII(+) Macrophages and Dendritic Cells. Immunity 2015; 42:1197-211.
401. Lindell DM, Berlin AA, Schaller MA, Lukacs NW. B cell antigen presentation promotes Th2 responses and immunopathology during chronic allergic lung disease. PLoS One 2008; 3:e3129.
402. Dullaers M, Schuijs MJ, Willart M, Fierens K, Van Moorleghem J, Hammad H, et al. House dust mite driven asthma and allergen specific T cells depend on B cells when the amount of inhaled allergen is limiting. J Allergy Clin Immunol 2016.
403. Crotty S. T follicular helper cell differentiation, function, and roles in disease. Immunity 2014; 41:529-42.
404. King IL, Mohrs M. IL-4-producing CD4+ T cells in reactive lymph nodes during helminth infection are T follicular helper cells. J Exp Med 2009; 206:1001-7.
405. Vogelzang A, McGuire HM, Yu D, Sprent J, Mackay CR, King C. A fundamental role for interleukin-21 in the generation of T follicular helper cells. Immunity 2008; 29:127-37.
406. Mukherjee S, Rasky AJ, Lundy PA, Kittan NA, Kunkel SL, Maillard IP, et al. STAT5-induced lunatic fringe during Th2 development alters delta-like 4-mediated Th2 cytokine production in respiratory syncytial virus-exacerbated airway allergic disease. J Immunol 2014; 192:996-1003.
407. Barnes PJ. Immunology of asthma and chronic obstructive pulmonary disease. Nat Rev Immunol 2008; 8:183-92.
408. Lambrecht BN, Hammad H. The immunology of asthma. Nat Immunol 2015; 16:45-56.
409. Tindemans I, Serafini N, Di Santo JP, Hendriks RW. GATA-3 function in innate and adaptive immunity. Immunity 2014; 41:191-206.
410. Napolitani G, Rinaldi A, Bertoni F, Sallusto F, Lanzavecchia A. Selected Toll-like receptor agonist combinations synergistically trigger a T helper type 1-polarizing program in dendritic cells. Nat Immunol 2005; 6:769-76.
411. Cheng P, Zhou J, Gabrilovich D. Regulation of dendritic cell differentiation and function by Notch and Wnt pathways. Immunological Reviewsl 2010; 234:105–19.
412. Guest RV, Boulter L, Dwyer BJ, Kendall TJ, Man TY, Minnis-Lyons SE, et al. Notch3 drives development and progression of cholangiocarcinoma. Proc Natl Acad Sci U S A 2016; 113:12250-5.
413. Verdine GI, Hilinski GJ. All-hydrocarbon stapled peptides as Synthetic Cell-Accessible Mini-Proteins. Drug Discov Today Technol 2012; 9:e41-e7.
414. Ashley JW, Ahn J, Hankenson KD. Notch signaling promotes osteoclast maturation and resorptive activity. J Cell Biochem 2015; 116:2598-609.
415. Mathieu M, Cotta-Grand N, Daudelin JF, Thébault P, Labrecque N. Notch signaling regulates PD-1 expression during CD8+ T-cell activation. Immunol Cell Biol 2013; 91:82-8.
416. Wolfe MS, Kopan R. Intramembrane proteotysis: Theme and variations. Science 2004; 305:1119-23.
417. KleinJan A, Godthelp T, van Toornenenbergen AW, Fokkens WJ. Allergen binding to specific IgE in the nasal mucosa of allergic patients. J Allergy Clin Immunol 1997; 99:515-21.
418. Rzepecka J, Siebeke I, Coltherd JC, Kean DE, Steiger CN, Al-Riyami L, et al. The helminth product, ES-62, protects against airway inflammation by resetting the Th cell phenotype. Int J Parasitol 2013; 43:211-23.

419. Tindemans I, Lukkes M, de Bruijn MJ, Li BW, van Nimwegen M, Amsen D, et al. Notch signaling in T cells is essential for allergic airway inflammation, but expression of Notch ligands Jagged1 and Jagged2 on dendritic cells is dispensable. J Allergy Clin Immunol 2017.
420. Krug N, Hohlfeld JM, Kirsten AM, Kornmann O, Beeh KM, Kappeler D, et al. Allergen-induced asthmatic responses modified by a GATA3-specific DNAzyme. N Engl J Med 2015; 372:1987-95.
421. Okamoto M, Takeda K, Joetham A, Ohnishi H, Matsuda H, Swasey CH, et al. Essential role of Notch signaling in effector memory CD8+ T cell-mediated airway hyperresponsiveness and inflammation. J Exp Med 2008; 205:1087-97.
422. Gu W, Xu W, Ding T, Guo X. Fringe controls naïve CD4[+]T cells differentiation through modulating notch signaling in asthmatic rat models. PLoS One 2012; 7:e47288.
423. Danahay H, Pessotti AD, Coote J, Montgomery BE, Xia D, Wilson A, et al. Notch2 is required for inflammatory cytokine-driven goblet cell metaplasia in the lung. Cell Rep 2015; 10:239-52.
424. Sundlisaeter E, Edelmann RJ, Hol J, Sponheim J, Kuchler AM, Weiss M, et al. The alarmin IL-33 is a notch target in quiescent endothelial cells. Am J Pathol 2012; 181:1099-111.
425. Lloyd CM, Saglani S. Epithelial cytokines and pulmonary allergic inflammation. Curr Opin Immunol 2015; 34:52-8.
426. Chen YR, Feng F, Wang L, Qu SY, Zhang ZQ, Liu L, et al. Deletion of RBP-J in dendritic cells compromises TLR-mediated DC activation accompanied by abnormal cytoskeleton reorganization. Mol Biol Rep 2013; 40:1531-9.
427. Erle DJ, Sheppard D. The cell biology of asthma. Journal of Cell Biology 2014; 205:621-31.
428. Coote J, Danahay H, Pessotti A, Xia D, Capodieci P, Doelemeyer A, et al. Therapeutic Notch2 Neutralization Reverses an Established Goblet Cell Formation in an IL-13 Mouse Model of Mucus Hypersecretion. Am J Respir Crit Care Med 2016; 193:A5889.
429. Maillard I, Adler SH, Pear WS. Notch and the immune system. Immunity 2003; 19:781-91.
430. McCright B, Lozier J, Gridley T. Generation of new Notch2 mutant alleles. Genesis 2006; 44:29-33.
431. Barnden MJ, Allison J, Heath WR, Carbone FR. Defective TCR expression in transgenic mice constructed using cDNA-based alpha- and beta-chain genes under the control of heterologous regulatory elements. Immunol Cell Biol 1998; 76:34-40.
432. Li BWS, Stadhouders R, de Bruijn MJW, Lukkes M, Beerens DMJM, Brem MD, et al. Group 2 Innate Lymphoid Cells Exhibit a Dynamic Phenotype in Allergic Airway Inflammation. Frontiers in Immunology 2017; 8.
433. Stadhouders R, Li BWS, de Bruijn MJW, Gomez A, Rao TN, Fehling HJ, et al. Epigenome analysis links gene regulatory elements in group 2 innate lymphocytes to asthma susceptibility. J Allergy Clin Immunol 2017; in press.
434. Vroman H, Bergen IM, Li BW, van Hulst JA, Lukkes M, van Uden D, et al. Development of eosinophilic inflammation is independent of B-T cell interaction in a chronic house dust mite-driven asthma model. Clin Exp Allergy 2016.
435. van Hamburg JP, de Bruijn MJ, Ribeiro de Almeida C, van Zwam M, van Meurs M, de Haas E, et al. Enforced expression of GATA3 allows differentiation of IL-17-producing cells, but constrains Th17-mediated pathology. Eur J Immunol 2008; 38:2573-86.
436. de Bruijn MJ, Rip J, van der Ploeg EK, van Greuningen LW, Ta VT, Kil LP, et al. Distinct and Overlapping Functions of TEC Kinase and BTK in B Cell Receptor Signaling. J Immunol 2017.
437. Picelli S, Bjorklund AK, Faridani OR, Sagasser S, Winberg G, Sandberg R. Smart-seq2 for sensitive full-length transcriptome profiling in single cells. Nat Methods 2013; 10:1096-8.
438. Kim D, Bae S, Park J, Kim E, Kim S, Yu HR, et al. Digenome-seq: genome-wide profiling of CRISPR-Cas9 off-target effects in human cells. Nat Methods 2015; 12:237-43, 1 p following 43.
439. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol 2014; 15:550.
440. Heinz S, Benner C, Spann N, Bertolino E, Lin YC, Laslo P, et al. Simple combinations of lineage-determining transcription factors prime cis-regulatory elements required for macrophage and B cell identities. Mol Cell 2010; 38:576-89.
441. Tanoue T, Atarashi K, Honda K. Development and maintenance of intestinal regulatory T cells. Nat Rev Immunol 2016; 16:295-309.
442. Oh H, Grinberg-Bleyer Y, Liao W, Maloney D, Wang P, Wu Z, et al. An NF-kappaB Transcription-Factor-Dependent Lineage-Specific Transcriptional Program Promotes Regulatory T Cell Identity and Function. Immunity 2017; 47:450-65.e5.
443. Tripathi S, Pohl MO, Zhou Y, Rodriguez-Frandsen A, Wang G, Stein DA, et al. Meta- and Orthogonal Integration of Influenza "OMICs" Data Defines a Role for UBR4 in Virus Budding. Cell Host Microbe 2015; 18:723-35.
444. Yuan JS, Kousis PC, Suliman S, Visan I, Guidos CJ. Functions of notch signaling in the immune system: consensus and controversies. Annu Rev Immunol 2010; 28:343-65.
445. Mionnet C, Buatois V, Kanda A, Milcent V, Fleury S, Lair D, et al. CX3CR1 is required for airway inflammation by promoting T helper cell survival and maintenance in inflamed lung. Nat Med 2010; 16:1305-12.
446. Groom JR. Moving to the suburbs: T-cell positioning within lymph nodes during activation and memory. Immunol Cell Biol 2015; 93:330-6.
447. Wang W, Yu S, Myers J, Wang Y, Xin WW, Albakri M, et al. Notch2 blockade enhances hematopoietic stem cell mobilization and homing. Haematologica 2017; 102:1785-95.
448. Thelen M. Dancing to the tune of chemokines. Nat Immunol 2001; 2:129-34.
449. Hynes RO. Integrins: versatility, modulation, and signaling in cell adhesion. Cell 1992; 69:11-25.
450. Nagato M, Heike T, Kato T, Yamanaka Y, Yoshimoto M, Shimazaki T, et al. Prospective characterization of neural stem cells by flow cytometry analysis using a combination of surface markers. J Neurosci Res 2005; 80:456-66.
451. Chen LH, Chang CH, Lee ZL, Niu CC, Lai PL, Tan CF, et al. Intervertebral disc herniation in adolescents. Chang Gung Med J 2004; 27:22-8.
452. Hodkinson PS, Elliott PA, Lad Y, McHugh BJ, MacKinnon AC, Haslett C, et al. Mammalian NOTCH-1 activates beta1 integrins via the small GTPase R-Ras. J Biol Chem 2007; 282:28991-9001.
453. Chong SW, Jiang YJ. Off limits--integrins holding boundaries in somitogenesis. Trends Cell Biol 2005; 15:453-7.
454. Julia V. CX3CL1 in allergic diseases: not just a chemotactic molecule. Allergy 2012; 67:1106-10.
455. Stadhouders R, Lubberts E, Hendriks RW. A cellular and molecular view of T helper 17 cell plasticity in autoimmunity. J Autoimmun 2017.
456. Nelson RP, Jr., DiNicolo R, Fernandez-Caldas E, Seleznick MJ, Lockey RF, Good RA. Allergen-specific IgE levels and mite allergen exposure in children with acute asthma first seen in an emergency department and in nonasthmatic control subjects. J Allergy Clin Immunol 1996; 98:258-63.
457. Hammad H, Chieppa M, Perros F, Willart MA, Germain RN, Lambrecht BN. House dust mite allergen induces asthma via Toll-like receptor 4 triggering of airway structural cells. Nat Med 2009; 15:410-6.
458. Kubo M. Innate and adaptive type 2 immunity in lung allergic inflammation. Immunol Rev 2017; 278:162-72.
459. Kopan R, Ilagan MX. The canonical Notch signaling pathway: unfolding the activation mechanism. Cell 2009; 137:216-33.
460. Hoyne GF, Le Roux I, Corsin-Jimenez M, Tan K, Dunne J, Forsyth LM, et al. Serrate1-induced notch signalling regulates the decision between immunity and tolerance made by peripheral CD4[+] T cells. Int Immunol 2000; 12:177-85.
461. Koyanagi A, Sekine C, Yagita H. Expression of Notch receptors and ligands on immature and mature T cells. Biochem Biophys Res Commun 2012; 418:799-805.
462. Le Friec G, Sheppard D, Whiteman P, Karsten CM, Shamoun SA, Laing A, et al. The CD46-Jagged1 interaction is critical for human TH1 immunity. Nat Immunol 2012; 13:1213-21.
463. Chai Q, Onder L, Scandella E, Gil-Cruz C, Perez-Shibayama C, Cupovic J, et al. Maturation of lymph node fibroblastic reticular cells from myofibroblastic precursors is critical for antiviral immunity. Immunity 2013; 38:1013-24.
464. Fletcher AL, Malhotra D, Turley SJ. Lymph node stroma broaden the peripheral tolerance paradigm. Trends Immunol 2011; 32:12-8.
465. Gasteiger G, Ataide M, Kastenmuller W. Lymph node - an organ for T-cell activation and pathogen defense. Immunol Rev 2016; 271:200-20.
466. Lund FE, Randall TD. Effector and regulatory B cells: modulators of CD4+ T cell immunity. Nat Rev Immunol 2010; 10:236-47.
467. Mohtashami M, Shah DK, Kianizad K, Awong G, Zuniga-Pflucker JC. Induction of T-cell development by Delta-like 4-expressing fibroblasts. Int Immunol 2013; 25:601-11.
468. Kowal K, Moller HJ, Dubuske LM, Moestrup SK, Bodzenta-Lukaszyk A. Differential expression of monocyte CD163 in single- and dual-asthmatic responders during allergen-induced bronchoconstriction. Clin Exp Allergy 2006; 36:1584-91.
469. Zhang S, Loch AJ, Radtke F, Egan SE, Xu K. Jagged1 is the major regulator of Notch-dependent cell fate in proximal airways. Dev Dyn 2013; 242:678-86.
470. Huang MT, Chen YL, Lien CI, Liu WL, Hsu LC, Yagita H, et al. Notch Ligand DLL4 Alleviates Allergic Airway Inflammation via Induction of a Homeostatic Regulatory Pathway. Sci Rep 2017; 7:43535.
471. Palmer WH, Deng WM. Ligand-Independent Mechanisms of Notch Activity. Trends Cell Biol 2015; 25:697-707.
472. Guy CS, Vignali KM, Temirov J, Bettini ML, Overacre AE, Smeltzer M, et al. Distinct TCR signaling pathways drive proliferation and cytokine production in T cells. Nat Immunol 2013; 14:262-70.
473. Croisant S. Epidemiology of asthma: prevalence and burden of disease. Adv Exp Med Biol 2014; 795:17-29.
474. Wenzel SE. Asthma phenotypes: the evolution from clinical to molecular approaches. Nat Med 2012; 18:716-25.
475. Holgate ST, Polosa R. The mechanisms, diagnosis, and management of severe asthma in adults. Lancet 2006; 368:780-93.
476. Fahy JV. Type 2 inflammation in asthma--present in most, absent in many. Nat Rev Immunol 2015; 15:57-65.
477. Woodruff PG, Modrek B, Choy DF, Jia G, Abbas AR, Ellwanger A, et al. T-helper type 2-driven inflammation defines major subphenotypes of asthma. Am J Respir Crit Care Med 2009; 180:388-95.
478. Anderson GP. Endotyping asthma: new insights into key pathogenic mechanisms in a complex, heterogeneous disease. Lancet 2008; 372:1107-19.
479. Lotvall J, Akdis CA, Bacharier LB, Bjermer L, Casale TB, Custovic A, et al. Asthma endotypes: a new approach to classification of disease entities within the asthma syndrome. J Allergy Clin Immunol 2011; 127:355-60.
480. Custovic A, Taggart S, Woodcock A, Arifhodzic N. Assessing exercise-induced bronchospasm. Chest 1994; 105:1624-5.
481. Vonk JM, Jongepier H, Panhuysen CI, Schouten JP, Bleecker ER, Postma DS. Risk factors associated with the presence of irreversible airflow limitation and reduced transfer coefficient in patients with asthma after 26 years of follow up. Thorax 2003; 58:322-7.
482. Pavord ID, Hilvering B, Shrimanker R. Emerging Biologics in Severe Asthma. Immunol Allergy Clin North Am 2016; 36:609-23.

483. Yick CY, Zwinderman AH, Kunst PW, Grunberg K, Mauad T, Dijkhuis A, et al. Transcriptome sequencing (RNA-Seq) of human endobronchial biopsies: asthma versus controls. Eur Respir J 2013; 42:662-70.
484. Brutsche MH, Joos L, Carlen Brutsche IE, Bissinger R, Tamm M, Custovic A, et al. Array-based diagnostic gene-expression score for atopy and asthma. J Allergy Clin Immunol 2002; 109:271-3.
485. Orsmark-Pietras C, James A, Konradsen JR, Nordlund B, Soderhall C, Pulkkinen V, et al. Transcriptome analysis reveals upregulation of bitter taste receptors in severe asthmatics. Eur Respir J 2013; 42:65-78.
486. Howrylak JA, Moll M, Weiss ST, Raby BA, Wu W, Xing EP. Gene expression profiling of asthma phenotypes demonstrates molecular signatures of atopy and asthma control. J Allergy Clin Immunol 2016; 137:1390-7 e6.
487. Seumois G, Zapardiel-Gonzalo J, White B, Singh D, Schulten V, Dillon M, et al. Transcriptional Profiling of Th2 Cells Identifies Pathogenic Features Associated with Asthma. J Immunol 2016; 197:655-64.
488. Bousquet J, Humbert M. GINA 2015: the latest iteration of a magnificent journey. Eur Respir J 2015; 46:579-82.
489. Ten Berge B, Kleinjan A, Muskens F, Hammad H, Hoogsteden HC, Hendriks RW, et al. Evidence for local dendritic cell activation in pulmonary sarcoidosis. Respir Res 2012; 13:33.
490. Juniper EF, Bousquet J, Abetz L, Bateman ED. Identifying 'well-controlled' and 'not well-controlled' asthma using the Asthma Control Questionnaire. Respir Med 2006; 100:616-21.
491. Wambre E, Bajzik V, DeLong JH, O'Brien K, Nguyen QA, Speake C, et al. A phenotypically and functionally distinct human TH2 cell subpopulation is associated with allergic disorders. Sci Transl Med 2017; 9.
492. Romagnani S. Cytokines and chemoattractants in allergic inflammation. Mol Immunol 2002; 38:881-5.
493. Pettipher R, Hansel TT, Armer R. Antagonism of the prostaglandin D2 receptors DP1 and CRTH2 as an approach to treat allergic diseases. Nat Rev Drug Discov 2007; 6:313-25.
494. Balzar S, Fajt ML, Comhair SA, Erzurum SC, Bleecker E, Busse WW, et al. Mast cell phenotype, location, and activation in severe asthma. Data from the Severe Asthma Research Program. Am J Respir Crit Care Med 2011; 183:299-309.
495. Barnes N, Pavord I, Chuchalin A, Bell J, Hunter M, Lewis T, et al. A randomized, double-blind, placebo-controlled study of the CRTH2 antagonist OC000459 in moderate persistent asthma. Clin Exp Allergy 2012; 42:38-48.
496. Berkova Z, Tao RH, Samaniego F. Milatuzumab - a promising new immunotherapeutic agent. Expert Opin Investig Drugs 2010; 19:141-9.
497. Wilkinson K, Velloso ER, Lopes LF, Lee C, Aster JC, Shipp MA, et al. Cloning of the t(1;5)(q23;q33) in a myeloproliferative disorder associated with eosinophilia: involvement of PDGFRB and response to imatinib. Blood 2003; 102:4187-90.
498. Barnes PJ. Severe asthma: advances in current management and future therapy. J Allergy Clin Immunol 2012; 129:48-59.
499. Diamant Z, Spina D. PDE4-inhibitors: a novel, targeted therapy for obstructive airways disease. Pulm Pharmacol Ther 2011; 24:353-60.
500. Guo B, McMillan BJ, Blacklow SC. Structure and function of the Mind bomb E3 ligase in the context of Notch signal transduction. Curr Opin Struct Biol 2016; 41:38-45.
501. Fukuyama Y, Tokuhara D, Sekine S, Kataoka K, Markham JD, Irwin AR, et al. Notch-ligand expression by NALT dendritic cells regulates mucosal Th1- and Th2-type responses. Biochem Biophys Res Commun 2012; 418:6-11.
502. Kaisho T, Hoshino K, Iwabe T, Takeuchi O, Yasui T, Akira S. Endotoxin can induce MyD88-deficient dendritic cells to support T(h)2 cell differentiation. Int Immunol 2002; 14:695-700.
503. Eisenbarth SC, Piggott DA, Huleatt JW, Visintin I, Herrick CA, Bottomly K. Lipopolysaccharide-enhanced, toll-like receptor 4-dependent T helper cell type 2 responses to inhaled antigen. J Exp Med 2002; 196:1645-51.
504. Schnare M, Barton GM, Holt AC, Takeda K, Akira S, Medzhitov R. Toll-like receptors control activation of adaptive immune responses. Nat Immunol 2001; 2:947-50.
505. Jiang Y, Zhao S, Yang X, Liu Y, Wang C. Dll4 in the DCs isolated from OVA-sensitized mice is involved in Th17 differentiation inhibition by 1,25-dihydroxyvitamin D3 *in vitro*. J Asthma 2015; 52:989-95.
506. Higashi T, Hashimoto K, Takagi R, Mizuno Y, Okazaki Y, Tanaka Y, et al. Curdlan induces DC-mediated Th17 polarization via Jagged1 activation in human dendritic cells. Allergol Int 2010; 59:161-6.
507. You P, Xing F, Mao C, Chen Z, Zhang H, Wang Y, et al. Jagged-1-HES-1 signaling inhibits the differentiation of TH17 cells via ROR gammat. J Biol Regul Homeost Agents 2013; 27:79-93.
508. Wang Y, Xing F, Ye S, Xiao J, Di J, Zeng S, et al. Jagged-1 signaling suppresses the IL-6 and TGF-beta treatment-induced Th17 cell differentiation via the reduction of RORgammat/IL-17A/IL-17F/IL-23a/IL-12rb1. Sci Rep 2015; 5:8234.
509. Jiao Z, Wang W, Hua S, Liu M, Wang H, Wang X, et al. Blockade of Notch signaling ameliorates murine collagen-induced arthritis via suppressing Th1 and Th17 cell responses. Am J Pathol 2014; 184:1085-93.
510. Ladi E, Nichols JT, Ge W, Miyamoto A, Yao C, Yang LT, et al. The divergent DSL ligand Dll3 does not activate Notch signaling but cell autonomously attenuates signaling induced by other DSL ligands. J Cell Biol 2005; 170:983-92.
511. Cahill EF, Tobin LM, Carty F, Mahon BP, English K. Jagged-1 is required for the expansion of $CD4^+$ $CD25^+$ $FoxP3^+$ regulatory T cells and tolerogenic dendritic cells by murine mesenchymal stromal cells. Stem Cell Res Ther 2015; 6:19.
512. Gopisetty A, Bhattacharya P, Haddad C, Bruno JC, Jr., Vasu C, Miele L, et al. OX40L/Jagged1 cosignaling by GM-CSF-induced bone marrow-derived dendritic cells is required for the expansion of functional regulatory T cells. J Immunol 2013; 190:5516-25.
513. Kared H, Adle-Biassette H, Fois E, Masson A, Bach JF, Chatenoud L, et al. Jagged2-expressing hematopoietic progenitors promote regulatory T cell expansion in the periphery through notch signaling. Immunity 2006; 25:823-34.
514. Mota C, Nunes-Silva V, Pires AR, Matoso P, Victorino RM, Sousa AE, et al. Delta-like 1-mediated Notch signaling enhances the *in vitro* conversion of human memory CD4 T cells into FOXP3-expressing regulatory T cells. J Immunol 2014; 193:5854-62.
515. Rutz S, Janke M, Kassner N, Hohnstein T, Krueger M, Scheffold A. Notch regulates IL-10 production by T helper 1 cells. Proc Natl Acad Sci U S A 2008; 105:3497-502.
516. Rutz S, Mordmuller B, Sakano S, Scheffold A. Notch ligands Delta-like1, Delta-like4 and Jagged1 differentially regulate activation of peripheral T helper cells. Eur J Immunol 2005; 35:2443-51.
517. Vigouroux S, Yvon E, Wagner HJ, Biagi E, Dotti G, Sili U, et al. Induction of antigen-specific regulatory T cells following overexpression of a Notch ligand by human B lymphocytes. J Virol 2003; 77:10872-80.
518. Yvon ES, Vigouroux S, Rousseau RF, Biagi E, Amrolia P, Dotti G, et al. Overexpression of the Notch ligand, Jagged-1, induces alloantigen-specific human regulatory T cells. Blood 2003; 102:3815-21.
519. Ou-Yang HF, Zhang HW, Wu CG, Zhang P, Zhang J, Li JC, et al. Notch signaling regulates the FOXP3 promoter through RBP-J- and Hes1-dependent mechanisms. Mol Cell Biochem 2009; 320:109-14.
520. Billiard F, Lobry C, Darrasse-Jeze G, Waite J, Liu X, Mouquet H, et al. Dll4-Notch signaling in Flt3-independent dendritic cell development and autoimmunity in mice. J Exp Med 2012; 209:1011-28.
521. Charbonnier LM, Wang S, Georgiev P, Sefik E, Chatila TA. Control of peripheral tolerance by regulatory T cell-intrinsic Notch signaling. Nat Immunol 2015; 16:1162-73.
522. Burghardt S, Erhardt A, Claass B, Huber S, Adler G, Jacobs T, et al. Hepatocytes contribute to immune regulation in the liver by activation of the Notch signaling pathway in T cells. J Immunol 2013; 191:5574-82.
523. Kassner N, Krueger M, Yagita H, Dzionek A, Hutloff A, Kroczek R, et al. Cutting edge: Plasmacytoid dendritic cells induce IL-10 production in T cells via the Delta-like-4/Notch axis. J Immunol 2010; 184:550-4.
524. Amsen D, Antov A, Flavell RA. The different faces of Notch in T-helper-cell differentiation. Nat Rev Immunol 2009; 9:116-24.
525. Ong CT, Cheng HT, Chang LW, Ohtsuka T, Kageyama R, Stormo GD, et al. Target selectivity of vertebrate notch proteins. Collaboration between discrete domains and CSL-binding site architecture determines activation probability. J Biol Chem 2006; 281:5106-19.
526. Arnett KL, Hass M, McArthur DG, Ilagan MX, Aster JC, Kopan R, et al. Structural and mechanistic insights into cooperative assembly of dimeric Notch transcription complexes. Nat Struct Mol Biol 2010; 17:1312-7.
527. Fiorini E, Merck E, Wilson A, Ferrero I, Jiang W, Koch U, et al. Dynamic regulation of notch 1 and notch 2 surface expression during T cell development and activation revealed by novel monoclonal antibodies. J Immunol 2009; 183:7212-22.
528. Xia M, Viera-Hutchins L, Garcia-Lloret M, Noval Rivas M, Wise P, McGhee SA, et al. Vehicular exhaust particles promote allergic airway inflammation through an aryl hydrocarbon receptor-notch signaling cascade. J Allergy Clin Immunol 2015; 136:441-53.
529. Cabrera CV. Lateral inhibition and cell fate during neurogenesis in Drosophila: the interactions between scute, Notch and Delta. Development 1990; 110:733-42.
530. Van de Walle I, Waegemans E, De Medts J, De Smet G, De Smedt M, Snauwaert S, et al. Specific Notch receptor-ligand interactions control human TCR-alphabeta/gammadelta development by inducing differential Notch signal strength. J Exp Med 2013; 210:683-97.
531. Mazzone M, Selfors LM, Albeck J, Overholtzer M, Sale S, Carroll DL, et al. Dose-dependent induction of distinct phenotypic responses to Notch pathway activation in mammary epithelial cells. Proc Natl Acad Sci U S A 2010; 107:5012-7.
532. Tao X, Constant S, Jorritsma P, Bottomly K. Strength of TCR signal determines the costimulatory requirements for Th1 and Th2 $CD4^+$ T cell differentiation. J Immunol 1997; 159:5956-63.
533. Constant S, Pfeiffer C, Woodard A, Pasqualini T, Bottomly K. Extent of T cell receptor ligation can determine the functional differentiation of naïve $CD4^+$ T cells. J Exp Med 1995; 182:1591-6.
534. Groth C, Fortini ME. Therapeutic approaches to modulating Notch signaling: current challenges and future prospects. Semin Cell Dev Biol 2012; 23:465-72.
535. Brown DR, Fowell DJ, Corry DB, Wynn TA, Moskowitz NH, Cheever AW, et al. Beta 2-microglobulin-dependent NK1.1$^+$ T cells are not essential for T helper cell 2 immune responses. J Exp Med 1996; 184:1295-304.
536. De Grove KC, Provoost S, Hendriks RW, McKenzie AN, Seys LJ, Kumar S, et al. Dysregulation of type 2 innate lymphoid cells and TH2 cells impairs pollutant-induced allergic airway responses. J Allergy Clin Immunol 2016.

English summary





Asthma is a chronic heterogeneous respiratory disease characterized by episodes of airway inflammation, bronchoconstriction, airway hyperreactivity and increased mucus production. It is estimated that worldwide over 300 million people have asthma. Since a major subgroup of asthma patients do not establish disease control using the currently prescribed medical treatment, it is important to develop new therapeutic approaches to treat patients. Allergic asthma is the most prevalently occurring type of asthma and is typically characterized by eosinophilia and T helper 2 (Th2) cell-mediated inflammation. Allergic asthma is triggered by inhaled allergens such as house-dust mite (HDM) that activate dendritic cells (DCs). These cells present the allergens to naïve T cells in the draining lymph nodes, which initiates Th2 cell differentiation. Th2 cells have a central role in the development of allergic asthma, since they typically produce the cytokines IL-4, IL-5 and IL-13 that explain many hallmarks of allergic asthma by inducing IgE production by plasma cells, eosinophilia and smooth muscle hyperreactivity and mucus production by goblet cells, respectively. Moreover, type 2 innate lymphoid cells (ILC2) contribute to the Th2 response in allergic asthma by producing IL-5 and IL-13 in response to epithelial pro-inflammatory cytokines such as TSLP, IL-25 and IL-33 (**Chapter 1**).

It was observed that depending on cytokine signals naïve T helper cells can differentiate into multiple T helper cell lineages including - in addition to Th2 cells - Th1, Th2, Th9, Th17, T follicular helper (Tfh) and T regulatory (Treg) cells. Each T helper cell subset is identified by a unique cytokine production profile and a key transcription factor (**Chapter 1**). Th2 cells express the key transcription factor Gata3, which controls expression of IL-4, IL-5 and IL-13. Moreover, Gata3 is required for T cell development as well as ILC2 development and function (**Chapter 2**). While Th2 cells are the main producers of IL-4, these cells are induced in response to IL-4 via signal transducer and activator of transcription 6 (STAT6) signaling. The initiation of Th2 cell differentiation via the IL-4/STAT6 axis is therefore suggestive of an autocrine loop and the origin of the initial IL-4 that induces Th2 cell differentiation remains unclear.

It was shown that expression of Gata3 and IL-4 can be directly regulated by Notch signaling. The highly conserved Notch signaling pathway mediates cell-cell contact-dependent signaling that regulates cell proliferation, apoptosis and a broad array of cell fate decisions in immune cell development and differentiation. Differentially expressed Notch ligands endow differentiation of naïve $CD4^+$ T helper cells: Delta-like (DLL) and Jagged ligands were shown to induce Th1 and Th2 differentiation, respectively. Notch signaling leads to the activation of a nuclear DNA-binding complex that contains recombination-signal-binding protein for immunoglobulin Jκ region (RBPjκ). In **chapter 3** of this thesis, we investigated the role of Notch signaling and Jagged ligands in allergic asthma using mice lacking Jagged ligands on DCs or mice lacking RBPJκ specifically in T cells. We induced allergic airway inflammation (AAI) by sensitizing and challenging mice intranasally with HDM. We show that HDM exposure promoted the expression of Jagged1, but not Jagged2, on DCs. Mice lacking Jagged1, Jagged2 or both of these Notch ligands specifically in DCs developed AAI characterized by eosinophilia and Th2 cell activation that was not different from the eosinophilic airway inflammation in wild-type (WT) control littermates. Importantly, RBPJκ-deficient mice failed to develop AAI and airway hyperreactivity. Therefore, our results demonstrate that the Notch signaling pathway in T cells is essential for inducing Th2-mediated AAI in a HDM-driven asthma model, while the expression of the Notch ligands Jagged1 and Jagged2 on DCs is dispensable.

Our findings that Notch signaling via RBPJκ in T cells is crucial for the induction of AAI, indicates that the Notch signaling pathway is a potential therapeutic target in allergic asthma. To investigate this, we treated HDM-exposed mice with the cell-permeable inhibitor stapled α-helical peptide derived from mastermind-like 1 (SAHM1) to target protein-protein interfaces in the Notch transactivation complex. Also, we investigated whether blocking Notch signaling is essential during the sensitization or during the challenge phase. Interestingly, SAHM1 treatment during only the challenge phase significantly reduced eosinophil numbers and decreased Th2 cell-mediated AAI in bronchoalveolar lavage, compared with control peptide-treated mice (**Chapter 4**). Also, SAHM1 therapy reduced serum IgE levels. Therapeutic intervention of Notch signaling by SAHM1 is therefore an appealing new topical treatment opportunity for asthmatic patients.

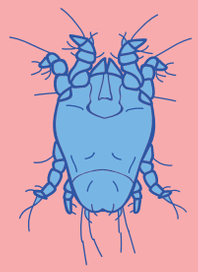
Since we found that inhibiting Notch signaling was mainly effective during the challenge phase, we wanted to further explore the function of the Notch receptors in Th2 cell inflammation. In **chapter 5**, we therefore exposed mice lacking either Notch1 or Notch2 or both receptors on T cells to acute and chronic house-dust mite (HDM)-driven models, as well as ovalbumine (OVA)-mediated models for AAI. Also, we assessed whether the lack of Notch1 and/or Notch2 can be rescued by enforced expression of Gata3. Although HDM exposure induced AAI in wild-type (WT) animals, conditional deletion of both Notch1 and Notch2 in T helper cells prevented development of eosinophilic airway inflammation, Th2 cytokine production, induction of serum IgE levels and airway hyperreactivity. Surprisingly, Gata3 overexpression in Notch-deficient T cells only partially rescued HDM-driven Th2 cell-driven AAI. Therefore, we questioned what other functions Notch signaling has during Th2 cell-mediated inflammation beyond the induction of Gata3. We found that Th2 cell polarization following sensitization to OVA and HDM was independent of Notch signaling. In contrast, Notch-deficient OVA-specific Th2 cells polarized *in vitro* showed reduced accumulation in the lung following transfer into wild-type mice that were subsequently exposed to OVA. Instead, we observed retention of Th2 cells in lung draining lymph nodes. We performed transcriptome analyses to identify genes that are controlled by Notch signaling in the context of AAI. Transcriptome comparisons of Notch-deficient and WT Th2 cells from lymph nodes revealed 692 differentially expressed genes, including genes encoding adhesion molecules, cytokines and cytokine receptors. Therefore we conclude that in a HDM-driven asthma model, Notch signaling in T cells is essential in AAI for efficient cytokine responsiveness, cell adhesion and migration of Th2 cells, in particular for lymph node egress.

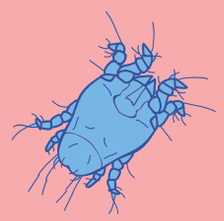
We have shown that mice lacking RBPjκ or the Notch1 and Notch2 receptors or mice that were treated with the inhibitory peptide SAHM1 to target the Notch transactivation complex failed to generate Th2 responses or eosinophilia in acute and chronic HDM-driven AAI mouse models (AAI) (**Chapter 3-5**). In contrast, we showed that mice lacking Jagged expression specifically on DCs still developed Th2 cell-mediated AAI (**Chapter 3**). In **chapter 6** we therefore investigated the role and function of the expression of the Jagged1 and jagged2 Notch ligands on B cells, lymph node follicular reticular cells (FRCs) and T cells. We demonstrated that although Notch ligand expression is induced in FRCs in the lymph nodes upon HDM stimulation, neither Jagged1 nor Jagged2 expressed on CCL19$^+$ FRC is required for the induction of AAI (**Chapter 6**). Also, Notch ligand expression on CD4$^+$ T cells was low and not required for the induction of AAI. We therefore conclude that the expression of the Notch ligands Jagged1 and Jagged2 on FRC, DCs or T cells is not critical for HDM-driven AAI *in vivo*. Lastly, intranasal blockade of DLL4 did not alter eosinophilia or Th2 cell-driven airway inflammation. We therefore hypothesize that either Jagged on other cells is required for Th2 cell mediated inflammation, or alternatively other Notch ligands such as DLL1 have the capacity to support Th2 cell-mediated responses.

A subgroup of asthma patients is unable to control their disease using corticosteroids. It is therefore important to obtain knowledge about the immunological differences between steroid-controlled and uncontrolled asthmatic patients to develop additional therapeutic approaches for asthma. Because of the evidence that Notch signaling is required during Th2 inflammation in AAI in mice, we questioned whether Notch expression is altered on Th2 cells in allergic asthma patients. In **chapter 7,** we performed flow-cytometry on peripheral blood mononuclear cells from asthmatic patients and healthy subjects. We found increased proportions of Notch1 and Notch2 expressing cells especially within the population of memory CD4$^+$ T cells from asthma patients with low asthma control. In addition, we performed genome-wide expression profiling of Th2 cells and identified several genes involved in lymphocyte activation that were higher expressed in Th2 cells from asthma patients than in healthy controls. Moreover, we identified many genes that were expressed at a lower level in Th2 cells from asthma patients than in healthy individuals, including genes involved in the activation of JUN kinase and in apoptosis. A fraction of these genes correlated with lung function (**Chapter 7**). The differentially expressed genes that we identified, together with the clinical phenotype of patients, may be useful in the future to classify patients and to predict the type of therapy they might respond to.

In summary, we found that Notch signaling is required for Th2 cell-mediated AAI in an HDM-driven model for AAI. Also, we showed that Notch expression was increased on memory T cells from asthmatic patients, which suggests that targeting the Notch signaling pathway is a promising therapeutic approach for asthma patients.

Nederlandse samenvatting





Astma is een heterogene chronische longziekte die gepaard gaat met ontsteking van de luchtwegen, luchtwegvernauwing, hyperreactiviteit van de luchtwegen en verhoogde slijmproductie. Wereldwijd zijn er ongeveer 300 miljoen mensen die lijden aan astma. Een deel van deze patiënten heeft nog steeds klachten ondanks het gebruik van ontstekingsremmende medicatie. Daarom is het nodig om op zoek te gaan naar nieuwe manieren om astmapatiënten te behandelen. Allergische astma is de meest voorkomende vorm van astma en wordt veroorzaakt doordat dendritische cellen allergenen zoals huisstofmijt herkennen in de luchtwegen en hiermee T cellen activeren. Na activatie door dendritische cellen ontwikkelen T cellen zich tot een specifieke soort T cellen, de zogenaamde T helper 2 (Th2) cellen. Th2 cellen produceren ontstekingsbevorderende eiwitten (cytokines IL-4, IL-5 en IL-13) die verantwoordelijk zijn voor het aantrekken van eosinofielen (een type ontstekingscellen dat kenmerkend is voor allergische astma), het verhogen van de slijmproductie door slijmbekercellen en hyperreactiviteit van glad spierweefsel (**Hoofdstuk 1**).

Er bestaan ook andere soorten T cellen, namelijk Th1 cellen, Th9 cellen, Th17 cellen, T folliculaire helper cellen en regulatoire T cellen. Iedere soort T helper cel heeft een specifieke functie en wordt gekarakteriseerd door de productie van een uniek profiel van cytokines, dat gereguleerd wordt door een zogenaamde transcriptiefactor (**Hoofdstuk 1**). Th2 cellen hebben de belangrijke transcriptiefactor Gata3 die cruciaal is voor de ontwikkeling van T cellen en ook voor de productie van de cytokines IL-4, IL-5 en IL-13. In **hoofdstuk 2** hebben we literatuuronderzoek gedaan naar de functie van Gata3 in de ontwikkeling en het functioneren van het afweersysteem. Terwijl Th2 cellen de voornaamste cellen zijn die IL-4 produceren is IL-4 ook nodig voor de ontwikkeling van Th2 cellen zelf. Dit betekent dat de ontwikkeling van Th2 cellen afhankelijk is van een stof die deze cellen zelf produceren. Het is onduidelijk wat de bron is van de initiële IL-4 die de ontwikkeling van Th2 cellen induceert.

Onderzoekers hebben gevonden dat signalering via Notch receptoren op T cellen direct kan leiden tot verhoging van Gata3 in T helper cellen en de productie van IL-4. De Notch signaleringsroute is een evolutionair geconserveerde route die belangrijk is voor de ontwikkeling van veel soorten afweercellen. Als een ligand voor Notch aan de Notch receptor bindt dan ontstaat er een reactie waarbij er in de T cel een eiwitcomplex wordt gevormd dat zorgt voor een verhoging van de aanmaak van diverse eiwitten waaronder Gata3 en IL-4. Dit eiwitcomplex bevat de transcriptie regulator RBPJκ. Wij hebben onderzocht wat de functie is van Notch signalering tijdens het ontstaan van astma door muizen te genereren die specifiek de RBPJκ regulator missen in T cellen. Hierdoor zijn er in deze T cellen geen effecten meer van Notch signalering. Deze muizen hebben we in een astmamodel herhaaldelijk blootgesteld aan huisstofmijt. Hierdoor ontstaat normaal gesproken een allergische ontsteking in de longen en luchtwegvernauwing, maar muizen zonder RBPJκ in T cellen ontwikkelen geen van deze astma verschijnselen (**Hoofdstuk 3**). Er bestaan twee verschillende soorten liganden voor Notch receptoren, namelijk Delta en Jagged. In de literatuur is beschreven dat het ligand Delta kan zorgen dat T cellen zich kunnen ontwikkelen tot Th1 cellen, terwijl Jagged ervoor zorgt dat T cellen zich kunnen ontwikkelen tot Th2 cellen. Deze bevindingen waren vooral gebaseerd op celkweek experimenten en de rol van deze verschillenden liganden bij allergische astma was onduidelijk. Wij hebben in **hoofdstuk 3** onderzocht of Jagged op dendritische cellen belangrijk is voor de ontwikkeling van allergische astma. In ons astma muismodel vonden we dat de expressie van Jagged op het celopperval van dendritische cellen omhoog ging nadat muizen werden blootgesteld aan huisstofmijt. Door gebruik te maken van muizen zonder Jagged

op het celoppervlak van hun dendritische cellen, konden we echter aantonen dat Jagged op dendritische cellen niet nodig is voor het ontstaan van een ontstekingsreactie in de longen. We vonden namelijk in deze muizen vergelijkbare aantallen ontstekingscellen in de longen als in muizen die wel Jagged op hun dendritische cellen hadden. Onze resultaten laten daarom zien dat de Notch signaleringsroute in T cellen cruciaal is voor de ontwikkeling van allergische astma in muizen, terwijl het ligand Jagged op dendritische cellen hiervoor niet nodig is.

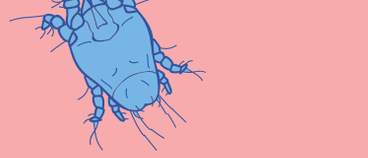
Onze bevinding dat de Notch signaleringsroute in T cellen nodig is voor het ontstaan van allergische astma in muizen suggereert dat Notch een aangrijpingspunt kan zijn voor de behandeling van allergische astma. Om dit te testen hebben we muizen behandeld met SAHM1, een remmer van RBPJκ, waardoor de Notch signaleringsroute niet meer effectief is. We vonden dat muizen die behandeld waren met SAHM1 geen astma meer ontwikkelden. We konden namelijk vaststellen dat het aantal eosinofielen en Th2 cellen in de longen verlaagd was en dat deze muizen verminderde IgE antistoffen in hun serum hadden. Deze antistoffen zijn kenmerkend voor een allergische immuunreactie. Het effect van het behandelen van de muizen met de Notch remmer was het sterkst tijdens herhaalde blootstelling aan huisstofmijt en niet in een vroege fase van de opbouw van allergie na de eerste blootstelling (**Hoofdstuk 4**). De bevindingen dat de Notch remmer met name werkt als muizen al astmaverschijnselen hebben, betekent dat het blokkeren van Notch ook bij astmapatiënten zou kunnen leiden tot een vermindering van astmasymptomen.

Omdat we hadden gevonden dat het blokkeren van de Notch signaleringsroute in allergische astma vooral effectief was tijdens een latere fase van de ziekte, na herhaaldelijke blootstelling aan huisstofmijt allergeen, wilden we vervolgens weten wat het onderliggende mechanisme is. Om dit te onderzoeken hebben we gebruik gemaakt van muizen die geen Notch receptoren op hun T cellen hebben. Deze muizen werden blootgesteld aan astmamodellen met huisstofmijt of het eiwit ovalbumine als allergeen. Daarnaast hebben we Notch-deficiënte muizen gekruist met muizen die verhoogd Gata3 eiwit in hun T cellen hebben, om te onderzoeken of verhoogde expressie van Gata3 het effect van verminderde Notch expressie op kan heffen. We vonden dat muizen zonder expressie van Notch receptoren op T cellen - net als RBPJκ deficiënte muizen - geen astma ontwikkelen. Muizen zonder Notch receptoren op T cellen maar met verhoogd Gata3 in T cellen hadden maar een lichte verhoging van allergische ontsteking in de longen. Dit betekent dat Notch signalering tijdens Th2 cel-gemedieerd astma niet alleen werkt via verhoging van de hoeveelheid Gata3, maar ook via andere mechanismes. In verder onderzoek vonden we dat Notch signalering niet nodig is voor de initiële activatie van T cellen bij de eerste allergeenblootstelling, maar dat Notch signalering wel nodig is tijdens een latere fase van de afweerreactie (wanneer allergische symptomen zichtbaar worden na herhaaldelijke allergeen blootstelling). We hebben namelijk gemeten dat de afwezigheid van Notch bij al geactiveerde Th2 cellen leidt tot een ophoping van Th2 cellen in de lymfeklieren en een verminderde hoeveelheid Th2 cellen in de longen. Dit is een sterke aanwijzing dat Th2 cellen die gevormd worden in de lymfeklier afhankelijk zijn van Notch signalering om op een efficiënte manier de lymfeklier te verlaten. Met een uitgebreide analyse van de verschillen tussen Th2 cellen met Notch receptoren en Th2 cellen zonder Notch receptoren hebben we gevonden dat Notch ervoor zorgt dat Th2 cellen beter kunnen reageren op cytokines en beter kunnen migreren naar andere weefsels (**Hoofdstuk 5**).

In hoofdstuk 3 tot en met 5 hebben we aangetoond dat muizen die Notch receptoren of de Notch-geassocieerde transcriptiefactor RBPJκ missen in hun T cellen of muizen die behandeld worden met SAHM1 nauwelijks astma ontwikkelen. Maar, we vonden ook dat muizen zonder het Notch ligand Jagged op dendritische cellen wel nog steeds astma ontwikkelden. Daarom onderzochten we de mogelijkheid dat Jagged belangrijk zou kunnen zijn op andere cellen dan dendritische cellen (**Hoofdstuk 6**). We vonden dat stromale cellen (steuncellen) in de lymfeklieren Jagged tot expressie brengen en dat de hoeveelheid Jagged verhoogd was als muizen werden blootgesteld aan huisstofmijt. Maar we vonden dat Jagged op deze lymfeklier stromale cellen niet noodzakelijk was voor de ontwikkeling van allergisch astma in de muis. Daarnaast hebben we aanwijzingen verkregen dat Jagged ook niet belangrijk is op alveolaire macrofagen, T cellen of B cellen. Deze uitkomsten suggereren dat de liganden die nodig zijn om Notch signalering in T cellen aan te zetten zich bevinden op andere cellen of dat het ligand Delta belangrijk is voor het aanzetten van de Notch signaleringsroute in T cellen.

Een subgroep van allergische astmapatiënten blijft klachten houden ondanks het gebruik van ontstekingsremmende medicatie. Daarom is de bevinding in ons muismodel dat een specifieke Notch remmer verschijnselen van allergische ontsteking kan verminderen, met name werkt als muizen al allergische luchtwegontsteking hebben ontwikkeld, van groot belang. Het maakt het namelijk aannemelijk dat het blokkeren van de Notch signaleringsroute ook bij astmapatiënten zou kunnen leiden tot een vermindering van astmasymptomen. Daarom hebben we T cellen in bloed onderzocht op de aanwezigheid van Notch receptoren. Hierbij vonden we dat het percentage van de T helper cellen dat Notch receptoren op hun oppervlak heeft bij astmapatiënten hoger is dan bij gezonde mensen. Daarnaast was de fractie Notch-positieve T helper cellen meer verhoogd in patiënten die slecht reageren op ontstekingsremmende medicatie dan bij patiënten waarbij de astmaverschijnselen goed onder controle te houden zijn met medicatie (**Hoofdstuk 7**). We hebben de Th2 cellen van astmapatiënten ook genoom-wijd in detail gekarakteriseerd om te onderzoeken of er nog meer verschillen zijn in Th2 cellen van astmapatiënten vergeleken met gezonde mensen. Hierbij hebben we verschillende genen gevonden die in sterkere mate actief zijn in Th2 cellen van astmapatiënten. Een aantal van deze genen is betrokken bij de activatie van T helper cellen en sommige van deze genen correleerden met een verminderde longfunctie. Toekomstig onderzoek moet uitwijzen of deze genen een voorspellende waarde kunnen hebben voor het ziektebeloop van astmapatiënten of dat deze genen een aanknopingspunt kunnen zijn voor de ontwikkeling van nieuwe medicijnen tegen astma.

Samenvattend hebben we gevonden dat de Notch signaleringsroute belangrijk is bij een Th2 cel-afhankelijk muismodel voor allergische astma gebaseerd op herhaalde blootstelling aan huisstofmijt. Daarnaast hebben we gevonden dat bij astmapatiënten de fractie van Notch-positieve T helper cellen in het bloed verhoogd is. Daarom is de Notch signaleringsroute een potentieel aangrijpingspunt voor toekomstige therapie.



Abbreviations

AAI	Allergic airway inflammation
ACQ	Asthma control questionaire
ADAM	A disintegrin and metalloproteinases
AHR	Airway hyperreactivity
AGM	Aorta-gonad-mesonephros
APC	Antigen presenting cell
BAL	Bronchoalveolar lavage
BATF3	Basic Leucine Zipper ATF-Like Transcription Factor 3
BCL	B-cell lymphoma protein
BEC	Blood endothelial cells
BHR	Bronchial hyperreactivity
BM	Bone marrow
bmDCs	Bone marrow-derived dendritic cells
CA	Controlled asthma
CCL	Chemokine (C-C motif) ligand
CCR	C-C chemokine receptor
CD	Cluster of differentiation
cDC	Conventional DC
CFSE	Carboxyfluorescein succinimidyl ester
Chd4	Chromodomain helicase DNA binding protein 4
Chip	Chromatin immunoprecipitation
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
CRTH2	Chemoattractant homologous receptor expressed on TH2 cells
CTCF	CCCTC-binding factor
CXCR	C-X-C chemokine receptor
DC	Dendritic cell
DE	Differentially expressed
Derp	Dermatophagoides pteronyssinus
DHS	DNAse I hypersensitive site
DLL	Delta-like ligand
DMSO	Dimethylsulfoxide
DN	Double-negative
DNA	Deoxyribonucleic acid
DP	Double-positive
DTR	Diphtheria toxin receptor
EAE	Experimental autoimmune encephalomyelitis
Ebf1	Early B cell factor-1
ELISA	Enzyme-linked immunoasorbent assay
Eomes	Eomesodermin
ETP	Early thymic progenitors
EYFP	Enhanced yellow fluorescent protein
FACS	Fluorescence-activated cell sorting

FeNO	Exhaled nitric oxide
FEV1	Forced Expiratory Volume in the first second
Flt3L	FMS-like tyrosine kinase 3 ligand
Fog	Friend of GATA
Foxp3	Forkhead box P3
FRC	Fibroblastic reticular cells
GINA	Global Initiative for Asthma
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GSI	γ-secretase inhibitors
GWAS	Genome-wide association studies
HAT	Histone acetyltransferease
HC	Healthy control
HDAC	Histone deacetylase
HDM	House-dust mite
HE	Haematoxylin/eosin
Hes	Hairy enhancer of split
HSC	Hematopoietic stem cell
ICOS	Inducible costimulatory
Id2	DNA-binding 2
IFA	Incomplete Freund's Adjuvant
Ig	Immunoglobulin
IL	Interleukin
ILC	Innate lymphoid cells
i.n.	Intranasal
i.p.	Intraperitoneal
i.t.	Intratracheal
i.v.	Intravenously
IFN	Interferon
IRF	Interferon-regulatory factor
Jg	Jagged
LCMV	Lymphocytic choriomeningitis virus
LCR	Locus control region
LEC	Lymphatic endothelial cells
LPS	Lipopolysaccharide
LTi	Lymphoid tissue-inducer
MAML1	Mastermind-like 1
MAPK	Mitogen-activated protein kinase
MedLN	Mediastinal lymph node
MFI	Mean fluorescent intensity
Mib	Mindbomb
MoDC	Monocyte-derived DC
MPP	Multipotent progenitor
mTOR	Mammalian target of rapamycin
N	Notch
NECD	Notch extracellular domain
Nfil3	Nuclear-factor interleukin-3 related
NICD	Notch intracellular domain
NK	Natural killer
NO	Nitric Oxide
OVA	Ovalbumin
PA	Partly controlled asthma
PAS	Periodic acid-Schiff
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
pDC	Plasmacytoid DC
PGD2	Prostaglandin D2
PI3K	Phosphatidylinositol 3-kinase
PMA	Phorbol 12-myristate 13-acetate
qRT-PCR	Quantitative real-time PCR
RBPjκ	Recombination-signal-binding protein for immunoglobulin Jκ region
RELB	V-rel avian reticuloendotheliosis viral oncogene homolog B
RNA	Ribonucleic acid
Rorγt	Retinoic acid receptor-related orphan nuclear receptor gamma
RPKM	Reads Per Kilobase Million
RSV	Respiratory syncytial virus
S1P	Sphingosine-1-phosphate
S1PR	Sphingosine-1-phosphate receptor
SATB	Special AT-rich binding protein
Seq	Sequencing
STAT	Signal transducer and activator of transcription
T-ALL	T cell acute lymphoblastic leukemia
T-bet	T-box-containing protein
TCR	T cell receptor
TF	Transcription factor
Tfh	T follicular helper
Tg	Transgene
Th	T helper
TGF-β	Transforming growth factor beta
TLR	Toll-like receptor
TNF-α	Tumor Necrosis Factor alpha
TNP-KLH	Tri-nitrophenol keyhole limpet hemagglutinin
Treg	Regulatory T cell
TSLP	Thymic stromal lymphopoietin
UA	Uncontrolled asthma
WT	Wild-type
YFP	Yellow fluorescent protein

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Department of Respiratory Medicine, Franciscus Gasthuis and Vlietland, Rotterdam, the Netherlands

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Menno van Nimwegen
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Department of Pediatric Medicine, Franciscus Gasthuis and Vlietland, Rotterdam

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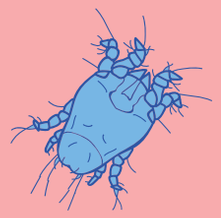
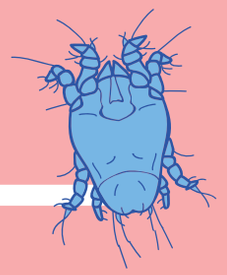
Dankwoord





De afgelopen jaren heb ik mogen samenwerken met fantastische mensen zonder wie dit proefschrift er niet hetzelfde had uitgezien. Ik wil graag iedereen bedanken die een bijdrage heeft geleverd aan mijn onderzoek.

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Ik heb de ruim vier jaar dat ik heb mogen meedraaien in de hectiek die longziekten heet als een hele fijne tijd ervaren, waarbij de jaren echt voorbij gevlogen zijn. Ik wil daarom iedereen van de afdeling longziekten (zelfs als ik je verder helemaal vergeet te noemen) ontzettend bedanken voor de samenwerking, maar vooral ook voor de gezelligheid tijdens de gezamenlijke lunches, koffiemomentjes, borrels, afdelingsfeesten, longdrinks, etentjes, bioscoopbezoekjes en congressen.

Lieve Heleen, best handig dat jij al wat verder was met je promotietraject toen ik begon;). Bedankt voor al je hulp met astmamodellen en DC kleuringen, maar vooral voor de samenwerking tijdens het opzetten en draaiende houden van de iPhase studie. Daarnaast wil ik je bedanken voor de gezelligheid tijdens meerdere cappuccino's en congressen en je poging om mij wat beter te leren skiën! Aangezien we nu allebei postdoc zijn in hetzelfde gebouw hoop ik dat de samenwerking, maar vooral de kopjes koffie nog niet voorbij zijn. Ik vind het ontzettend leuk dat je mijn paranimf wilt zijn!

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chocolademelkmomentjes en vooral voor je vrolijke noot tijdens de lange dagen, ook al moest ik daar 's ochtends vroeg soms nog even aan wennen. Beste Melanie, bedankt voor je hulp met ongeveer alle (mega) experimenten en miljoenen genotyperingen. Zonder jou had mijn boekje niet zo dik kunnen zijn en ik wens je veel succes met je nieuwe taken in het Thorr-team! Ralph, de korte tijd dat we hebben samengewerkt is ontzettend waardevol geweest. Bedankt dat je me wat hebt bijgebracht over het werken met grote datasets en alle brainstormsessies. Ik blijf graag op de hoogte van het vervolg van je plannen met de iPhase studie en mogelijk zelfs het vervolg van het Notch werk. Beste Mirjam, bedankt voor al je adviezen over de verschillende astma muismodellen, DC subsets en de meerdere keren dat je voor mij de literatuur in bent gegaan om mee te denken welke antigeen concentratie of markers ik moest gebruiken. Ook wil ik je bedanken voor je hulp met het opzetten van de iPhase studie. Menno, Ingrid en Jennifer, bedankt voor jullie hulp als ik (alweer) een te groot experimentje had bedacht. Karolina, bedankt voor je hulp met histologie.

Mijn tijd bij longziekten was ook vooral een gezellige tijd. Beste Bobby, door jou voelde ik me meteen welkom op de afdeling en met name in het begin heb je me vaak tot in de late uurtjes (zolang ik maar pizza bestelde) geholpen met mijn experimenten. Ook wil ik je bedanken voor het zorgen voor mijn katten als ik weer eens op vakantie ging, het stofzuigen als ze weer eens een plant omgegooid hadden en zelfs het voorkomen dat de brandweer onze voordeur sloopte! Pauline, bedankt voor je adviezen voor analyses met R, de vele carrière brainstormmomentjes, je skilessen en het organiseren van de longdrinks! Odilia, toetje? Bedankt voor een ontzettend gezellige tijd in Japan, de meerdere Japanse dinnerdates ter voorbereiding hiervan en dat je me wat hebt bijgebracht over whiskey! Floris, gelukkig hield jij wel ALLE literatuur bij, bedankt dat je mij hiervan op de hoogte hield en bedankt voor het organiseren van fantastische bierproeverijen. Tridib, bedankt dat je me op de hoogte hield van alle "awesome" nieuwe hotspots in Rotterdam en voor een gezellige tijd in Tours! Laten we een cocktail gaan drinken (wel goed het hele menu lezen!) als jij ook je boekje af hebt! Jasper, bedankt voor je hulp met phosphoflow kleuringen en voor het organiseren van de bierborrel. Saravanan, thanks for the yoga lessons, Indian food and the travel advise for visiting India! Margaretha, Koen en Myrthe, bedankt voor de gezelligheid tijdens alle longdrinks. Simar, Denise, Esmee, Sabine, Thomas and Peter, thank you for a great time in the lab and good luck with finishing your PhD studies, of course I am looking forward to receiving your theses;).

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Eventjes wat bloed verzamelen van astmapatiënten bleek toch best complex te zijn, zeker in een ander ziekenhuis, en was niet zomaar gelukt zonder de hulp van vele mensen. Gert-Jan Braunstahl bedankt voor je enthousiasme en ontzettend waardevolle imput. Alle longartsen en arts-assistenten van het Franciscus Gasthuis, bedankt voor jullie hulp met het includeren van patiënten. Caroline, bedankt voor je adviezen. Annemarie en Marjolein, bedankt voor jullie hulp met alle formulieren (stukje meer dan wij hadden verwacht) en het zorgen dat wij ons aan alle regeltjes hielden. Benvinda, Simone en Ingrid, bedankt voor jullie hulp met het opzetten en draaiende houden van de iPhase studie en met name voor de vele sputuminducties! Bianca, de studie ging een stuk beter lopen toen jij kon gaan helpen met het bellen en inplannen van patiënten. Linda en Femke, bedankt voor jullie hulp met Open Clinica. Geertje en Gerdien, bedankt voor de samenwerking tijdens de Breathe studie, waardoor ik voor mijn proefschrift data kon verzamelen van een stuk meer patiënten. Uiteraard wil ik ook alle patiënten en vrijwilligers bedanken voor het doneren van bloed en sputum.

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Lieve Manon, tumtumtumtumtumtumtumtumtumtum, hoe fantastisch is het dat we na samen op te groeien in Mierlo, nu allebei in Rotterdam wonen? Bedankt voor de etentjes en festivalweekendjes, maar vooral voor alle Mojitos! Ik vind het super dat je mijn paranimf wilt zijn!

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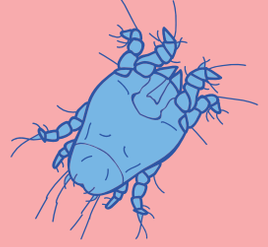
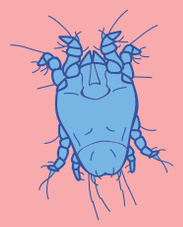
Beste vrienden, bedankt voor alle afleiding tijdens etentjes, festivals, drankjes, stapavondjes, shopdagjes, concertbezoekjes, vakanties en het samen maken van muziek.

Lieve mama en Emiel, bedankt voor jullie onvoorwaardelijke support, het is altijd fijn om thuis te komen.

Lieve Paul, ik ben blij met jou!





Portfolio

PhD student:	Irma Tindemans
Erasmus MC Department:	Pulmonary Medicine
Research school:	Molecular Medicine
PhD period:	2013 – 2017
Promotor:	Prof. Dr. R.W. Hendriks

Courses:

2014	Advanced Immunology
2015	Scientific Integrity
2016	Presenting skills for junior researchers
2016	Medische immunologie
2016	Basic course on R
2017	Ingenuity Pathway Analysis

National conferences:

2013	3rd international Lymphoid Tissue meeting, Rotterdam
2013	3rd NRS symposium on Animal Models in Respiratory Research, Utrecht
2013	NRS 5th Young Investigator Symposium, Amsterdam
2013	Advances in comparative pathology, Rotterdam
2013	Mini-symposium: ''B or T cells in Auto-Immune Disease: Who are the Bad Guys?''
2013	NVVI annual meeting, Noordwijkerhout
2014	18th Molecular Medicine Day, Rotterdam (**poster presentation**)
2014	Mini-symposium: ''Immune Regulation by Dendritic Cells at Epithelial Interfaces'', Rotterdam
2014	NVVI-symposium: Mucosal Immunology; Crossing Borders, Lunteren
2014	Landsteiner lecture and masterclass: "Hollywood Comes to Biology: Moviemaking that Reveals the Inner Workings of the Innate and Adaptive Immune System" by Ron Germain, Amsterdam
2014	4rd NRS symposium on Animal Models in Respiratory Research, Utrecht
2014	NVVI 50th Anniversary Congress: ''a Future Heritage'', Kaatsheuvel (**poster presentation**)
2015	19th Molecular Medicine Day, Rotterdam (**poster presentation**)
2015	NVVI-symposium: Immunity and Science Fiction; the Next 50 Years in Immunology, Lunteren
2015	6th Symposium & master classes on Mucosal Immunology: Cells Living On the Edge, Rotterdam
2015	5th NRS symposium on Animal Models in Respiratory Research, Utrecht (**oral presentation**)
2015	Erasmus MC Rheumatology Symposium: Cytokines, T cells and Drug-Resistance: Implications for Rheumatic Diseases, Rotterdam
2016	NVVI-symposium: The immune system strikes back, Lunteren
2016	6th NRS symposium on Animal Models in Respiratory Research, Utrecht
2017	NVVI annual meeting, Noordwijkerhout (**oral presentation**)

International conferences:

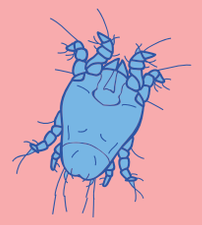
2013 16th International Congress of Mucosal Immunology (ICMI 2013), Vancouver, Canada
2013 4th International Symposium on Regulators of Adaptive Immunity, Erlangen, Germany
2014 12th EAACI Immunology Winter School, Poiana Brasov, Romania (**oral presentation**)
2014 EMBO Conference on Lymphocyte Signalling, Bertinoro, Italy (**poster presentation**)
2014 13th International Symposium on Dendritic Cells (DC2014), Tours, France (**oral presentation**)
2014 Cell symposia: The Multifaceted Roles of Type 2 Immunity, Bruges, Belgium (**poster presentation**)
2015 17th International Congress of Mucosal Immunology (ICMI 2015), Berlin, Germany (**oral presentation**)
2016 5th NIF Winter School on Advanced Immunology, Awaji Island and Osaka, Japan (**oral and poster presentation**)
2016 BSI / NVVI annual congress, Liverpool, UK (**2 oral presentations**)
2017 Keystone symposium: Asthma: From Pathway Biology to Precision Therapeutics, Keystone, Colorado, USA (**oral and poster presentation**)

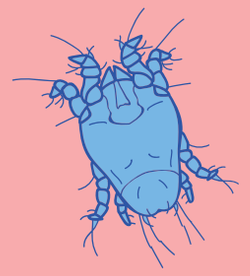
Teaching:

2015 Bachelor student (Biomedical Sciences, Leiden) 5 months
2016 Master student (Infection and Immunity, Rotterdam) 12 months
2016 Master student (Molecular Medicine, Rotterdam), guiding in writing a literature review

Scholarschips, grants and prizes:

2014 Travelgrant EAACI Winter School
2014 NRS travelgrant to attend the EMBO Conference on Lymphocyte Signalling, Bertinoro, Italy, €1250
2016 Travelgrant IFReC-SIgN Winter School
2016 NVVI travelgrant to attend the BSI / NVVI annual congress, Liverpool, UK, €200
2016 BSI's third-placed 'PhD bright spark' of 2016 at the BSI/NVVI Congress 2016, Liverpool, UK
2017 NRS travelgrant to attend the Keystone symposium: Asthma: From Pathway Biology to Precision Therapeutics, Keystone, Colorado, USA, €1250
2017 Keystone symposium scholarschip to attend the Keystone symposium: Asthma: From Pathway Biology to Precision Therapeutics, Keystone, $1200





List of publications

2014 **Tindemans, I.**, Serafini, N., Di Santo, J. P., & Hendriks, R. W. GATA-3 function in innate and adaptive immunity. Immunity, 41(2), 191-206

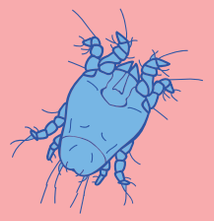
2016 Li, B. W., de Bruijn, M. J., **Tindemans, I.**, Lukkes, M., KleinJan, A., Hoogsteden, H. C., & Hendriks, R. W. T cells are necessary for ILC2 activation in house dust mite-induced allergic airway inflammation in mice. Eur J Immunol, 46(6), 1392-1403.

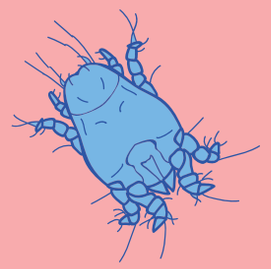
2017 **Tindemans, I.**, Lukkes, M., De Bruijn, M. J. W., Li, B. W. S., Van Nimwegen, M., Amsen, D., KleinJan, A., & Hendriks, R. W. Notch signaling in T cells is essential for allergic airway inflammation, but expression of Notch ligands Jagged1 and Jagged2 on dendritic cells is dispensable. J Allergy Clin Immunol, 140(4), 1079-1089.

2017 **Tindemans, I.**, Peeters, M.J.W., & Hendriks, R. W. Notch Signaling in T Helper Cell Subsets: Instructor or Unbiased Amplifier? Front Immunol, 8, 419.

2017 KleinJan, A., **Tindemans, I.**, Montgomery, J. E., Lukkes, M., de Bruijn, M. J. W., van Nimwegen, M., Bergen, I., Moellering, R. E., Hoogsteden, H. C., Boon, L., Amsen, D., & Hendriks, R. W. (2017). The Notch Pathway Inhibitor SAHM1 Abrogates the Hallmarks of Allergic Asthma. J Allergy Clin Immunol. *In press.*

Curriculum vitae





Irma Tindemans was born on November 7th 1989 in Breda, the Netherlands, and grew up in Mierlo. After completing her secondary education at the Strabrecht College in Geldrop in 2007, she studied Applied Sciences at Fontys University for Applied Sciences in Eindhoven. During her bachelor, she did an internship in the group of prof. Chris Franco at the Department of Medical Biotechnology at Flinders University of South Australia. Here she studied the production of antibiotics by actinobacteria isolated from barley and wheat. She performed her second internship in the Department of Pediatrics at Maastricht University in the group of prof. Boris Kramer, where she worked on the damaging effects of chorioamnionitis on the developing gut of preterm sheep. After obtaining her Bachelor of Applied Sciences degree in 2011, she was admitted to the research master program Infection and Immunity at Erasmus University Rotterdam. During the master program, she performed an internship in the Department of Immunology at Erasmus MC Rotterdam in the group of dr. Wim Dik. In this internship she studied interactions between mast cells and orbital fibroblasts in Graves' Ophthalmopathy. She did her second internship in the group of dr. Janneke Samsom in the Laboratory of Pediatric Gastroenterology and Nutrition where she studied the immunological function of myofibroblasts in celiac disease. After her graduation in 2013, she started her PhD project in the group of prof. Rudi Hendriks in the Department of Pulmonary Medicine, Erasmus MC Rotterdam. In these PhD studies she investigated the function of Notch signaling in house-dust mite-driven T helper 2 cell-mediated allergic airway inflammation in mice as well as in peripheral blood T cells from allergic asthma patients. The results of her PhD project are described in this thesis and will be defended in May of 2018. In October of 2017, Irma returned to the laboratory of Pediatric Gastroenterology and Nutrition to work as a postdoc focusing on inflammatory markers in pediatric patients with inflammatory bowel disease.

Notch signaling during T helper 2 cell-mediated inflammation in allergic asthma