



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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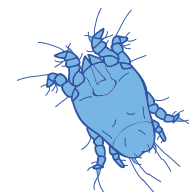
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Chapter 1

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, β_2 -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.

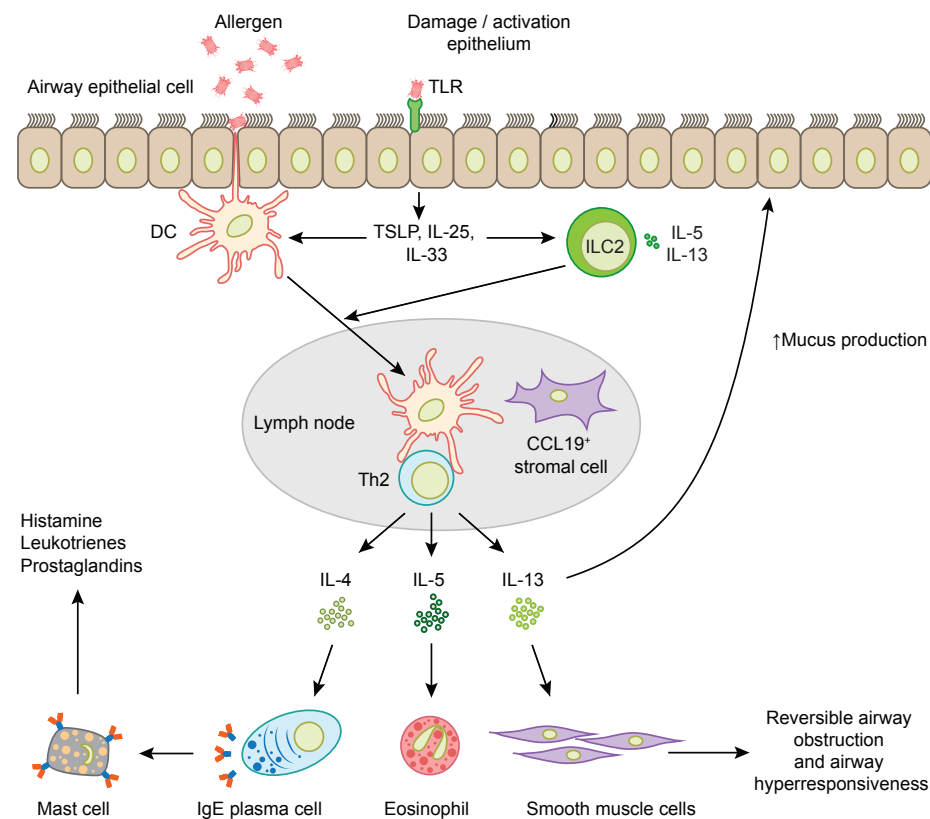


Figure 1. The central role of Th2 cells in allergic asthma

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 inflammation³⁻⁵. 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 cells⁷. 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 of allergic asthma. IL-4 induces Th2 cell differentiation as well as IgE class switching of B cells, development of mast cells and mucous metaplasia⁸. IL-5 is required for the growth, maturation and activation of eosinophils⁹. IL-13 causes smooth muscle hyperreactivity and goblet cell hyperplasia¹⁰ (**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 stimulation¹⁵.

T helper cell differentiation

Activated migratory DCs mature and enter tissue draining lymph nodes where they activate naïve CD4⁺ T cells¹⁶. 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 cells 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 interest¹⁹. 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 source²⁰. Other chemokine receptors that have been implicated in the homing of Th2 cells to the lungs are CCR5, CXCR3, CCR6 and CCR8²¹. 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 cells²³. 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 resolved²¹. Therefore, the differential expression of chemokine receptors can be helpful in identifying distinct T helper subtypes (**Figure 2**).

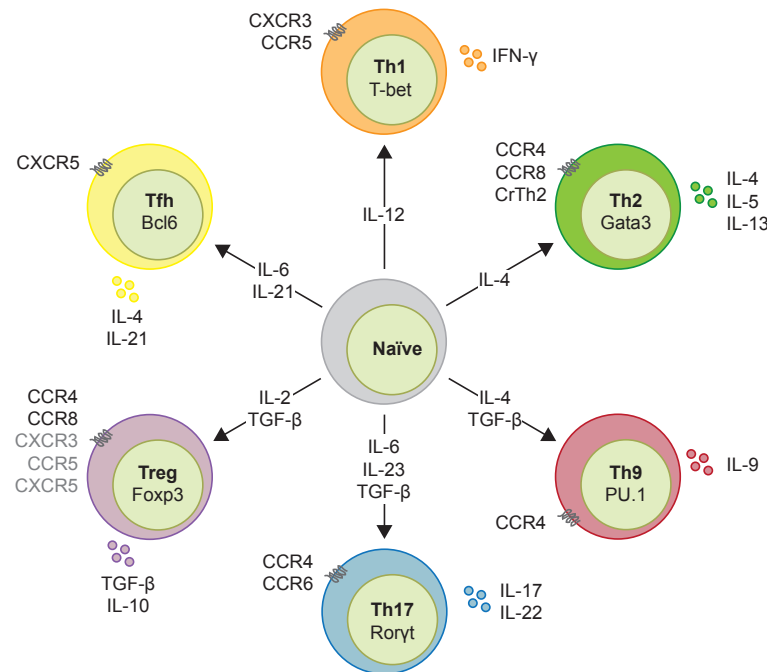


Figure 2. T helper cell differentiation

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 opsonization²⁷. 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 immunity³⁰. 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-4³³⁻⁴⁰. 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 infections⁴³. 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 diseases⁴⁵. Th17 cells play a role in moderate to severe asthma⁴⁷. Th17 cells are induced in response to IL-6, IL-23, and TGF- β ⁴⁸⁻⁵¹. Via STAT3, IL-6 induces expression of retinoic acid receptor-related orphan nuclear receptor gamma (Ror γ t), 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 tolerance⁵⁴⁻⁵⁷. Differentiation of Tregs requires high concentrations of TGF- β , with the absence of proinflammatory cytokines⁵⁸. 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 formation⁶¹. Tfhs are characterized by expression of the transcription factor BCL-6⁶²⁻⁶⁴ and by CXCR5 by which Tfhs are attracted towards CXCL13 present in the B cell zone⁶⁵. Tfh differentiation requires activation of the inducible costimulator (ICOS) and IL-21, IL-6 and STAT3 signaling⁶⁶. Recent studies have indicated that Tfhs, depending on the stimulus, can differentiate into effector cells⁶⁷. 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 Ror γ t 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 reaction⁷⁰.

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 MHCII⁷². 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 tumors⁷³⁻⁷⁵. In contrast, cDC1s were shown to dampen Th2 and Th17 responses⁷⁶⁻⁷⁸.

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 (RBPJ κ ; 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 priming⁷⁹⁻⁸².

pDC development depends on E2-2 and these cells are involved in anti-viral responses by producing type I interferons⁸³⁻⁸⁵. In addition, pDCs were implicated in the induction of Tregs⁸⁶⁻⁸⁸ 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 Fc ϵ RI on the cell surface, are poor at migrating but are involved in locally amplifying inflammation⁸⁰.

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 DCs⁹². 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 Jagged⁹³⁻⁹⁵.

The Notch signaling pathway

The Notch signaling pathway is highly conserved and was first identified 100 years ago in fruit flies⁹⁶. 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 DLL⁹⁹. 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

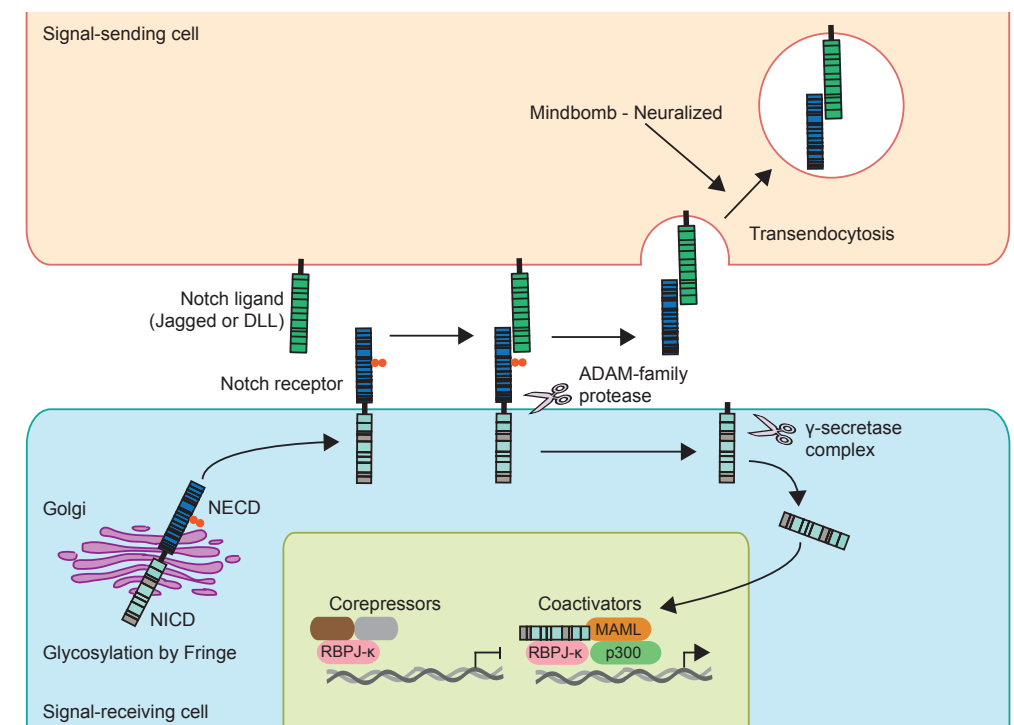


Figure 3. The Notch signaling pathway

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 neutralized 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 RBPJ κ 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; RBPJ κ , Recombination-signal-binding protein for immunoglobulin J κ 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 RBPjk. NICD binding to RBPjk 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 Hairly enhancer of split (Hes) or Hairly 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 PKC θ , NF- κ B, hypoxia, glucose uptake, IL-2R or TGF- β R) and thereby broaden the spectrum of target genes that are influenced by Notch signaling¹⁰⁵⁻¹¹⁵.

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 cells¹¹⁷⁻¹²³. For this, DLL4 expression by thymic epithelial cells is crucial¹²⁴⁻¹²⁶. Notch1 is especially required to restrict developing $\alpha\beta$ T cells to the T cell lineage during early developmental stages up to the double-negative 3 (DN3) stage¹²⁷. In this CD44⁺CD25⁺DN3 stage, first the TCR β gene locus is rearranged and functionality of the TCR β 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 TCR α 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 signaling¹²⁸.

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 ligation¹³⁸. Also, Th17 cell differentiation is decreased when Notch signaling is blocked and the *Rorc*, *Il17* and *Il23r* gene promoters are identified as direct Notch targets¹³⁹⁻¹⁴³. 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, respectively⁹³. 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 outcomes¹³⁵. 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.

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

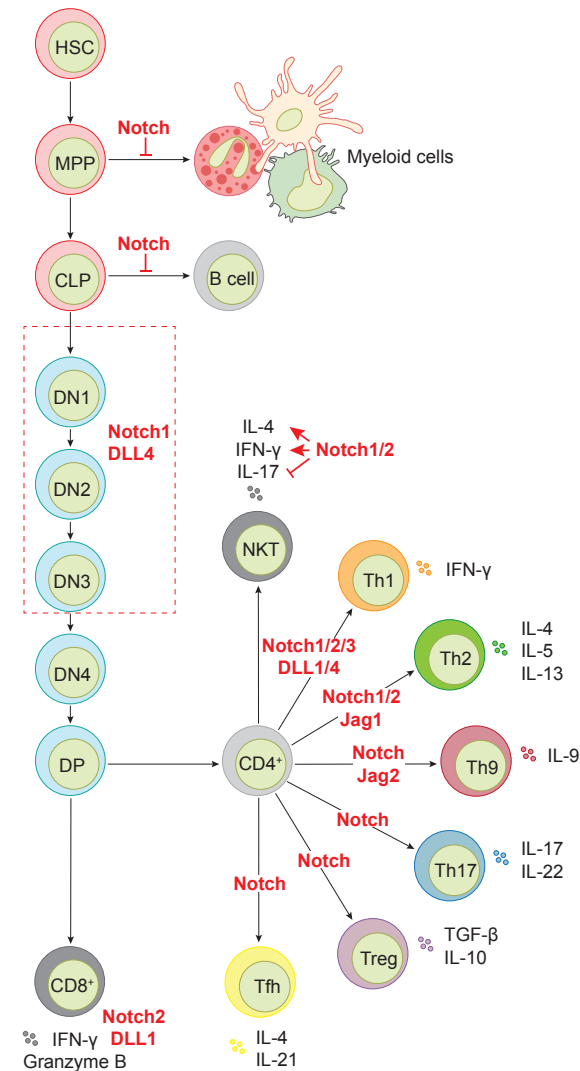


Figure 4. The function of Notch signaling during T cell development, differentiation and function

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.

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^{153, 153}. It was shown in mice with a conditional deletion for Notch1 and Notch2 or RBPjk that Notch signaling coordinates NKT cell differentiation and function by positively regulating IFN- γ and IL-4 expression, and impairing IL-17 secretion^{154, 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 expression¹⁵⁵. In contrast to T cells, ILCs lack antigen-specific receptors. ILC2 are dependent on ROR α 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 DLL1¹⁵⁶⁻¹⁵⁸. This has however not yet been confirmed *in vivo*. Natural killer (NK) cells belong to the group 1 ILCs and have cytotoxic activity and functions¹⁵⁵. 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 cells¹⁵⁹⁻¹⁶⁵. 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 ROR γ ¹⁵⁵. Notch1 and Notch2 are required for the generation of fetal α 4 β 7⁺ LTi cell progenitors before upregulation of ROR γ t. However, Notch signaling has to be subsequently downregulated again to allow the expression of ROR γ t and the final maturation of LTi cells¹⁶⁶. IL-22 producing NKp46⁺ ILC3 are, like LTi cells, dependent on ROR γ t¹⁵⁵. The differentiation of NKp46⁺ ILC3 into NKp46⁺ ILC3 was shown to depend on DLL1 signals¹⁶⁷⁻¹⁶⁹. 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 patches¹⁷⁰.

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 cells¹⁷³⁻¹⁷⁶, 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 pathogens¹⁷⁷⁻¹⁸¹. In addition, Notch signaling via DLL1 is required for optimal DC maturation and activation since RBPjk 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 asthma¹⁸³.

During macrophage activation, Notch signaling was shown to induce transcription of proinflammatory cytokines including IL-1 β , IL-6, TNF- α and IL-12¹⁸⁴⁻¹⁸⁶. Lastly, *in vitro* studies have implied functions for Notch in the differentiation of granulocytes¹⁸⁷ and Notch signaling is required for *in vitro* survival, differentiation and cytokine production by basophils¹⁸⁸.

In addition to its role in immune cell differentiation, Notch also drives lung organogenesis and alveologenesis¹⁸⁹. 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 cells¹⁹⁰. While some studies describe that Notch signaling induces goblet cell differentiation¹⁹¹, others found an inhibitory role for Notch in the differentiation of secretory cells into goblet cells¹⁹².

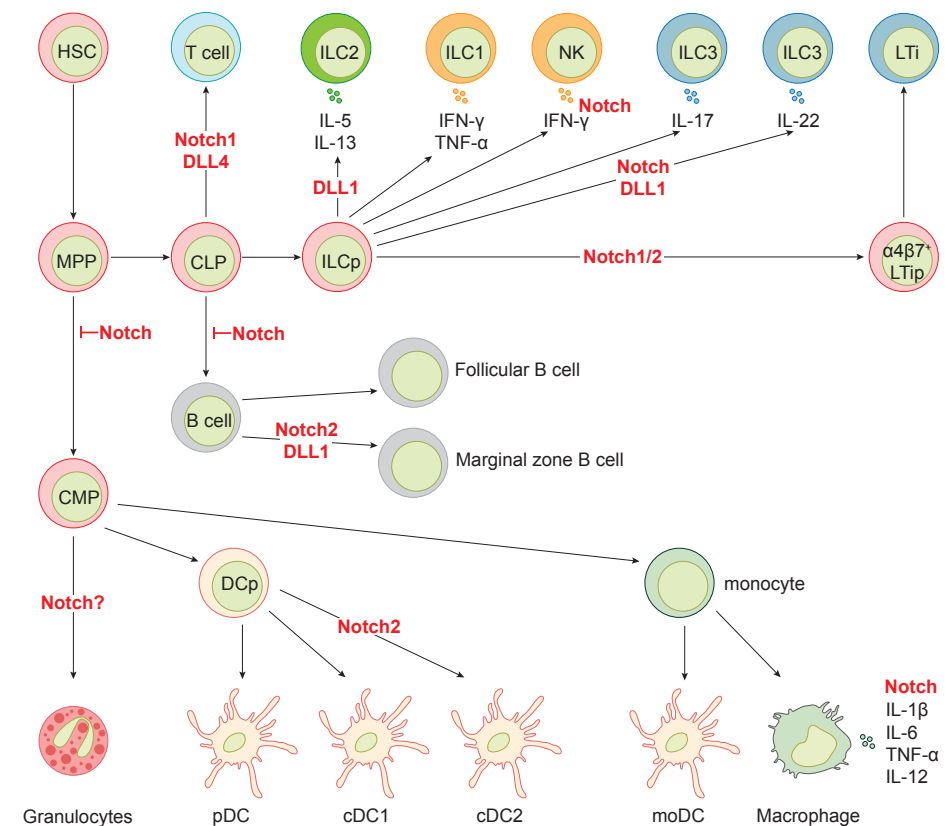


Figure 5. The role of Notch signaling during lymphoid and myeloid cell development and function

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 mice¹⁹³. 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- κ B and NFAT¹⁹⁶. 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 allergies¹⁹⁷. 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 cadherins¹⁹⁸. Synthetic, cell-permeable stabilized peptides that specifically target the Notch transactivation complex¹⁹⁹⁻²⁰¹, as well as specific antibodies targeting Notch receptors²⁰²⁻²⁰⁴ 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 RBPJ κ 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 addition²³¹⁻²³³.

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

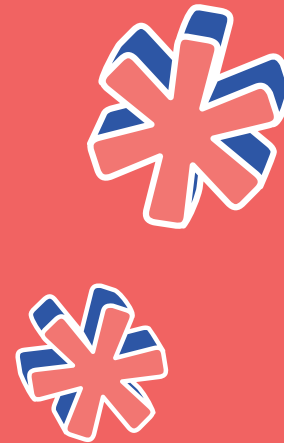
asthma models was sufficient to decrease Th2 cytokine production^{132, 133}. Effects of GSI are not limited to Notch signaling and can induce unwanted side-effects including gastrointestinal toxicity²³⁴. Therefore, we aimed to investigate whether cell-permeable stabilized peptides that more specifically 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 SAHM1¹⁹⁹⁻²⁰¹ 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**.



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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 transcription²³⁷. 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 production²³⁸⁻²⁴¹. 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 cytokines²⁴²⁻²⁴⁴. 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 function²⁴⁵⁻²⁴⁷. Beyond the T cell lineage, GATA-3 is also involved in hematopoietic stem cell (HSC) self-renewal and maintenance²⁴⁸⁻²⁵⁰ 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 cells²⁵³. 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 subsets²⁵⁹⁻²⁶¹, 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 Cys⁴ 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/C), 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 embryos²⁶⁷ 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 cells²⁶⁸.

GATA-1 is critically involved in the development of erythrocytes, megakaryocytes, mast cells, dendritic cells (DC), basophils and eosinophils (**Figure 1**). GATA-2 is indispensable 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 marrow²⁶⁹. 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 specification²⁷¹. 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 acetyltransferase [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 <i>Il5</i> 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 hematopoiesis²⁷². 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 dams²⁷³. 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 mutations²⁷⁴. 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 cancer²⁷⁶.

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 gene²⁷⁷, 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 p38²⁸¹. Finally, it is known that the acetylation status of GATA-3 affects its transactivation ability. The GATA-3 mutant KRR-GATA-3 is hypoacetylated and shows hypomorphic functions, resulting in reduced T cell survival and altered lymphocyte homing²⁸².

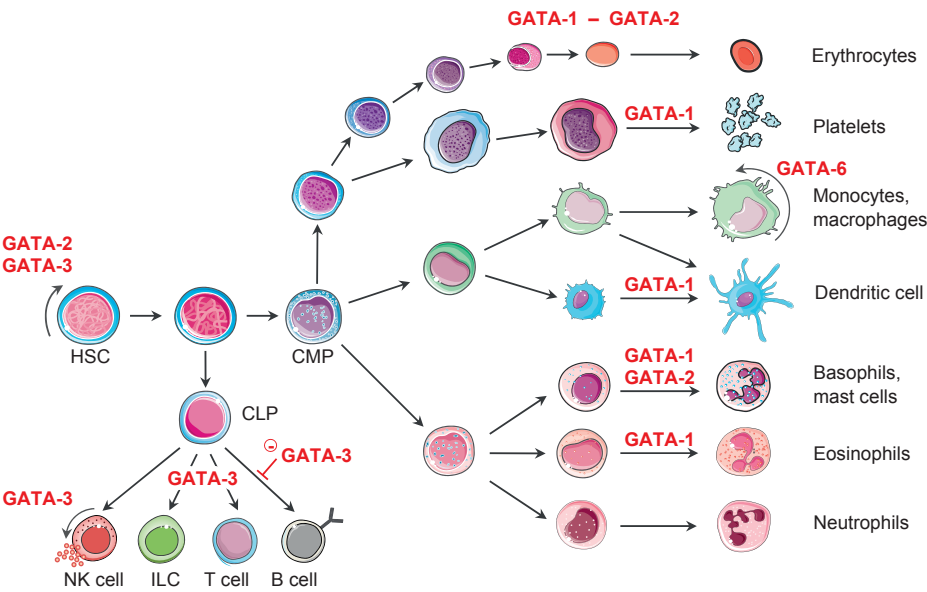


Figure 1. Function of GATA factors in hematopoietic development. Overview of hematopoiesis, showing the main lineage commitment steps from HSC to fully matured and functional blood cells. GATA transcription factors that are required for these processes are indicated in red: GATA-1 and GATA-2 are important 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 protein²⁸³. 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 region²⁸⁵. 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 directly²⁸⁶ or via the transcription factor Dec2²⁸⁷. 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 cells²⁹². 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 differentiation²⁹³. 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 reviewed¹⁴⁶. 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, respectively⁹³. 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 Jk region (RBPjk). In the absence of GATA-3, Notch no longer induces Th2 cells but instead induces Th1 cell differentiation. Mice lacking RBPjk or the Notch1 and Notch2 receptors fail to generate robust Th2 responses to parasite antigens¹²⁹. 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

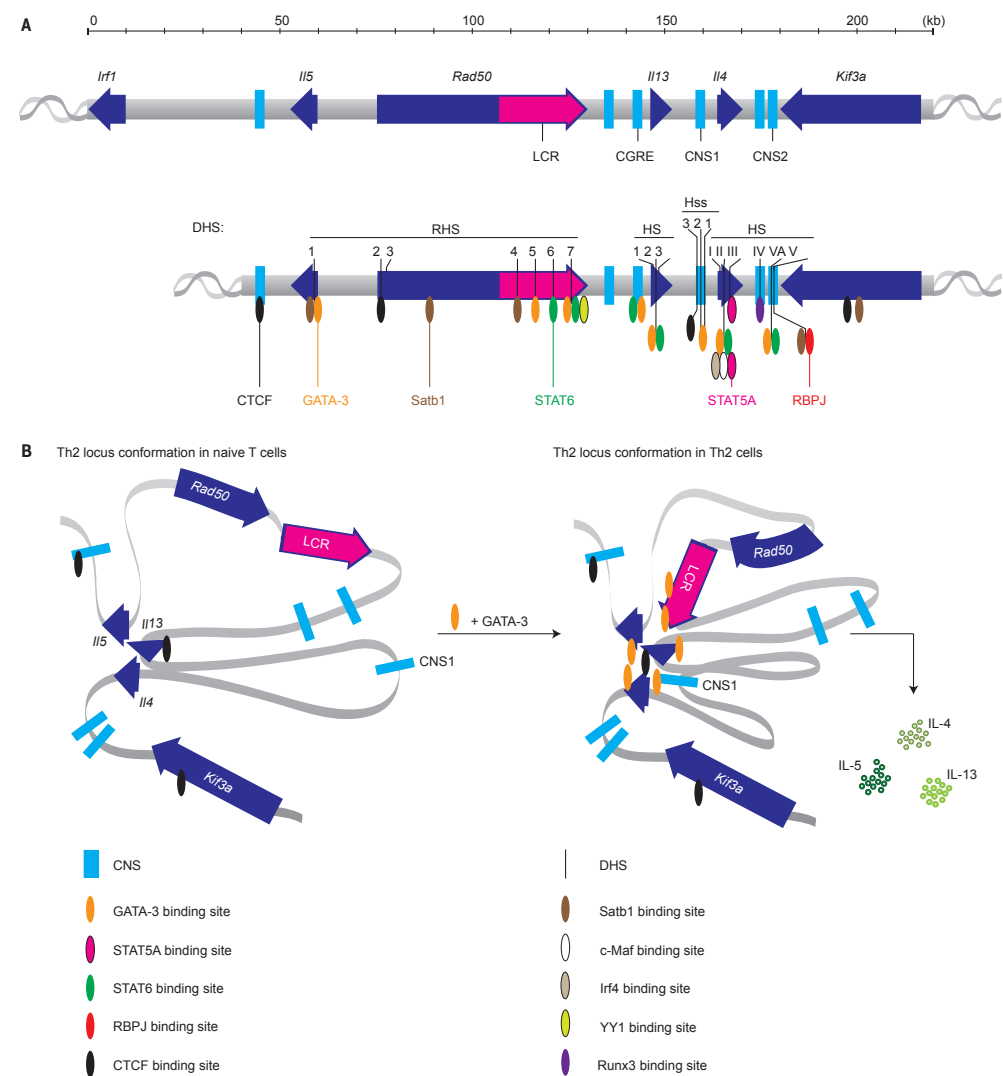


Figure 2. GATA-3-mediated regulation of the Th2 cytokine locus.

[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* gene³⁷⁵. 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, HSIVa 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* gene³⁷⁹ in a STAT6-dependent manner³⁰⁵. 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 naive 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 CTCF³⁸⁰. 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; RBPjk, recombination-signal-binding protein for immunoglobulin Jk 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-3²⁶⁰. 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 TGF β alone¹³⁸.

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-12R β 2 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-12R β 2 mRNA²⁶⁰. 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) infection²⁹⁷⁻³⁰⁰. 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, ROR γ t, 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* gene³⁰². GATA-3 can induce the latter DHS in Th1 cells, which clearly demonstrates that GATA-3 is associated with chromatin remodeling activity³⁰³. 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 remodeling²⁶⁰. 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 configuration²⁸³. GATA-3 and STAT6 have the capacity to directly remodel the LCR³⁰⁵ 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 cohesin³⁰⁶⁻³⁰⁹ (**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 cells³¹⁰, 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 mice²⁷², 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 marrow³¹³. 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 Bcl11b³¹⁴. 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 triggering²⁵² (**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 commitment³¹⁸. 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 development²⁵¹. In the absence of Ebf1, lymphoid progenitors exhibit increased T cell lineage potential associated with increased *Gata3* gene transcription²⁵¹. Ebf1 ablation can divert lymphoid precursors into the ILC pathway as well³¹⁹. As such, the relative balance between GATA-3 and Ebf1 pathways critically determines the B versus T cell or ILC cell fate decision.

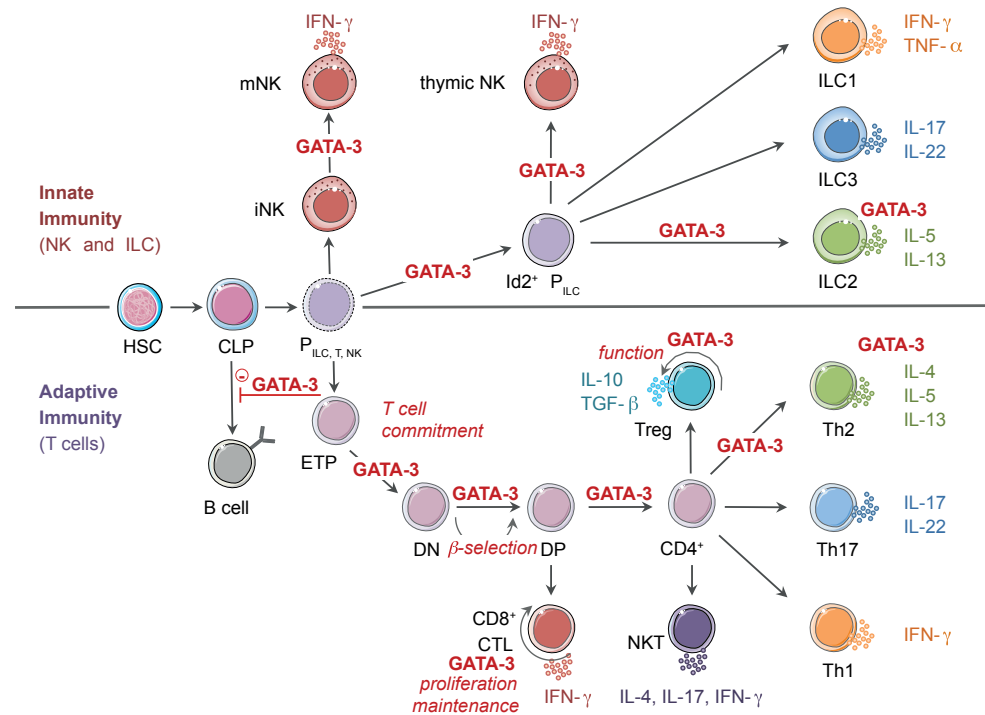


Figure 3. GATA-3 has multiple roles in ILC and T cell development and function

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 is 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 (TCR β)-expressing DN4 thymocytes³²⁰. This is consistent with findings in GATA-3 reporter mice that demonstrate up-regulation of GATA-3 during pre-TCR-mediated β -selection³⁴¹. 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 stages³²³. 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 choice³²⁰. 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 inefficiently³²⁴. 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 ThPOK³²⁴. 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 CD3 δ and CD3 ϵ 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 production²⁴⁶ (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 differentiation³³⁰.

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-3²⁴⁸, 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 differentiation²⁵⁰. 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 system²⁴⁹.

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 subsets²⁵⁷. 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 Ror γ t 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 homeostasis²⁵⁸.

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-7R α ⁺ NK cells²⁵³ 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 gut²⁵⁶, but not in the liver³³⁶. 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 cells²⁵⁴. 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 humans³³⁹ 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-7R α , 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 man³⁴¹ (**Figure 3**). ILC2 are generated from CLP *in vivo*^{256, 257} 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 number²⁴⁰. The transition from CLP to ILC2 is associated with up-regulation of the transcription factors inhibitor of DNA-binding 2 (Id2) and ROR α , 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 precursors³²³, inactivation of GATA-3 in mature ILC2 did not affect the expression of Id2 or ROR α ²⁴¹. *In vitro* generation of ILC2 from CLP is dependent on Notch signaling^{156, 257}, 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 development³⁴⁴, with Tcf1 being a Notch1-induced target for T cell specification³¹⁶ 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 ILC2³⁴⁶. 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 asthma³⁴⁷. Moreover, ILC2-derived IL-13 can promote migration of activated lung DCs that drive differentiation of naïve T cells into Th2 cells¹³.

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 clusters³⁴⁸. 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 defense³⁴⁹. 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 inflammation³⁵⁴⁻³⁵⁶. 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 LT α cells and T-bet $^{+}$ ILC3²⁴¹. 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 infection²³. Conditional *Gata3* ablation using Vav1-Cre generates a similar defect in ILC3 development²⁴¹. Moreover, in the fetus GATA-3 is critical for differentiation of CD135 $^{+}$ α 4 β 7 $^{+}$ CLP-like cells and cell-intrinsic GATA-3 expression is essential to generate fetal liver ROR γ t ^{hi} IL7R α ^{hi} precursor cells²⁵⁵. 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 ILC3²⁴¹. 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 homeostasis²³⁹.

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 α 4 β 7 $^{+}$ fetal liver precursor (Lin $^{-}$ IL-7R α $^{+}$ Sca-1 ^{intc} -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 α 4 β 7 $^{+}$ Lin-IL-7R α $^{+}$ bone marrow subset exists, although it includes more mature ILC2^{239, 240}. Recently, two reports identified that this α 4 β 7 $^{+}$ 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 $^{+}$ α 4 β 7 $^{+}$ cells co-expressed GATA-3 protein, suggesting a link between GATA-3 expression in these ILC precursors and their cell fate potential²⁵⁴.

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 RBPjk, Id2, Tcf1, Nfil3, T-bet or ROR γ 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-1³⁶². 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, respectively³⁶³. 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 motifs²⁶⁰. 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 DNA³⁶⁴. 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 motifs²⁶¹. 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 cells³⁶⁵. 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 Ror γ t 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 expression²⁴⁵. 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 binding³⁰⁸. 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 cells²⁶⁰. 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 elements²⁶¹. 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 significant 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*, *Il1h* (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-33R³⁶⁸.

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 *Ilhl1*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, ROR α , ROR γ , 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 mutations³⁷⁰. Likewise, recent genome-wide germline SNP analysis identified *GATA3* gene variants that influence susceptibility to Philadelphia chromosome-positive acute lymphoblastic leukemia and risk of relapse³⁷¹. It will be important to understand how dysregulated GATA-3 influences neoplastic transformation in hematopoietic cells and non-hematopoietic lineages²⁷⁵.

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 sensors³⁷². 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 nucleus⁴⁹, could be an excellent starting point for drug discovery strategies.

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Chapter 3

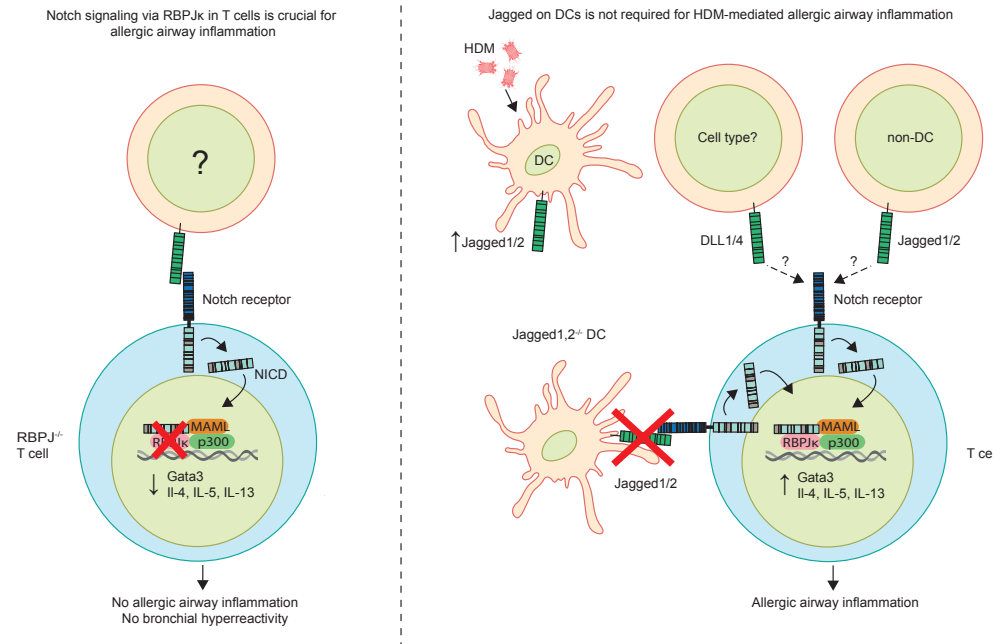
Notch signaling in T cells is essential for allergic airway inflammation, but expression of Notch ligands Jagged1 and Jagged2 on dendritic cells is dispensable

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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 RBPJ κ , 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, RBPJ κ -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 exposure³⁸⁵. 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 Gata3²⁸⁵. 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 J κ region (RBPJ κ) 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 RBPJ κ or the Notch1 and Notch2 receptors¹²⁹ and in mice expressing dominant-negative MAML¹³¹. 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 model¹³³.

Several lines of research support that Notch ligands Delta-like ligand (DLL) and Jagged instruct Th1 and Th2 cell differentiation, respectively²⁰⁷. 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 generation⁹³. 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 cells²²⁰, 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)DCs²⁰⁸. 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}* mice³⁸⁹, *Jag2^{fl/fl}* mice³⁹⁰ and Rosa26-stop-EYFP reporter (ROSA^{EYFP}) (Jackson Laboratories, Bar Harbor, ME, USA) mice were bred with CD11c-cre transgenic mice¹⁷⁷. RBPJ^{K^{fl/fl}} mice³⁹¹ were crossed with CD4-cre transgenic mice³⁹². 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 described³⁴⁷. 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 described³⁹³. 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^{dCD11c/dCD11c}*) 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'-cgctcatcctttcagttcg-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'-gtccatacaggatgcaatgt-3', probe 3). mRNA levels of genes of interest were normalized to glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) (forward: 5'-TTCACCAACCATGGAGAAGGC-3', reverse: 5'-GGCATGGACTGTGCTCATGA-3', probe TGCATCCTGCACCACTG). 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^{ΔCD11c}* 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 described⁸⁰. 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 before³⁹⁴. 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 described³⁹⁵. For immunoglobulin analysis, blood was drawn from the tail vein 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 previously³⁹⁶. 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 H₂O. 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% NaN₃ in PBS as described previously³⁹⁷. 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 (golgi stop, 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 immunoassorbent 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 described³⁹⁸.

Levels of TNP-KLH specific IgM, IgG1 and IgG2a were determined as described³⁹⁹. 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

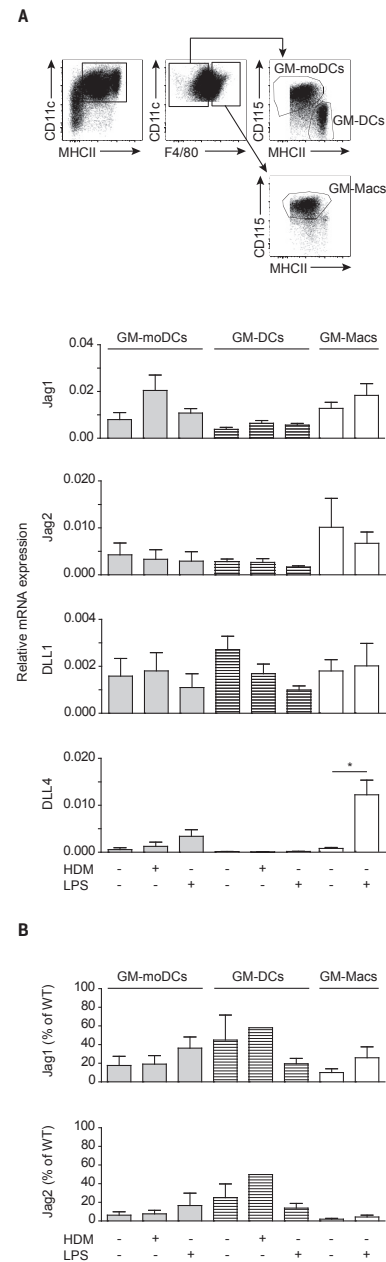
Because several research groups have shown a role for Jagged in the orchestration of T cell responses by employing GM-CSF bmDCs^{93, 208-210, 387}, we first investigated the expression of Notch ligands on bmDCs upon stimulation with the pro-Th2 stimulus HDM and the pro-Th1 stimulus LPS. GM-CSF bmDCs 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^{ΔCD11c/ΔCD11c}*) 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^{ΔCD11c/ΔCD11c}*, compared with *Jg1Jg2^{+/+}* mice (**Supplementary figure 1B**).



To confirm that Jagged expression on DCs is essential for allergic airway inflammation (AAI) induction by intratracheal transfer of allergen-pulsed GM-CSF bmDC, we sensitized WT mice with HDM-pulsed total GM-CSF bmDCs from *Jg1Jg2^{ΔCD11c/ΔCD11c}* 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^{ΔCD11c/ΔCD11c}* 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^{ΔCD11c/ΔCD11c}* 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^{ΔCD11c/ΔCD11c}* 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

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^{ΔCD11c/ΔCD11c}* 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.

required during the challenge phase of AAI induction, *Jg1Jg2^{ΔCD11c/ΔCD11c}* or *Jg1Jg2^{+/+}* mice were sensitized with WT GM-CSF bmDCs and challenged with HDM. We found comparable AAI induction in *Jg1Jg2^{ΔCD11c/ΔCD11c}* 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.

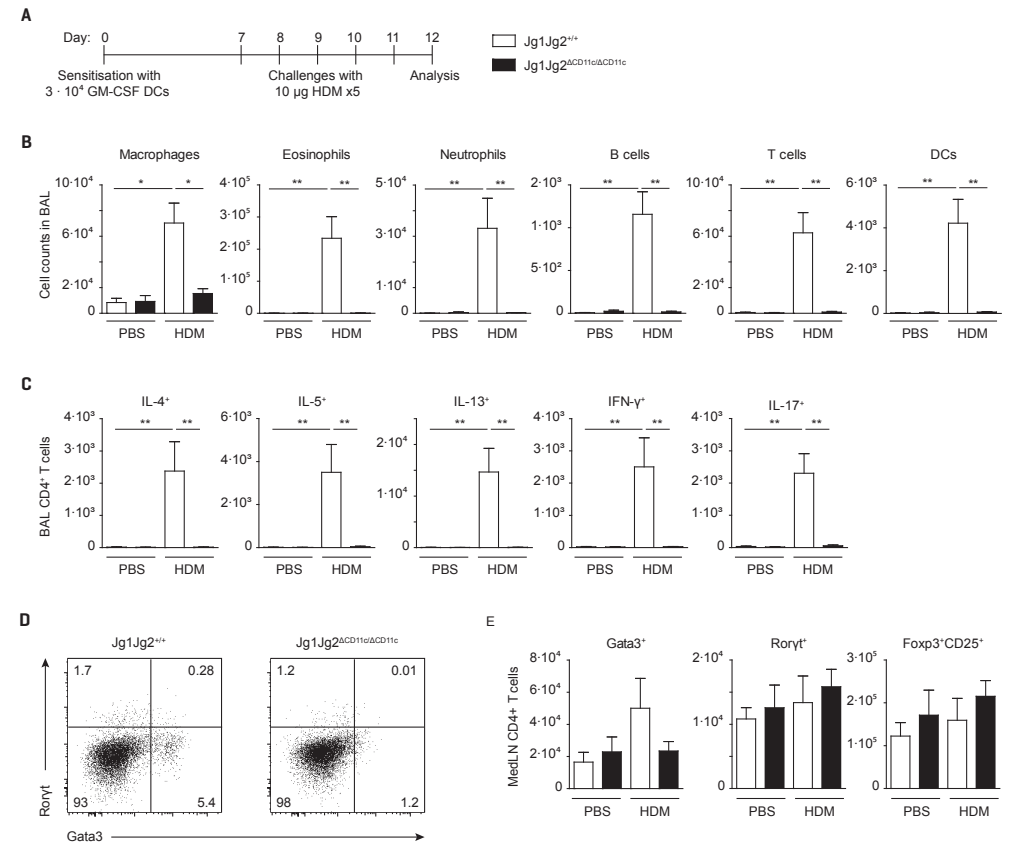


Figure 2. Jagged1 and Jagged2 are crucial during the sensitization phase when using GM-CSF bmDCs to induce AAI

(A) Sensitization and challenge scheme of HDM-driven AAI in mice, using cultured total bmDCs. (B) Numbers of macrophages [FSC^{high}SSC^{high}auto-fluorescent⁺CD11c⁺Siglec-F⁺], eosinophils [FSC^{int}SSC^{high}Siglec-F⁺], neutrophils [Ly-6G⁺], B cells [CD19⁺], T cells [CD3⁺], DCs [CD11c⁺MHCII⁺] in BAL in mice treated with either PBS-pulsed or HDM-pulsed bmDCs from *Jg1Jg2^{ΔCD11c/ΔCD11c}* 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^{ΔCD11c/ΔCD11c}* 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^{ΔCD11c/ΔCD11c}* 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 phase⁸⁰. 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).

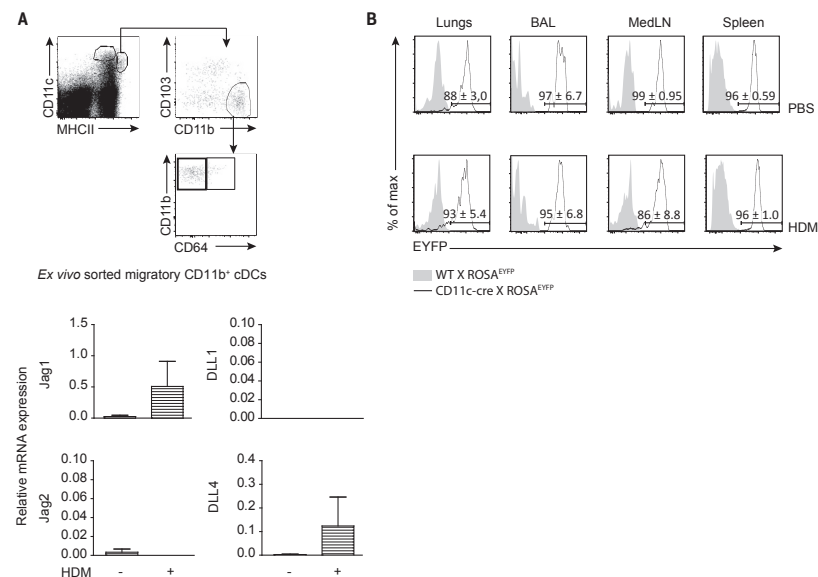


Figure 3. Jagged1 is upregulated on migratory CD11b⁺ cDCs upon HDM exposure and CD11c-cre is effective in *in vivo* DCs
 [A] Gating strategy of *ex vivo* sorted DC subsets from C57BL/6 mice that were i.n. treated with 50 µg HDM or PBS [top]. mRNA expression of the indicated Notch ligands, as determined by qRT-PCR, in DAPI⁺MHCII⁺CD11b⁺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⁺ 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.

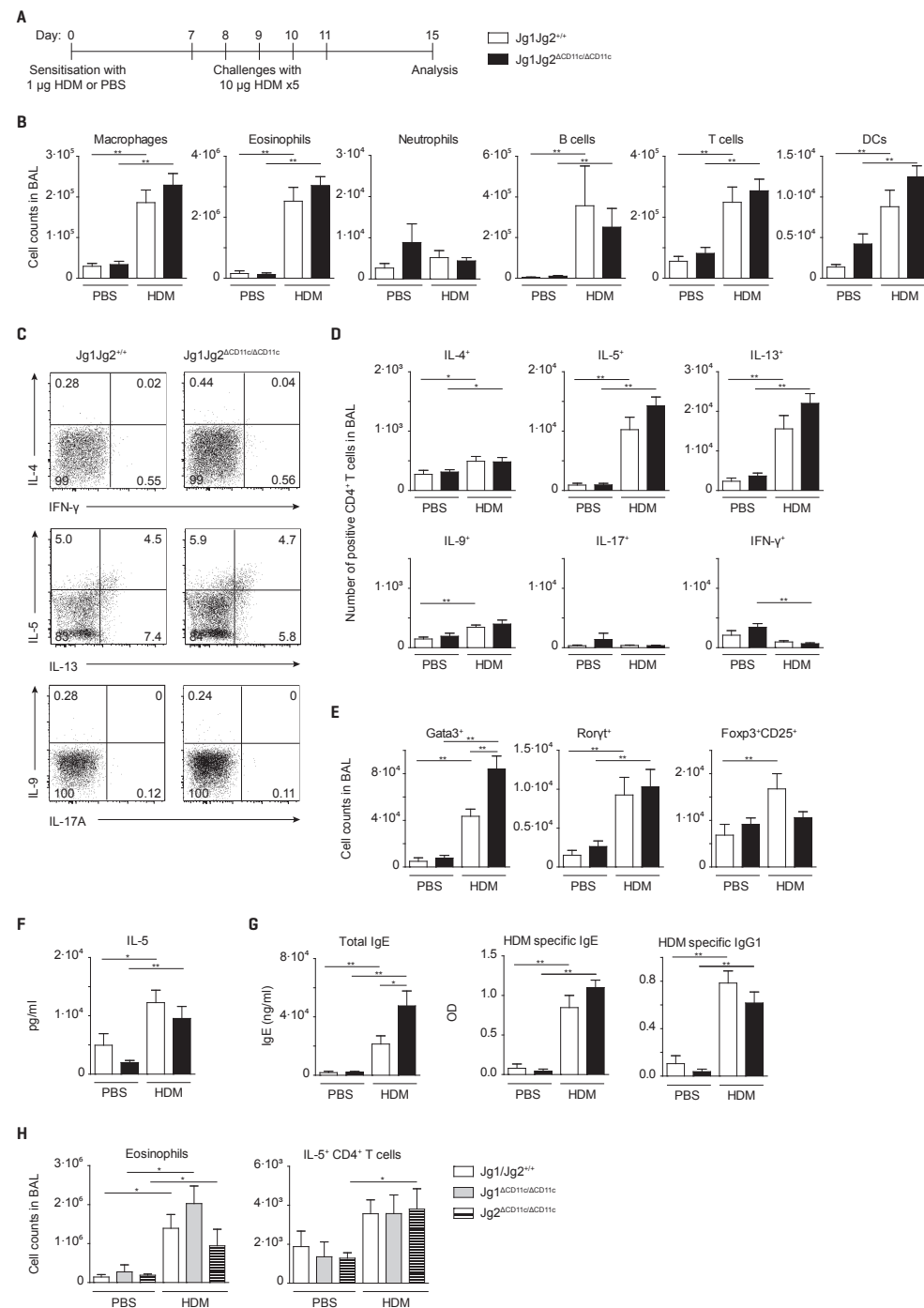


Figure 4. Jagged1 and Jagged2 expression on DCs is dispensable for the development of AAI *in vivo*

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^{ΔCD11c/ΔCD11c} and Jg1Jg2^{+/+} mice. Likewise, no differences were found in T cell-dependent B cell responses, because total or high affinity TNP-KLH-specific IgM, Th2-driven IgG1 and Th1-driven IgG2c levels were similar in Jg1Jg2^{ΔCD11c/ΔCD11c} 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 RBPJκ⁹³ were studied. We exposed CD4-Cre transgenic RBPJκ^{fl/fl} mice (termed RBPJκ^{ΔCD4/ΔCD4}) 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^{ΔCD4/ΔCD4} 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^{ΔCD4/ΔCD4} 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^{ΔCD4/ΔCD4} 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^{ΔCD11c/ΔCD11c} or Jg1Jg2^{+/+} mice.
(C) Intracellular flow cytometric 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^{ΔCD11c/ΔCD11c} 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^{ΔCD11c} and Jg2^{ΔCD11c} 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.

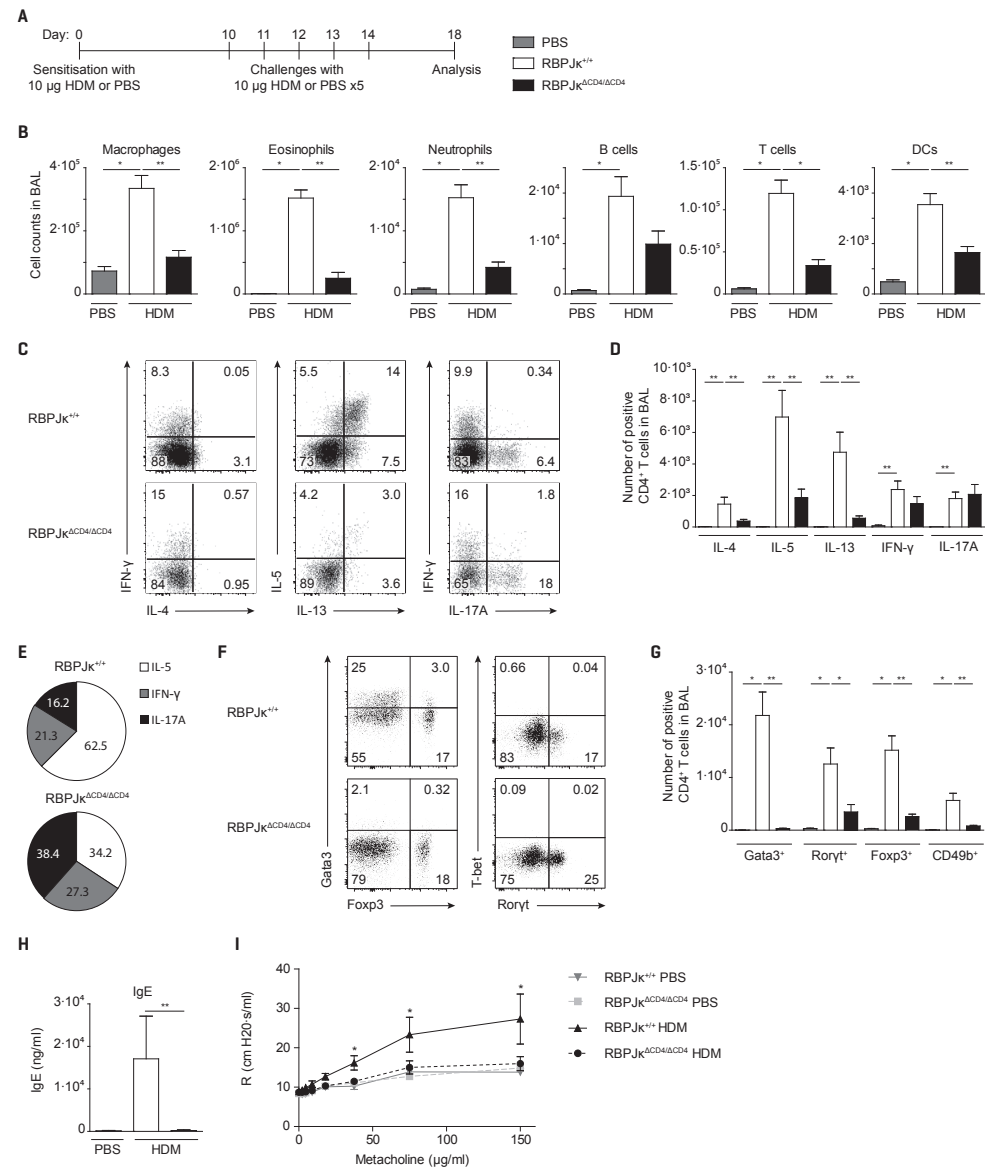


Figure 5. Notch signaling in CD4⁺ T cells is crucial for the induction of AAI

(A) Scheme of HDM-mediated AAI induction in RBPJk^{ACD4/ACD4} and RBPJk^{+/+} mice.
 (B) Total numbers of the indicated cell populations in BAL from PBS- or HDM-treated mice.
 (C-E) Intracellular flow cytometric 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 OVA¹³³. 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 bmDCs²⁰⁸, 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 macrophages⁴⁰⁰. 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 responses²⁰⁷. 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, respectively⁹³. 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 proliferation⁸⁰. 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^{ΔCD11c/ΔCD11c}* 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) cells⁴⁰³, which play an important role in AAI by secreting IL-4 and IL-2^{334, 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 allergen²¹⁷, and when DLL4-pretreated bmDCs stimulated with OVA were adoptively transferred to induce AAI²¹⁸. On the other hand, Th2 responses were decreased when DLL4 was neutralized *in vivo* in a mouse model for RSV-mediated allergic asthma exacerbations⁴⁰⁶. 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.

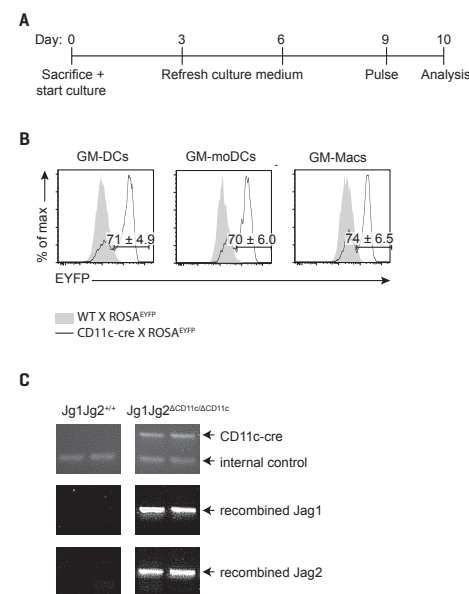
In summary, we showed that Notch signaling is crucial for the induction of HDM-mediated eosinophilia, Th2 responses and airway hyperreactivity *in vivo*, indicating that Notch on T cells could be a potential therapeutic target in allergic asthma patients. In addition, our data indicate that there is 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

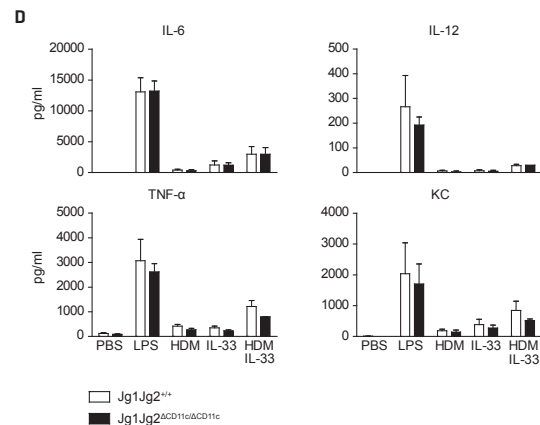
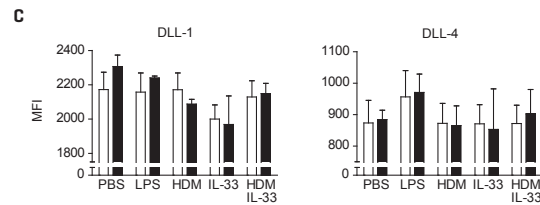
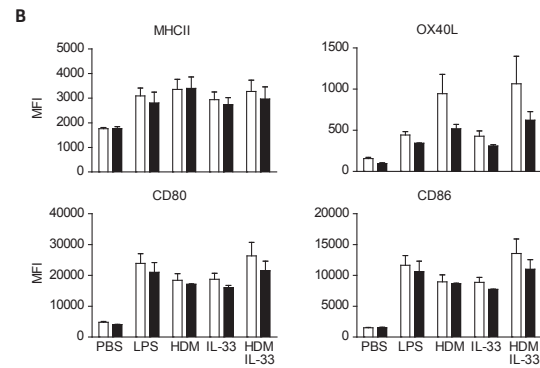
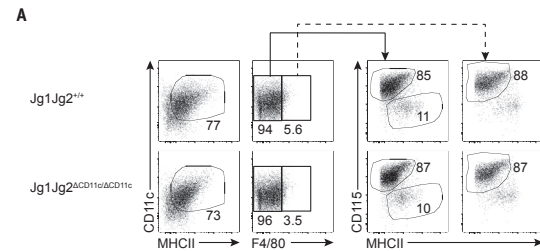


Supplementary figure 1. Generation of Jagged deficient bmDCs

(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.



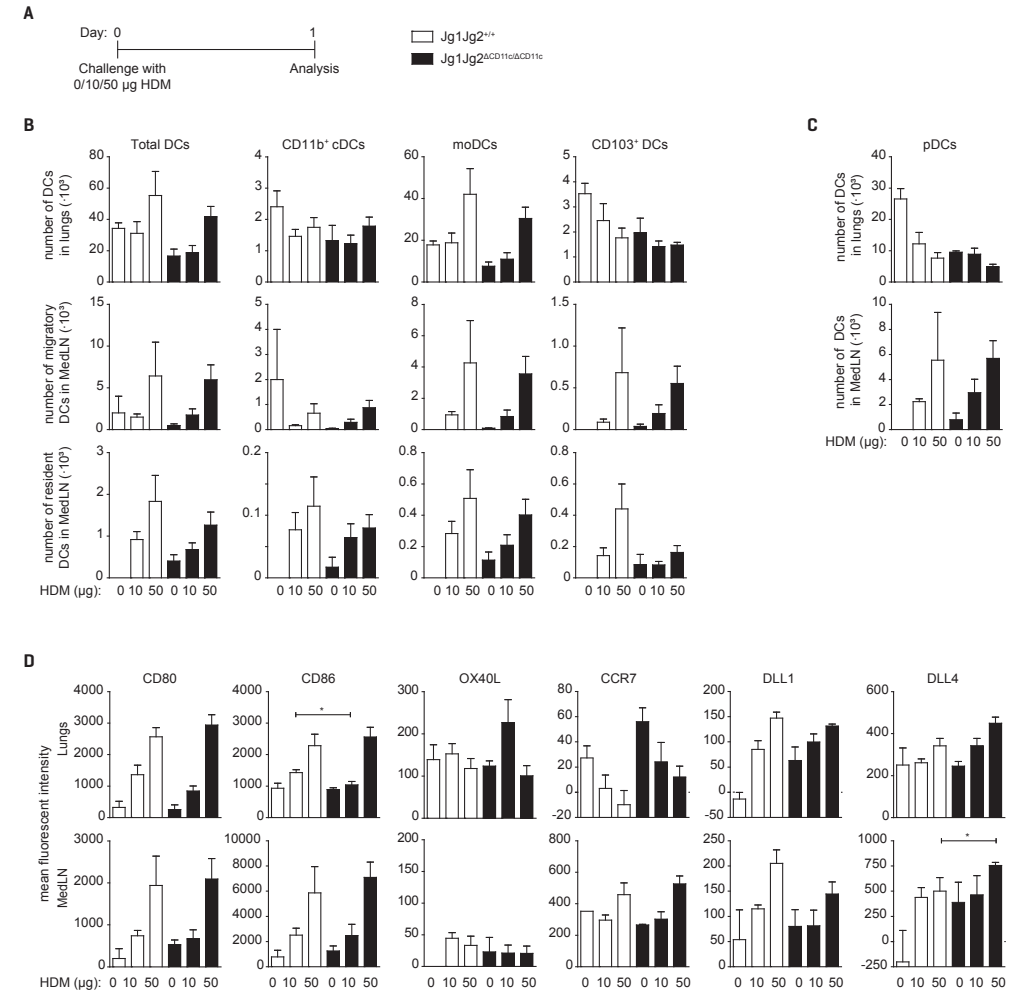
Supplementary figure 2. BmDCs from Jg1Jg2^{ΔCD11c/ΔCD11c} and Jg1Jg2^{+/+} mice are similar in expression of co-stimulatory molecules, DLL ligands and pro-inflammatory cytokines

[A] Flow cytometric gating strategy for bmDC subsets from Jg1Jg2^{ΔCD11c/ΔCD11c} 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^{ΔCD11c/ΔCD11c} and Jg1Jg2^{+/+} bmDCs, using Mann-Whitney U-tests.



Supplementary figure 3. DCs from Jg1Jg2^{+/+} and Jg1Jg2^{ΔCD11c/ΔCD11c} mice are similar in numbers and expression of co-stimulatory factors in an innate response to HDM

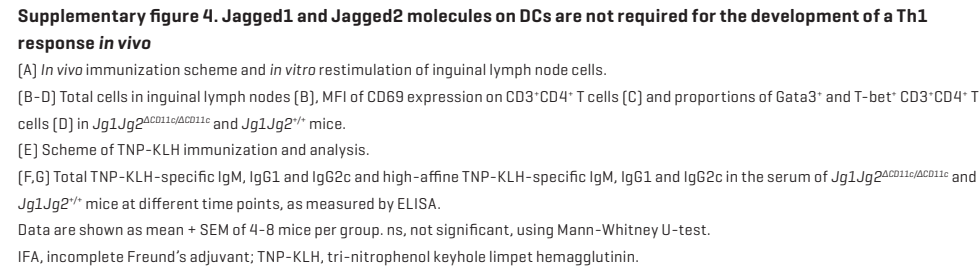
[A] Scheme of innate DC activation: Jg1Jg2^{+/+} and Jg1Jg2^{ΔCD11c/ΔCD11c} mice were i.n. challenged with the indicated amounts of HDM and analyzed one day later by flow cytometry.

[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⁺] 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^{int}) 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^{ΔCD11c/ΔCD11c} mice. Differences were not significant unless indicated; * p < 0.05, using Mann-Whitney U-test.



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	eBio103
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	XM61.2
IFN-γ	PE-Cy7	eBioscience	XM61.2
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	D9302C12
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
Roryt	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

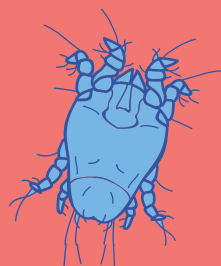
Supplementary Table 2. CD11c-cre activity in *in vivo* DCs

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.

4

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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-acid-related orphan receptor γ 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-4⁹³. 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 Ig κ region (RBPj) in T cells¹⁰⁸. 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 cytokines¹³⁵, potentiates T cell receptor and CD28 signaling, and stimulates metabolic reprogramming and IL-2 secretion during priming of naïve T cells¹⁰⁸. 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 cells¹⁰⁸. RBPj in T cells affected the ability of Th17 cells to adequately respond to IL-23¹⁴¹.

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 maturation⁴¹¹.

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 model¹³³. 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-103⁴¹². 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 0³⁴⁶. Ten days later, anesthetized mice were challenged with 10 µg of HDM in 50 µL 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 described³⁴⁶. 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 described⁴⁷. 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).

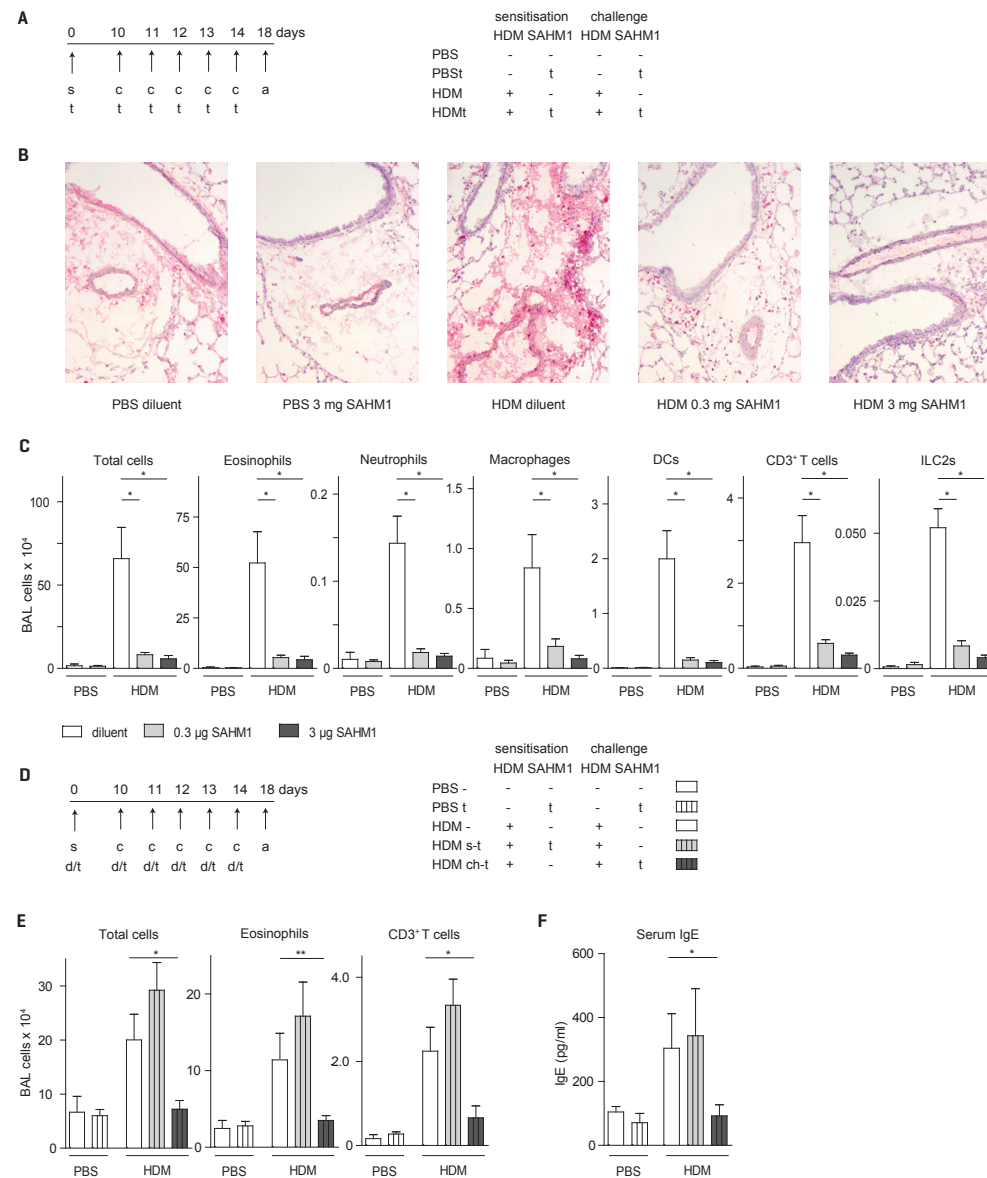


Figure 1. SAHM1 abrogates eosinophilic airway inflammation.

(A) Experimental HDM-driven asthma design, showing intratracheal sensitization [s] and challenge [c] of 10 μ g 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 [t]. 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 $\pm$ 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 μ g 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 $\pm$ 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 D1¹⁹⁹ 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 γ t (Roryt)⁺ and forkhead box P3 (Foxp3)⁺ T cells in BAL fluid (Figure 3). For Roryt 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 whether SAHM1 enhances the recovery after establishment of asthma.

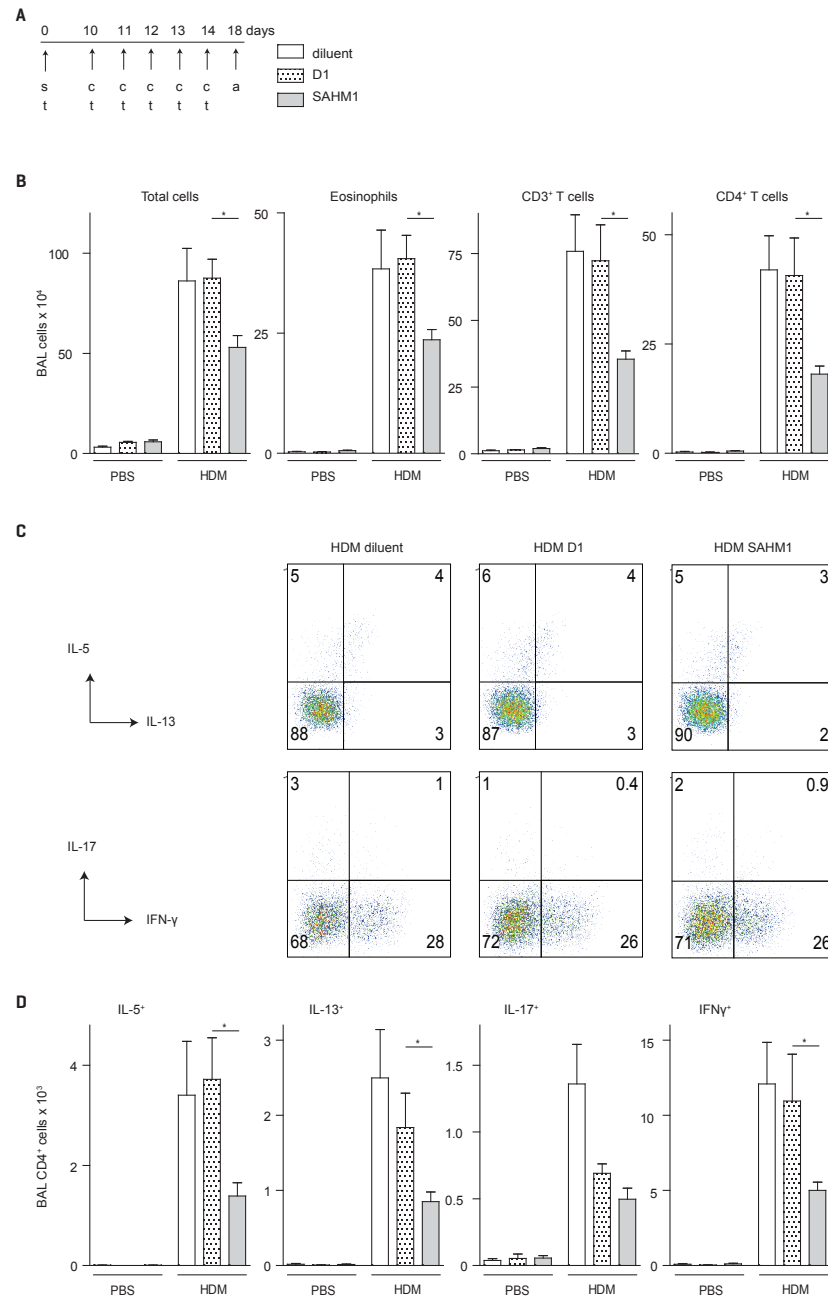


Figure 2. SAHM1, but not the control peptide D1, abrogates eosinophilic airway inflammation.

[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 3 mg of SAHM1. PBS admixed with diluents, 3 mg 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.

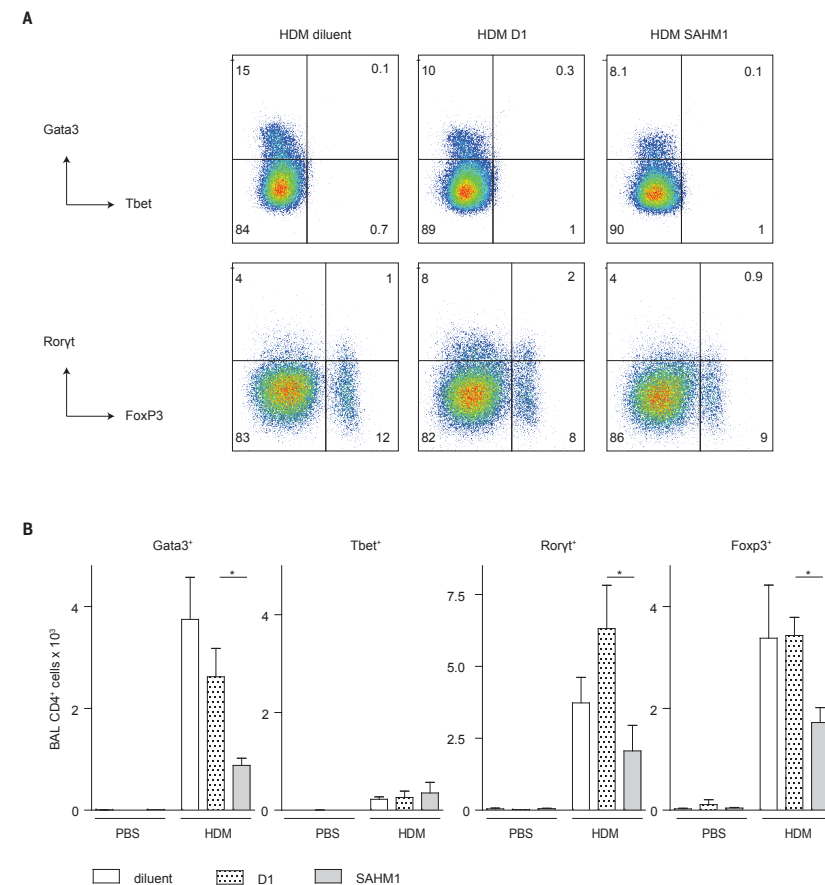


Figure 3. SAHM1, but not D1, reduces key T cell subset TF expression.

[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-γ³⁵. 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.

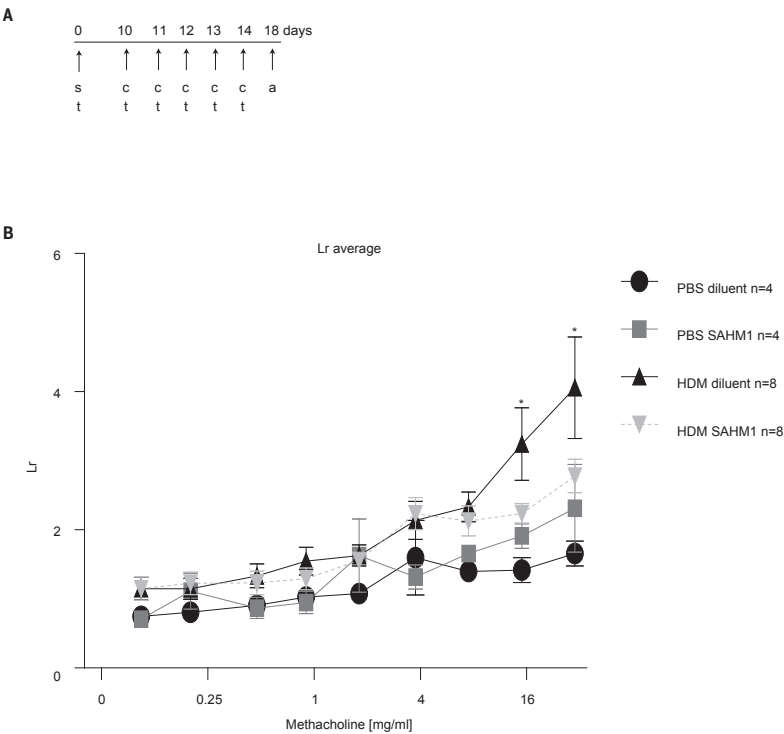


Figure 4. SAHM1 treatment abrogates airway BHR.

[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^{35, 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

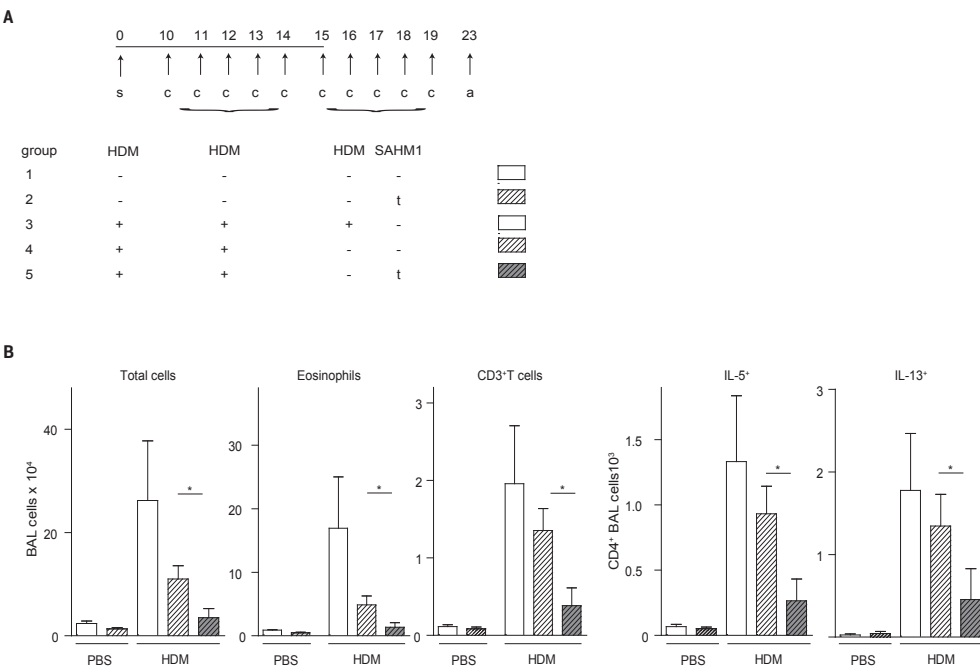


Figure 5. SAHM1 improves recovery from AAI

[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. Analyses [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 γ 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 γ 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.

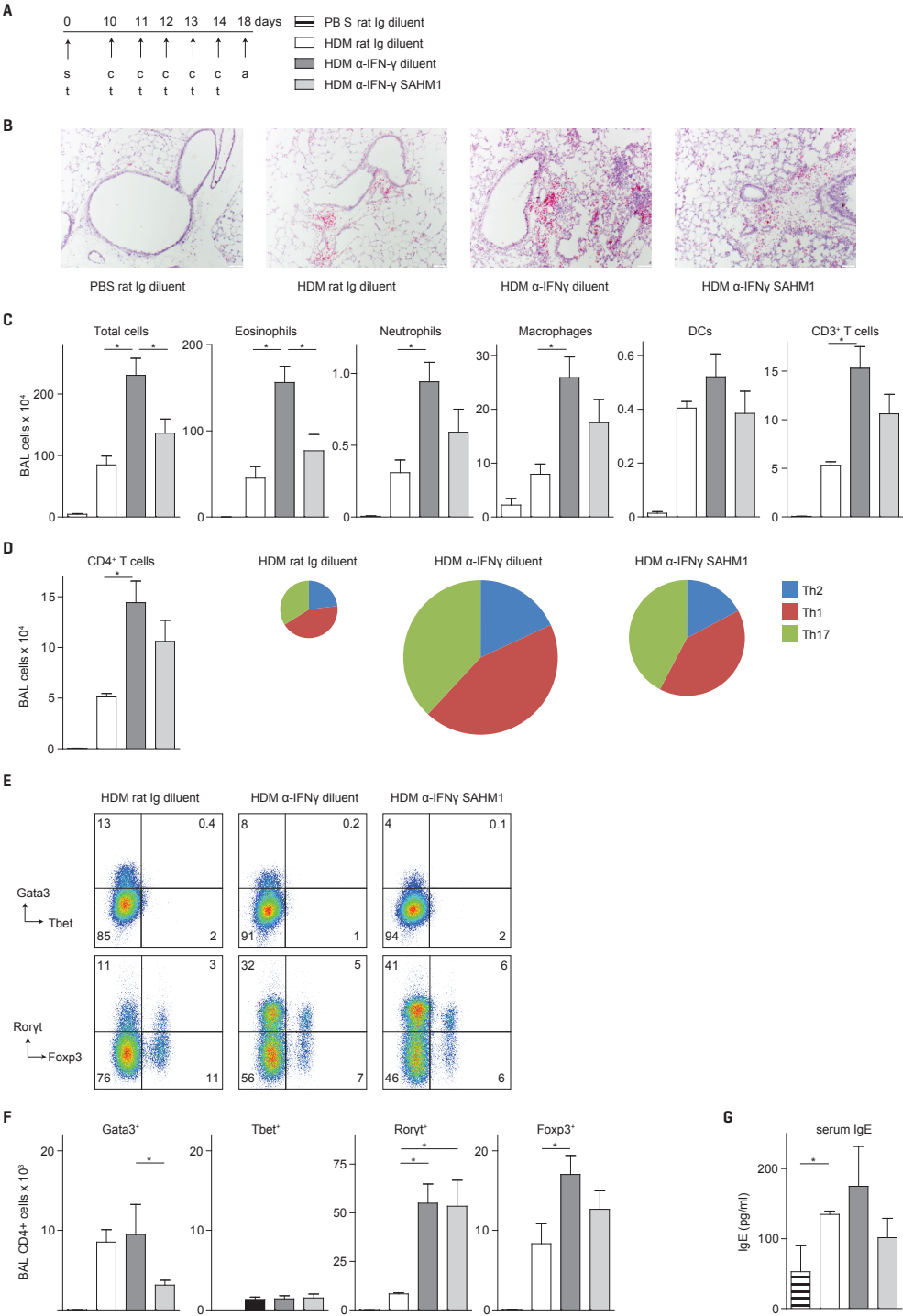


Figure 6. SAHM1 treatment suppresses α -IFN- γ induced asthma exacerbation

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 DNzyme that specifically targets *Gata3* mRNA⁴²⁰. 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.

GISs, 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 program¹³⁵. 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 DCs⁴²³. Because in endothelial cells the *Il33* gene is a Notch target⁴²⁴, 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 AAI⁴²⁵. Notch is a common differentiation signal for T cell priming of CD11b⁺ DC subsets in the spleen and intestine¹⁷⁸, and deletion of RBPJ resulted in a reduced capacity of DCs to activate T cells⁴²⁶. 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 responses²²⁰. 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 beneficial⁴²⁷. In this context Notch and STAT6 signaling operate in parallel and independent pathways to regulate mucous metaplasia¹⁹¹. Thus, pharmacological targeting of Notch signaling in epithelial cells, such as by SAHM1, can be considered in patients with airway diseases associated with mucous metaplasia¹⁹¹. 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 cells⁴¹⁹.

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 5

Notch signaling supports the Th2 cell response in allergic airway inflammation by stimulating lymph node egress

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Abstract

Background: Allergic asthma is mediated by a T helper 2 (Th2) response to inhaled allergens. Allergic airway inflammation (AAI) in mice is dependent on Notch signaling in T cells. Although *in vitro* experiments indicated that Notch signaling promotes transcription of the key Th2 transcription factor Gata3, the function of Notch signaling in AAI *in vivo* remains unclear.

Objective: To explore mechanisms by which Notch signaling supports AAI in mice.

Methods: We used acute and chronic house-dust mite (HDM)-driven models as well as ovalbumine (OVA)-mediated AAI models to investigate the role of Notch1 and Notch2 in Th2 differentiation *in vivo*. We performed transcriptome analysis of T cells to identify genes that are controlled by Notch signaling in the context of AAI.

Results: Whereas HDM exposure resulted in the development of AAI in wild-type (WT) animals, conditional deletion of Notch1 and Notch2 in T cells prevented eosinophilic airway inflammation. Hereby Th2 cytokine production, induction of serum IgE levels and airway hyperreactivity were significantly reduced. Gata3 overexpression in Notch-deficient T cells only partially rescued HDM-driven Th2 cell-driven AAI. We found that Th2 polarization following allergen sensitization was independent of Notch signaling. However, Notch-deficient OVA-specific *in vitro* polarized Th2 cells showed reduced accumulation in the lung during the OVA allergen challenge phase. Instead, we observed retention of Th2 cells in lung draining lymph nodes. 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.

Conclusion: Notch signaling in T cells is essential for the Th2 response in AAI and lymph node egress of Th2 cells.

Introduction

Allergic asthma is the most prevalent occurring type of asthma and is characterized by a type-2 immune response. Many hallmarks of allergic asthma can be attributed to cytokines produced by T helper 2 (Th2) cells or group 2 innate lymphoid cells (ILC2): IL-4 induces IgE class-switching of B cells, IL-5 recruits eosinophils and IL-13 provokes smooth muscle hyperreactivity and goblet cell hyperplasia. Together, this leads to a disease of the conducting airways involving chronic airway inflammation, airway hyperreactivity and episodes of bronchoconstriction. It is thought that IL-4 signaling via STAT6 is the main driver of Th2 cell differentiation via enhancing expression of the Th2 master transcription factor Gata3²⁸⁵ (**Chapter 2**). Although IL-4 is important in the differentiation and activation of Th2 cells, Th2 responses remain present in IL-4/STAT6 deficient mice⁴¹. This indicates a role for STAT6-independent mechanisms in the induction and re-activation of Th2 cells.

Notch signaling is capable of inducing Th2 cell differentiation by either binding to the upstream *Gata3* gene promoter or by directly regulating *Il4* gene transcription through activation of a 3' enhancer^{93, 129, 130}. In mammals, four Notch receptors have been identified (Notch1, Notch2, Notch3 and Notch4) that can bind to five transmembrane Notch-ligands (DLL1, DLL3, DLL4, Jagged1 and Jagged2). On the genetic level, *Notch1* and *Notch2* are closely related, while *Notch3* and *Notch4* are more divergent⁴²⁹. Upon ligand binding, the Notch intracellular domain (NICD) is generated by two membranous cleavage events. NICD thereafter translocates to the nucleus where it interacts with the transcriptional co-repressor RBPJ κ . This converts RBPJ κ into an activator complex and leads to the recruitment of co-activators, including p300 and Mastermind-like (MAML) proteins, which results in the transcriptional activation of various target genes. Notch signaling in CD4⁺ T cells is required for physiological Th2 responses to parasite antigens or house-dust mite (HDM). This was shown in mice deficient for RBPJ κ or both the Notch1 and Notch2 receptors¹²⁹ (**Chapter 3**) and in mice expressing dominant-negative MAML^{130, 131}, which showed decreased airway hyperreactivity, reduced numbers of eosinophils as well as lower levels of Gata3 and type 2 cytokine expression in T cells. Moreover, pharmacological inhibition of Notch signaling using γ -secretase inhibitors (GSI) or the cell-permeable peptide SAHM1, led to decreased Th2 cytokine production after immunization with HDM or ovalbumin (OVA) in asthma or food allergy models^{133, 134} (**Chapter 4**). Absence of Gata3 turned Notch from a Th2 inducer into a powerful inducer of Th1 differentiation^{129, 130}. Although previous studies reported that the Notch ligands Jagged and DLL instruct Th2 and Th1 cell differentiation, respectively^{93, 207}, others 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 outcomes¹³⁵. This suggests that in a physiological model, Notch signaling together with cytokine signaling synergizes to promote Th cell responses. In addition, studies using GSI or SAHM1 found that blocking Notch signaling only during the challenge phase led to decreased features of allergic airway inflammation (AAI)^{133, 134} (**chapter 4**), suggesting a role for Notch signaling in optimizing immune responses rather than inducing initial Th2 cell differentiation. Hence, the precise role of Notch signaling during Th2 cell polarization and activation remains controversial.

In this chapter we investigated which Notch receptor (Notch1 or Notch2) is required during Th2 responses in both acute and chronic models for HDM-driven AAI *in vivo*. We assessed whether the lack of Notch1 and/or Notch2 can be rescued by enforced expression of Gata3. We also investigated whether Notch signaling is required for the priming of T cells or during the challenge phase. Finally, we studied whether Notch is required for proliferation, differentiation or migration of Th2 cells in a mouse model of AAI.

Methods

Mice

Wildtype (WT) mice were purchased from Envigo (Indianapolis, USA). *Notch1*^{fl/fl} mice¹¹⁷, *Notch2*^{fl/fl} mice⁴³⁰ and RBPJκ^{fl/fl} mice³⁹¹ were crossed with CD4-cre transgenic mice³⁹², with CD2-Gata3 Tg mice³⁶⁸, with OTII mice⁴³¹ or with GATIR mice. The latter contains an IRES-YFP sequence introduced into the 3' untranslated region of the *Gata3* gene, resulting in the concomitant production of both Gata3 and YFP^{432, 433} (kindly provided by HJ Fehling, Ulm). 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^{117, 368, 391, 392, 430}. Both male and female 6-14-week-old mice were used in experiments. 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, 419}. Briefly, directly after harvest, spleen, lymph nodes, thymus and lungs were mechanically disrupted in a 100 µm cell strainer (BD Falcon, Bedford, MA, USA). 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 after which cells were separated from bones using a cell strainer. Erythrocytes in BM and lung were lysed with osmotic lysis buffer for 1 minute.

Mouse studies

Acute HDM-mediated AAI was induced as described before³⁹⁴. In short, mice were sensitized with 1 or 10 µg (as indicated in the figures) HDM (Greer, Lenoir, NC, USA)(endotoxin; 1397.5 EU/vial, protein; 5.59 mg/vial) i.n. in 40 µl or with PBS (Invitrogen, Carlsbad, CA, USA). 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. Broncho-alveolar lavage fluid (BAL) was obtained by flushing the lungs three times with 1 mL PBS containing 0.5 mM EDTA (Sigma-Aldrich, Darmstadt, Germany).

Chronic HDM-mediated AAI was induced and lung function was measured at day 37 as described before⁴³⁴.

In vivo OVA-specific T cell responses were induced as described³⁹⁴. In brief, CD4⁺ T cells were isolated from spleen and lymph nodes of OTII mice using a CD4⁺ T cell MACS isolation kit (Miltenyi Biotec) and were stained with 0.5 mM CFSE (Thermo Fisher Scientific, Waltham, MA, USA) for 10 minutes at 37°C. A total of 2·10⁶ OTII cells were transferred intravenously into WT recipients. The next day, mice were exposed intranasally to 5 or 20 µg OVA (Endotoxin-free, Hyglos, Bernried am Starnberger See, Germany) and 50 µg HDM and sacrificed 72 hours later for FACS analyses. To study the role of Notch in maintaining Th2 responses, 10·10⁶ *in vitro* polarized Th2 OTII cells⁴³⁵, were injected in WT recipients. Next, mice were challenged intratracheally (i.t) for 4 consecutive days with 50 µg OVA and 10 µg HDM. Mice were analyzed 1 day after the last challenge.

DC-OTII cell co-cultures

GM-CSF bmDCs were generated as described^{393, 419} and stimulated overnight with 5 µg/ml HDM or 100 ng/ml LPS (Enzo life sciences, Farmingdale, NY, USA) in combination with variable concentrations of endotoxin-free OVA as indicated in the figure legends. Naïve T cells were isolated from spleen and lymph nodes of OTII mice using a CD4⁺ T cell MACS isolation kit and were subsequently 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 CD4⁺CD62L⁺. A list of all used fluorochrome labeled antibodies can be found in **Supplementary table 1**. BmDCs (5·10³) were cultured with 1·10⁵ CFSE labeled naïve T cells for four days at 37°C, after which cells were analyzed using flow cytometry.

T helper cell cultures

Naïve T cells were obtained as described above and were polarized in Th1, Th2 and Th17 conditions as described⁴³⁵.

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% NaN₃ in PBS as described previously^{394, 419}. A list of all fluorochrome labeled antibodies that were used can be found in **Supplementary table 1**. 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).

To measure phosphorylation of S6 ribosomal protein, total spleen cells were stained for extracellular markers and stimulated for 3 hours with combinations of anti-CD3 and anti-CD28 (both BD biosciences). Staining was performed as described⁴³⁶.

Histology

Lung sections were obtained and stained using haematoxylin/eosin (HE), periodic acid-Schiff (PAS) or Masson-trichrome (Sigma-Aldrich) as described⁴³⁴.

Cytokine and immunoglobulin measurements

Cytokines were quantified by commercial enzyme-linked immunoassorbent assay (ELISA) for IL-5 (eBioscience), IL-13 (R&D, Minneapolis, MN, USA), IgE (BD biosciences) and IgG1 (BD biosciences) according to manufacturers' protocol. HDM-specific IgE and IgG1 (antibodies from BD biosciences) were measured as described³⁹⁸.

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). 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 *Dtx1*³⁷⁰, *Itgb1* (forward: 5'-ATGCAGGTTGCGGTTTGT-3', reverse: 5'-CATCCGTGGAAAACACCAG-3', probe 19), *Cx3cr1* (forward: 5'-AAGTTCCTTCCCATCTGCT-3', reverse: 5'-CAAAATTCTCTAGATCCACTTCAGG-3', probe 10), *Sell* (forward: 5'-GGAGCATCTGGAAACTGGTC-3', reverse: 5'-TTGATCTTTGAGAACTTCTGTTTG-3', probe 21), *Cd47* (forward: 5'-AATCATTCTGCTGCTCGTTG-3', reverse: 5'-GCATTCTTCACGGGCTTTT-3', probe 100) and *Slc2a1* (forward: 5'-CAGCCGGCACAGCTAGAG-3', reverse: 5'-AGCACTGCTCCTCCACA-3', probe 3). mRNA levels of genes of interest were normalized to glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) (**Chapter 3**). Primers were checked for specificity and efficacy using standard criteria.

RNA-sequencing

RNA sequencing was performed and analyzed as described before^{432, 433}. Library preparation was performed using the Smart-seq2 methodology⁴³⁷ and sequenced on an Illumina HiSeq2500 (single read mode, 51 bp read length) according to the Illumina TruSeq Rapid v2 protocol. HISAT2 was used to align reads to the mouse genome (mm10 build)⁴³⁸. Scaling of samples as well as statistical analysis was executed using the R package DESeq2⁴³⁹ as implemented in HOMER⁴⁴⁰; genes with >0.5 absolute log2 fold change and adjusted P < 0.05 (Wald test) were considered differentially expressed. Genes with average Reads Per Kilobase Million (RPKM) > 1 were considered expressed. Since a small subset of Tregs also expresses Gata3 and could therefore contaminate our Th2 cell population⁴⁴¹, genes that were similarly affected (>2 fold) in Tregs⁴⁴² compared to all other Th cells were excluded from our analysis. Pathway analyses on differentially expressed genes were performed using Metascape (<http://metascape.org>)⁴⁴³.

Statistical analysis

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

Notch1 and Notch2 expression on CD4⁺ T cells is required for the induction of acute AAI

To delete *Notch1* and *Notch2* specifically in T cells, we crossed *Notch1*^{fl/fl} and *Notch2*^{fl/fl} mice (containing *Notch* loci with loxP sites flanking critical exons^{117, 430} with transgenic mice expressing Cre recombinase under the control of the T cell-specific CD4 promoter (CD4-Cre). Notch signaling is required at various stages during early T cell development⁴⁴⁴. However, since thymocytes immediately downregulate Notch1 expression after successfully passing β -selection, double-positive (CD4⁺CD8⁺) thymocytes have very low levels of Notch signaling¹²⁸, which is why CD4-Cre-mediated Notch deletion should not affect T cell development³⁹². We analyzed T cell development in thymus and spleen from CD4-Cre transgenic *Notch1*^{fl/fl}*Notch2*^{fl/fl} mice (NiN2 ^{Δ CD4/ Δ CD4}) and non CD4-Cre expressing NiN2^{+/+} (referred to as WT) control mice (**Supplementary figure 1A**). Consistent with published findings¹¹⁶, mice with a conditional deletion of Notch receptors had similar numbers of thymocytes compared to wild-type (WT) littermates (**Supplementary figure 1B**). In addition, percentages and absolute counts of thymocytes in different developmental stages were similar between NiN2 ^{Δ CD4/ Δ CD4} and WT mice (**Supplementary figure 1B-E**). Moreover, total splenocytes as

well as percentages and absolute counts of CD4 and CD8 T cells in spleen were unaltered in NiN2 ^{Δ CD4/ Δ CD4} mice (**Supplementary figure 1F-H**). Together, these findings confirm that T cell development is unaltered in the absence of Notch1 and Notch2 signaling in T cells.

Next, we induced AAI in Notch1 and Notch2 single and double-deficient mice (Ni ^{Δ CD4/ Δ CD4} Ni2 ^{Δ CD4/ Δ CD4} and NiN2 ^{Δ CD4/ Δ CD4}) and WT littermates through sensitization and challenge with HDM. Four days after the last challenge, mice were analyzed (**Figure 1A**). In the absence of both Notch1 and Notch2 in T cells, mice displayed a significant decrease in the total number of eosinophils, neutrophils, B cells, T cells and DCs in BAL fluid compared to WT littermates (**Figure 1B**). Interestingly, Ni ^{Δ CD4/ Δ CD4} mice developed a milder form of AAI compared to WT mice, characterized by a reduction in eosinophils. In contrast, Ni2 ^{Δ CD4/ Δ CD4} mice developed AAI that was similar to WT mice (**Figure 1B**). In both Ni ^{Δ CD4/ Δ CD4} and NiN2 ^{Δ CD4/ Δ CD4} mice, we found lower total numbers and proportions of IL-4⁺, IL-5⁺, IL-13⁺ IL-9⁺, IL-17⁺ and IFN- γ ⁺ CD4⁺ T cells in BAL fluid, mediastinal lymph nodes (MedLN) and lungs compared to WT mice (**Figure 1C** and data not shown). In addition, Gata3 induction was impaired in Ni ^{Δ CD4/ Δ CD4} and NiN2 ^{Δ CD4/ Δ CD4} mice in BAL fluid, MedLN and lung CD4⁺ cells compared to WT mice, whereas Gata3 induction was not affected in Ni2 ^{Δ CD4/ Δ CD4} mice (**Figure 1D-F** and data not shown). Accordingly, HDM-restimulated MedLN cells from NiN2 ^{Δ CD4/ Δ CD4} showed severely reduced production of IL-5 and IL-13. A trend towards reduced cytokine production was observed in HDM-restimulated MedLN cells from Ni ^{Δ CD4/ Δ CD4} and Ni2 ^{Δ CD4/ Δ CD4} mice (**Figure 1G**). Finally, total levels of IgG1 and IgE in serum were significantly lower in Ni ^{Δ CD4/ Δ CD4} and NiN2 ^{Δ CD4/ Δ CD4} mice compared to WT mice. In Ni2 ^{Δ CD4/ Δ CD4} mice only total serum IgE was significantly decreased (**Figure 1H**). Regarding HDM-specific antibodies, we found significantly reduced IgG1 and IgE in the serum of NiN2 ^{Δ CD4/ Δ CD4} mice.

Taken together, these findings show that Ni ^{Δ CD4/ Δ CD4} mice manifested only a minor reduction of HDM-driven AAI and that the absence of Notch2 did not affect AAI development. Because in NiN2 ^{Δ CD4/ Δ CD4} mice AAI was strongly reduced, we conclude that the expression of either Notch1 or Notch2 on CD4⁺ T cells is required to induce acute AAI *in vivo*.

Notch signaling in CD4⁺ T cells is required for the induction of chronic AAI and airway hyperreactivity

Since Notch signaling is important to induce acute AAI, we questioned whether Notch signaling is also required for airway remodeling and bronchial hyperreactivity during chronic AAI. Therefore, we subjected NiN2 ^{Δ CD4/ Δ CD4} and WT mice to a chronic HDM-induced asthma model and investigated whether Notch-deficient mice develop AAI following chronic HDM challenge for 5 consecutive weeks (**Figure 2A**). Similar to the acute model, we found that in the absence of both Notch1 and Notch2 in T cells, mice displayed a significant decrease in the number of inflammatory cells, such as eosinophils, T cells and ILC2s, in BAL fluid compared to WT littermates (**Figure 2B**). In addition, the presence of cellular infiltrates and collagen in the lungs as well as airway resistance to methacholine was lower in NiN2 ^{Δ CD4/ Δ CD4} mice compared to HDM-treated WT mice (**Figure 2C and 2D**, respectively). In addition, both total and HDM-specific IgG1 and IgE levels in serum were decreased in NiN2 ^{Δ CD4/ Δ CD4} mice, compared to WT mice (**Figure 2E**). In contrast, CD4⁺ T cell numbers in the MedLN were unaltered and showed increased positivity for CD25, Gata3 and Ror γ t (**Supplementary figure 2**). Together, these results show that Notch signaling in CD4⁺ T cells is required for the induction of chronic allergic inflammation and remodeling in the lung, but not for the induction of Th2 or Th17 cells in MedLN.

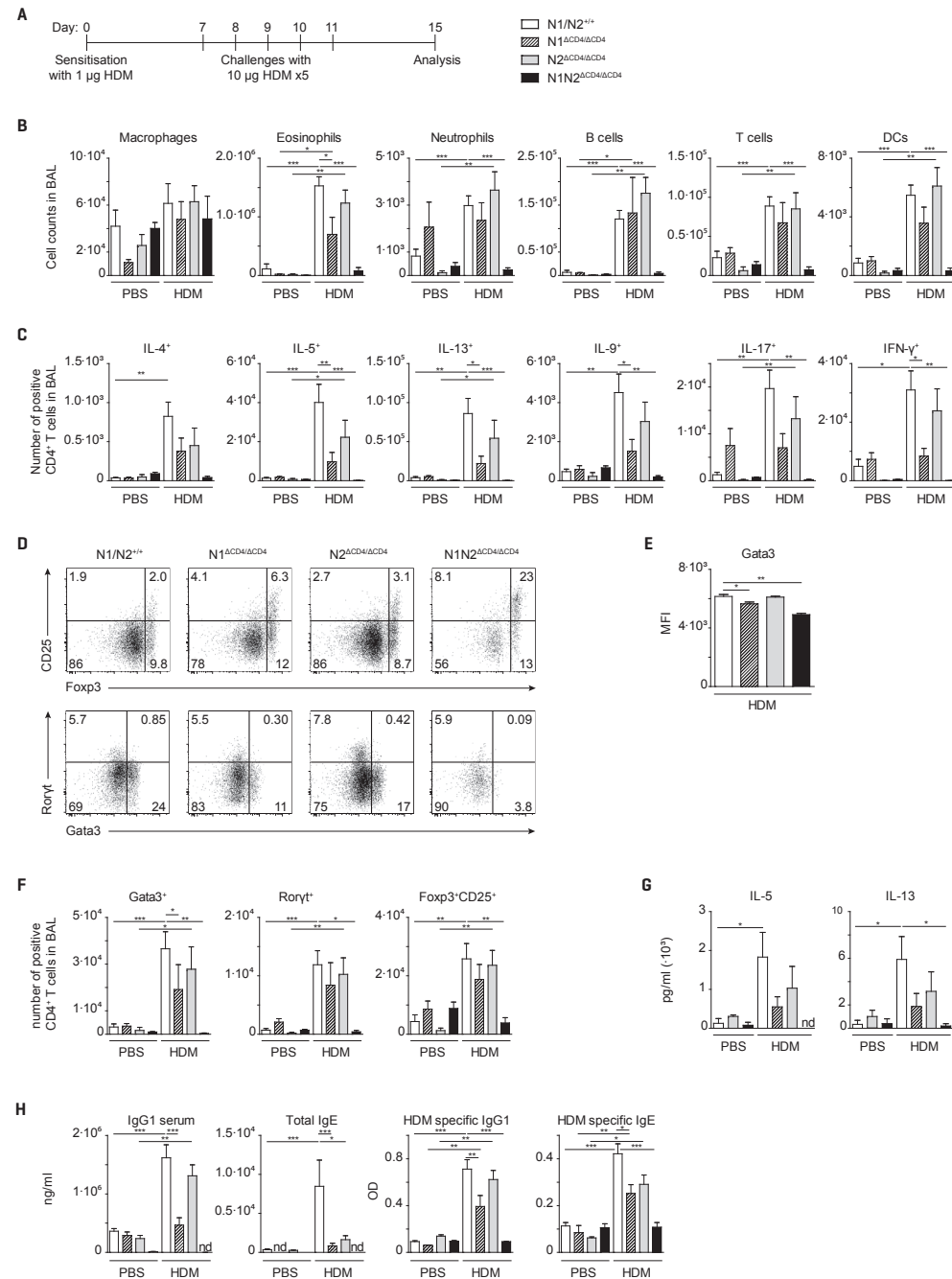


Figure 1. Expression of both Notch1 and Notch2 on CD4⁺ T cells is required for the induction of acute AAI

Figure 1. Expression of both Notch1 and Notch2 on CD4⁺ T cells is required for the induction of acute AAI

[A] Scheme of acute HDM-mediated AAI induction in *N1N2^{ΔCD4/ΔCD4}* and *N1N2^{+/+}* [WT] mice.

[B] Numbers of macrophages [FSC^{high}SSC^{high}auto fluorescent⁺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 PBS- or HDM-sensitized mice.

[C] Quantification of the total numbers of IL-4⁺, IL-5⁺, IL-13⁺, IFN-γ⁺ and IL-17A⁺ by intracellular flow cytometry in CD3⁺CD4⁺ T cells in BAL of the indicated mice.

[D-E] Flow cytometry profile of transcription factor expression in CD3⁺CD4⁺ T cells in BAL from mice treated with HDM.

[F] Quantification of Gata3⁺, Roryt⁺ and Foxp3⁺CD25⁺ CD4⁺ T cells BAL.

[G] 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.

[H] Total IgG1 and IgE levels and levels of HDM-specific IgG1 and IgE in serum, as determined by ELISA.

Data are shown as mean + SEM of 3-16 mice per group and are combined from 2 independent experiments. * p<0.05, ** p<0.01, ***p<0.001, using Mann-Whitney U-test.

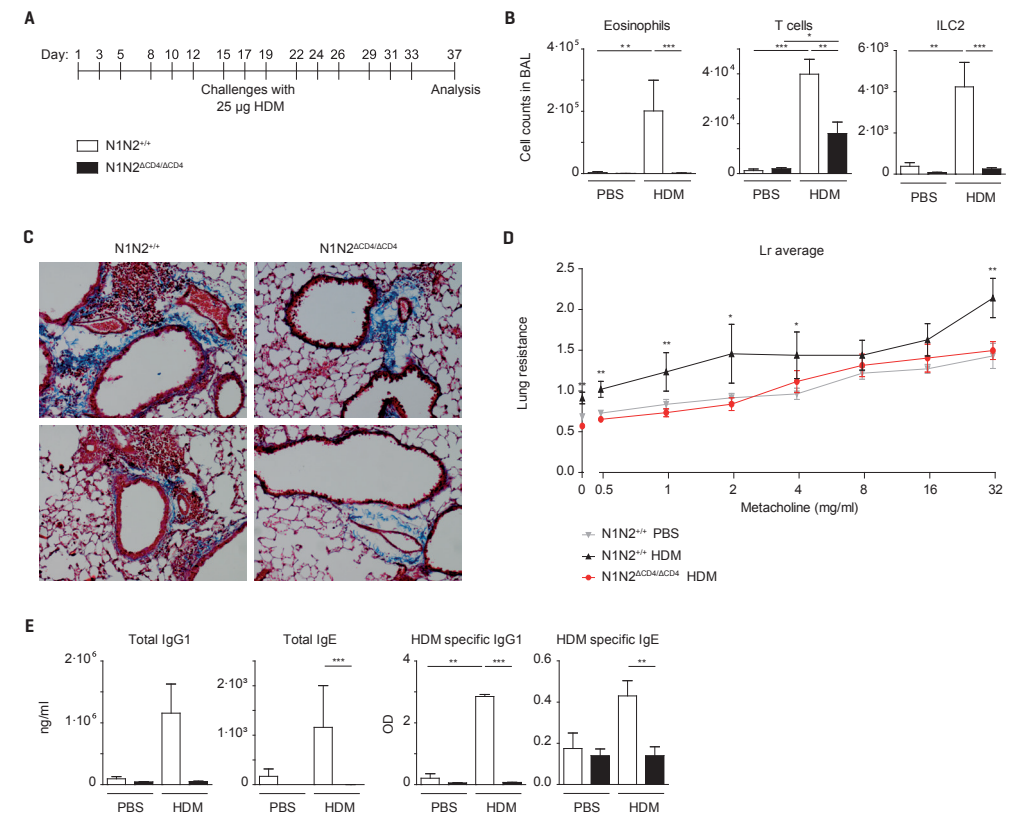


Figure 2. Notch signaling in CD4⁺ T cells is required for the induction of chronic AAI

[A] Sensitization and challenge scheme of chronic HDM-driven AAI in mice

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

[C] Masson-trichrome staining on lung tissue from HDM treated *N1N2^{ΔCD4/ΔCD4}* and WT mice. Blue staining indicates the presence of connective tissue, nuclei are stained in dark red and cytoplasm is pink.

[D] Airway hyperresponsiveness was measured upon increasing doses of inhaled methacholine in the indicated mouse groups.

[E] Levels of total IgG1 and IgE and levels of HDM-specific IgE and IgG1 in serum, as determined by ELISA.

Data are shown as mean + SEM of 6-8 mice per group. * p<0.05, ** p<0.01, ***p<0.001, using Mann-Whitney U-test.

Enforced Gata3 expression partly rescues AAI induction in $N1^{\Delta CD4/\Delta CD4}$ and $N1N2^{\Delta CD4/\Delta CD4}$ mice

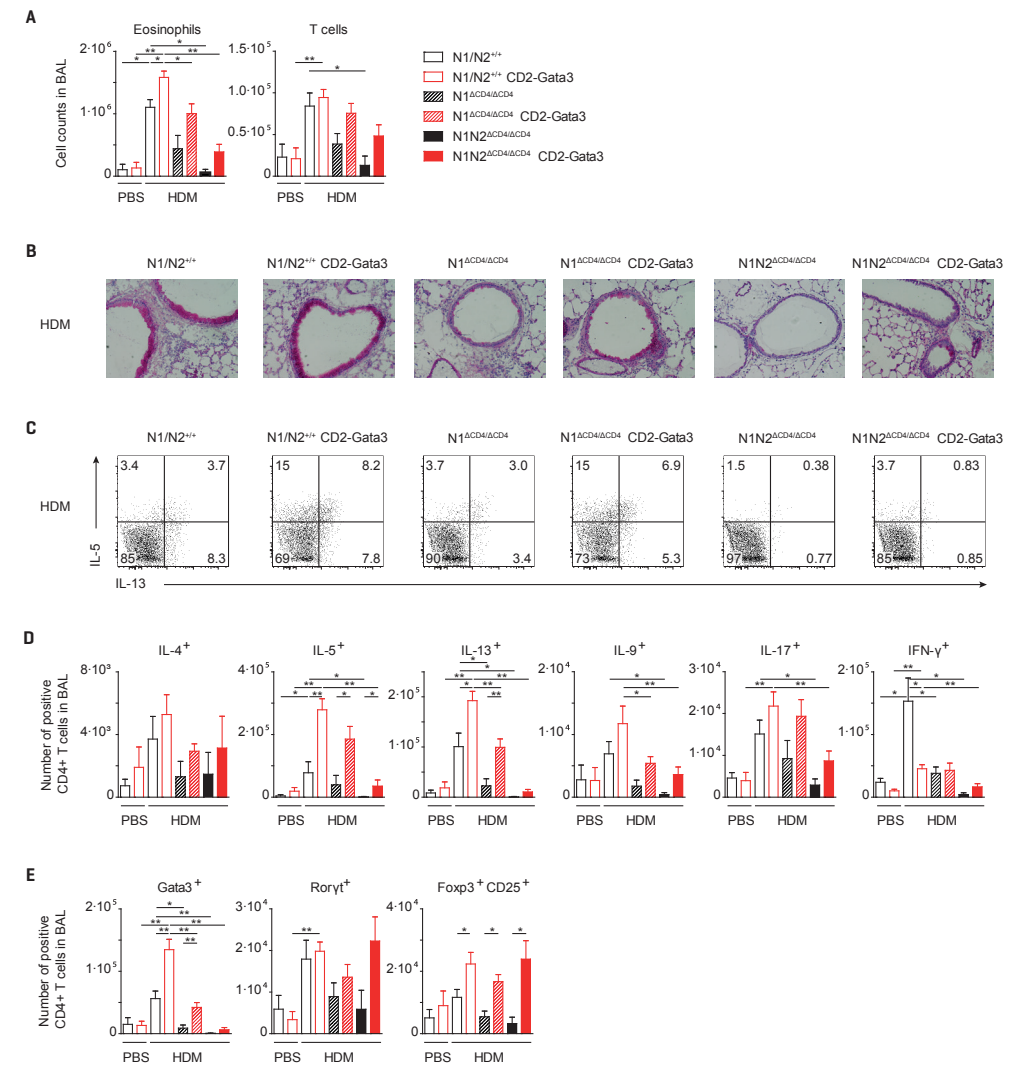
Notch-induced Th2 differentiation is shown to be dependent on Gata3, whereby an upstream promotor of Gata3 was identified as a direct Notch target^{129, 130}. Therefore, we wondered whether enforced Gata3 expression could rescue AAI induction in the absence of Notch signaling. To investigate this, we crossed $N1^{\Delta CD4/\Delta CD4}$ and $N1N2^{\Delta CD4/\Delta CD4}$ mice with mice that harbor a *Gata3* transgene under the control of the human CD2 promotor, termed CD2-*Gata3* mice, which constitutively express Gata3 in all T cell subsets³⁶⁸. We investigated CD2-*Gata3* Tg and non-Tg mice that were either $N1^{\Delta CD4/\Delta CD4}$, $N1N2^{\Delta CD4/\Delta CD4}$ or Notch WT in our HDM-driven acute AAI model. In accordance with our previous findings³⁴⁶, CD2-*Gata3* mice developed elevated AAI compared to WT mice, characterized by increased eosinophilia in BAL fluid (Figure 3A and Supplementary figure 3A). We found that enforced Gata3 expression increased the numbers of eosinophils and T cells in BAL fluid of $N1^{\Delta CD4/\Delta CD4}$ and $N1N2^{\Delta CD4/\Delta CD4}$ mice, although not statistically significant. Mucus production by goblet cells was enhanced in $N1^{\Delta CD4/\Delta CD4}$ and $N1N2^{\Delta CD4/\Delta CD4}$ mice that carried a *Gata3*-transgene (Figure 3B). Accordingly, Gata3 overexpression led to significantly enhanced numbers of IL-5 and IL-13-producing CD4⁺ T cells in BAL fluid of both WT, $N1^{\Delta CD4/\Delta CD4}$ and mice. Also a trend towards increased IL-4⁺, IL-9⁺ and IL-17A⁺ T cells in BAL was observed, but numbers of IFN- γ ⁺ T cells in BAL fluid were unaffected by enforced Gata3 expression (Figure 3C, 3D and data not shown). The ratio of cytokine-producing T cells shifted to an increasingly Th2-dominated phenotype in the presence of the CD2-*Gata3* transgene in WT, $N1^{\Delta CD4/\Delta CD4}$ and $N1N2^{\Delta CD4/\Delta CD4}$ mice (Supplementary figure 3B). Also, enforced Gata3 expression in $N1^{\Delta CD4/\Delta CD4}$ mice led to a recovery of Gata3⁺ T cells numbers to a similar level as WT mice, but this was not the case in $N1N2^{\Delta CD4/\Delta CD4}$ mice. Treg numbers increased in the BAL fluid of all CD2-*Gata3* mice (Figure 3E). Gata3 overexpression did not appear to affect serum IgG1 levels in any of the mouse groups tested. By contrast, it did increase total and HDM-specific IgE levels in WT and $N1^{\Delta CD4/\Delta CD4}$ mice, but not in $N1N2^{\Delta CD4/\Delta CD4}$ mice (Supplementary figure 3C). In general, compared to AAI in WT mice, constitutive Gata3 expression largely enhanced AAI in $N1^{\Delta CD4/\Delta CD4}$ mice, but only had minor effects in $N1N2^{\Delta CD4/\Delta CD4}$ mice.

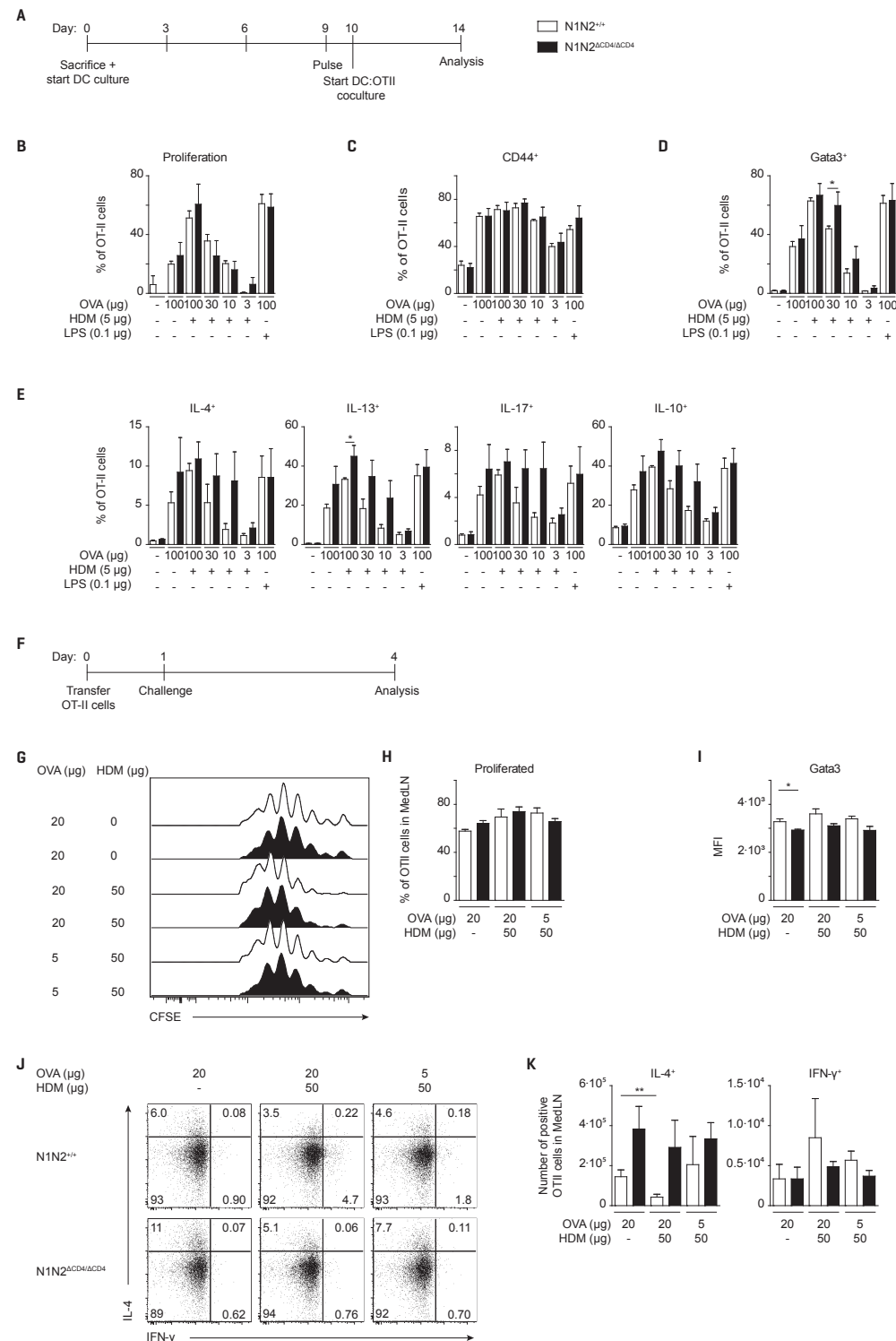
To confirm that enforced Gata3 expression cannot rescue development of AAI in Notch signaling-deficient mice, we compared HDM-driven AAI development in $RBPJ^{\Delta CD4/\Delta CD4}$ mice that did or did not harbor the CD2-*Gata3* transgene. Indeed, we did not find evidence for rescued AAI development in $RBPJ^{\Delta CD4/\Delta CD4}$ CD2-*Gata3* mice compared to $RBPJ^{\Delta CD4/\Delta CD4}$ mice when measuring eosinophilia, cytokine production or IgE levels in serum (Supplementary figure 4).

Altogether, we conclude that Gata3 overexpression largely rescued AAI induction in mice that have Notch1-deficient T cells, but had limited effects mice with T cells lacking both Notch1 and Notch2 or the downstream effector RBPJ κ . These finding indicate that Notch signaling in CD4⁺ T cells has additional critical functions in type 2 immunity beyond Gata3 induction.

Notch1 and Notch2 expression on T cells is not required for the activation, proliferation and differentiation of CD4⁺ T cells *in vitro*

Next, we explored the mechanisms by which Notch signaling supports Th2 inflammation. To this end, we crossed $N1N2^{\Delta CD4/\Delta CD4}$ mice with OTII transgenic mice, which express a chicken ovalbumin (OVA)-specific T cell receptor (TCR) on CD4⁺ T cells. To investigate the proliferative capacity of Notch-deficient CD4⁺ T cells *in vitro*, CFSE-labeled naïve CD4⁺ OTII cells from $N1N2^{\Delta CD4/\Delta CD4}$ and WT mice were cultured in the





presence of bone marrow-derived DCs that were activated with HDM or LPS and loaded with a range of OVA concentrations (**Figure 4A**). No differences were found in proliferation (**Figure 4B**) or in the expression of CD44, which signifies cellular activation (**Figure 4C**), when comparing OTII cells from $N1N2^{\Delta CD4/\Delta CD4}$ and WT mice. Th2 polarization was not reduced in the absence of Notch signaling; even a modest increase in Gata3 expression or cytokine production was noticed, particularly at lower antigen concentrations (**Figure 4D and 4E**). Along these lines, *in vitro* activation and polarization of naïve T cells from $RBPJ^{\Delta CD4/\Delta CD4}$ and WT mice using anti-CD3 and anti-CD28 antibodies in combination with cytokines and anti-cytokine antibodies⁴³⁵ did not affect cell expansion or Th subset differentiation (**Supplementary figure 5A-D**).

Notch signaling was described to potentiate phosphatidylinositol 3-kinase (PI3K)-dependent signaling downstream of the TCR and CD28 by inducing activation of Akt kinase and mammalian target of rapamycin (mTOR), which enhances T cell effector functions and survival and allows them to respond to lower antigen doses^{108, 111, 112}. Therefore, we studied phosphorylation of S6 ribosomal protein, a downstream target of Akt, by stimulating splenic cells from $N1N2^{\Delta CD4/\Delta CD4}$ and WT mice for 3 hours with a range of anti-CD3 and anti-CD28 antibody concentrations. However, we did not detect any defect in phosphorylation of S6 ribosomal protein in T cells from $N1N2^{\Delta CD4/\Delta CD4}$ mice (**Supplementary figure 5E**).

To further validate these findings, we investigated if Notch signaling is required for activation of naïve T cells *in vivo*. CFSE-labeled OTII transgenic T cells from $N1N2^{\Delta CD4/\Delta CD4}$ and WT mice were injected intravenously into WT recipients that 1 day later were intranasally challenged with different doses of OVA and HDM, as indicated in **Figure 4F**. At day 4, we did not observe significant differences in *in vivo* proliferation between $N1N2^{\Delta CD4/\Delta CD4}$ and WT OTII transgenic T cells (**Figure 4G and 4H**). Although the median levels of Gata3 expression appeared slightly decreased in $N1N2^{\Delta CD4/\Delta CD4}$ OTII cells present in the draining MedLN (**Figure 4I**), these $N1N2^{\Delta CD4/\Delta CD4}$ OTII cells were still capable of producing IL-4 and IFN- γ (**Figure 4J and 4K**).

In conclusion, whereas Notch signaling in T cells is required for the induction of AAI *in vivo*, our findings indicate that Notch signaling is not involved for the initial *in vitro* or *in vivo* expansion, activation or Th2 polarization of naïve CD4⁺ T cells upon TCR stimulation.

Figure 4. Notch1 and Notch2 expression on T cells is not required for the activation, proliferation and differentiation of CD4⁺ T cells *in vitro*

(A) Scheme of DC-OTII cell co-culture using WT OTII cells and $N1N2^{\Delta CD4/\Delta CD4}$ OTII cells. WT GM-CSF bmDCs were stimulated 1 day prior to co-culture in the presence or absence of the indicated concentrations of OVA, HDM and LPS. (B) Proliferation of OTII cells from the indicated mice, measured using CFSE. (C-E) Percentage of CD44⁺, (D) Gata3⁺ and (E) cytokine positive OTII cells in culture, measured using flow cytometry. Data are shown as mean + SEM of 4-5 mice per group and are representative of 3 independent experiments. * $p < 0.05$, using Mann-Whitney U-test. (F) Scheme of OTII cell transfer, using purified WT OTII cells and $N1N2^{\Delta CD4/\Delta CD4}$ OTII cells, and challenge with the indicated amounts of OVA and HDM. (G-H) flow cytometric analysis of proliferation of OTII cells in MedLN, measured using CFSE (H) percentage of proliferated cells of WT and $N1N2^{\Delta CD4/\Delta CD4}$ OTII cells in MedLN from mice treated with the indicated amounts of OVA and HDM. (I) Mean fluorescent intensity (MFI) of Gata3 expression from OTII cells. (J-K) Flow cytometry profile of IL-4 and IFN- γ expression in OTII cells and (K) quantification of cytokine positive OTII cells in MedLN from mice treated with the indicated concentrations of OVA and HDM. Data are shown as mean + SEM of 5 mice per group. * $p < 0.05$, ** $p < 0.01$, using Mann-Whitney U-test.

Notch signaling mediates lymph node egress of *in vitro* polarized Th2 cells

Since our findings indicate that Notch signaling was not required for the initiation of a Th2 response, we next investigated the function of Notch in the activation of polarized Th2 cells. We transferred *in vitro* polarized WT and $N1N2^{\Delta CD4/\Delta CD4}$ OTII CD4⁺ T cells intravenously into WT recipients who were subsequently challenged with OVA and HDM for four consecutive days (Figure 5A). On day 5, mice that received $N1N2^{\Delta CD4/\Delta CD4}$ Th2 cells had similar total cell counts in BAL fluid and MedLN, but lower cellular influx in the lungs compared to mice who received WT Th2 cells. Eosinophilia in BAL fluid and lungs was comparable between the two groups (Figure 5B). Interestingly, mice that received Notch-deficient OTII CD4⁺ T cells had lower number of OTII CD4⁺ T cells in BAL fluid and lungs, but significantly higher numbers of OTII CD4⁺ T cells in MedLN (Figure 5C). Moreover, IL-13 producing $N1N2^{\Delta CD4/\Delta CD4}$ OTII CD4⁺ T cells were less abundant in lungs and more abundant in MedLN, compared to WT OTII cells. Differences in IL-4 and IFN- γ producing cells followed the same trend (Supplementary figure 6). In the lungs and BAL, the WT and $N1N2^{\Delta CD4/\Delta CD4}$ OTII CD4⁺ T cell populations did not differ significantly in the proportions of IL-4⁺ or IL-13⁺ cells (Figure 5D). However, because mice that received Notch-deficient OTII CD4⁺ T cells had lower numbers of these cells in lungs, the absolute numbers of IL-4⁺ or IL-13⁺ OTII CD4⁺ T cells in lungs were decreased (Supplementary figure 6).

To migrate from the draining lymph nodes into the lungs, Th2 cells require expression of CCR4²⁰. This chemokine receptor binds to its ligands CCL17 and CCL22, which are highly expressed in inflamed airways²⁰. Another chemokine receptor that has been implicated in Th2 cell inflammation is CCR8, which is upregulated upon multiple allergen exposures²¹. We found that the surface expression levels of CCR8 were higher in MedLN in $N1N2^{\Delta CD4/\Delta CD4}$ OTII cells, compared to WT OTII cells. Total numbers of CCR4⁺ T cells were increased in MedLN and decreased in lungs but the proportions of CCR4⁺ cells did not differ (Figure 5E and 5F). WT and $N1N2^{\Delta CD4/\Delta CD4}$ OTII cells showed similar expression of the CD44 memory marker and similar proliferation rates (Figure 5G and 5H).

To further explore how Notch signaling in Th2 cells affects T cell migration, we sorted WT and $N1N2^{\Delta CD4/\Delta CD4}$ Th2 cells from MedLN at day 5 after *in vivo* transfer and measured the expression of genes involved in T cell adhesion and migration. In these experiments, we included *in vitro* polarized Th2 cells that were not transferred, to define if differences were present at baseline or were induced upon allergen challenges *in vivo*. As expected, the Notch target Deltex1 (*Dtx1*) was significantly downregulated in Th2 cells in MedLN in the absence of Notch (Figure 5I). The $\beta 1$ integrin subunit *Itgb1* (CD29) and *Cx3cr1*, which has been implicated in T cell migration and T cell survival in the inflamed lung⁴⁴⁵, were significantly increased in Notch-deficient Th2 cells from MedLN compared to WT Th2 cells. In contrast, expression of *Cd47*, which enhances

Figure 5. Notch signaling mediates lymph node egress of already established Th2 cells

(A) Scheme of OTII cell transfer, using *in vitro* polarized WT and $N1N2^{\Delta CD4/\Delta CD4}$ OTII cells, and challenge with OVA and HDM.
(B–C) Numbers of the total cell counts, eosinophils and [C] OTII cells in BAL, lungs and MedLN in mice treated with HDM and OVA.
(D) Percentages of cytokine-positive CD3⁺CD4⁺ T cells in the indicated tissues.
(E) Flow cytometry profile of CCR4 and CCR8 expression in OTII T cells in MedLN from mice treated with HDM and OVA.
(F) Quantification of CCR4⁺ OTII cells and MFI of CCR8 expression on OTII cells in lungs and MedLN.
(G) Quantification of CD44⁺ OTII cells in lungs and MedLN.
(H) Proliferation of OTII cells and proliferation index in lungs and MedLN from the indicated mice, measured using CFSE at day 3.
(I) mRNA expression of the indicated genes, quantified by qRT-PCR in OTII cells sorted from MedLN at day 5.
Data are shown as mean + SEM of 12 [B–C and E–G] or 6 [D and H] mice per group and are representative of 3 independent experiments. * p<0.05, **p<0.01, ***p<0.001, using Mann-Whitney U-test.

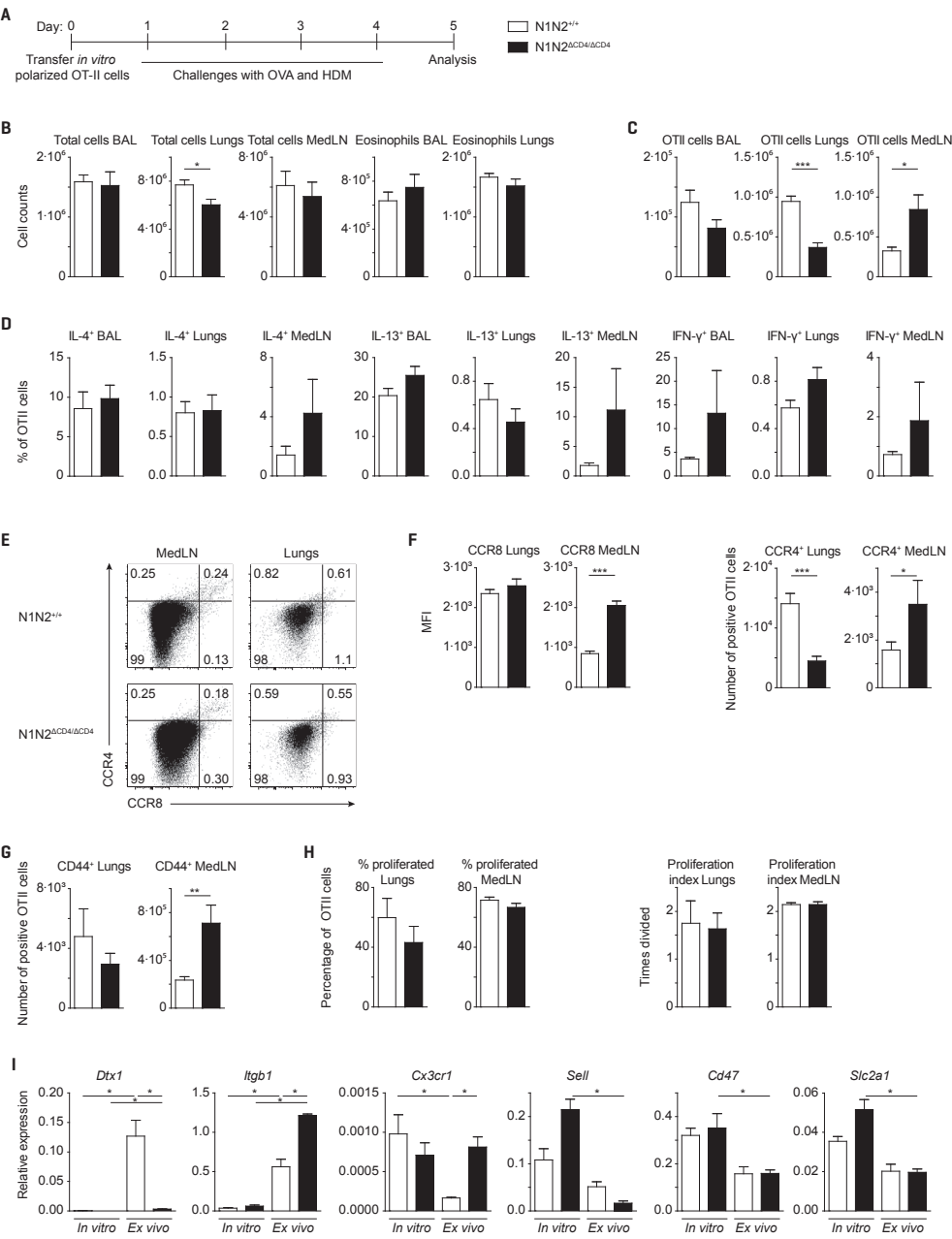


Figure 5. Notch signaling mediates lymph node egress of already established Th2 cells

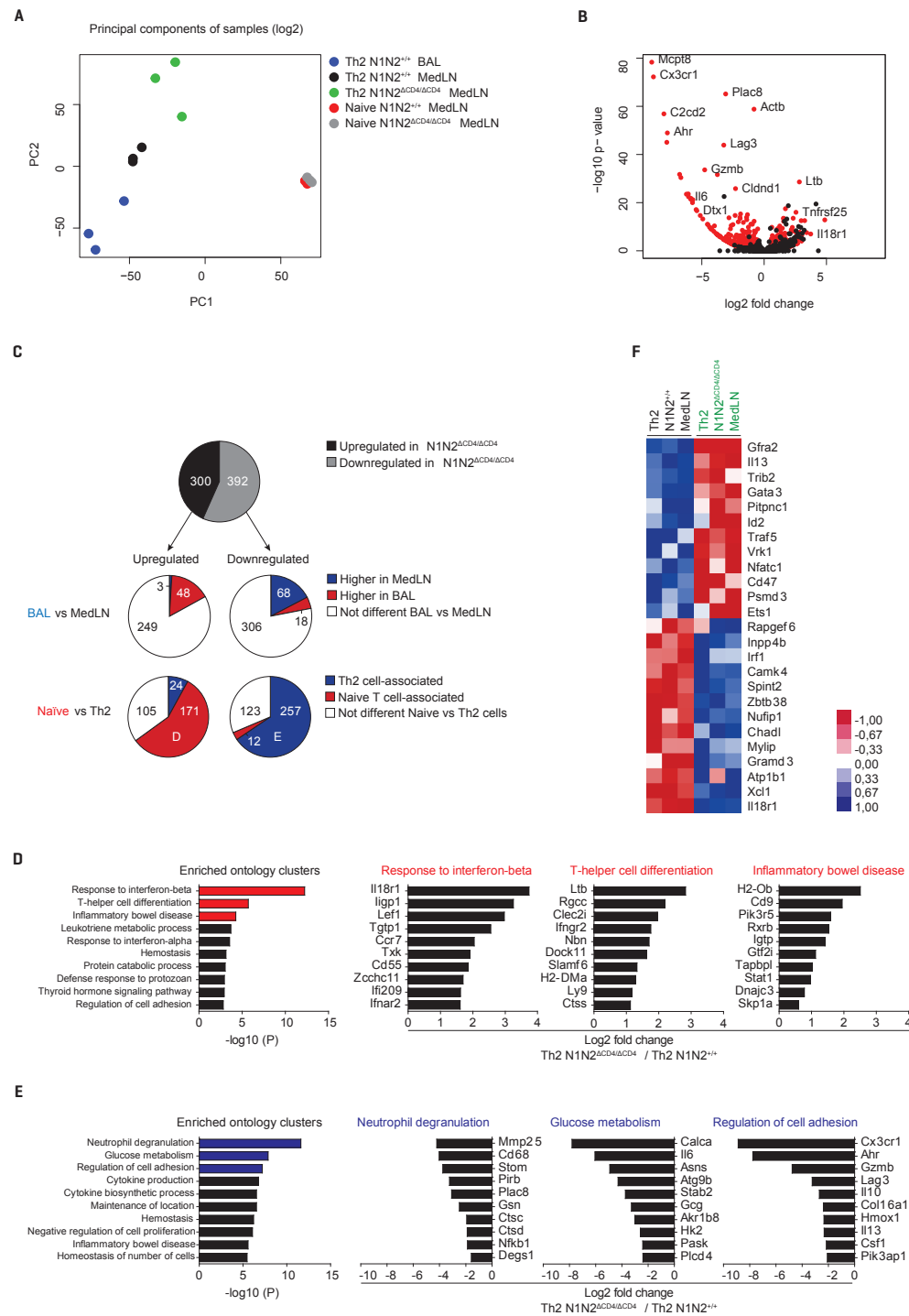


Figure 6. Notch signaling affects expression of cytokines and adhesion molecules

intracellular calcium concentration upon cell adhesion to extracellular matrix, *Sell* (CD62L), which is required for T cells to enter and stay in the lymph nodes⁴⁴⁶, and *Slc2a1* (Glut1), which was shown to be regulated by RBPJ κ in memory CD4⁺ T cells¹⁰⁹ were unaltered in the absence of Notch signaling (**Figure 5I**).

Taken together, these findings suggest that Notch signaling is involved in lymph node egress of polarized Th2 cells in AAI. Accumulating polarized Th2 cells in the MedLN in the absence of Notch signaling show increased expression of IL-13, the CCR8 chemokine receptor and the β 1 integrin subunit.

Genome-wide analysis of the effects of Notch signaling in Th2 cells

To explore the molecular mechanism how Notch affects lymph node egress of T cells, we performed genome-wide RNA sequencing of naive T cells and Th2 cells sorted from MedLN on day 12 of HDM-induced AAI (**Supplementary figure 7A**). To facilitate the identification of Th2 cells, we crossed WT and *N1N2^{ΔCD4/ΔCD4}* mice with Gata3-YFP reporter mice ('GATIR' mice)^{432, 433}. Interestingly, we found that in the absence of Notch signaling, the total numbers of YFP⁺ or CXCR4⁺ CD4⁺ T cells were reduced in the MedLN, but the numbers of CCR8⁺ T cells were increased (**Supplementary figure 7B**). CXCR4, which was recently identified as a Notch target⁴⁴⁷, is involved in chemotaxis and triggering cell adhesion⁴⁴⁸.

For RNA-sequencing, naive T cells were defined as CD3⁺CD4⁺YFP⁺CD62L⁺CD44^{low}, while Th2 cells were sorted as CD3⁺CD4⁺YFP⁺ from MedLN and BAL (**Supplementary figure 7C**). Interestingly, principle component analysis showed that gene expression in naive MedLN CD4⁺ T cells was similar between WT and *N1N2^{ΔCD4/ΔCD4}* mice (**Figure 6A**). In contrast, many genes were differentially expressed (DE, see Methods for criteria used) when comparing Th2 cells sorted from MedLN from the two mouse groups. This was also evident from a correlation and principal component analysis (**Supplementary figure 7D, Figure 6A**). The low numbers of Th2 cells in the BAL fluid of *N1N2^{ΔCD4/ΔCD4}* mice precluded their expression analysis, but we did include WT BAL Th2 cells and found that they differed substantially from WT MLN Th2 cells (**Figure 6A; Supplementary figure 7D**).

These findings indicate that differences between WT and *N1N2^{ΔCD4/ΔCD4}* Th2 cells involve genes of which the expression is specifically altered during T cell activation. Of the 692 DE genes (**Figure 6B; Supplementary Table 2**), 392 were downregulated and 300 were upregulated in *N1N2^{ΔCD4/ΔCD4}* Th2 cells compared to WT Th2 cells (**Figure 6C**). The majority of these 692 DE genes were not MedLN-specific, as was evident from the comparison of WT Th2 cells from MedLN and BAL fluid. Interestingly, 48 of the 300 genes upregulated in Notch-deficient Th2 cells represent genes that were higher in WT BAL Th2 cells than in WT MedLN Th2

Figure 6. Notch signaling affects expression of cytokines and adhesion molecules

(A) Principle component analysis on Log2 transformed RPKM values from RNA-sequencing of naive T cells and Th2 cells sorted from BAL and MedLN of *N1N2^{ΔCD4/ΔCD4}* and WT GATIR mice exposed to the acute HDM-mediated AAI protocol.

(B) Volcano plot of all genes >1 RPKM. Differentially expressed (DE) genes are shown in red.

(C) Percentage of upregulated and downregulated genes within the 692 DE genes when comparing *N1N2^{ΔCD4/ΔCD4}* Th2 cells with WT Th2 cells and distribution of Notch-regulated genes when comparing WT Th2 cells from BAL to MedLN or when comparing WT naive T cells to Th2 cells.

(D) Pathway analysis on genes that are upregulated in *N1N2^{ΔCD4/ΔCD4}* Th2 cells compared to WT Th2 cells and downregulated in WT Th2 cells compared to WT naive T cells. The top 10 enriched pathways and the top 10 of genes of the top 3 pathways are shown.

(E) Pathway analysis on genes that are downregulated in *N1N2^{ΔCD4/ΔCD4}* Th2 cells compared to WT Th2 cells and upregulated in WT Th2 cells compared to WT naive T cells. The top 10 enriched pathways and the top 10 of genes of the top 3 pathways are shown.

(F) Heatmap of GWAS genes that are DE when comparing *N1N2^{ΔCD4/ΔCD4}* Th2 cells with WT Th2 cells.

For RNA-sequencing, 2-3 mice were pooled per group to obtain 3 samples.

cells. Conversely, 68 of the 393 genes downregulated in Notch-deficient Th2 cells were expressed at lower levels in WT BAL Th2 cells than in WT MedLN Th2 cells (**Figure 6C**). To investigate whether the 692 DE genes were specific for Th2 cells, we compared them with genes that were different between WT MedLN naïve T cells and Th2 cells. The majority (171) of the 300 upregulated DE genes in NiN2^{ΔCD4/ΔCD4} Th2 cells were normally downregulated during Th2 cell differentiation. Pathway analysis on these 171 upregulated naïve T cell genes revealed genes involved in response to IFN- α and IFN- β and genes in T helper cell differentiation, including *Ccr7* (**Figure 6D**). *Ccr7* is a gene that is normally downregulated upon T cell activation and is essential for T cells to enter and stay in the lymph node and could therefore contribute to retention of Notch-deficient T cells in the MedLN. In contrast, the majority of the 392 downregulated genes were normally upregulated during Th2 cell differentiation (**Figure 6C**). Together, these data show that NiN2^{ΔCD4/ΔCD4} Th2 cells failed to adopt a proper Th2-associated gene expression program. Pathways that are enriched in the 257 downregulated Th2 cell genes included glucose metabolism, cell-adhesion and cytokine production (**Figure 6E**). As expected, downregulated genes included *Gata3* and *Il13*, but also *Il6*, *Il10* and *Cx3cr1*. Finally, we detected the misregulation of several genes (25 out of 665) that were previously linked to asthma in genome-wide association studies (GWAS)⁴³³ (**Figure 6F**).

In summary, our data indicate that Notch signaling enhances lymph node egress of Th2 cell, whereby the absence of Notch signaling affects cytokine production, glucose metabolism and the expression of adhesion molecules.

Discussion

Th2 cells play a central role in the development of allergic asthma via the production of the Th2 cytokines IL-4, IL-5 and IL-13, whose expression is controlled by Gata3. Notch signaling was described to promote Th2 cell differentiation by directly inducing *Gata3* or *Il4* transcription^{93, 129, 130}. While it is assumed that Notch signaling induces Th2 cell differentiation in a STAT6-independent manner, we have previously shown that blocking Notch signaling during only the challenge phase in a HDM-driven model for AAI leads to a reduction of eosinophilia, Th2 cell induction and bronchial hyperreactivity (BHR) (**chapter 4**). Therefore, the mechanism of how Notch signaling steers Th2 cell mediated inflammation remained unclear. Here, we show that Notch signaling is not required for the proliferation of naïve T cells upon antigenic stimulation or for their polarization towards Th2 cells. Although we found that Gata3 induction is reduced in the absence of Notch signaling, Gata3 overexpression did not completely rescue AAI induction in the absence of Notch signaling. These findings indicate that Notch signaling controls additional aspects of Th2 cell-mediated AAI beyond the induction of Gata3.

Importantly, in the absence of Notch signaling established Th2 cells accumulated in draining lymph nodes, while reduced numbers were detected in BAL and lungs. In this context, it is of note that several studies have demonstrated parallels between developmental processes controlled by Notch and integrin-mediated cell adhesion. Integrins are heterodimeric transmembrane receptors that mediate interactions between cells and the extracellular matrix⁴⁴⁹. Processes that are regulated by $\alpha 4\beta 1$, $\alpha 5\beta 1$ and $\alpha 6\beta 1$ integrins include survival, migration, and differentiation^{450, 451}. Notch1 activates $\beta 1$ integrins in mammalian cells⁴⁵², suggesting that Notch may affect integrin activation and thereby modulates cell migration by alternating

cell-matrix interactions⁴⁵³. Indeed, we found that RNA expression of the $\beta 1$ subunit *Itgb1* (CD29) was significantly lower in Notch1 and Notch2 double-deficient Th2 cells compared to WT Th2 cells in the acute HDM-driven AAI model. Genes that are associated with cell adhesion pathways were strongly affected in the absence of Notch signaling. In addition to *Itgb1*, other adhesion molecules that were modulated by Notch signaling include RNA expression of 1) *Cx3cr1*, which has been implicated in T cell migration to the lungs in airway inflammation⁴⁵⁴, 2) *Icam5* that binds to the adhesion molecule LFA-1, 3) *Cd47* that enhances intracellular calcium concentration upon cell adhesion to extracellular matrix and 4) *Cd53* that interacts with integrins. Genes involved in cellular adhesion and migration that were upregulated in the absence of Notch included L1 Cell Adhesion Molecule *L1cam* and *Cd9*, as well as *Sell* (CD62L) and *Ccr7*. The latter two genes are required for T cells to enter and stay in the lymph nodes⁴⁴⁶. As such, we hypothesize that Notch signaling regulates the expression of critical adhesion molecules on Th2 cells, facilitating their exit from lymph nodes and migration into the lungs to orchestrate airway inflammation.

We found conflicting results when performing gene expression analysis on (1) Th2 cells that were *in vitro* polarized and transferred to WT mice that were subsequently exposed to HDM and OVA (**Figure 5**) and (2) Th2 cells from WT and Notch-deficient mice with HDM-driven AAI (**Figure 6**). These include e.g. expression levels of *Itgb1*, *Sell* and *Cx3cr1*. It is conceivable that these differences are a consequence of the procedure of T cell activation. In the first model, Th2 cell differentiation was induced *in vitro* using strong anti-CD3/CD28 stimulation and IL-4, which leads to Th2 cell differentiation in the absence of Notch signaling (**Supplementary figure 5**). In contrast, T cells that were induced by HDM-activated DCs may have received less strong activation signals *in vivo* whereby Notch-deficient T cells showed reduced Gata3 expression. In the OTII T cell transfer model, we robustly polarized these cells towards Gata3⁺ Th2 cells *in vitro*, while in the *in vivo* HDM-model, there is also production of IL-17 and IFN- γ , as shown in **Figure 1C**. In addition, there is evidence for CD4⁺ T cell plasticity as Th2 cells may transdifferentiate from follicular T helper cells and “hybrid” subsets exist - including IL-17 or IFN- γ -producing Th2 cells, and a subpopulation of activated Tregs also expresses high Gata3 levels^{245, 455}. Although we did not detect a role for Notch signaling during the sensitization phase in models using OVA, we cannot exclude that HDM-driven Th2 cells displayed altered gene expression due to the lack of a Notch stimulus during HDM-induced priming of naïve T cells. In addition, Notch signaling could function by providing anti-apoptotic signals to antigen-experienced T cells¹⁰⁹. In this context, it is important that there are differences in kinetics between the two models: in the OTII Th2 cell transfer model allergen challenges were performed one day after transfer of polarized Th2 cells, whereas in the HDM model allergen challenges were done 7 days after sensitization. It is possible that after these 7 days fewer Th2 cells survived due to the lack of Notch signaling. Therefore, the OTII Th2 cell transfer model is suitable to study the role of Notch signaling specifically during the challenge phase, while characteristics of Th2 cells from the HDM-driven model depend on both the sensitization and the challenge phase. Genome-wide transcription profiling experiments on *in vitro* polarized OTII Th2 cells and challenged *in vivo* should help in understanding the role of Notch signaling during the challenge phase.

Our studies showed that Notch receptors or RBPJ κ (unpublished observations) are dispensable for T cell activation, differentiation or expansion *in vitro* using anti-CD3/CD28 or activated GM-CSF bmDCs and *in vivo* using OVA. In contrast, others have shown that blocking Notch signaling with GSI reduces proliferation and CD25 expression, but not expression of CD44 and CD69, on T cells^{107, 110}. In addition, Laky *et al.* showed that DLL4-mediated Notch signaling increases cell-surface expression of glucose transport proteins (Glut1)

in naïve T cells due to increased phosphorylation of the PI3K-Akt-mTOR pathway¹⁰⁸. We could not detect an effect of Notch1/Notch2 deletion on S6 phosphorylation upon CD3/CD28 stimulation, indicating that activation of the PI3K-Akt-mTOR pathway is independent of Notch1 and Notch2. Although other Notch receptors might be involved, it has been shown that TCR stimulation can induce interaction of NICD with other proteins in the cell in a non-canonical, RBPJ κ -independent pathway that leads to NF κ B activation^{114, 115}. However, we did find that *Slc2a1* (Glut1) and glucagon (*Gcg*) expression were reduced in Notch-deficient Th2 cells compared to WT Th2 cells, in addition to many other genes involved in glucose metabolism. Consistent with these data, it was shown that RBPJ κ is required for upregulation of Glut1 in memory CD4⁺ T cells¹⁰⁹. Therefore, although Notch signaling appears important for the proper regulation of metabolic genes, we did not find evidence that Notch acts via the Akt-mTOR pathway.

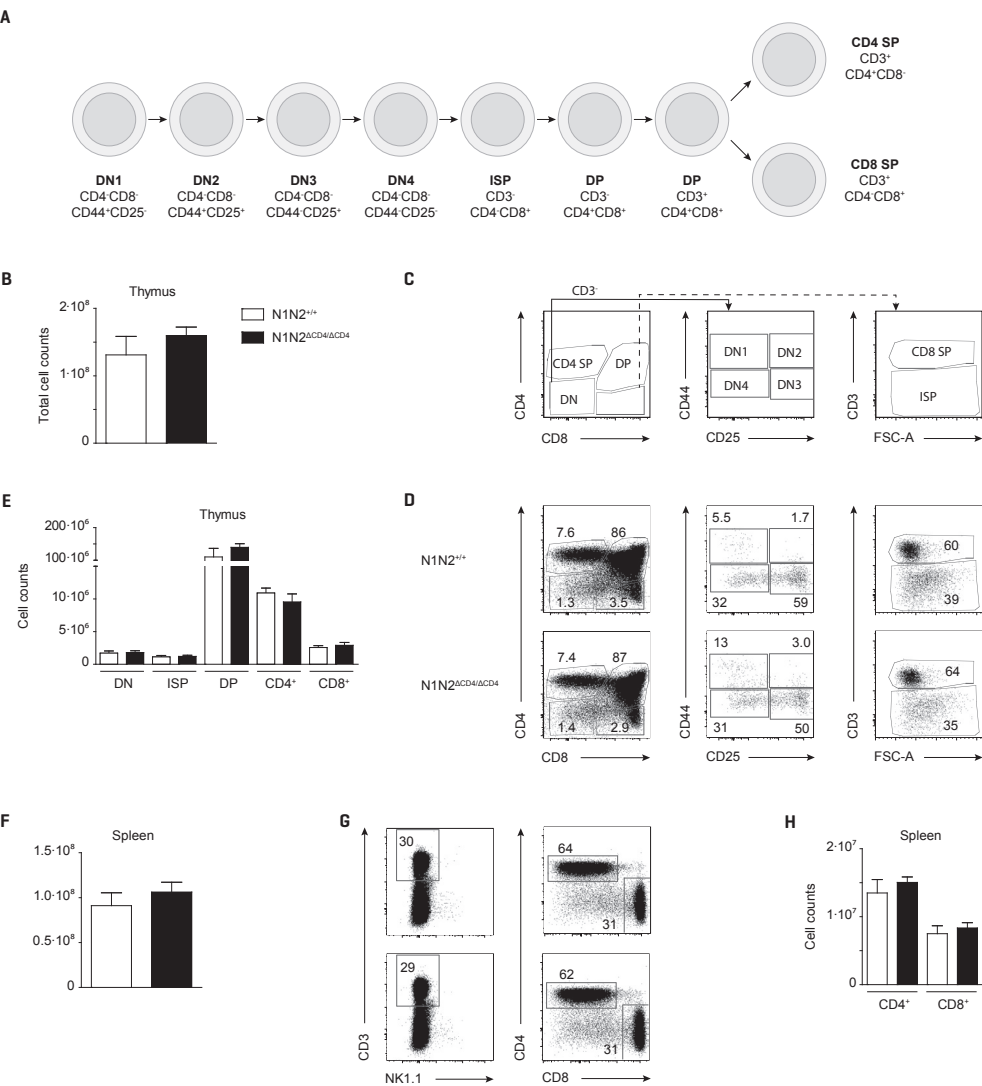
Notch signaling was shown to only drive T cell differentiation in the presence of polarizing cytokines¹³⁵. We found that genes that were misregulated in the absence of Notch signaling are involved in cytokine production and cellular responses to cytokine stimuli. Genes that were downregulated in the absence of Notch include the expected Th2 genes e.g. *Gata3*, *Ilh3* and *Ilh0*, but also *Cr1f2* that encodes the TSLP receptor and *Il2rg* that encodes the common gamma chain, the latter being essential for interleukin -2, -4, -7 and -21 signaling. Moreover, *Il6* was downregulated in Notch deficient Th2 cells. These observations suggest that Notch signaling optimizes T cell sensitivity to cytokines. It would be interesting to determine whether the misregulation of adhesion molecules is a direct effect of the absence of Notch signaling or indirectly caused by altered cytokine signaling.

Inhibiting Th2 responses by blocking Notch signaling was recently shown to increase T-bet and IFN- γ expression by T cells in a food allergy model¹³⁴, in line with restored Th2 responses in Notch- deficient mice that received anti-IFN- γ antibodies or high amounts of exogenous IL-4¹³⁵ (**Chapter 4**). These studies suggest that Notch signaling regulates the Th1/Th2 differentiation balance. Also in our analyses, many Th1 associated genes were upregulated in Th2 cells in the absence of Notch signaling, including *Irf1*, members of the Tnf superfamily, *Stat1*, *Cxcr3*, *Ifnar2* and *Ifngr*. This suggests that the role of Notch signaling during Th2 cell-mediated AAI is Th2-specific and does not lead to a defect in other T helper subsets. Altogether, we show that Notch signaling is required for optimizing rather than inducing Th2 responses by promoting cytokine responsiveness, cell adhesion and migration. The current study, combined with our findings that inhibiting Notch signaling specifically during the challenge phase led to reduced allergic airway inflammation and BHR^{133, 134} (**chapter 4**), indicates that blocking the Notch signaling pathway could represent a non-invasive and specific strategy to decrease Th2 cell-mediated inflammation in allergic asthma.

Acknowledgements

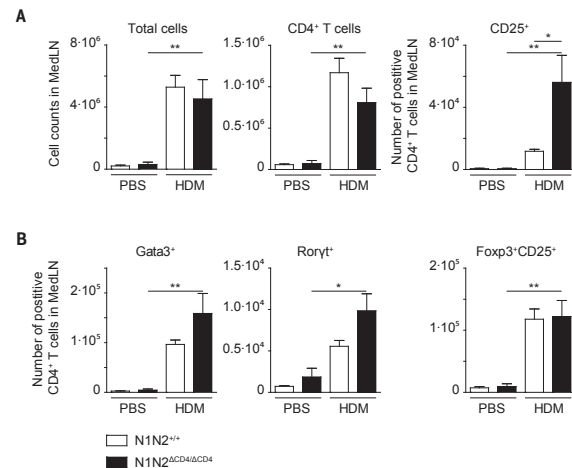
The authors like to thank O.B.J. Corneth, J. Rip, M. van Nimwegen and W.M.E. Hendriks (Erasmus MC Rotterdam) for their technical assistance. This study is partly supported by Lung Foundation Netherlands Grants 3.2.12.087 and 3.2.12.067.

Supplementary figures and tables



Supplementary figure 1. T cell development is unaffected in the absence of Notch signaling in CD4⁺ T cells

[A] Schematic representation of T cell development and the accompanying expression of surface receptors during the different stages.
[B] Number of total cells in thymus in WT and *N1N2 Δ CD4/ Δ CD4* mice.
[C-D] Flow cytometric analysis of T cell developmental stages in thymus from WT and *N1N2 Δ CD4/ Δ CD4* mice.
[E] Quantification of the total numbers of double-negative [DN], immature single positive [ISP], double-positive [DP] and CD4⁺ and CD8⁺ single positive thymocytes
[F] Number of total cells in thymus in WT and *N1N2 Δ CD4/ Δ CD4* mice.
[G] Flow cytometric analysis of CD4⁺ and CD8⁺ T cells in spleen from WT and *N1N2 Δ CD4/ Δ CD4* mice.
[H] Quantification of the total numbers of CD4⁺ and CD8⁺ single positive splenocytes.
Data are shown as mean + SEM of 3 mice per group.



Supplementary figure 2. Notch signaling in CD4⁺ T cells is not required for the induction of Th2 cell differentiation in the lymph nodes in chronic AAI

(A) Total cells and number of CD3⁺CD4⁺ T cells and CD25⁺CD4⁺ T cells in MedLN from PBS- or HDM-treated mice.

(B) Quantification of Gata3⁺, Rorγt⁺ and Foxp3⁺CD25⁺ CD4⁺ T cells MedLN.

Data are shown as mean + SEM of 6-8 mice per group. * p<0.05, **p <0.01, using Mann-Whitney U-test.

Supplementary figure 3. Enforced Gata3 expression partly enhances AAI in N1^{ΔCD4/ΔCD4} and N1N2^{ΔCD4/ΔCD4} mice

(A) Numbers of the indicated cell populations in BAL of the indicated mice, sensitized with PBS or HDM.

(B) distribution of Th1, Th2 and Th17 cells, as signified by their key cytokines IL-5 or IL-13, IFN-γ and IL-17A, in BAL of the indicated HDM-sensitized mice.

(C) Total IgG1 and IgE levels and levels of HDM-specific IgG1 and IgE in serum, as quantified by ELISA.

Data are shown as mean + SEM of 3-6 mice per group and are representative of 3 independent experiments. * p<0.05, **p <0.01, using Mann-Whitney U-test.

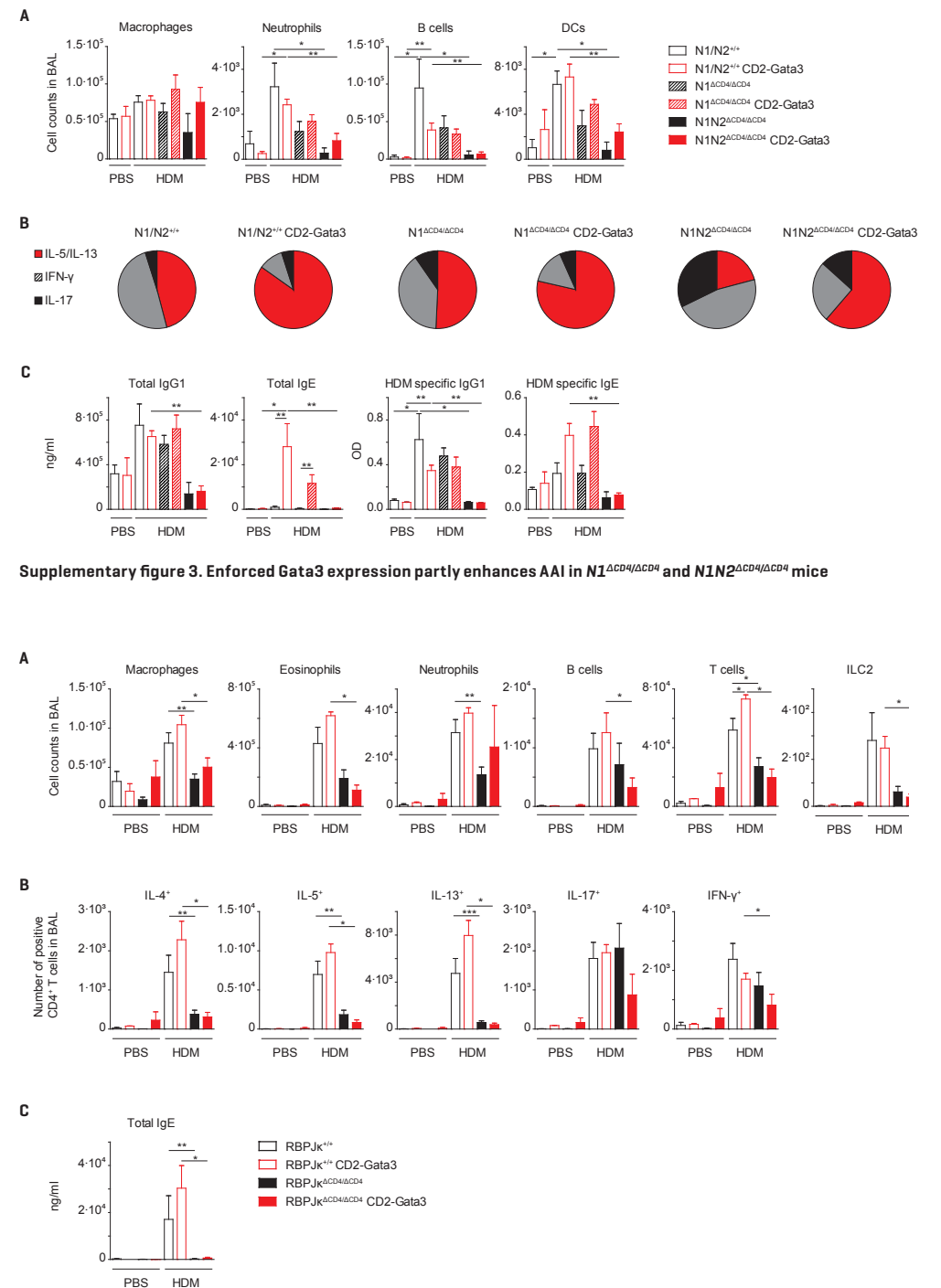
Supplementary figure 4. Enforced Gata3 expression does not alter AAI in RBPJ^{ΔCD4/ΔCD4} mice

(A) Numbers of the indicated cell populations in BAL of the indicated mice, sensitized with PBS or HDM.

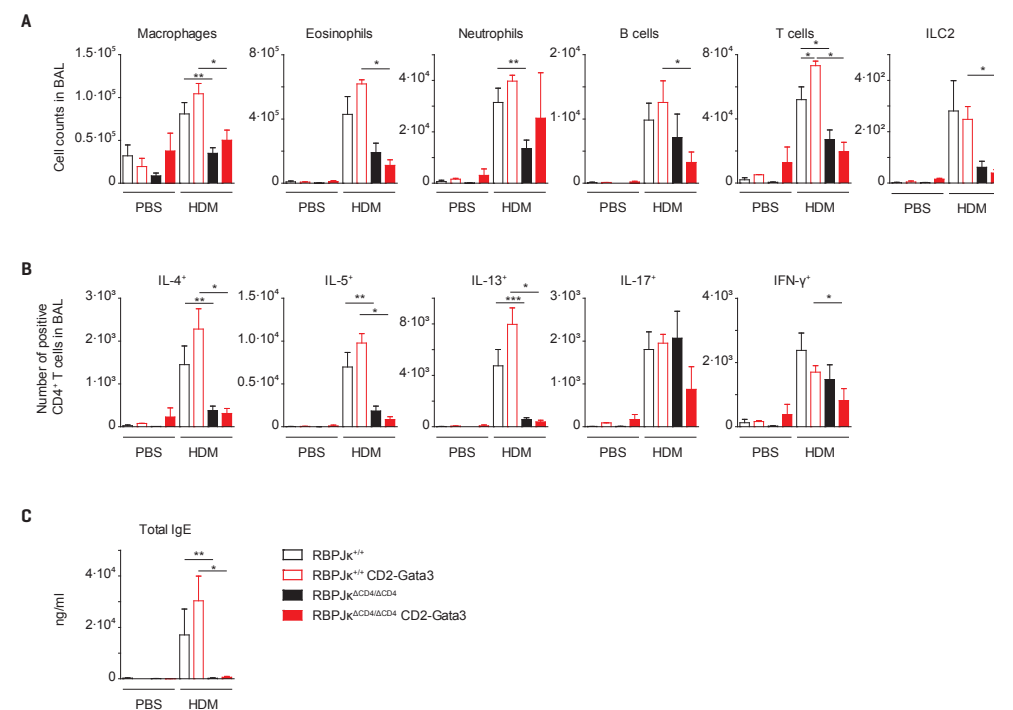
(B) Quantification of the total numbers of cytokine-positive CD3⁺CD4⁺ T cells in BAL.

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

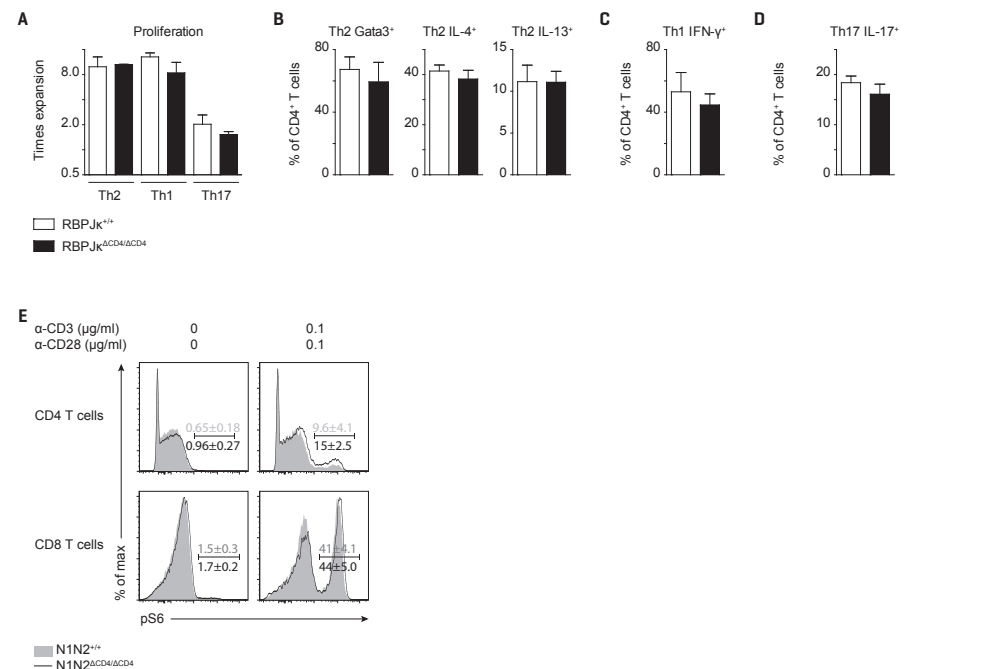
Data are shown as mean + SEM of 2-7 mice per group and are representative of 3 independent experiments. * p<0.05, **p <0.01, ***p<0.001 using Mann-Whitney U-test.



Supplementary figure 3. Enforced Gata3 expression partly enhances AAI in N1^{ΔCD4/ΔCD4} and N1N2^{ΔCD4/ΔCD4} mice



Supplementary figure 4. Enforced Gata3 expression does not alter AAI in RBPJ^{ΔCD4/ΔCD4} mice



Supplementary figure 5. Notch signaling is not required for *in vitro* proliferation and differentiation of CD4⁺ T cells

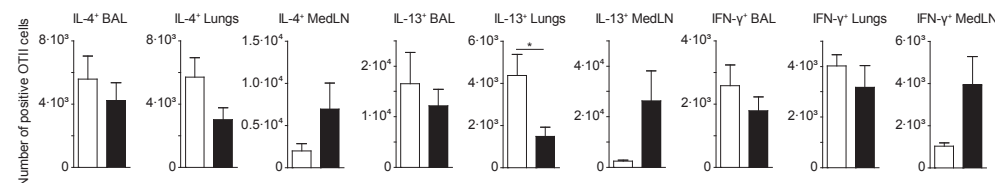
[A] Expansion of *in vitro* polarized CD4⁺ T cells with anti-CD3 and anti-CD28 antibodies from RBPJk^{+/+} and RBPJk^{ΔCD4/ΔCD4} mice.

[B–D] Percentage of Gata3 and cytokine positive CD4⁺ T cells from *in vitro* polarized Th2 cells, [C] Th1 cells and [D] Th17 cells from RBPJk^{+/+} and RBPJk^{ΔCD4/ΔCD4} mice.

Data are shown as mean + SEM of 2–4 mice per group and are representative of 2 independent experiments.

[E] Total spleen cells from WT OTII cells and N1N2^{ΔCD4/ΔCD4} mice were stimulated with the indicated concentrations of α-CD3 and α-CD28 for 3 hours after which phosphorylation of ribosomal S6 in CD4⁺ and CD8⁺ T cells was measured using flow cytometry.

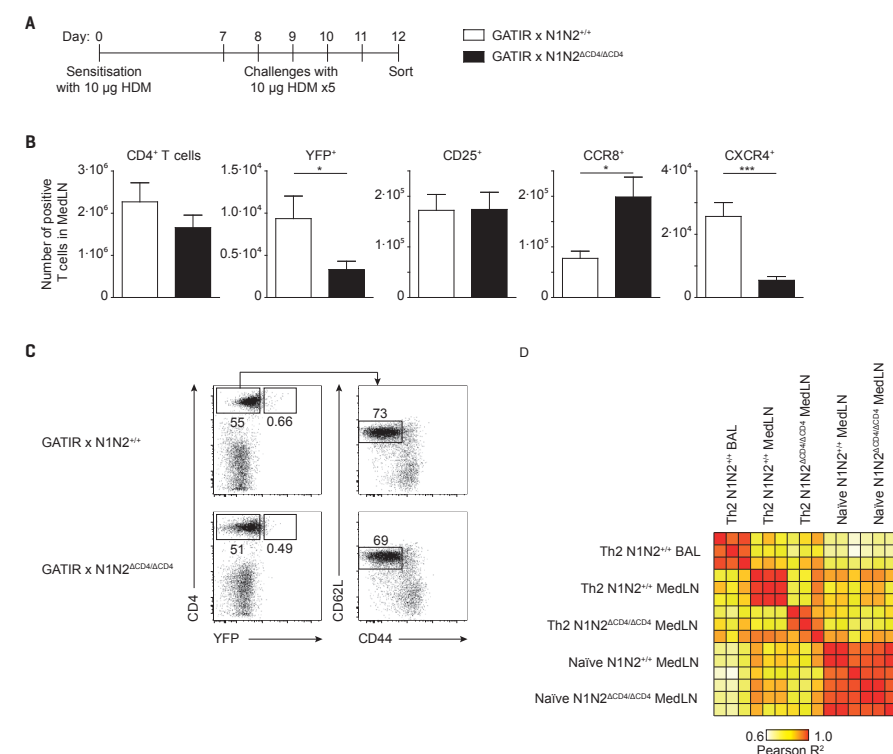
Data are shown as mean + SEM of 4–5 mice per group and are representative of 3 independent experiments. * p<0.05, using Mann-Whitney U-test.



Supplementary figure 6. Notch signaling mediates lymph node egress of already established Th2 cells

Quantification of the total numbers of cytokine-positive CD3⁺CD4⁺ *in vitro* polarized WT and N1N2^{ΔCD4/ΔCD4} OTII cells in the indicated tissues from mice challenged with OVA and HDM.

Data are shown as mean + SEM of 6 mice per group and are representative of 3 independent experiments. Statistics were performed using Mann-Whitney U-test.



Supplementary figure 7. Characterization of Th2 cells from N1N2^{ΔCD4/ΔCD4} and WT mice in an acute AAI model

[A] Scheme of acute HDM-mediated AAI induction in N1N2^{ΔCD4/ΔCD4} and WT GATIR mice.

[B] Quantification of CD4⁺, YFP⁺, CD25⁺, CCR8⁺, and CXCR4⁺ T cells in and MedLN.

[C] Gating strategy for sorting naive T cells [CD3⁺CD4⁺YFP-CD62L⁺CD44^{low}] and Th2 cells [CD3⁺CD4⁺YFP⁺].

[D] Correlation matrix of RNA-sequencing of sorted naive T cells and Th2 cells from BAL and MedLN.

Data are shown as mean + SEM of 8 mice per group of one experiment. For RNA-sequencing, 2–3 mice were pooled per group to obtain 3 samples. * p<0.05, **p<0.01, ***p<0.001 using Mann-Whitney U-test.

Supplementary Table 1. Antibodies used for flow cytometry

Target	Conjugate	Company	Clone
B220	APC	BD Biosciences	RA3-6B2
B220	PE	eBioscience	RA3-6B2
CCR4	BV421	Biolegend	SA214G2
CCR8	PE-Cy7	Biolegend	2G12
CD3	APC-ef780	eBioscience	17A2
CD3	BV421	BD Biosciences	145-2C11
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	BV711	BD Biosciences	RM4-5
CD4	FITC	eBioscience	RM4-5
CD4	PE	eBioscience	RM4-5
CD4	PerCP-Cy5.5	eBioscience	RM4-5
CD5	PE	eBioscience	53-7.3
CD8a	FITC	eBioscience	Ly-2
CD8	PE	eBioscience	Ly-2
CD8a	PE-Cy7	eBioscience	53-6.7
CD11b	APC	eBioscience	M1/70
CD11b	PE	eBioscience	M1/70
CD11c	BV786	BD Biosciences	HL3
CD11c	PE	eBioscience	N418
CD19	AF700	eBioscience	eBio1D3
CD19	APC-ef780	eBioscience	1D3
CD19	PE	BD Biosciences	1D3
CD25	PE-Cy7	eBioscience	PC61.5
CD25	PerCP-Cy5.5	eBioscience	PC61.5
CD44	APC	BD Biosciences	IM7
CD44	APC-Cy7	BD Biosciences	IM7
CD44	FITC	eBioscience	IM7
CD44	PerCP-Cy5.5	eBioscience	IM7
CD62L	APC	eBioscience	MEL-14
CD127	e450	eBioscience	A7R34
CD127	PE-Cy7	eBioscience	A7R34
CXCR4	APC	BD Biosciences	2B11
FcεRIα	PE	eBioscience	MAR-1
Foxp3	AF488	eBioscience	FJK-16s
Foxp3	PE-Cy7	eBioscience	FJK-16s
Gata3	ef660	eBioscience	TWAJ-14

IFN-γ	BV650	BD Biosciences	XM61.2
IFN-γ	e450	eBioscience	XM61.2
IFN-γ	PE-Cy7	eBioscience	XM61.2
IL-4	BV711	BD Biosciences	11B11
IL-4	PE	BD Biosciences	11B11
IL-5	APC	BD Biosciences	TRFK-5
IL-5	PE	BD Biosciences	TRFK-5
IL-5	Biotin	BD Biosciences	TRFK4
IL-9	PE	BD Biosciences	D9302C12
IL-10	AF488	eBioscience	JES5-16E3
IL-10	PerCP-Cy5.5	eBioscience	JES5-16E3
IL-13	ef450	eBioscience	eBio13A
IL-13	ef660	eBioscience	eBio13A
IL-17A	AF700	BD Biosciences	TC11-18H10.1
Live/Dead	Amcyan	Invitrogen	
Ly-6A/Ly-6E	BV786	BD Biosciences	D7
Ly-6G	PE-Cy7	BD Biosciences	1A8
Ly-6C/Ly-6G	APC-ef780	eBioscience	RB6-8C5
Ly-6C/Ly-6G	PE	eBioscience	RB6-8C5
MHC class II	AF700	eBioscience	M5/114.15.3
NK1.1	Horizon 450	BD Biosciences	PK136
NK1.1	PE	eBioscience	PK136
Rorγt	PE	BD Biosciences	Q31-378
Siglec-F	PE	BD Biosciences	E50-2440
Streptavidin	APC-ef780	eBioscience	
Streptavidin	BV711	BD Biosciences	
Streptavidin	BV785	BD Biosciences	
Streptavidin	PE-Cy7	eBioscience	
T1/ST2 (IL-33R)	Biotin	mdbiosciences, Zürich, Swiss	DJ8
T1/ST2 (IL-33R)	FITC	mdbiosciences	DJ8
T-bet	BV421	BD Biosciences	O4-46
TCR vα2	APC-Cy7	BD Biosciences	B20.1
TCR vα2	Biotin	eBioscience	B20.1
TCR vβ5.1, 5.2	Biotin	BD Biosciences	MR9-4
TCR vβ5.1, 5.2	BV785	BD Biosciences	MR9-4
TER-119	PE	eBioscience	TER119

			BAL	MLN				Naïve										
			Th2	WT1	WT2	WT3	WT1	WT2	WT3	K04	K05	K06	WT1	WT2	WT3	K04	K05	K06
	Annotation	Log2Fold Change	p-value	WT1	WT2	WT3	WT1	WT2	WT3	K04	K05	K06	WT1	WT2	WT3	K04	K05	K06
	Atpb3	1.15	1.89E-09	127.91	172.30	158.93	143.48	113.57	139.21	261.17	323.68	303.38	664.65	694.96	754.19	773.00	919.27	838.17
	Atxv1	-1.54	1.83E-03	19.22	17.43	11.72	16.29	20.33	23.32	3.56	8.93	5.63	2.10	1.54	1.13	1.30	1.57	1.31
	AW011738	-2.45	2.84E-03	3.55	0.00	2.77	2.07	4.42	3.37	0.00	0.00	0.00	0.789	2.46	6.03	1.08	1.42	0.84
	AW112010	0.96	6.70E-04	2007.90	1666.93	2333.20	848.92	874.14	750.16	1411.81	2108.91	1355.19	375.46	342.52	280.11	248.75	330.14	247.02
	B3gnt11	1.33	1.96E-02	6.24	10.03	4.01	12.40	9.65	11.16	26.03	47.32	19.61	6.36	5.75	8.19	12.12	5.08	4.40
	Bolp3	1.52	6.92E-04	1.44	2.51	5.04	3.14	4.45	2.47	11.85	9.61	10.48	1.39	2.00	2.12	1.66	2.08	1.69
	Baz1a	-2.14	2.12E-03	30.07	15.38	27.92	44.30	62.19	78.09	1.55	11.74	12.67	3.65	2.78	2.68	4.18	4.63	3.13
	Baz2b	2.45	3.12E-05	2.60	1.23	4.27	2.18	0.93	0.52	9.50	9.89	9.64	5.83	6.50	6.07	6.76	7.45	4.92
	BC031181	-0.73	7.44E-03	127.41	138.67	100.43	96.74	113.69	106.76	57.47	62.20	69.00	110.01	95.14	103.57	135.39	103.85	112.36
	Bedn3d	-2.05	1.96E-02	0.00	26.50	10.63	11.44	9.23	12.80	0.00	0.00	0.28	15.71	22.03	19.76	11.80	18.88	23.59
	Bcl10	-0.94	3.00E-03	7.91	11.46	22.41	22.74	25.88	25.78	11.92	12.43	13.49	32.36	21.76	24.42	29.13	29.36	28.74
	Bhlhe40	-1.02	1.42E-03	169.33	139.94	185.99	80.29	79.79	106.08	34.31	39.87	52.81	0.89	0.25	1.22	2.04	0.55	1.22
	Bin2	0.61	4.03E-03	536.22	488.51	468.21	373.45	441.69	486.53	755.53	569.92	672.76	688.08	674.10	637.96	503.19	566.22	711.03
	Blmh	-0.92	3.65E-02	31.02	58.08	55.53	97.82	66.97	90.95	47.03	28.04	54.76	20.71	23.89	25.06	28.67	25.92	27.98
	Bms1	0.93	4.61E-03	11.13	8.03	8.97	14.23	15.38	10.27	25.33	25.47	26.86	27.21	18.41	19.95	16.57	22.89	18.13
	Bsg	-0.70	4.20E-05	126.71	127.73	159.14	224.43	201.88	231.55	152.67	121.11	128.73	144.41	138.86	114.29	120.00	113.66	150.27
	Btn15-ps	2.30	5.25E-03	0.00	0.00	1.54	1.38	0.81	0.07	10.68	17.67	8.08	0.00	0.00	0.00	0.00	0.00	0.00
	Bzrep1	-1.75	1.32E-02	2.08	0.00	0.56	5.30	11.85	7.40	1.27	2.98	1.03	0.33	0.20	0.22	0.00	0.00	0.42
	C2cd2	-8.04	1.35E-57	5.26	0.50	0.73	13.82	10.38	15.74	0.01	0.00	0.00	0.00	0.07	0.47	0.00	0.00	0.00
	Calca	-7.81	8.80E-46	698.18	458.48	481.45	77.66	183.87	183.90	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Colm3	-1.39	1.22E-13	85.55	85.45	92.71	186.36	202.22	192.03	64.87	81.64	69.69	56.10	44.06	53.63	61.84	50.24	57.61
	Comk4	0.94	4.48E-04	4.44	6.67	6.63	4.85	3.43	4.40	8.78	7.95	8.07	10.30	9.26	10.18	11.46	10.06	10.04
	Capn2	-1.19	5.08E-03	130.62	168.06	190.77	114.63	134.93	143.42	52.11	32.73	78.63	25.38	24.34	23.84	30.37	37.70	40.24
	Car7	-4.25	7.65E-10	35.92	0.00	3.83	7.38	14.21	16.62	0.00	0.00	0.00	0.19	0.81	2.36	0.00	1.32	1.29
	Cast	0.84	8.58E-04	55.13	108.69	90.56	69.45	58.43	58.26	122.62	84.24	132.29	95.13	92.95	73.28	89.35	100.11	101.25
	Cat	-0.90	1.91E-02	17.77	39.92	47.64	44.47	46.45	50.21	19.60	21.49	31.92	50.51	51.91	45.13	53.13	57.76	54.83
	Cbl	1.82	1.24E-02	5.78	0.30	11.47	3.92	1.55	5.72	20.05	28.84	8.25	7.07	5.39	9.39	8.55	8.81	7.06
	Cbx7	1.76	3.59E-04	4.45	43.08	19.86	12.95	13.35	14.79	27.90	54.66	78.91	24.89	19.86	25.71	28.13	28.99	21.57
	Cc2d1b	1.46	4.41E-10	16.89	26.28	22.87	17.26	23.01	21.77	64.22	54.54	57.02	23.73	16.25	32.53	21.27	21.78	24.67
	Ccdc117	1.02	4.66E-02	9.43	38.53	14.39	6.92	9.36	6.54	17.87	11.60	20.01	15.58	16.16	12.49	15.33	15.95	13.32
	Ccdc174	1.42	3.18E-02	7.18	24.40	14.10	7.88	11.16	11.86	38.07	15.36	46.58	23.16	26.82	22.53	25.98	30.66	25.53
	Ccdc22	1.13	2.21E-03	11.31	0.97	24.24	14.59	10.32	15.63	36.05	26.35	30.51	38.76	36.72	35.49	27.84	29.58	43.75
	Ccdc66	1.69	5.24E-03	10.08	25.97	17.17	8.04	11.86	10.27	64.84	17.24	40.14	12.82	7.11	8.31	12.67	10.30	7.37
	Ccdc84	1.10	8.44E-03	44.18	18.82	32.99	24.16	16.20	19.71	48.30	49.80	36.92	24.19	23.54	23.23	21.68	22.33	21.81

Ccdc92	1.26	3.32E-03	5.41	12.04	11.27	9.47	11.46	6.72	26.50	22.09	22.34	14.05	11.28	9.24	14.86	15.14	22.48
Cc2	-1.07	1.40E-04	163.56	190.59	222.36	77.86	84.83	111.26	44.91	33.80	48.34	0.95	0.39	0.05	0.07	0.12	0.00
Cc7	2.05	9.21E-05	0.00	58.50	28.69	25.58	11.12	19.02	119.56	46.92	120.68	777.63	755.94	743.84	855.05	893.79	1221.68
Cc4	-0.76	3.36E-02	495.36	501.32	373.24	496.71	666.28	647.31	274.43	454.92	313.64	311.98	298.95	319.37	325.75	304.17	379.71
Cc47	-0.80	8.54E-04	226.20	231.23	287.47	220.77	210.02	189.86	103.27	103.98	149.68	252.03	244.77	249.85	329.16	311.61	253.47
Cc52	0.67	2.80E-05	2098.71	2117.57	1973.88	1822.19	1664.25	1506.78	2406.26	2553.61	2996.58	2827.85	2469.74	3090.32	4432.61	3915.24	4101.82
Cc53	-1.34	9.90E-16	573.89	721.46	719.03	440.31	510.20	541.93	226.65	175.46	180.37	369.36	398.44	379.57	463.26	452.89	359.38
Cc55	1.86	1.67E-04	0.00	2.96	1.67	7.72	3.71	4.17	24.54	24.26	17.45	31.25	35.67	24.58	33.81	45.59	50.68
Cc68	-4.04	8.05E-09	117.43	95.81	35.44	13.26	9.85	12.05	0.00	0.00	0.00	1.30	1.11	1.59	0.47	2.80	0.21
Cc84	1.01	3.22E-02	25.93	21.25	35.55	24.84	20.64	30.73	77.27	36.67	49.27	30.80	34.47	34.31	31.28	37.46	37.35
Cc9	1.95	1.74E-03	45.81	189.82	54.15	9.93	3.21	6.35	24.49	39.61	35.81	89.85	122.80	85.05	99.95	109.05	93.65
Cdc34	-1.15	1.15E-04	6.13	13.87	18.33	33.67	33.84	30.37	14.64	12.89	15.11	15.53	13.94	22.52	14.67	11.59	15.75
CdK6	-1.55	1.48E-08	7.35	10.48	10.32	13.05	12.04	16.67	5.18	4.59	3.88	1.93	1.87	1.32	2.10	1.27	2.20
Cdk1a	-1.70	1.17E-02	49.82	37.24	61.94	23.09	31.73	45.43	8.75	11.06	3.40	0.28	0.12	0.55	1.22	0.83	0.83
Cdkn2b	-2.31	6.03E-03	6.28	0.00	7.80	3.09	18.91	10.28	0.00	0.00	0.00	0.38	0.00	0.29	0.00	0.00	0.00
Cep135	1.37	4.63E-02	10.33	2.90	4.53	2.99	2.25	3.08	9.32	3.95	12.74	3.96	3.45	5.25	3.21	3.48	1.62
Cep290	1.66	4.27E-02	0.00	0.00	0.81	0.19	0.19	0.49	1.09	1.56	1.50	0.35	0.41	0.35	0.29	0.23	0.36
Cep295	1.70	4.48E-02	0.32	0.08	3.27	4.04	0.53	1.91	5.67	10.43	18.04	3.89	3.96	4.18	4.24	4.14	1.98
Cers5	-0.79	7.76E-03	48.68	81.20	69.76	67.65	52.24	75.78	36.36	34.40	39.99	34.28	33.48	28.78	30.26	26.58	35.58
Cfan36	1.06	2.89E-02	0.08	17.54	21.55	15.24	25.28	22.78	55.08	47.78	37.88	11.12	11.32	8.51	18.30	18.46	16.79
Cf1	-0.78	1.18E-05	516.38	609.25	669.90	1061.28	1058.42	1103.39	712.74	489.24	662.53	1390.95	1318.10	1505.10	1380.12	1509.16	
Cflar	-1.40	4.43E-02	13.20	12.95	15.87	14.18	17.60	12.39	9.97	7.40	11.65	11.27	10.88	9.41	11.41	17.12	13.29
Cfhd1	1.60	2.04E-02	0.19	0.60	2.05	1.13	1.52	1.19	3.43	6.40	5.00	1.13	0.71	1.03	0.82	0.77	0.96
Cfhd6	1.72	2.30E-03	0.00	0.00	4.51	3.64	5.16	6.50	19.18	24.26	17.15	3.93	4.29	4.43	6.42	5.31	6.23
Cfhp2	-1.33	6.56E-03	4.80	19.05	26.37	20.05	15.62	26.02	9.99	7.04	5.33	11.58	7.94	15.36	10.50	13.37	14.56
Cfb2	2.68	2.27E-05	16.31	79.31	30.97	2.60	3.28	6.73	27.80	68.26	44.05	0.00	0.00	0.00	0.00	0.00	0.00
Cfcd1	-1.09	1.53E-02	123.51	76.63	73.57	101.43	96.74	124.89	38.54	63.42	40.59	44.46	37.04	35.96	36.92	43.99	44.20
Cldn1	-2.30	1.50E-26	82.87	91.83	77.51	149.46	156.29	179.18	33.77	24.72	36.37	22.37	22.55	12.42	20.55	21.15	17.65
Clec2l	1.95	5.36E-05	0.00	2.53	1.75	7.63	6.99	5.10	19.02	28.68	42.28	23.91	27.41	22.06	28.66	27.15	16.90
Cmln	-3.49	2.04E-06	0.00	0.00	0.00	17.14	23.57	7.99	0.00	0.00	0.00	1.14	0.00	0.00	0.00	0.00	0.00
Cmt1	1.48	2.01E-02	4.03	1.46	1.01	1.25	0.92	0.80	3.35	2.23	4.39	0.16	0.00	0.42	0.40	0.02	0.01
Coo3	-1.12	3.38E-02	84.24	119.71	123.02	121.77	140.71	178.42	61.84	39.23	87.95	177.60	167.13	129.79	149.53	127.54	157.01
Col16a1	-2.39	3.96E-03	0.00	0.00	0.00	1.06	1.71	2.58	0.00	0.00	0.00	0.00	0.00	0.31	0.00	0.00	0.00
Cops7a	0.84	4.52E-02	54.23	36.75	24.29	42.28	36.77	41.91	99.68	51.53	74.54	60.87	45.26	48.25	54.05	48.62	50.55
Caq3	-1.90	3.38E-02	0.00	23.34	12.50	41.11	14.76	13.59	0.00	0.00	0.20	8.61	6.22	5.92	5.60	4.50	3.75
Cat1	-0.91	1.26E-03	409.08	413.29	411.39	510.69	581.95	493.43	218.25	251.48	357.85	285.88	230.28	295.57	400.70	357.04	373.67
ox17	-0.85	4.28E-02	338.48	322.53	304.21	355.36	393.89	356.06	135.65	222.84	228.71	141.00	127.66	118.41	189.16	168.23	138.18

		BAL	MLN					Naive									
			Th2	WT1	WT2	WT3	WT1	WT2	WT3	K04	K05	K06	WT1	WT2	WT3	K04	K05
Annotation	Log2Fold Change	p-value	WT1	WT2	WT3	WT1	WT2	WT3	K04	K05	K06	WT1	WT2	WT3	K04	K05	K06
Cox6a1	-0.52	2.05E-02	274.16	327.19	413.80	456.70	468.55	502.13	300.33	311.20	374.59	449.06	373.58	473.17	516.04	485.48	501.90
Crf2	-1.00	3.07E-02	11.90	17.38	27.61	30.88	34.96	24.25	16.64	13.75	12.15	11.60	6.19	11.80	11.83	16.36	11.50
Csf1	-2.17	4.56E-03	100.57	67.37	27.61	101.10	140.18	125.45	34.24	6.54	2.23	0.60	0.59	0.00	0.36	0.25	0.34
Csf7	-1.92	1.35E-03	92.33	97.05	112.58	306.26	462.22	407.56	155.96	40.42	42.13	26.23	15.73	34.39	5.45	2.87	6.11
Ctla2a	2.33	3.02E-14	147.61	168.60	189.79	12.03	13.65	14.08	88.88	54.41	76.72	1.23	0.81	0.35	1.14	1.47	0.47
Ctla2b	2.47	9.61E-09	24.13	30.27	27.36	3.62	2.99	3.97	20.51	29.96	21.01	0.28	0.34	0.00	0.67	0.80	0.00
Ctzb	-0.64	6.45E-03	234.31	354.48	258.30	251.92	273.52	308.51	176.00	202.93	150.47	93.64	86.16	68.57	77.76	72.82	99.11
Ctsc	-1.94	1.32E-14	58.52	121.94	53.03	66.78	98.80	92.70	24.38	27.40	16.84	25.46	27.44	22.90	22.42	25.25	20.86
Ctsd	-1.89	4.94E-16	1164.24	1054.58	788.82	588.48	639.68	884.93	172.58	207.92	163.84	239.66	212.56	226.35	279.56	250.88	314.80
Ctss	1.12	1.41E-04	361.10	489.26	375.27	125.65	87.72	73.56	214.06	194.94	231.38	283.20	267.36	233.79	314.20	290.21	312.98
Ctsw	3.02	3.65E-07	52.26	96.75	67.27	21.29	3.31	7.81	159.53	162.49	129.87	80.30	62.87	72.33	159.61	164.27	218.73
Cx3cr1	-0.88	5.98E-73	36.35	9.81	32.05	31.00	35.42	46.37	0.00	0.00	0.00	0.02	0.00	0.17	0.00	0.00	0.00
Cxcr3	2.19	8.29E-03	30.63	20.92	37.29	14.63	3.78	2.19	16.67	185.99	56.36	0.51	1.51	0.00	2.66	0.34	0.00
Cyb5b	-1.10	1.07E-06	20.62	23.10	38.62	51.28	45.68	43.43	18.28	22.29	23.60	37.00	28.83	31.88	34.05	28.87	38.94
Cyfp1	-1.18	3.42E-04	14.38	44.92	31.44	36.67	26.73	32.14	10.73	12.14	17.45	3.17	3.51	2.11	2.68	3.52	3.34
Cyld	-1.14	8.44E-03	16.82	13.89	16.70	20.99	12.92	17.01	8.58	4.75	8.38	18.50	17.05	19.50	21.23	21.73	19.09
Cyp4f13	1.77	2.18E-03	30.25	0.00	5.52	5.94	8.19	5.64	16.88	38.70	25.81	33.35	33.41	29.83	22.64	24.51	37.18
Cytl4	1.71	3.37E-18	165.53	152.73	166.95	88.27	86.34	76.79	227.57	290.30	320.29	119.58	103.99	91.91	104.45	110.24	151.89
Daird3	0.95	3.23E-02	90.63	59.37	52.85	32.08	43.06	33.70	54.29	71.22	93.93	23.28	28.11	22.16	19.84	26.50	27.29
Dap3	1.14	1.24E-04	15.52	24.26	14.17	22.40	14.05	15.37	43.61	34.76	39.08	30.09	33.14	26.15	23.27	30.23	28.99
Dapl1	1.68	2.66E-02	0.00	0.00	0.00	5.76	6.37	5.12	12.51	28.84	36.20	282.29	357.51	297.92	728.02	716.67	625.83
Dappp1	-0.98	1.46E-02	33.45	90.25	50.22	113.94	110.16	104.81	66.39	59.82	33.27	20.75	23.56	24.90	19.61	21.17	17.56
Dbi	-0.83	2.58E-04	135.71	171.23	139.90	279.09	285.24	294.85	139.35	172.57	161.48	107.92	108.17	96.38	105.99	94.23	97.55
Dcaf17	1.13	4.58E-02	17.55	25.78	20.32	18.00	11.74	15.59	45.88	46.33	25.63	27.01	18.79	22.65	15.47	15.73	19.99
Ddb1	-0.76	4.54E-02	53.46	36.07	58.06	86.44	96.70	92.11	43.12	42.94	72.91	62.20	54.32	52.25	50.21	46.95	71.02
Ddb2	1.60	6.98E-03	14.79	60.22	15.02	34.67	15.83	32.55	52.98	146.61	97.08	48.95	55.88	64.05	51.55	56.22	47.03
Ddit4	-1.37	2.24E-03	11.73	11.05	12.83	41.70	55.65	41.73	22.95	12.21	14.39	36.17	16.11	36.38	40.73	54.64	26.47
Dder1	1.36	2.05E-02	5.18	0.00	8.23	14.64	7.83	9.98	48.63	26.45	20.03	5.66	3.25	6.26	4.58	4.02	5.16
Dggs1	-1.58	4.59E-06	304.98	245.41	270.63	182.00	322.33	288.38	65.36	95.52	87.03	69.96	70.19	68.69	81.06	75.74	74.16
Dmdm6b	0.95	1.99E-02	0.27	3.22	1.85	4.99	3.88	4.59	8.29	10.22	8.28	8.93	7.94	5.62	4.96	5.84	6.99
Dnac5	-3.58	8.91E-07	2.49	0.00	0.08	10.13	7.27	4.31	0.00	0.00	0.00	0.69	0.37	0.38	0.39	0.20	0.00
Dnka	0.97	1.69E-02	148.58	167.17	147.23	204.28	165.17	151.43	222.55	463.14	359.51	480.43	484.45	557.84	487.25	489.62	538.62
Dnkd	1.83	1.44E-04	4.77	10.37	9.22	10.60	10.05	7.23	63.72	26.71	25.86	31.18	28.10	34.71	32.15	33.61	39.63
Dnuk	0.84	1.48E-03	86.63	19.00	64.17	59.22	59.75	55.00	89.68	111.64	108.79	100.44	109.10	97.11	88.87	95.63	97.44

Dnrc24	-2.27	7.05E-03	3.33	0.02	1.56	1.80	1.56	1.99	0.00	0.00	0.00	1.33	0.66	0.62	1.11	0.74	0.63
Dnrg2	-1.94	2.94E-02	5.68	0.01	1.81	1.40	1.14	0.69	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Dnrgb9	-1.54	3.63E-03	7.63	16.89	19.39	7.70	9.22	9.11	2.67	2.93	2.15	11.68	16.58	13.68	18.11	12.67	12.30
Dnrgc3	0.78	4.58E-02	24.98	14.10	17.42	26.37	21.30	17.95	45.42	40.86	29.05	36.46	32.04	34.20	38.28	21.31	30.46
Dnrc11	1.62	1.45E-04	7.10	13.98	7.42	4.38	7.68	6.66	15.61	25.04	22.83	13.09	15.86	17.27	16.22	18.79	14.55
Dnrc2	-1.27	1.45E-10	223.78	248.48	233.24	363.06	467.96	385.36	155.35	152.87	189.08	126.06	124.47	105.59	98.96	97.30	137.79
Dnrg5	-1.39	9.52E-03	14.15	41.06	16.89	18.58	17.38	31.86	8.49	7.53	7.00	72.91	80.36	69.72	70.67	55.54	76.86
Dnrg19l1	-3.58	6.67E-07	0.14	3.24	1.90	3.22	2.90	2.05	0.00	0.00	0.00	1.25	0.99	0.91	1.75	2.55	2.09
Dnrga2	-0.88	6.63E-03	43.51	49.09	53.12	62.60	71.97	85.34	31.62	45.74	38.98	62.68	62.47	34.69	77.23	64.96	43.76
Dnrc1	1.48	7.14E-03	18.40	10.58	8.55	6.00	5.45	10.60	26.06	22.19	22.31	19.76	22.95	14.64	14.68	11.83	16.16
Dnrc1	-5.40	2.28E-17	0.00	0.00	0.00	12.36	5.90	6.70	0.00	0.00	0.00	32.93	14.42	35.79	0.12	0.07	0.02
Dnrc2	-1.44	1.74E-08	29.70	77.61	35.60	68.86	71.88	60.26	26.78	19.05	25.68	12.79	7.36	15.71	14.14	11.78	17.66
Dnrc1b	-1.86	4.11E-02	0.00	5.62	3.16	1.85	2.61	3.80	0.00	0.07	0.00	1.91	1.72	1.29	3.23	1.28	0.12
Dnrg3	-2.58	1.26E-03	0.75	3.13	3.09	1.82	0.68	2.57	0.09	0.01	0.06	0.31	0.99	1.07	1.11	1.96	0.68
Dnrg4	-1.23	3.83E-02	35.85	15.38	20.22	25.65	39.37	46.50	8.88	17.90	14.81	11.70	11.96	12.81	16.34	14.88	13.21
Dnrg1	-2.33	2.13E-15	14.21	15.36	15.30	19.47	22.94	19.12	4.57	3.52	3.21	9.00	7.46	8.67	9.39	9.73	8.82
Dnrg	-0.91	4.87E-02	57.87	72.07	94.05	77.77	91.41	84.22	48.53	26.87	54.68	58.78	49.59	52.21	34.71	39.06	34.24
Dnrg1	0.87	4.10E-02	25.85	50.88	52.28	32.77	34.40	42.82	60.05	60.71	87.51	56.87	43.94	42.61	47.74	44.96	63.40
Dnrg1	-1.65	1.81E-04	27.89	33.47	29.55	76.67	82.47	99.00	14.27	23.34	35.97	34.14	28.48	31.10	31.34	32.86	26.53
Dnrg1l1	-1.67	1.69E-12	36.22	55.30	57.99	74.50	79.06	77.20	22.30	18.97	28.73	10.07	8.99	8.55	9.32	7.79	9.12
Dnrg1	-1.66	3.64E-02	12.59	19.84	30.21	38.28	61.49	57.99	2.32	11.81	19.58	3.01	0.82	4.73	2.71	4.65	2.06
Dnrg1	-0.67	4.31E-03	63.41	79.43	67.71	120.63	111.46	127.30	87.11	71.10	65.01	139.49	126.07	120.78	124.90	123.05	120.50
Dnrg4	-0.87	4.74E-05	16.69	29.88	19.45	34.26	25.65	31.19	16.45	15.96	16.70	25.97	27.50	20.83	25.03	25.63	23.47
Dnrg1	-0.89	4.41E-02	6.68	13.46	21.95	35.13	30.18	23.50	20.09	10.56	15.68	26.21	24.77	26.96	35.55	32.17	25.88
Dnrg1	-0.98	1.86E-02	73.31	106.33	62.05	98.46	87.19	91.15	27.32	45.29	60.75	60.58	68.54	59.25	45.14	50.69	61.46
Dnrg2	-2.64	3.38E-11	32.99	67.75	38.36	38.53	50.24	55.82	3.94	8.61	6.40	19.88	18.53	18.41	11.70	19.23	27.34
Dnrg4	0.54	3.42E-02	46.16	73.47	62.50	48.22	54.03	46.01	71.18	80.52	64.66	25.98	27.23	30.65	27.79	24.43	25.83
Dnrg1	-1.01	3.90E-02	198.81	154.88	194.65	139.63	143.19	150.42	103.71	41.05	58.82	48.92	45.26	41.94	37.49	41.43	39.99
Dnrg3	2.00	9.59E-08	24.03	7.57	14.68	10.88	10.06	15.65	69.84	53.23	39.34	27.43	33.91	28.43	29.62	25.80	26.44
Dnrg1	0.68	1.68E-04	109.76	95.37	134.57	186.80	153.67	166.19	277.30	286.05	250.50	356.86	381.57	301.65	286.99	305.22	371.23
Dnrg4	1.68	5.23E-08	0.25	11.52	6.37	4.34	4.13	3.40	14.91	10.57	14.38	4.28	5.28	3.14	2.84	3.97	2.67
Dnrg4	1.90	4.68E-03	11.35	5.62	2.84	6.09	2.98	1.34	25.21	14.65	13.77	5.12	4.39	7.55	5.21	7.28	8.43
Dnrg2	3.00	9.94E-05	0.00	0.00	0.00	0.00	0.00	0.00	5.80	1.95	8.36	0.00	0.00	0.00	0.00	0.00	0.00
Dnrg1	1.72	4.10E-02	3.42	1.12	7.61	0.66	1.05	3.02	13.15	7.40	5.34	3.46	2.99	3.42	2.27	3.33	3.52
Dnrg1	-2.17	1.14E-02	3.14	0.00	0.28	1.06	3.85	7.12	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Dnrg1	-3.17	2.72E-05	12.32	0.00	4.98	3.70	2.00	3.03	0.00	0.00	0.00	0.44	0.20	0.35	0.70	0.14	0.24
Dnrg1	-1.49	9.09E-03	77.93	36.00	83.05	59.66	73.47	91.75	21.86	35.63	10.54	9.11	9.04	9.80	10.85	14.19	11.65

			BAL	MLN			Naïve											
			Th2	WT1	WT2	WT3	WT1	WT2	WT3	K04	K05	K06	WT1	WT2	WT3	K04	K05	K06
Annotation	Log2Fold Change	p-value																
<i>Erf1l</i>	-2.58	1.39E-03	16.68	0.00	1.96	3.21	1.97	5.24	0.00	0.00	0.00	3.01	2.02	2.24	1.73	2.90	0.62	
<i>Egr1l</i>	-1.15	7.48E-06	90.51	117.21	158.28	138.49	206.16	154.06	70.73	79.49	69.35	80.41	64.03	102.42	92.18	98.23	121.84	
<i>Etfb</i>	-1.01	5.35E-03	229.81	264.22	197.64	303.01	416.79	336.34	221.37	121.25	167.29	94.09	81.57	70.81	78.34	75.13	102.04	
<i>Ets1</i>	-0.52	4.47E-02	159.63	249.55	214.29	214.15	189.64	245.96	183.59	131.81	133.23	438.09	430.91	308.31	361.79	358.64	380.64	
<i>Etv6</i>	-2.25	2.60E-03	9.88	8.28	7.48	8.51	4.90	8.20	0.19	0.98	0.81	3.47	1.13	1.80	1.43	1.31	0.33	
<i>Exosc1</i>	0.92	3.18E-02	6.89	7.39	31.17	16.77	18.15	19.10	45.83	27.37	33.96	43.18	47.29	34.63	43.00	50.62	50.13	
<i>Exl1</i>	-1.84	2.85E-02	7.81	8.37	5.82	12.83	5.32	3.38	1.01	0.46	1.85	0.78	1.68	2.23	2.33	3.18	1.69	
<i>Fahh</i>	0.94	1.22E-02	96.34	86.03	74.01	53.92	47.02	48.04	90.08	131.30	71.83	72.92	66.87	70.62	59.91	59.30	71.01	
<i>Fabp5</i>	-1.33	1.73E-02	29.78	27.87	18.55	23.65	22.30	23.57	5.85	8.17	10.47	0.87	1.19	2.03	2.46	1.87	2.21	
<i>Fam118a</i>	1.64	3.40E-02	3.66	6.31	13.29	7.21	4.25	7.28	10.79	18.85	53.34	23.80	23.98	23.80	21.40	24.06	19.18	
<i>Fam169b</i>	1.15	1.83E-03	39.89	74.63	68.10	22.73	33.99	30.09	51.91	71.84	78.10	73.34	73.30	53.47	67.92	77.69	89.80	
<i>Fam189b</i>	1.35	8.02E-08	26.90	71.11	42.25	32.56	32.85	30.89	95.81	91.76	65.18	118.94	126.86	106.06	112.04	113.79	133.88	
<i>Fam234a</i>	-1.72	6.91E-05	22.74	24.79	36.01	33.00	36.97	30.15	9.56	5.20	12.25	9.11	10.30	11.27	9.82	9.79	17.75	
<i>Fam69c</i>	-2.85	2.68E-04	0.00	0.00	3.82	5.12	4.25	3.69	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	
<i>Fam83g</i>	-3.24	1.54E-05	0.00	0.00	1.69	3.92	3.04	5.75	0.00	0.00	0.05	0.05	0.00	0.02	0.00	0.00	0.00	
<i>Fam89b</i>	-0.78	8.54E-03	47.80	59.33	90.82	87.07	90.23	78.24	56.74	47.57	41.76	57.94	73.68	74.14	69.08	73.15	81.25	
<i>Fbx15</i>	1.24	3.07E-02	23.27	40.76	33.63	15.42	10.79	17.84	56.98	36.39	22.65	32.24	39.87	30.40	34.21	36.55	40.08	
<i>Fbx16</i>	1.12	3.29E-02	42.64	32.77	67.16	43.32	30.16	57.01	81.34	139.12	81.31	28.46	19.69	39.65	36.86	31.47	38.09	
<i>Fcgr2b</i>	-1.81	4.66E-02	10.51	27.06	10.87	4.02	4.39	2.73	0.00	0.00	0.00	2.18	0.44	0.50	0.00	0.53	0.36	
<i>Fdps</i>	-1.32	2.05E-04	71.27	29.26	50.91	66.80	50.30	58.33	16.52	22.62	26.34	32.67	30.82	33.00	26.65	21.48	33.04	
<i>Fdx1</i>	-2.13	4.18E-07	14.47	25.61	18.99	109.57	152.65	135.32	33.09	29.69	15.15	13.79	17.35	16.65	18.97	21.46	15.88	
<i>Fech</i>	1.23	4.44E-02	3.85	30.08	7.71	7.93	8.12	11.65	25.13	33.20	14.16	24.69	21.47	16.39	15.69	18.24	21.62	
<i>Ferm13</i>	-0.82	1.10E-02	217.65	283.85	225.26	288.37	320.75	357.01	168.40	131.09	237.69	182.51	164.84	179.45	149.03	172.98	211.29	
<i>Fez1</i>	-2.86	2.56E-04	0.00	0.00	0.00	6.20	7.64	8.31	0.00	0.00	0.00	0.00	0.00	0.59	0.00	0.00	0.00	
<i>Fgd2</i>	-3.74	1.92E-07	0.02	0.00	0.00	6.20	3.72	6.99	0.00	0.00	0.00	2.88	1.45	1.12	0.10	0.33	0.00	
<i>Fgl9</i>	-3.61	6.69E-07	0.43	0.52	0.38	2.19	5.75	5.02	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	
<i>Filp</i>	1.22	4.75E-02	51.46	14.90	40.66	41.62	32.87	32.48	103.02	49.21	136.72	47.00	45.31	59.32	63.15	50.31	77.34	
<i>Fikbp3</i>	0.99	1.83E-02	133.09	137.54	128.44	172.44	149.06	150.90	242.21	450.58	272.39	271.77	278.60	230.91	314.73	312.92	282.64	
<i>Fliat1</i>	-0.89	9.04E-03	198.28	219.83	176.66	96.47	120.29	109.38	71.30	55.08	44.83	34.83	29.15	29.99	28.52	22.43	36.25	
<i>Foxj3</i>	-3.53	1.23E-06	0.32	4.24	5.49	4.51	1.49	7.07	0.00	0.04	0.00	5.35	4.13	6.96	7.38	5.64	4.87	
<i>Foxo1</i>	1.76	1.17E-02	0.33	6.86	4.24	3.98	2.20	1.10	6.51	14.23	12.59	18.81	15.32	17.61	18.60	19.22	23.79	
<i>Frmf4a</i>	-2.68	8.11E-04	0.02	0.00	0.06	1.63	1.59	0.63	0.00	0.01	0.00	0.35	0.05	0.30	0.65	0.16	0.54	
<i>G3bp2</i>	-0.66	1.48E-03	41.56	40.40	38.82	60.80	49.28	56.61	36.97	29.93	37.48	59.41	62.26	64.22	82.01	77.06	54.29	
<i>Gatc3</i>	-1.70	1.85E-06	435.42	541.37	393.01	416.07	520.10	521.54	188.46	148.63	78.56	47.54	39.14	30.26	45.34	38.80	28.88	

Gba	-1.31	2.17E-05	30.24	88.62	71.58	70.62	82.33	75.41	25.99	24.88	36.92	59.03	61.49	48.10	42.33	49.78	64.47
Gbp2	0.94	1.15E-02	118.09	66.97	110.57	34.53	36.08	34.13	83.34	75.16	48.53	101.00	55.50	59.47	80.34	83.28	66.92
Gbp6	1.70	1.77E-02	41.52	30.23	39.82	7.41	4.97	3.36	17.56	40.43	10.30	18.74	8.16	6.25	9.41	8.98	10.40
Gcg	-3.29	1.23E-05	0.00	0.00	1.30	143.85	124.53	7.76	0.12	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Gfra2	-5.89	3.85E-22	0.00	0.00	0.00	8.74	7.58	11.55	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Gmcp3	1.52	2.17E-07	220.78	149.48	172.56	242.96	167.57	176.74	406.94	658.88	686.94	840.87	885.73	685.55	716.42	784.46	841.22
Gmcp4	1.56	5.79E-13	181.85	183.89	126.37	256.63	160.48	173.30	567.79	507.59	709.25	650.73	685.60	605.07	703.19	797.16	665.92
Gmcp6	1.57	3.97E-14	251.10	206.03	168.24	226.86	161.87	199.24	721.95	481.71	591.68	800.50	798.24	689.93	755.16	746.59	904.21
Gmcp7	1.44	5.91E-04	43.75	63.41	33.75	110.93	71.14	50.21	178.05	254.18	244.59	190.54	168.12	64.74	98.80	127.66	100.46
Gmcp9	0.87	1.89E-02	123.74	104.45	88.88	75.22	80.13	98.56	119.62	163.18	192.03	204.80	203.26	151.45	193.67	188.28	191.97
Gbl1	-1.29	1.79E-05	55.43	53.84	61.55	72.02	65.01	73.37	36.88	24.46	23.60	27.09	24.53	26.88	24.61	27.19	27.68
Glpr2	-0.91	1.68E-02	122.41	121.18	113.69	105.19	101.66	86.80	35.88	56.74	58.07	82.84	65.60	89.02	64.84	62.00	79.81
Gm11532	2.85	2.03E-08	3.10	0.97	1.88	0.57	0.72	0.85	11.04	5.29	5.52	4.63	2.44	3.47	5.76	4.89	5.03
Gm14005	1.22	2.97E-02	60.72	58.48	65.57	48.73	56.33	68.37	29.77	21.26	16.01	0.51	0.20	0.00	0.00	0.00	0.00
Gm14308	1.38	6.52E-03	0.07	0.25	2.57	2.02	1.50	1.66	4.34	6.01	4.56	2.89	2.74	2.74	2.64	3.96	3.08
Gm14430	1.38	6.52E-03	0.07	0.25	2.57	2.02	1.50	1.66	4.34	6.01	4.56	2.89	2.74	2.74	2.64	3.96	3.08
Gm14434	1.38	6.52E-03	0.07	0.25	2.57	2.02	1.50	1.66	4.34	6.01	4.56	2.89	2.74	2.74	2.64	3.96	3.08
Gm15441	1.20	1.25E-02	12.45	8.40	9.61	7.58	12.44	10.51	22.25	25.98	27.45	31.39	22.79	56.47	50.15	50.10	27.22
Gm15506	2.08	1.35E-02	0.78	0.16	0.05	0.21	0.15	0.04	1.72	2.16	0.33	0.47	0.37	0.43	0.56	0.40	0.43
Gm16712	1.22	3.01E-02	1.47	1.82	2.04	3.07	3.10	2.60	8.22	8.00	6.34	0.95	1.45	1.33	1.43	0.99	1.14
Gm2663	2.68	7.95E-04	0.00	0.00	0.00	0.00	0.00	0.00	6.57	26.62	17.47	0.00	0.00	0.00	0.00	0.00	0.00
Gm28042	1.97	5.84E-05	3.70	10.20	9.34	2.43	2.84	3.02	9.78	18.80	9.68	4.68	4.17	4.35	2.53	2.29	4.08
Gm2a	-1.41	1.83E-03	136.35	264.01	249.41	289.88	400.27	428.46	65.95	179.55	136.88	75.09	82.46	59.09	95.52	85.40	88.57
Gm4724	1.38	6.52E-03	0.07	0.25	2.57	2.02	1.50	1.66	4.34	6.01	4.56	2.89	2.74	2.74	2.64	3.96	3.08
Gm5424	0.86	3.35E-02	15.69	27.33	26.65	36.51	23.56	27.17	49.14	49.06	63.87	47.83	38.46	48.62	54.78	53.80	58.09
Gna14	-5.13	2.18E-15	0.00	0.00	5.05	5.40	8.37	6.42	0.00	0.00	0.00	0.05	0.03	0.00	0.00	0.00	0.00
Gna1	1.32	1.87E-02	14.73	15.48	13.44	4.47	5.03	7.33	16.98	20.28	9.56	12.58	12.53	10.38	9.84	11.48	14.05
Gnaq	-1.69	3.20E-03	11.53	10.75	7.49	15.79	19.37	17.56	5.91	5.51	1.98	1.00	0.92	0.81	0.83	0.63	0.80
Gnb1	-0.84	1.97E-04	59.59	134.48	94.38	76.78	83.70	100.29	46.56	50.36	46.72	89.13	110.11	68.10	104.49	109.91	78.79
Gnl2	0.98	6.82E-03	23.50	6.28	39.80	30.43	27.64	33.38	72.81	42.24	71.88	66.90	66.85	59.53	48.75	50.41	70.78
Gnpat1	1.84	1.13E-04	5.10	7.94	9.17	4.08	5.72	6.37	29.32	22.55	15.39	11.65	15.71	14.62	23.37	21.87	11.51
Gnptab	-3.73	2.16E-07	2.64	5.56	1.74	3.78	4.16	1.81	0.00	0.00	0.00	2.86	1.46	3.34	1.18	2.49	2.88
Gpat4	0.94	1.44E-02	19.95	59.84	32.04	20.09	20.21	28.59	56.56	40.90	37.72	38.46	26.61	24.90	26.93	27.71	24.05
Gpl1	-0.71	6.56E-03	93.40	82.88	92.38	141.44	140.06	168.77	86.32	73.32	109.64	79.11	59.52	84.32	62.05	66.91	74.20
Gpx4	0.65	2.08E-02	320.31	358.84	314.37	186.18	209.44	175.48	264.97	344.88	290.10	200.04	186.55	203.55	253.80	246.84	256.41
Gramd3	1.97	1.77E-02	3.62	5.10	6.15	9.89	1.04	1.96	52.86	26.48	21.99	130.70	128.21	95.60	114.49	165.25	132.63
Grr	-1.65	3.16E-04	151.67	131.58	186.34	136.77	121.20	125.47	58.57	28.67	21.06	10.71	3.86	8.74	6.07	6.16	9.70

			BAL	MLN			Naive											
			Th2	WT1	WT2	WT3	WT1	WT2	WT3	K04	K05	K06	WT1	WT2	WT3	K04	K05	K06
	Log2Fold Change	p-value																
<i>Gsn</i>	-2.50	1.31E-11	146.35	92.03	145.42	381.09	469.11	432.82	49.86	100.36	43.70	70.88	60.68	55.66	54.22	50.23	79.92	
<i>Gstp1</i>	-0.91	1.31E-05	181.11	113.67	128.67	298.23	244.97	246.81	138.04	148.13	126.78	139.95	130.66	130.59	108.07	114.18	135.53	
<i>Gstp3</i>	1.07	1.35E-02	9.24	13.02	35.15	55.99	49.31	41.84	87.14	135.36	100.17	57.93	52.47	71.63	54.84	50.16	73.00	
<i>Gf2i</i>	1.13	3.07E-02	174.6	44.08	17.83	20.57	22.73	15.10	68.90	40.77	28.52	91.00	85.75	91.17	79.23	93.52	93.06	
<i>Gf3c5</i>	1.16	6.97E-04	24.59	26.15	5.48	17.96	19.41	13.31	36.47	34.16	46.72	13.30	11.41	10.07	8.47	9.80	16.01	
<i>Gtppp2</i>	1.65	1.33E-06	34.14	20.42	27.40	13.98	19.81	23.93	79.20	57.33	58.60	24.77	22.45	26.14	17.41	20.71	28.79	
<i>Gzmb</i>	-4.77	2.46E-34	32.55	47.44	59.84	423.01	668.47	730.25	25.10	12.65	9.25	0.00	0.37	1.04	0.04	0.00	0.00	
<i>H2-DMa</i>	1.28	1.27E-03	69.13	92.10	38.65	36.31	29.65	51.25	122.24	73.91	106.20	181.45	95.03	131.52	50.42	50.38	70.10	
<i>H2-K1</i>	0.58	2.28E-02	3296.87	3196.02	3826.62	2237.09	2232.72	2042.74	3314.06	3892.94	2542.37	2740.75	2529.17	2749.98	2448.07	2948.80	3139.71	
<i>H2-Ob</i>	2.51	7.30E-05	0.00	9.83	0.00	1.44	2.32	6.59	36.30	23.69	37.44	48.49	52.63	40.69	46.88	48.56	62.91	
<i>H2-Q5</i>	1.44	1.48E-02	16.63	24.48	27.19	17.31	16.79	13.97	27.69	69.44	52.08	10.88	11.32	6.77	11.20	15.18	11.37	
<i>H2-Q6</i>	1.13	7.05E-03	144.02	149.16	143.07	99.63	88.10	62.79	146.11	246.00	182.99	55.78	44.03	45.91	40.28	69.42	39.06	
<i>H2-Q7</i>	1.09	5.44E-07	405.88	348.64	381.81	237.65	243.47	211.90	439.73	578.87	467.99	295.84	236.68	315.27	268.47	341.17	257.25	
<i>H2-Q8</i>	1.14	7.04E-03	139.90	145.81	139.20	97.34	84.86	61.15	142.01	240.64	177.93	54.58	42.60	44.42	39.14	67.17	38.06	
<i>H2-Q9</i>	1.09	5.44E-07	352.56	300.33	321.77	200.62	203.58	178.27	373.81	498.87	383.99	247.89	198.22	264.52	219.31	280.05	213.61	
<i>H6pd</i>	1.71	4.57E-03	0.03	0.00	1.38	1.79	2.28	2.75	7.83	13.76	5.66	2.12	4.26	3.73	5.14	2.94	2.44	
<i>Hdac10</i>	0.88	2.09E-06	28.20	34.61	29.43	39.26	37.46	42.22	67.99	77.28	73.85	18.37	20.45	22.95	18.72	26.92	27.90	
<i>Hectd3</i>	-1.47	4.61E-05	13.67	54.81	29.20	33.50	30.10	26.83	12.78	10.73	7.03	16.93	14.37	19.39	12.63	9.50	20.56	
<i>Heg1</i>	-1.57	2.21E-03	4.39	0.00	2.73	3.62	4.97	5.88	1.50	1.48	1.29	0.75	0.66	1.37	1.45	2.38	1.63	
<i>Hemk1</i>	1.06	1.19E-02	32.72	3.69	19.06	15.84	19.26	18.58	43.98	45.03	28.23	10.85	8.31	5.28	5.99	6.49	2.77	
<i>Hk1</i>	-0.84	1.04E-02	28.13	59.89	58.32	47.16	68.64	71.25	33.61	30.78	38.18	42.99	40.30	35.44	37.70	36.37	39.64	
<i>Hk2</i>	-2.59	1.30E-03	0.28	0.20	4.06	2.88	1.65	1.12	0.00	0.00	0.00	0.00	0.24	0.11	0.02	0.00	0.00	
<i>Hlf</i>	-5.75	1.04E-20	4.01	2.31	8.96	4.43	6.37	4.39	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	
<i>Hmg20a</i>	1.25	9.27E-04	20.51	1.85	21.11	15.37	11.05	12.38	26.72	40.72	28.77	33.86	32.71	31.72	28.70	35.73	36.84	
<i>Hmox1</i>	-2.34	5.04E-03	0.00	8.37	15.53	2.81	8.23	10.06	0.00	0.00	0.00	0.78	0.00	0.37	0.76	0.00	1.26	
<i>Hn1i</i>	-1.22	1.54E-03	10.85	11.64	11.02	46.74	26.59	32.17	15.77	12.11	14.93	27.59	19.59	15.19	19.54	18.58	22.22	
<i>Hnrnp1</i>	-1.15	4.72E-02	20.82	23.25	15.83	20.76	29.56	24.86	15.18	7.47	8.23	13.13	16.37	14.12	28.75	24.39	10.52	
<i>Hprt</i>	-0.81	5.21E-04	40.34	59.69	61.97	97.43	83.64	87.80	59.49	45.02	47.54	70.71	67.45	63.47	57.96	74.81	70.86	
<i>Hps3</i>	1.42	3.65E-02	2.91	3.66	3.80	2.56	3.58	4.31	18.84	10.43	6.61	7.64	10.92	10.64	10.38	8.13	10.75	
<i>Hras</i>	-1.81	4.78E-02	0.03	0.13	1.74	2.89	1.71	1.39	0.00	0.00	0.05	1.26	0.62	2.00	0.86	1.15	0.54	
<i>Hsd11b1</i>	1.69	3.86E-04	8.75	21.96	20.65	30.44	21.45	27.05	56.62	133.70	97.82	202.46	200.05	154.66	170.73	188.33	217.11	
<i>Hsd3b7</i>	1.89	1.54E-03	11.06	25.30	11.59	7.20	11.37	6.04	19.79	49.81	48.08	2.61	1.27	1.51	0.73	0.84	1.00	
<i>Hsp90b1</i>	-0.69	3.02E-03	91.78	184.40	127.83	354.47	242.69	283.10	209.11	161.91	166.29	113.07	124.52	122.14	124.37	128.13	130.61	
<i>Hsp94</i>	-0.73	2.20E-02	54.63	83.57	58.28	72.34	64.42	73.97	49.45	29.34	46.13	39.93	42.70	39.55	35.69	35.07	27.50	

<i>Hyal2</i>	-4.29	4.41E-10	11.24	15.08	10.45	12.84	6.91	8.93	0.00	0.00	0.00	8.04	7.36	4.11	10.43	1.99	11.80
<i>Iba57</i>	-1.86	4.10E-02	0.00	5.14	0.00	1.48	2.31	2.53	0.00	0.06	0.00	2.27	1.33	2.13	2.66	2.32	1.66
<i>Icam5</i>	-1.93	3.06E-02	6.52	0.00	1.30	1.99	1.55	2.91	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.21	0.00
<i>Id2</i>	-0.98	3.35E-02	660.95	746.21	700.36	522.73	674.11	716.56	481.76	225.38	224.29	58.59	74.82	61.56	68.49	64.69	52.09
<i>Idk</i>	0.87	7.48E-04	74.70	59.82	54.67	35.21	33.31	31.34	71.83	53.23	59.61	75.09	67.89	62.11	71.70	72.35	66.60
<i>Ier3</i>	-1.59	4.53E-02	23.89	31.99	47.94	62.34	95.33	116.91	8.07	41.06	13.78	0.32	0.21	0.16	0.41	0.00	0.00
<i>Ifi209</i>	1.62	1.30E-05	59.02	59.71	79.03	24.14	11.73	15.33	62.30	57.58	48.86	81.76	67.10	53.44	60.57	59.49	56.02
<i>Ifnar2</i>	1.61	2.41E-02	64.60	12.78	18.73	16.44	16.68	8.29	89.69	32.69	31.09	22.01	20.71	26.45	27.30	25.78	27.28
<i>Ifng1</i>	1.64	6.57E-08	545.00	628.48	517.20	94.46	119.41	112.77	281.72	473.79	308.63	128.04	146.02	94.50	136.60	155.56	138.83
<i>Ifng2</i>	1.75	2.31E-03	7.78	5.92	14.32	8.39	10.01	6.93	35.31	17.26	53.15	74.11	60.36	56.88	55.34	47.91	61.63
<i>Igtp</i>	1.42	2.85E-06	211.70	294.75	256.67	98.43	70.14	66.04	278.39	208.35	167.52	315.90	254.15	140.27	237.16	263.24	271.46
<i>Ilgp1</i>	3.25	2.91E-13	6.37	2.35	17.19	1.17	1.56	0.78	15.68	12.04	17.54	18.03	7.45	3.99	15.28	17.73	6.66
<i>Il10</i>	-2.70	7.47E-10	298.93	258.83	186.14	295.29	415.63	473.81	22.98	60.63	60.35	0.05	0.61	0.81	0.00	0.00	0.00
<i>Il13</i>	-2.32	5.93E-03	49.88	38.53	32.89	71.69	146.01	117.85	4.84	1.61	0.00	0.00	0.09	0.00	0.05	0.00	0.00
<i>Il16</i>	0.70	9.52E-03	89.42	41.99	77.08	79.25	88.32	60.05	133.69	86.63	122.50	164.57	185.94	176.22	143.37	163.82	197.32
<i>Il18</i>	3.74	1.05E-07	0.00	16.75	4.31	0.80	0.24	0.00	22.30	19.97	19.40	4.73	10.14	6.31	9.17	7.54	10.76
<i>Il1r1</i>	-3.57	9.83E-07	0.00	4.37	0.11	4.16	1.83	3.41	0.00	0.00	0.00	0.07	0.00	0.00	0.00	0.00	0.00
<i>Il1r2</i>	-1.72	1.95E-02	290.37	230.60	325.00	157.18	156.61	223.44	16.72	78.16	15.44	0.44	0.00	0.00	0.00	0.00	0.04
<i>Il2rg</i>	-0.87	2.30E-05	1329.81	1692.53	1223.79	1494.33	1796.03	1771.17	990.28	995.06	736.04	1759.81	1652.37	1094.88	1189.84	1191.11	1603.38
<i>Il6</i>	-6.07	3.70E-23	43.46	41.29	34.19	24.24	49.24	63.80	0.00	0.00	0.00	0.00	0.00	0.00	0.05	0.00	0.00
<i>Inpp4b</i>	0.85	1.35E-05	26.40	29.07	32.11	30.41	40.64	36.19	72.85	68.09	61.63	63.76	70.79	82.96	50.54	61.02	55.43
<i>Ipmk</i>	-6.24	3.06E-24	16.03	21.15	14.05	6.79	22.02	28.24	0.00	0.00	0.00	1.09	0.85	0.76	0.66	1.25	1.68
<i>Irk2</i>	1.58	1.39E-03	17.17	31.01	17.58	7.29	16.35	15.34	35.70	43.04	54.03	9.86	13.08	8.07	12.13	12.68	15.33
<i>Irf1</i>	0.92	3.36E-02	166.48	53.74	90.54	85.86	85.44	66.01	228.86	120.95	120.28	126.87	114.06	150.56	141.08	127.32	141.23
<i>Irf2</i>	0.83	3.83E-02	30.83	17.49	19.06	19.09	15.20	13.65	24.55	28.84	34.05	37.59	36.45	36.30	42.82	40.97	34.96
<i>Irf9</i>	-3.21	1.97E-05	0.01	0.00	1.10	2.47	1.41	0.95	0.00	0.00	0.01	1.81	1.12	1.15	0.69	1.22	2.41
<i>Itgb1</i>	-0.64	3.89E-02	109.75	157.30	156.76	330.47	249.66	211.12	181.28	130.29	190.18	5.80	2.08	8.32	10.50	8.04	10.32
<i>Itpk1</i>	-1.12	2.67E-02	33.45	31.12	15.67	33.25	28.39	41.92	11.47	11.54	21.18	19.29	14.46	18.93	12.62	15.99	24.91
<i>Itpkc</i>	-1.93	3.04E-02	4.57	6.37	1.28	1.46	1.79	2.79	0.00	0.00	0.00	3.19	2.03	1.20	0.39	0.44	1.81
<i>Itpr1p1</i>	-0.64	3.65E-02	24.33	34.72	24.73	32.45	30.99	25.69	18.72	20.37	17.50	36.26	39.72	21.02	33.42	33.20	30.39
<i>Izumo4</i>	1.22	1.54E-02	54.44	41.36	30.99	27.71	29.95	21.69	44.83	75.05	80.65	38.31	36.76	26.40	42.89	50.42	39.11
<i>Kbtbd11</i>	1.53	2.19E-04	10.86	11.71	5.33	6.29	4.92	4.56	10.88	19.11	19.29	32.68	26.40	40.94	40.53	40.68	34.26
<i>Kenab2</i>	-1.24	2.27E-06	29.01	26.01	24.86	55.00	41.85	37.90	16.74	16.00	20.41	35.74	27.22	46.68	45.87	37.04	45.18
<i>Kctd11</i>	-2.08	1.70E-02	0.57	0.00	6.59	3.46	1.40	6.47	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.74	0.58
<i>Kctd21</i>	-2.19	1.05E-02	0.02	0.00	0.00	1.99	1.36	3.06	0.00	0.04	0.00	0.67	0.57	0.61	1.31	0.00	1.03
<i>Klf10</i>	-2.05	6.32E-06	19.16	42.19	55.85	53.03	60.06	65.88	18.35	6.28	12.24	12.82	7.21	8.36	15.86	9.63	7.52

		BAL		MLN				Naive									
		Th2	WT1	WT2	WT3	WT1	WT2	WT3	K04	K05	K06	WT1	WT2	WT3	K04	K05	K06
	Log2Fold Change	P-value	WT1	WT2	WT3	WT1	WT2	WT3	K04	K05	K06	WT1	WT2	WT3	K04	K05	K06
	Kl68	1.82E-02	26.66	75.75	42.43	97.77	128.09	121.24	50.03	31.71	11.84	50.83	40.02	56.68	43.73	38.99	53.78
	Kmt2b	4.63E-02	1.33	5.41	5.24	3.08	2.33	2.73	7.13	4.85	4.27	3.59	2.81	2.92	1.85	3.43	4.36
	Kpna3	1.40E-02	10.44	13.89	10.09	11.57	10.83	13.27	22.44	19.03	18.27	9.27	5.67	7.28	10.20	9.73	9.60
	Krt17	1.50E-13	0.00	0.00	0.00	0.00	0.00	0.00	13.20	16.71	10.48	0.00	0.00	0.00	0.00	0.00	0.00
	L1cam	6.03E-03	4.34	15.76	22.54	10.48	8.72	9.15	17.40	13.76	16.20	4.78	4.24	7.59	6.67	6.14	9.80
	Lg3	1.30E-44	702.67	630.29	665.54	842.57	1210.44	1269.24	102.53	91.09	133.77	6.82	4.46	4.41	0.69	1.50	1.90
	Lme2	3.18E-05	0.00	0.00	1.44	3.99	1.44	2.66	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Lnc1	5.21E-04	12.77	17.59	10.92	13.70	13.89	10.19	24.29	25.03	29.84	15.15	14.72	14.74	12.46	13.37	16.43
	Lptm5	7.21E-09	1137.32	1243.44	1307.65	1276.43	1653.17	1522.26	772.95	491.74	562.55	841.81	741.20	859.83	824.59	756.95	931.92
	Lat	9.52E-04	744.08	934.21	853.17	888.07	945.11	932.95	704.55	523.33	663.30	1117.48	1075.42	1062.34	943.59	1038.33	1284.41
	Lax1	3.04E-02	38.40	11.24	21.07	61.37	50.92	73.87	17.33	35.81	26.11	39.93	36.69	32.56	21.25	30.13	33.38
	Lha	8.58E-12	1664.45	1479.98	1661.63	1882.01	1717.86	1992.85	992.38	904.72	736.00	767.03	755.43	750.00	647.40	653.08	826.09
	Ldhb	1.42E-05	0.00	21.09	8.15	6.56	15.06	21.85	104.76	174.90	64.92	86.59	74.37	57.46	107.33	85.37	93.51
	Lef1	3.06E-13	7.88	8.56	11.96	13.78	6.28	7.92	111.00	58.96	79.98	325.59	387.93	310.86	454.27	466.63	371.43
	Leprot	3.30E-03	78.09	117.92	87.57	121.57	118.34	111.81	55.61	77.07	82.86	33.76	36.33	27.87	34.96	38.01	41.73
	Lgals1	4.85E-08	1095.22	1290.96	1366.04	1411.08	1867.77	1632.98	380.44	405.11	695.44	99.54	80.28	95.67	179.97	104.69	136.35
	Lgals3	9.29E-04	309.09	470.04	371.86	211.39	240.06	182.66	49.56	100.96	82.18	3.42	0.45	1.83	0.37	0.00	0.54
	Lgals3bp	1.74E-05	233.82	162.28	328.72	130.86	143.98	166.74	84.67	80.28	84.59	120.07	78.09	77.34	73.43	52.25	78.69
	Lgals6	1.89E-02	122.79	65.13	52.34	47.11	62.67	43.57	23.95	26.13	20.41	16.43	20.61	16.82	7.33	14.74	13.76
	Lgals7	1.23E-06	180.44	198.66	90.27	382.82	429.89	318.11	19.85	43.63	5.49	2.28	0.29	3.74	1.70	0.00	2.30
	Lgalsl	2.60E-06	7.51	0.21	1.32	17.60	23.08	17.59	2.77	1.76	4.67	0.32	0.20	0.23	0.07	0.47	0.37
	Lmd2	1.08E-05	139.41	178.50	172.93	239.79	234.09	223.93	396.56	306.84	344.73	709.14	643.76	688.18	854.55	838.44	914.68
	Lms1	1.74E-05	8.91	1.39	1.04	1.49	3.78	5.92	0.00	0.00	0.00	4.51	3.38	2.62	2.69	3.23	3.78
	Lman1	4.45E-02	46.17	84.89	90.43	96.98	82.85	97.83	50.73	19.78	21.42	16.81	11.30	9.30	14.30	16.86	13.32
	Lpar3	2.07E-04	1.46	13.79	9.08	3.20	4.33	10.09	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Lpar6	6.52E-03	59.40	67.08	50.98	36.36	42.45	51.85	14.93	17.85	24.32	22.11	22.21	20.64	32.12	31.67	24.07
	Lpxn	8.37E-03	321.60	289.45	258.09	206.36	231.18	212.04	135.75	96.68	152.95	59.71	70.75	34.85	61.74	67.75	82.65
	Lrrc39	1.53E-02	0.00	0.00	0.97	1.25	1.31	1.16	6.15	4.64	3.03	1.94	1.70	2.97	3.86	1.98	2.09
	Lsp1	2.03E-02	529.87	536.75	430.98	528.20	349.99	342.82	612.97	807.81	620.32	607.74	565.16	514.84	555.27	531.80	766.81
	Lsq4h	1.42E-03	72.82	67.46	69.90	93.63	85.29	75.34	181.19	117.63	180.25	182.88	188.73	156.44	149.34	167.49	179.05
	Ltb	2.51E-29	215.55	236.85	218.44	231.00	140.42	133.85	1126.66	1402.79	1294.48	1757.46	1736.61	1146.56	1695.02	1866.30	2558.14
	Luzp1	1.46E-02	0.00	3.59	1.13	1.52	2.18	0.48	0.00	0.00	0.00	0.55	0.51	0.83	0.43	0.43	0.00
	Ly6a	4.56E-03	2305.18	1341.94	1805.05	711.02	728.40	769.62	342.94	488.16	292.24	192.09	123.83	128.55	179.06	255.50	163.86
	Ly6e	1.26E-03	371.65	269.51	382.43	369.74	338.36	316.64	520.55	632.96	473.70	489.40	328.12	387.91	421.58	497.06	416.06

Ly9	1.17	4.77E-02	96.41	37.61	66.13	104.07	41.05	52.75	112.67	226.77	146.36	167.28	182.21	141.75	120.20
Lypl2	-0.58	3.69E-02	55.66	93.80	81.49	98.07	122.25	107.40	73.43	64.01	80.03	81.24	61.60	86.67	94.04
Lyrm4	-1.68	3.94E-07	79.55	46.87	55.73	101.73	149.93	173.66	40.85	35.16	48.50	32.06	35.28	25.48	31.25
Macf1	1.18	4.95E-02	3.06	10.99	6.87	5.66	5.89	4.99	5.57	17.68	16.20	19.84	25.85	22.79	16.87
Maf	-2.23	2.89E-07	12.24	19.72	26.21	24.05	25.19	28.29	3.12	6.82	4.25	0.16	0.02	0.06	0.02
Man1a	1.62	1.57E-02	2.44	0.95	6.14	2.74	1.61	2.12	4.99	12.57	7.26	4.17	4.11	7.05	6.94
Man2b1	-0.57	2.21E-02	86.45	57.27	77.30	99.83	78.47	81.26	64.94	58.23	49.03	47.31	40.27	52.28	44.90
Mopk1p1	-0.73	4.93E-02	8.43	14.85	9.59	11.54	13.74	11.63	7.99	7.35	6.50	16.22	16.80	18.93	21.93
Mpre2	-1.27	3.17E-07	53.74	75.30	49.77	66.06	85.11	63.94	25.34	20.64	32.87	10.91	18.71	14.17	11.35
March7	0.82	1.32E-03	22.26	1.62	15.01	14.43	12.35	12.92	25.53	23.53	24.83	21.35	23.57	33.74	30.78
Mboat7	-1.04	1.61E-02	30.41	46.87	56.78	38.68	44.22	51.11	28.32	18.44	15.44	16.28	21.37	20.32	20.91
Mcat	-2.48	2.43E-03	0.06	0.00	4.00	13.26	4.06	1.99	0.03	0.00	0.00	6.17	2.27	2.16	1.43
Mcat8	-9.02	4.59E-79	29.80	9.91	3.88	142.81	164.29	170.70	0.00	0.00	0.00	0.00	0.00	0.07	0.00
Mdh1	-0.55	3.72E-02	223.84	234.76	245.22	279.97	334.06	342.74	240.03	226.88	181.04	170.10	178.15	168.08	167.28
Med19	-1.74	3.23E-03	1.74	7.85	11.84	10.52	8.83	12.44	2.92	1.51	3.28	16.46	21.39	15.12	18.41
Meiap1d	1.49	1.71E-02	9.86	9.60	0.70	5.74	4.96	6.79	17.63	26.83	13.35	11.55	15.84	17.69	14.36
Met15	1.81	7.35E-03	8.55	4.28	4.79	4.93	2.89	2.31	15.10	8.96	24.45	2.99	7.83	2.47	5.71
Mfsd2a	-1.98	2.54E-02	0.00	0.00	1.21	6.31	1.81	3.37	0.00	0.00	0.00	0.47	0.82	0.08	0.00
Mgst2	2.64	1.09E-07	9.78	10.70	9.35	21.56	11.52	10.57	80.39	117.30	163.23	171.21	167.37	139.21	178.70
Mic3	0.81	6.73E-03	16.02	11.76	10.65	12.01	10.26	14.59	23.63	18.67	23.26	20.40	18.03	20.65	21.76
Mical1	1.52	1.17E-02	3.77	7.23	3.05	2.67	4.16	1.50	10.59	10.09	7.66	6.07	5.51	6.07	6.14
Micu2	-0.73	4.28E-02	36.83	55.66	72.05	80.95	97.88	94.95	70.19	46.76	45.56	34.93	37.07	41.30	42.86
Miga2	-3.03	7.67E-05	0.95	0.00	0.19	3.36	2.37	6.39	0.00	0.00	0.00	4.55	4.14	4.54	6.56
Mis12	1.05	4.66E-02	21.20	13.87	14.08	17.28	16.92	15.16	37.10	48.60	22.58	27.44	27.98	26.21	30.29
Mmp25	-4.22	1.11E-09	0.00	0.91	1.87	8.90	6.05	3.27	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Mndal	1.15	1.75E-02	20.58	67.71	64.86	38.94	26.51	27.34	49.57	97.11	71.41	57.71	56.65	49.31	62.08
Mns1	-2.51	1.03E-04	33.11	28.56	39.38	63.12	87.86	65.98	15.80	2.84	4.43	3.52	6.11	3.54	12.53
Mprd	2.12	2.24E-03	8.31	0.45	11.68	2.15	1.97	0.87	17.66	8.52	8.25	13.39	9.05	8.18	6.70
Mpps1	1.26	4.12E-02	65.48	69.07	70.28	14.33	32.51	35.77	71.90	99.33	53.80	68.44	52.48	58.38	54.17
Mrip1	1.62	3.84E-02	19.32	1.03	4.71	5.88	3.95	13.05	52.09	26.42	21.12	9.16	3.13	3.86	8.06
Mtrf1	-1.47	1.68E-03	3.31	21.13	22.15	13.77	26.27	21.64	7.40	6.82	6.16	19.25	23.92	21.32	18.40
Mychp2	1.81	2.77E-05	4.96	4.87	5.32	2.14	3.22	4.19	12.48	16.02	9.20	13.66	11.88	10.32	10.52
Myh3	-2.21	9.32E-03	0.00	6.17	0.52	3.68	0.57	3.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00
My12a	-0.64	1.81E-02	244.43	293.49	287.48	300.15	292.71	316.06	199.93	145.26	233.44	319.27	311.86	254.08	333.02
My1p	1.60	4.38E-02	0.10	12.86	1.73	1.54	4.28	4.27	25.56	9.81	8.95	31.60	32.84	22.88	32.01
Myo1g	-0.70	1.27E-08	214.31	145.56	197.51	220.87	246.80	226.72	145.55	131.39	149.54	87.09	92.01	97.39	85.52
Nan10	-0.82	7.70E-03	59.62	78.50	89.65	166.01	194.62	168.93	114.82	103.09	78.26	95.69	70.58	87.47	89.23

				BAL	MLN			Naive											
				Th2	WT1	WT2	WT3	WT1	WT2	WT3	K04	K05	K06	WT1	WT2	WT3	K04	K05	K06
	Annotation	Log2Fold Change	p-value																
	Nob1	-2.08	9.35E-13	11.33	12.87	17.83	24.50	27.03	32.64	5.95	5.03	7.64	5.32	5.84	6.01	6.20	3.44	3.44	3.24
	Nap1l1	-0.92	4.37E-02	9.77	16.14	8.15	23.39	14.48	15.39	9.41	9.92	12.63	5.38	8.32	8.96	13.82	9.60	6.10	6.10
	Nbn	1.70	7.41E-03	6.77	0.00	3.20	5.10	5.89	3.28	27.45	9.43	22.72	23.01	21.61	18.20	19.41	18.81	16.27	16.27
	Nds1	-2.49	7.37E-04	7.63	0.50	1.50	5.75	2.79	1.03	0.32	0.21	0.20	1.98	1.24	1.18	0.86	1.54	0.71	0.71
	Neb	-1.93	4.94E-07	20.35	23.01	20.25	14.69	19.08	13.90	5.46	2.29	3.54	0.33	0.50	0.76	0.12	0.17	0.22	0.22
	Nek1	-1.81	4.66E-02	0.00	1.46	1.23	1.60	0.64	1.27	0.00	0.00	0.00	1.33	1.82	2.36	2.19	1.75	2.18	2.18
	Nek6	2.10	8.57E-03	6.15	8.73	2.34	1.91	0.23	2.07	8.70	15.58	12.60	0.00	0.02	0.35	0.40	0.33	0.54	0.54
	Nfatc1	-0.87	2.01E-02	37.91	26.34	43.88	84.29	114.81	94.46	44.54	67.13	43.90	24.08	18.81	21.48	17.07	21.51	21.87	21.87
	Nfatc1	-1.88	2.14E-05	220.07	216.96	210.15	203.81	250.69	249.90	27.64	83.75	54.03	21.09	15.25	12.46	11.27	9.24	14.23	14.23
	Nkg7	-2.82	2.00E-14	523.34	882.81	359.70	739.92	1259.92	1131.91	91.86	179.21	101.53	15.89	9.62	9.16	28.19	9.68	9.80	9.80
	Nkirs2	-1.42	1.13E-04	26.15	30.89	41.61	23.94	21.60	26.61	8.93	9.55	6.82	19.08	16.54	18.61	17.85	15.30	23.59	23.59
	Nme1	-0.57	2.63E-02	130.21	199.01	198.76	405.37	267.52	342.59	227.82	223.57	222.26	360.47	303.80	273.27	232.63	226.29	302.54	302.54
	Nmi	0.76	8.10E-03	60.32	76.74	52.62	47.86	42.21	56.43	82.83	88.20	79.92	99.94	90.70	95.19	91.53	87.53	81.49	81.49
	Nn18	1.42	3.35E-02	7.61	6.62	10.52	3.58	7.50	4.81	18.89	23.09	9.06	14.13	15.36	14.62	16.13	15.17	13.38	13.38
	Npel1	1.66	3.07E-02	13.54	0.00	15.04	10.94	4.53	12.14	61.96	48.44	13.67	22.51	7.35	19.70	18.10	10.84	12.70	12.70
	Nras	-1.73	2.42E-05	193.76	98.08	118.59	142.76	131.11	170.07	31.08	24.71	61.20	146.08	152.56	110.41	87.78	84.31	89.00	89.00
	Nsmc1	1.01	2.66E-02	55.16	15.81	46.08	68.34	43.48	39.75	127.89	109.38	83.19	173.80	142.14	160.08	157.87	171.13	171.44	171.44
	Nt5c3b	0.74	3.55E-05	38.34	112.27	68.43	92.08	85.42	88.40	138.02	141.96	167.02	92.85	92.19	94.00	102.83	85.55	112.28	112.28
	Nuff1	1.41	1.88E-02	0.00	0.00	6.73	2.95	8.14	5.57	18.89	16.00	16.71	10.24	10.07	10.74	9.52	9.74	16.02	16.02
	Nup107	-1.09	2.70E-02	7.21	11.06	9.52	46.88	47.13	25.98	14.74	15.33	22.54	31.89	28.17	19.54	24.52	21.75	27.18	27.18
	Nup214	0.92	4.58E-02	4.05	6.98	7.21	9.07	6.30	5.87	16.63	9.66	15.90	10.18	8.62	11.08	9.65	9.44	9.07	9.07
	Nxpe3	1.55	3.14E-14	2.68	9.63	11.45	7.36	6.43	8.06	20.63	22.09	22.43	19.50	16.97	15.94	18.63	19.66	21.00	21.00
	Oard1	0.91	3.21E-02	17.13	21.86	21.65	31.50	33.84	42.13	82.76	72.72	52.35	57.73	59.41	48.92	70.06	66.96	57.72	57.72
	Oik3	-6.09	2.47E-24	6.54	11.78	19.03	10.12	11.32	10.51	0.00	0.00	0.00	0.02	0.00	0.86	0.00	0.25	0.00	0.00
	Oma1	1.23	2.85E-02	5.06	40.41	19.79	17.38	15.64	17.51	43.68	61.19	25.38	22.41	21.29	26.86	25.64	27.18	28.88	28.88
	Otu4	-1.73	3.12E-02	2.03	2.46	2.42	1.08	0.95	1.45	0.14	0.33	0.18	2.61	2.12	3.87	3.62	2.76	2.40	2.40
	P2ry14	-2.73	5.80E-04	40.41	42.23	36.07	5.40	7.48	4.05	0.00	0.00	0.00	1.53	1.05	1.63	1.33	1.97	2.21	2.21
	P3h1	-2.84	2.78E-04	0.00	748	3.06	3.28	2.38	2.38	4.65	0.00	0.00	4.33	2.96	5.55	3.53	3.67	3.26	3.26
	Pold	-3.22	1.83E-05	1.79	0.79	3.22	3.10	3.47	1.62	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Perp4	0.97	1.13E-02	7.70	4.82	4.39	3.13	3.13	2.77	7.30	5.17	5.97	2.52	2.35	3.23	3.29	2.39	2.43	2.43
	Porp6	1.40	1.12E-02	12.36	2.12	12.30	10.42	5.52	7.75	17.77	31.78	20.51	12.69	15.60	16.87	15.26	15.73	10.97	10.97
	Porp8	2.06	1.94E-02	0.00	0.06	0.00	0.06	1.34	1.42	24.66	10.29	7.43	13.47	6.47	13.00	12.84	7.32	9.85	9.85
	Psk	-2.42	3.39E-03	5.73	0.00	1.63	7.96	1.13	2.40	0.00	0.00	0.00	0.06	0.00	0.23	0.12	0.00	0.09	0.09
	Ptdhga11	-2.59	1.37E-03	1.26	1.55	2.48	0.95	1.42	4.31	0.01	0.00	0.00	0.26	0.57	0.77	0.83	0.80	0.34	0.34

Pcdhga6	-1.81	4.65E-02	0.00	1.61	2.57	0.64	1.86	4.47	0.00	0.00	0.00	0.00	0.27	0.60	0.80	0.86	0.84	0.25
Pcdhga7	-1.95	2.84E-02	0.00	1.57	2.50	0.63	2.66	4.43	0.00	0.00	0.00	0.00	0.26	0.58	0.78	0.84	0.81	0.43
Pcdhgb4	-1.81	4.65E-02	0.00	1.63	3.13	0.65	1.89	4.53	0.00	0.00	0.00	0.00	0.28	0.60	0.81	1.01	0.85	0.25
Pcdhga7	-1.94	2.97E-02	0.00	1.61	2.58	0.77	1.89	4.48	0.00	0.00	0.00	0.00	0.29	0.68	1.03	1.03	1.15	0.25
Pdca4	0.79	4.66E-03	20.46	29.75	23.67	26.83	32.36	35.00	56.97	49.09	59.41	157.23	167.13	119.38	189.17	220.20	96.91	
Pdk1	1.09	3.87E-02	3.39	4.14	4.18	8.27	5.79	5.73	13.97	20.00	10.75	74.08	61.15	82.06	85.15	100.98	75.67	
Pdlm1	1.78	9.80E-04	10.95	5.03	4.31	9.96	6.38	11.52	57.35	30.16	27.64	87.86	81.35	87.78	80.06	83.59	90.84	
Pecm1	1.26	1.40E-03	13.28	13.80	19.69	22.85	12.70	11.99	39.96	39.60	38.80	86.08	87.63	76.99	87.49	81.69	113.72	
Penk	-2.34	2.96E-06	26.78	61.41	53.40	180.82	566.77	304.90	52.33	72.79	37.39	4.24	4.48	6.78	6.70	4.58	6.94	
Pex12	-2.17	1.12E-02	3.89	0.00	6.05	8.53	3.87	1.49	0.00	0.00	0.00	4.48	6.78	6.70	6.70	4.58	6.94	
Pfkp	-0.86	3.77E-02	108.86	93.73	105.04	91.65	135.07	130.27	45.57	63.79	80.79	68.10	55.42	53.27	46.37	47.50	50.13	
Pfn1	-0.84	8.97E-05	1793.74	2357.17	2285.13	3958.53	3658.61	4973.62	2177.98	1694.24	2739.96	5027.25	4615.36	3801.14	5423.63	5158.88	5556.81	
Pgm2	-1.06	1.81E-02	40.34	52.55	28.06	30.50	33.53	53.74	18.19	18.66	16.29	32.84	23.78	28.87	31.84	23.48	32.67	
Pik3ap1	-2.09	1.75E-02	18.07	39.02	23.87	46.89	58.06	56.81	3.33	0.00	0.67	1.35	0.67	0.20	0.24	0.21	0.48	
Pik3v5	1.59	1.54E-02	18.58	4.87	14.98	7.85	2.48	3.22	13.32	20.92	16.00	31.54	31.12	39.88	24.39	31.77	40.59	
Pip4k2b	-1.93	3.40E-02	0.05	1.67	1.13	0.92	0.68	1.76	0.03	0.04	0.16	0.72	1.02	0.68	2.09	1.15	0.45	
Pirb	-3.25	1.44E-05	1.05	11.37	6.29	2.49	6.41	3.10	0.00	0.00	0.02	0.07	0.04	0.02	0.05	0.32	0.31	
Pitrac1	-1.22	4.50E-02	23.66	28.94	30.07	25.54	46.33	45.36	21.92	7.36	16.18	37.65	44.67	28.92	45.24	40.17	30.13	
Pwif12	-3.05	6.87E-05	0.00	0.00	0.78	1.71	3.86	2.18	0.00	0.00	0.00	0.07	0.00	0.00	0.00	0.00	0.00	
Pkn	-0.94	9.29E-12	295.67	453.52	366.46	778.48	783.47	877.98	459.01	353.20	449.75	470.51	385.41	381.35	356.41	367.73	480.30	
Pkn3	-1.99	2.37E-02	0.65	8.66	0.42	1.18	1.83	7.28	0.42	0.22	0.28	0.10	0.23	0.32	1.36	0.27	0.76	
Pkp3	-1.09	3.50E-02	237.18	206.59	191.06	260.00	338.77	304.42	65.65	187.79	136.05	83.30	82.42	64.89	63.95	77.41	81.82	
Ploc8	-3.09	7.36E-66	1664.13	1240.95	2129.98	393.37	473.95	469.34	50.99	51.29	48.05	7.05	6.04	4.82	6.11	6.02	2.87	
Pldc4	-2.40	3.71E-03	0.00	0.82	0.02	2.74	2.12	4.45	0.00	0.00	0.00	0.15	0.02	0.06	0.23	0.00	0.08	
Plekkg3	-6.78	1.86E-32	3.00	0.26	5.00	7.28	11.50	9.29	0.00	0.00	0.00	0.01	0.00	0.00	0.01	0.00	0.00	
Ppp2	-3.49	1.81E-06	0.00	18.51	2.34	4.00	7.45	8.57	0.00	0.04	0.00	1.68	0.77	0.58	2.20	0.00	0.67	
Ppple2	-1.64	1.61E-03	8.44	7.92	24.26	21.24	26.34	16.39	8.16	5.76	3.87	12.54	6.35	18.06	14.30	15.31	8.27	
Pppl7	1.35	2.78E-02	23.79	3.22	25.94	14.91	11.80	5.42	21.02	30.92	42.29	31.73	20.85	26.33	22.86	24.75	24.57	
Ppnl1	-1.30	1.21E-03	68.59	26.65	50.55	71.13	116.88	88.95	28.99	43.98	31.88	2.72	3.16	1.86	1.40	0.94	0.63	
Ppnlp3	-0.76	7.69E-03	14.73	32.74	42.81	43.53	46.84	39.12	21.96	24.07	29.10	36.96	39.11	38.31	31.54	32.02	37.17	
Ppl	1.09	3.71E-02	25.96	3.03	13.73	5.77	6.00	7.30	20.80	11.51	16.25	18.25	16.86	19.65	12.14	15.20	13.38	
Ppl2e	0.54	1.15E-02	202.14	254.57	224.88	208.24	201.74	171.24	276.17	300.39	270.65	198.49	162.65	162.92	182.79	160.13	183.37	
Ppdc4	1.56	4.25E-03	7.65	23.09	25.70	17.77	27.13	31.10	144.21	62.19	54.96	49.31	40.85	40.76	34.74	42.70	43.54	
Ppflp2	-4.25	7.79E-10	0.00	6.99	0.00	3.79	4.68	7.29	0.00	0.00	0.00	0.20	0.00	0.00	0.00	0.07	0.00	
Ppplca	-0.55	2.70E-05	950.26	1147.47	883.97	1278.79	1185.62	1144.52	837.38	867.70	744.76	917.04	916.51	809.47	802.43	814.47	939.37	
Ppp1r3b	-2.48	2.40E-03	0.00	0.00	1.00	2.64	1.34	2.24	0.00	0.00	0.00	2.93	2.87	3.23	5.32	1.98	3.15	
Ppp1r3fos	1.73	4.66E-02	1.12	5.63	3.14	3.20	0.62	0.54	6.70	8.93	10.18	2.71	2.49	1.01	0.89	1.52	1.49	

			BAL	MLN				Naïve										
			Th2	WT1	WT2	WT3	WT1	WT2	WT3	K04	K05	K06	WT1	WT2	WT3	K04	K05	K06
	Log2Fold Change	P-value																
Annotation																		
Ppp2r1a	-0.55	9.97E-03	66.30	132.28	143.72	143.72	170.71	183.74	142.08	119.68	108.22	109.11	104.12	107.82	133.32	108.47	107.29	124.04
Ppp6r2	-1.66	8.21E-03	0.00	8.55	2.47	2.47	7.71	10.16	8.57	1.28	2.06	3.35	2.28	3.33	3.78	3.37	3.63	4.62
Pqlc3	-1.25	2.69E-05	38.52	119.20	63.44	63.44	74.35	82.34	93.97	33.52	26.72	41.76	15.99	13.37	13.07	26.26	20.32	23.94
Prag1	-2.49	2.24E-03	0.00	0.00	0.36	0.36	1.43	5.99	2.00	0.00	0.00	0.00	5.56	3.34	5.42	0.96	0.50	1.34
Prdx1	-0.59	1.77E-02	200.50	249.81	215.00	215.00	323.13	222.20	253.21	183.50	175.47	162.72	148.21	135.24	147.53	136.96	139.13	126.08
Prim1	1.01	6.64E-03	7.04	9.92	19.21	19.21	46.15	36.18	40.17	114.44	68.75	72.77	13.27	18.49	16.53	13.74	13.03	11.99
Prim2	-3.08	1.57E-05	0.91	0.00	11.95	11.95	31.33	7.18	16.11	0.16	0.96	0.88	4.45	4.94	5.97	4.49	7.96	
Pkr1a1a	-0.77	3.13E-03	344.31	352.68	320.90	320.90	300.39	292.96	355.49	141.55	203.27	203.78	177.36	182.68	159.59	190.49	189.45	186.38
Pkr42	0.72	1.68E-02	43.85	23.96	41.15	41.15	44.87	52.70	35.87	73.43	68.74	84.26	115.40	114.61	116.40	96.35	114.93	116.46
Pkrf6	0.74	3.34E-03	39.60	34.85	29.42	29.42	42.18	35.50	35.39	70.99	66.39	53.70	51.34	51.73	54.85	48.97	38.91	52.76
Pp3a13	1.16	4.84E-02	9.53	16.89	11.33	11.33	10.21	10.91	8.78	22.24	36.21	14.66	26.23	28.34	30.38	28.55	25.18	20.56
Prr13	-0.79	1.20E-04	482.41	618.64	628.41	628.41	407.06	470.38	498.31	236.03	255.88	294.80	137.12	139.97	82.07	84.73	97.21	116.38
Prune2	-6.68	3.92E-31	1.22	0.84	1.55	1.55	3.49	4.41	2.71	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Psmad1	-0.84	5.44E-07	44.32	30.56	40.22	40.22	81.31	71.27	79.84	48.01	40.98	39.33	48.57	48.35	39.17	37.93	38.35	45.81
Psmad3	-0.80	4.21E-02	22.66	58.00	52.74	52.74	98.36	67.25	75.43	37.96	54.56	41.08	59.70	58.98	72.78	67.58	69.07	76.82
Psmad5	-0.64	1.77E-02	45.66	51.54	46.33	46.33	68.68	55.13	62.40	39.17	34.84	45.05	100.05	99.05	69.56	73.74	94.71	110.25
Ptns	-1.18	1.98E-02	11.59	24.31	26.02	26.02	20.81	29.70	26.29	12.13	10.62	8.59	1.99	1.32	4.11	5.15	5.05	4.18
Ptp4a2	-0.70	1.65E-05	140.27	190.41	183.12	183.12	165.55	193.93	201.33	119.16	106.50	118.49	232.56	235.32	163.83	235.28	226.35	199.74
Ptpn12	1.86	3.25E-02	5.78	4.57	2.16	2.16	0.55	0.82	0.21	6.05	1.62	4.15	3.50	5.42	2.98	3.76	1.07	1.89
Ptpn13	-2.01	1.31E-05	13.23	28.05	22.74	22.74	41.26	35.91	28.80	4.83	11.70	5.53	0.52	0.52	0.64	0.00	0.02	0.09
Ptpa	0.80	3.11E-02	13.09	20.30	9.88	9.88	13.78	14.54	10.42	26.30	22.31	20.56	34.24	42.56	35.08	28.37	34.15	37.92
Pus3	1.19	4.46E-02	8.88	13.10	21.19	21.19	9.37	11.20	20.11	42.00	25.68	35.99	29.04	28.13	29.10	20.39	25.25	24.62
Pv1	1.46	2.85E-02	6.37	3.26	5.48	5.48	4.15	3.47	5.01	15.39	19.70	7.00	6.85	8.22	6.07	5.06	4.52	5.10
Qcct	2.21	4.30E-04	0.00	0.00	2.66	2.66	1.57	1.52	0.97	14.15	5.95	7.69	5.68	6.18	4.32	2.36	2.59	6.89
Qsot1	-1.32	8.02E-08	20.66	57.32	36.74	36.74	75.87	85.39	59.54	29.19	30.53	25.46	21.84	21.80	21.38	27.26	24.73	31.08
Rab1b	-0.59	3.29E-02	126.35	146.39	167.41	167.41	171.93	154.98	184.28	100.66	129.97	103.94	172.72	136.79	134.04	140.33	129.88	185.01
Rab32	-1.93	3.07E-02	18.29	0.00	4.20	4.20	1.31	6.84	11.22	0.00	0.00	0.00	5.23	4.26	5.99	5.49	3.99	3.48
Rab43	-1.65	3.06E-02	8.23	11.72	6.82	6.82	6.08	5.65	5.84	2.05	1.59	0.50	4.20	1.82	3.47	2.06	2.21	3.65
Rab4a	-1.82	7.17E-07	51.24	77.91	44.02	44.02	49.96	59.82	52.81	13.33	10.45	18.40	22.13	24.04	19.72	19.45	27.08	31.15
Rabb1	-0.65	8.10E-03	63.41	62.85	48.57	48.57	34.14	37.16	33.96	25.78	18.94	21.87	16.05	9.40	14.48	15.77	10.56	11.26
Rac1	-1.23	1.09E-03	122.95	118.57	179.20	179.20	225.94	307.59	284.82	162.71	86.31	83.83	114.60	86.25	159.54	177.85	179.45	155.97
Rad51b	1.63	9.52E-03	0.03	0.00	1.08	1.08	2.67	3.15	3.93	16.56	13.28	7.57	0.41	0.34	0.00	0.41	0.43	0.00
Ramp1	2.18	1.81E-09	52.70	88.29	65.83	65.83	23.62	45.99	49.96	217.10	220.44	168.66	50.42	48.54	43.91	63.31	48.34	76.03
Rappef6	0.68	1.44E-02	45.36	48.61	62.97	62.97	40.52	26.25	33.72	38.04	57.25	64.42	81.63	90.22	86.99	79.77	78.30	92.08

Rbp7	0.66	2.88E-02	31.87	113.40	81.17	183.63	129.55	146.96	198.95	275.27	258.77	132.65	149.47	118.22	122.73	107.93	131.45
Rbm27	-1.30	4.54E-02	5.66	18.59	3.50	3.96	4.70	4.70	0.97	2.14	1.51	8.47	6.49	6.64	7.86	6.59	7.07
Reep5	0.68	4.38E-02	46.72	60.73	63.34	44.77	40.48	32.88	68.54	69.77	52.67	29.33	30.29	27.45	39.01	38.88	37.95
Repn1	-2.60	1.23E-03	0.00	7.73	0.00	5.78	4.83	1.72	0.00	0.00	0.00	2.76	0.32	1.05	0.19	0.10	1.01
Rfx7	1.89	1.76E-02	0.32	0.00	3.80	0.98	1.46	1.12	7.50	13.16	1.88	3.33	3.54	2.14	4.29	2.71	2.96
Rgcc	2.19	3.19E-05	34.30	90.18	28.99	13.07	17.15	16.41	147.83	75.46	51.02	120.25	127.84	105.19	226.14	178.32	272.18
Rgs1	-0.60	1.92E-03	3268.18	3304.24	2682.35	685.29	840.11	706.81	554.87	437.88	478.25	41.77	37.59	38.01	40.66	38.06	36.26
Rgs2	1.51	6.37E-06	123.97	122.23	92.72	17.11	26.23	17.73	65.08	68.05	51.77	11.55	8.49	10.80	22.57	15.05	16.47
Rhl	-0.87	3.90E-02	78.85	84.23	59.52	89.62	96.78	90.40	67.23	31.39	48.37	34.74	36.87	44.96	42.71	38.71	37.38
Rh45s	-0.52	8.42E-10	1721.26	1740.56	4393.08	3672.38	3133.01	3234.87	2493.42	2309.01	2243.63	3231.11	3339.71	8099.19	6669.54	6152.80	4584.34
Rhf114	0.73	2.42E-05	143.17	189.46	171.49	90.94	99.03	90.42	179.96	138.10	149.60	254.81	201.96	178.52	152.99	155.31	197.36
Rhf25	1.13	4.19E-06	129.59	160.57	116.28	50.67	60.34	49.68	139.33	109.25	110.37	38.19	40.14	39.11	31.61	34.89	33.54
Rhf28	-1.56	4.35E-02	26.19	36.57	40.76	14.31	26.96	21.85	1.93	8.19	5.29	0.00	0.00	0.02	0.25	0.22	0.00
Rhnp	-1.04	1.03E-02	61.56	67.77	57.66	96.63	72.75	72.25	52.38	30.27	29.68	35.18	34.03	23.43	21.83	27.69	23.78
Rp9	1.17	3.76E-04	45.65	40.35	44.66	31.85	26.86	37.48	78.23	81.41	63.77	67.28	69.17	59.49	83.64	74.50	64.28
Rpap1	1.30	1.86E-05	0.00	4.36	12.38	9.45	12.91	12.22	26.29	27.65	34.77	12.72	7.95	10.80	9.47	10.47	10.73
Rpl13	0.66	3.49E-02	104.18	95.76	129.36	139.40	97.16	100.87	195.31	170.78	171.99	258.43	238.93	323.78	277.06	330.92	272.39
Rpl1	-0.72	7.32E-04	54.89	67.53	65.74	117.57	82.35	93.50	61.78	56.45	57.11	57.74	56.60	56.85	59.31	45.46	56.99
Rps27	1.51	4.41E-02	19.24	8.38	11.18	8.23	6.11	7.42	16.37	40.33	23.56	15.38	16.26	18.45	17.72	20.74	12.27
Rps3a1	1.09	1.62E-02	22.10	49.39	38.83	34.80	46.23	24.97	73.84	82.23	77.58	47.46	111.50	94.94	116.67	168.15	90.95
Rps6a5	-1.69	3.35E-02	7.54	0.00	3.42	2.03	3.50	3.11	0.32	0.97	0.49	0.28	0.40	0.80	0.67	1.69	0.67
Rrad	1.74	1.87E-03	22.91	11.80	29.17	22.61	9.45	12.92	77.29	67.05	36.57	51.64	42.45	32.67	52.74	47.10	61.73
Rrm2	-0.68	2.69E-02	29.44	11.01	46.40	258.96	207.35	202.34	108.85	159.83	138.41	6.37	3.45	4.14	22.52	3.29	5.47
Rsl1d1	0.90	1.26E-05	28.91	83.49	68.65	97.89	79.81	70.48	161.37	156.21	150.16	92.27	107.26	105.78	98.49	124.34	100.61
Rtn3	-0.87	4.73E-03	96.63	90.75	102.52	123.14	90.18	111.17	48.55	70.14	53.46	33.30	37.87	47.79	45.06	44.21	44.32
Rtn4	2.19	1.40E-04	6.88	1.86	9.48	1.42	2.22	2.02	13.83	14.70	6.26	1.85	1.55	1.16	1.41	1.70	1.89
Rum3	-1.27	4.48E-02	17.95	17.02	15.46	16.21	17.36	12.10	2.96	7.03	6.45	12.33	16.45	10.61	13.97	9.21	9.52
Rxb	1.53	1.10E-02	5.96	0.00	5.73	3.19	3.57	4.07	14.98	7.01	15.79	13.31	8.08	11.06	11.64	10.89	10.83
S100a13	-0.94	3.07E-02	149.82	158.43	200.29	137.09	205.77	181.90	68.37	88.85	105.25	90.42	87.21	86.70	110.88	102.70	93.32
S1pr1	1.48	1.74E-05	13.94	12.68	14.87	43.76	26.74	27.08	86.86	77.76	122.31	233.19	280.95	275.04	329.98	332.56	362.45
Sumhd1	1.25	5.03E-11	340.18	353.83	443.20	106.52	86.95	97.37	289.44	210.77	203.27	142.90	117.13	83.91	121.62	117.73	146.35
Sat1	0.79	1.86E-04	440.35	457.65	343.43	161.17	201.50	181.89	301.86	335.94	315.91	249.72	261.81	219.84	227.41	246.55	232.54
Sbk1	-2.20	9.88E-03	0.00	5.70	1.28	1.37	1.66	2.32	0.00	0.00	0.00	4.31	3.80	6.25	6.49	6.06	4.20
Scaf11	1.05	1.13E-02	8.19	18.14	11.75	8.14	9.04	8.18	23.92	18.67	12.44	16.80	17.20	15.25	14.35	12.24	17.27
Scin	-2.66	9.11E-04	0.00	0.00	0.00	1.92	4.54	4.49	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00
Scmh1	2.62	1.15E-03	0.00	0.00	0.00	0.02	0.05	0.00	10.96	1.11	3.78	4.53	9.09	5.33	5.66	5.75	6.93
dhaf1	1.64	7.91E-04	36.65	34.22	23.08	42.66	24.20	31.88	83.65	163.22	97.62	68.99	64.49	54.34	67.92	91.55	78.07

				BAL	MLN			Naive													
				Th2	WT1	WT2	WT3	WT1	WT2	WT3	K04	K05	K06	WT1	WT2	WT3	K04	K05	K06		
Log2Fold Change	Annotation	p-value	Sec24d	WT1	WT2	WT3	WT1	WT2	WT3	WT1	WT2	WT3	K04	K05	K06	WT1	WT2	WT3	K04	K05	K06
-2.21	1.97E-04	20.42	8.61	12.71	21.49	15.74	17.06	1.87	1.55	5.05	7.24	5.55	4.36	3.92	8.10	7.39	8.10	7.39	8.10	7.39	
-1.59	4.90E-02	2.50	3.51	5.10	12.23	12.24	12.82	1.42	5.50	1.41	7.00	9.27	6.87	10.51	10.00	7.39	10.51	10.00	7.39	7.39	
1.50	3.01E-02	28.22	9.86	10.34	103.35	22.50	26.24	181.72	146.47	203.35	567.68	564.02	625.99	639.35	649.14	670.61	639.35	649.14	670.61	670.61	
-0.90	1.51E-04	133.75	171.88	159.95	214.21	216.51	239.40	93.33	130.74	129.11	143.33	128.02	162.55	160.44	158.05	160.17	160.44	158.05	160.17	160.17	
1.99	1.42E-03	11.97	9.48	7.64	6.75	5.46	2.48	43.57	17.30	18.47	4.67	4.90	5.53	4.64	5.33	5.47	4.64	5.33	5.47	5.47	
2.26	1.90E-10	11.24	21.98	19.60	15.09	19.05	12.89	75.62	108.09	61.60	59.22	66.11	50.96	72.97	96.43	73.13	72.97	96.43	73.13	73.13	
1.78	7.44E-03	12.72	0.00	3.85	3.92	3.08	1.25	15.60	13.01	8.79	8.84	10.34	7.21	7.60	7.41	5.55	7.60	7.41	5.55	5.55	
0.62	1.75E-10	1397.70	1424.77	1675.94	1129.07	1060.74	1082.74	1589.13	1751.93	1687.00	2546.76	2377.57	2312.45	2235.69	2272.94	2404.37	2235.69	2272.94	2404.37	2404.37	
-0.71	4.75E-02	114.04	87.29	132.67	127.95	102.00	102.40	85.16	59.32	54.87	42.03	47.23	41.29	40.59	33.98	50.64	275.26	263.42	182.45	182.45	
0.61	1.59E-03	78.26	84.59	91.24	104.53	94.03	108.09	167.75	134.50	166.94	186.36	199.40	185.20	275.26	263.42	182.45	185.20	275.26	263.42	182.45	
1.32	5.27E-03	75.18	10.01	31.92	98.04	50.07	65.53	121.47	237.16	220.44	149.43	132.03	131.71	54.06	53.20	53.39	131.71	54.06	53.20	53.39	
-1.18	2.23E-02	29.35	87.08	43.69	40.99	52.79	45.74	12.15	18.35	25.88	23.33	23.85	20.20	20.28	20.77	22.07	20.28	20.77	22.07	22.07	
-1.94	2.94E-02	0.00	0.00	2.57	4.13	2.00	4.14	0.00	0.00	0.00	18.57	16.95	12.37	6.36	10.26	13.42	12.37	6.36	10.26	13.42	
-0.79	2.55E-04	201.66	287.88	264.42	320.44	239.15	261.83	184.54	143.73	152.08	113.42	103.73	103.61	143.67	148.42	156.00	103.61	143.67	148.42	156.00	
1.36	4.50E-02	0.00	8.01	8.87	8.47	4.59	4.38	21.16	22.91	8.99	4.25	4.20	7.86	3.52	4.57	5.98	3.52	4.57	5.98	5.98	
-1.14	1.96E-02	12.43	12.18	31.32	39.36	36.18	55.53	21.86	11.58	22.20	34.90	31.49	24.01	38.10	39.70	40.52	24.01	38.10	39.70	40.52	
-0.93	1.83E-02	70.74	30.61	45.54	68.79	51.92	48.54	28.37	20.01	37.49	35.58	31.56	27.00	28.44	28.04	40.65	27.00	28.44	28.04	40.65	
-0.86	3.78E-03	50.39	77.36	58.53	45.81	50.17	58.14	32.29	26.32	24.28	40.92	45.58	28.98	41.39	34.30	39.85	28.98	41.39	34.30	39.85	
1.91	1.71E-03	14.47	4.39	11.08	3.02	3.84	4.54	8.24	23.37	23.41	4.24	5.96	6.08	6.53	5.38	6.16	6.08	6.53	5.38	6.16	
1.46	1.16E-02	20.38	27.93	40.65	12.64	10.39	11.52	17.05	52.33	44.42	58.35	44.38	37.74	37.07	33.42	27.18	37.07	33.42	27.18	27.18	
1.14	3.86E-04	42.83	34.96	23.69	61.46	43.08	34.50	116.51	83.98	115.35	99.10	111.94	89.83	103.06	114.84	72.32	89.83	103.06	114.84	72.32	
-1.25	8.13E-04	45.73	35.40	36.63	62.46	70.47	76.96	27.02	22.07	35.04	11.52	9.34	9.04	6.08	7.86	11.36	6.08	7.86	11.36	11.36	
-0.90	1.04E-03	197.27	152.80	218.29	260.41	308.11	298.69	150.71	171.25	131.06	145.96	123.97	117.72	137.27	141.22	157.14	117.72	137.27	141.22	157.14	
-0.78	1.20E-02	31.25	44.04	42.90	56.61	50.34	62.63	40.92	27.40	28.89	56.24	53.56	48.29	56.53	56.30	68.47	48.29	56.53	56.30	68.47	
-0.52	3.29E-02	78.57	27.07	99.37	111.37	82.99	99.35	67.04	64.81	71.43	94.06	103.98	83.74	81.55	91.93	97.27	83.74	81.55	91.93	97.27	
0.52	3.11E-02	118.14	199.62	220.64	129.89	120.08	107.63	172.35	154.35	194.66	401.20	410.18	337.04	331.52	350.59	393.08	331.52	350.59	393.08	393.08	
1.24	5.63E-04	43.47	24.49	43.33	30.48	26.68	20.76	80.68	48.79	63.66	59.90	55.94	56.10	64.26	66.04	64.80	56.10	64.26	66.04	64.80	
0.52	3.29E-02	58.71	27.61	59.04	51.76	44.04	50.80	70.04	75.77	64.04	65.58	58.11	49.97	69.15	70.60	73.95	49.97	69.15	70.60	73.95	
-2.39	3.96E-03	0.00	0.00	0.00	2.25	3.84	2.70	0.00	0.00	0.00	3.94	1.22	3.27	0.34	0.31	2.38	3.27	0.34	0.31	2.38	
1.49	1.77E-02	0.00	0.00	1.89	2.82	4.98	4.23	16.19	13.73	10.82	5.44	5.27	3.26	5.02	4.78	8.42	3.26	5.02	4.78	8.42	
1.11	2.89E-07	81.11	98.44	55.82	61.93	52.76	69.04	147.24	132.52	124.16	69.33	75.76	85.47	98.73	92.38	98.01	75.76	85.47	98.73	92.38	
-1.40	1.40E-05	48.23	68.55	41.78	89.87	76.73	74.79	16.20	28.10	28.60	20.11	24.59	35.10	28.52	23.61	25.00	24.59	35.10	28.52	23.61	
-1.62	3.82E-03	18.09	2.88	8.01	15.47	8.24	11.13	1.98	3.07	4.43	10.59	7.94	8.26	9.50	7.72	11.00	7.94	8.26	9.50	7.72	
-1.11	6.73E-03	36.22	39.29	65.24	51.57	51.11	58.65	15.94	22.13	32.85	31.45	25.61	46.63	41.47	46.31	39.77	25.61	46.63	41.47	46.31	

Slgnp3	-2.29	6.56E-03	0.00	2.73	0.67	1.46	3.26	0.51	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00
Slgn	-1.09	2.83E-03	988.25	891.82	1041.64	1329.95	1618.51	1556.13	701.89	892.94	428.70	188.25	203.88	205.01	227.11	228.63	146.32	146.32	146.32
Ssr1	-0.71	3.00E-05	11.55	17.70	20.82	22.59	19.48	22.74	12.75	12.41	14.08	18.33	18.47	14.24	19.09	20.12	13.36	13.36	13.36
Stab2	-3.74	2.16E-07	0.00	0.00	0.00	1.41	2.02	4.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Star7	-1.43	4.74E-02	40.02	19.56	11.27	30.19	19.15	19.74	3.01	5.94	11.46	18.82	28.00	32.55	23.58	25.59	31.07	31.07	31.07
Star1	0.97	1.23E-02	196.55	187.82	199.53	160.07	100.17	71.15	248.99	187.12	234.28	305.09	241.20	152.57	301.96	280.43	320.96	320.96	320.96
Stom	-3.76	1.82E-07	13.86	12.88	7.59	13.81	3.51	8.49	0.00	0.00	0.00	0.50	0.28	0.02	0.20	0.00	0.00	0.00	0.00
Sufu	-1.46	3.72E-04	5.02	0.91	4.57	17.00	10.59	11.21	4.82	3.42	4.77	5.80	6.31	7.92	6.09	8.24	7.90	7.90	7.90
Supr71	-3.21	2.14E-05	0.36	0.00	10.86	26.92	4.49	6.87	0.00	0.00	0.00	18.81	19.45	14.78	13.49	15.38	20.61	20.61	20.61
Syme1	-1.94	1.22E-02	0.05	2.45	1.83	0.42	4.50	1.15	1.40	0.00	0.16	1.80	3.44	1.37	3.80	1.12	2.73	2.73	2.73
Syng3	-3.20	2.16E-05	8.88	17.61	6.55	17.69	7.68	4.46	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Togln2	-0.87	1.32E-03	353.10	653.16	484.15	336.04	485.16	397.25	186.31	248.29	193.98	129.86	137.69	148.97	223.75	228.59	250.22	250.22	250.22
Tap1	0.78	1.12E-03	159.97	160.80	225.14	131.28	129.07	91.18	195.17	195.09	220.63	134.91	119.01	124.64	123.65	156.71	151.74	151.74	151.74
Tapbp1	1.02	2.21E-02	36.97	60.87	71.50	35.24	51.17	28.05	67.34	80.52	97.17	77.01	59.61	64.95	72.06	57.69	62.25	62.25	62.25
Tbcl1d17	0.91	4.60E-02	29.54	43.71	24.29	22.87	23.33	32.49	69.36	45.85	39.88	35.35	24.78	31.67	35.80	26.31	45.96	45.96	45.96
Tbcl1d19	-3.28	1.26E-05	5.61	0.00	1.01	5.59	3.56	3.79	0.00	0.00	0.00	1.56	0.93	1.11	3.07	1.90	0.78	0.78	0.78
Tcp1J12	0.66	1.61E-02	58.18	102.90	80.73	38.16	38.71	35.51	65.68	48.05	66.02	127.75	160.94	101.62	119.60	127.97	153.00	153.00	153.00
Tgfb3	-2.07	1.75E-02	0.00	0.00	2.79	2.48	1.40	9.25	0.00	0.00	0.00	0.31	0.50	0.57	0.17	0.16	0.02	0.02	0.02
Tgfp2	1.63	3.78E-02	4.41	5.74	2.93	4.77	3.71	2.14	16.35	24.09	5.70	11.64	13.15	10.07	11.95	11.30	12.64	12.64	12.64
Tgfp1	2.55	6.92E-04	3.20	0.79	4.99	1.21	0.28	0.47	13.77	12.79	3.29	34.88	26.84	19.09	30.00	25.33	32.07	32.07	32.07
Tgfp2	1.38	8.84E-03	45.27	29.81	34.28	10.76	6.66	6.07	18.43	30.18	19.26	43.12	32.53	19.21	29.39	30.52	36.79	36.79	36.79
Thap3	0.97	9.52E-03	9.67	68.01	53.38	34.24	24.01	30.94	64.59	69.95	52.51	79.59	90.79	68.21	78.88	82.06	95.39	95.39	95.39
Timm10	0.88	2.24E-02	32.07	24.02	43.85	29.72	24.45	30.57	58.81	46.99	54.48	42.51	29.82	40.95	26.52	33.52	37.53	37.53	37.53
Tipr1	-0.52	3.06E-02	24.60	23.81	24.69	21.20	21.58	19.61	15.01	14.38	13.84	22.87	23.05	20.89	24.97	26.19	22.24	22.24	22.24
Tm74f3	-1.61	2.66E-02	112.07	64.30	80.79	32.77	74.16	61.63	19.70	17.56	4.36	5.41	6.79	5.51	7.39	4.20	5.93	5.93	5.93
Tmed2	-0.57	2.28E-02	105.45	125.72	148.47	120.66	115.97	154.46	90.19	80.62	89.29	155.33	173.38	130.87	189.60	190.69	148.33	148.33	148.33
Tmem14c	-0.97	1.81E-04	17.31	102.66	120.16	149.08	142.35	128.54	67.67	59.22	83.35	79.63	65.82	73.13	71.69	67.30	57.52	57.52	57.52
Tmem191c	1.70	9.52E-04	18.33	17.40	23.11	7.76	12.02	10.88	41.65	24.05	51.38	14.80	11.69	14.68	11.79	13.38	16.35	16.35	16.35
Tmem2	-5.72	6.28E-22	7.23	8.88	4.85	12.49	17.56	10.17	0.03	0.00	0.08	0.36	0.18	0.00	0.31	0.26	0.09	0.09	0.09
Tmem203	-1.87	1.01E-04	10.82	14.87	12.28	17.29	19.89	19.52	4.88	4.63	3.63	18.12	16.12	23.90	30.21	35.57	29.40	29.40	29.40
Tmem57	1.75	4.60E-02	0.00	1.39	3.58	0.86	1.61	0.54	11.89	4.69	2.24	0.81	2.30	2.03	2.84	2.11	2.39	2.39	2.39
Tmtc4	-2.09	1.69E-02	5.20	0.00	2.40	3.77	1.93	2.98	0.00	0.00	0.07	2.90	4.19	5.34	2.70	4.26	1.98	1.98	1.98
Tmx1	-0.67	2.22E-02	36.19	63.74	46.24	48.50	43.49	49.68	34.99	26.28	26.23	44.28	55.51	37.50	53.18	66.40	48.36	48.36	48.36
Tnfrsf13b	1.65	5.56E-03	2.63	4.70	11.79	13.92	6.16	12.67	32.53	46.02	45.30	19.65	13.07	14.85	19.57	14.09	20.93	20.93	20.93
Tnfrsf25	2.55	1.02E-16	8.08	20.41	13.74	19.13	15.71	21.15	118.98	138.78	88.35	51.53	59.84	42.62	57.58	61.26	72.41	72.41	72.41
Tnfrsf8	-1.68	3.40E-02	318.84	262.70	243.70	243.80	222.98	283.83	6.85	63.56	85.57	24.21	18.71	28.27	22.31	15.33	16.63	16.63	16.63
Tnk	1.13	9.87E-03	8.34	5.91	4.22	8.39	6.69	4.57	16.13	15.75	23.41	11.55	12.31	12.41	13.79	11.50	11.23	11.23	11.23

			BAL	MLN			Naïve											
			Th2	WT1	WT2	WT3	WT1	WT2	WT3	K04	K05	K06	WT1	WT2	WT3	K04	K05	K06
Annotation	Log2Fold Change	P-value																
Tnfr3	-1.03	1.13E-02	63.68	57.24	22.78	39.31	27.35	34.05	16.63	11.03	19.79	33.52	41.28	35.12	29.51	27.58	34.12	
Tnfr2b	0.91	3.38E-02	2.97	4.20	7.84	6.36	7.62	6.27	10.12	15.03	14.32	18.16	17.30	16.95	24.01	21.24	20.27	
Tnfr3b	1.53	7.65E-03	21.67	25.78	23.20	10.48	31.26	19.45	81.70	80.21	47.65	35.10	26.37	23.53	22.87	20.04	32.74	
Tph1	-5.87	1.68E-22	0.00	0.00	0.68	16.54	8.18	10.68	0.00	0.00	0.10	0.00	0.00	0.00	0.00	0.00	0.00	
Tpr	0.66	4.84E-07	19.54	18.08	29.61	23.86	22.53	21.57	36.04	33.23	38.17	40.87	37.30	35.57	43.37	47.36	44.26	
Traf5	-0.96	7.67E-03	20.91	16.39	9.69	56.81	61.22	39.82	24.98	28.07	25.48	58.66	75.76	50.80	47.26	48.96	52.49	
Tnfr2	-1.91	2.57E-03	29.71	15.86	29.32	85.88	104.73	117.55	17.07	6.11	38.47	179.61	153.89	169.78	110.96	99.43	115.66	
Trim14	1.08	2.88E-02	36.45	19.07	44.25	25.77	42.30	22.84	76.04	79.63	49.11	35.90	30.21	35.28	22.71	34.64	33.57	
Trim30a	1.05	3.17E-02	56.00	28.49	59.39	15.20	14.61	8.06	24.17	25.62	33.68	35.00	25.65	23.58	22.03	20.65	21.20	
Trim36	-1.89	4.04E-02	0.73	6.59	2.60	4.28	6.42	8.89	0.53	0.00	0.28	2.14	0.72	0.86	1.75	1.43	1.38	
Tsc22d2	2.21	7.48E-09	0.97	0.00	0.89	1.01	0.72	0.95	3.91	5.52	4.62	1.88	1.31	1.05	2.16	2.64	1.13	
Tspon3	1.71	5.94E-03	23.65	18.16	16.38	27.14	11.57	14.03	82.60	95.40	35.03	15.15	16.13	14.01	21.37	26.47	19.68	
Tspon32	1.07	6.86E-10	77.12	98.73	105.38	202.38	169.40	166.29	338.83	376.18	425.92	416.63	413.61	343.32	322.44	329.18	481.23	
Txk	1.92	5.72E-03	2.18	0.00	1.83	9.05	2.28	6.36	45.31	17.14	33.62	180.90	173.33	155.20	140.31	132.46	164.09	
Txnip	1.28	8.81E-10	38.72	26.72	35.08	32.89	42.82	35.99	88.28	92.82	95.42	115.05	91.82	185.42	180.34	167.82	95.34	
Ube2q1	-1.41	1.97E-02	5.67	7.41	6.78	13.06	7.51	9.39	3.07	2.16	4.43	6.11	6.20	3.76	3.79	4.52	4.46	
Ubxn11	2.15	7.92E-05	40.61	19.27	12.69	8.19	4.67	5.97	41.36	46.35	19.40	32.61	32.86	17.69	37.42	35.02	38.25	
Ucp2	-0.79	6.22E-09	450.92	324.31	396.42	948.25	1036.10	852.72	568.83	487.58	572.55	390.97	286.44	537.34	470.05	456.07	442.94	
Ucp2	-1.74	1.74E-05	28.87	26.53	38.28	29.95	36.29	37.92	7.12	7.95	13.33	11.36	10.13	11.91	12.40	12.23	7.97	
Ukl1	-4.55	9.29E-12	7.52	9.96	5.27	4.15	2.05	3.85	0.01	0.00	0.00	1.98	1.30	2.30	0.91	2.89	1.43	
Upf1	-1.82	3.29E-02	4.99	3.54	1.44	1.95	1.32	0.98	0.15	0.14	0.35	1.39	1.18	1.57	2.18	1.81	1.88	
Urb2	-4.51	1.35E-11	0.01	1.83	0.00	2.57	2.99	2.02	0.01	0.00	0.00	3.00	3.25	3.81	3.33	0.84	1.40	
Usp14	-0.87	9.29E-06	11.44	20.99	12.13	29.99	26.55	30.14	17.14	13.95	15.71	14.47	17.16	17.46	18.73	20.56	18.78	
Usp47	0.96	1.66E-02	4.02	40.73	12.97	10.16	14.73	10.05	20.52	27.12	22.78	19.94	16.51	18.05	17.11	17.22	17.16	
Usp53	2.21	6.59E-03	0.02	3.74	4.56	0.66	1.12	1.47	29.44	2.73	6.56	4.75	4.79	6.18	3.55	4.08	4.17	
Vamp1	1.18	1.49E-02	9.98	6.36	15.96	6.70	9.32	7.74	26.60	15.28	16.43	5.91	8.44	15.94	7.31	7.02	5.06	
Vdr	-3.75	2.29E-32	87.20	72.53	48.63	85.89	117.79	165.46	6.40	7.33	9.42	0.00	0.00	0.01	0.00	0.00	0.27	
Vps28b	-0.89	3.44E-02	12.02	13.54	13.50	15.82	18.75	15.95	8.31	11.03	6.78	13.24	17.26	15.99	16.28	18.03	17.24	
Vrk1	-0.88	2.09E-03	22.96	70.80	58.08	100.91	70.23	106.26	48.39	54.67	44.00	31.82	37.51	27.50	28.45	33.90	34.22	
Vsir	-1.75	3.03E-09	36.15	53.00	44.33	85.48	109.65	114.32	25.34	37.05	24.09	52.03	50.07	69.36	73.54	65.45	85.60	
Wbp2	-1.40	4.89E-05	100.62	72.38	99.70	101.21	115.34	134.13	35.97	39.19	56.75	87.14	77.57	95.61	106.77	114.03	103.92	
Wdr13	1.46	7.16E-04	2.46	5.06	9.53	4.90	4.13	6.99	12.78	19.07	17.20	6.02	3.11	4.37	5.33	6.06	6.04	
Wdr78	-1.92	3.11E-02	0.00	0.00	0.76	4.16	1.05	1.67	0.00	0.00	0.00	1.63	1.18	0.76	1.85	1.26	1.90	
Xcl1	2.67	8.54E-04	0.00	0.00	0.00	0.00	0.00	0.73	36.97	62.61	76.53	0.12	0.00	0.00	0.89	0.00	0.00	

Xpo5	1.14	8.22E-03	9.55	5.66	3.62	5.25	6.78	5.28	10.88	13.67	15.83	10.37	7.21	11.90	8.21	6.73	10.95
Ybe1	-2.08	1.17E-02	0.21	0.17	0.77	1.94	0.81	1.01	0.09	0.10	0.19	1.14	0.84	1.11	1.09	0.38	1.63
Yipf4	-0.81	2.99E-02	23.55	45.91	28.99	34.53	26.07	33.82	17.32	14.81	20.41	27.58	27.96	29.69	36.02	34.55	30.66
Yhdhc1	1.25	3.18E-02	7.11	9.82	8.14	7.97	7.22	3.73	13.53	18.80	17.47	11.90	13.12	15.07	12.95	18.85	13.95
Ywhae	-1.12	3.39E-04	75.61	92.20	95.31	141.67	124.93	119.72	59.92	39.97	72.30	92.79	98.23	87.82	140.16	122.60	98.87
Ywhaz	-0.75	3.76E-08	153.12	211.90	226.62	299.05	287.22	301.87	194.92	147.13	183.46	229.97	239.37	210.92	316.88	309.42	221.49
Zfp70	-1.18	1.18E-08	420.78	446.29	359.34	482.03	545.85	724.42	270.01	240.46	250.57	263.25	241.69	202.14	195.67	186.80	246.80
Zbtb38	1.37	3.59E-08	22.90	22.41	15.68	13.22	13.53	15.36	36.72	42.34	32.60	8.05	8.37	9.24	8.92	8.80	9.92
Zcche10	-1.29	1.78E-06	66.78	115.23	81.26	56.54	82.69	59.81	24.37	19.72	26.65	13.48	13.18	16.29	21.85	17.95	13.60
Zcche11	1.70	4.85E-02	4.19	1.65	3.99	0.20	1.49	1.08	5.73	3.94	5.78	4.07	5.23	3.65	5.51	3.47	4.24
Zfp141	0.96	4.10E-02	0.00	17.07	5.49	4.99	6.44	7.16	15.33	12.14	10.47	6.80	8.27	7.91	7.18	8.96	6.40
Zfp148	0.86	3.23E-03	6.15	6.35	8.95	10.81	8.41	8.78	20.15	17.30	14.19	12.33	11.53	11.03	11.26	13.19	9.87
Zfp236	1.59	3.35E-02	1.89	3.67	2.23	0.56	1.16	0.36	3.76	2.32	2.52	2.31	2.62	2.91	2.38	1.99	3.53
Zfp27	-1.82	4.49E-02	0.00	0.00	2.25	1.06	1.78	5.69	0.00	0.00	0.00	3.53	3.46	1.84	2.89	2.41	5.17
Zfp324	-4.38	1.44E-10	6.04	6.27	0.96	3.60	3.32	5.06	0.00	0.00	0.00	3.34	1.10	0.73	0.65	1.25	1.99
Zfp385c	-1.37	1.63E-03	10.43	12.46	15.09	8.61	7.71	9.82	3.22	3.62	2.46	6.83	5.72	7.18	6.11	5.83	8.79
Zfp606	2.51	6.19E-05	2.75	0.00	0.43	0.44	0.38	1.05	7.91	4.91	4.40	2.76	2.73	3.69	4.87	1.66	3.06
Zfp935	-2.11	6.35E-03	11.27	2.23	7.12	7.62	8.42	11.73	0.34	1.93	1.08	4.58	6.27	5.62	5.91	6.16	5.96
Zfp938	1.22	4.88E-02	1.53	0.92	5.00	4.87	4.05	2.69	10.31	11.55	8.56	5.81	9.09	6.21	4.44	8.05	3.57
Zfp942	1.13	3.03E-03	0.67	1.61	2.76	4.68	4.76	5.20	9.57	11.91	11.87	8.85	7.90	6.49	11.95	6.43	6.52
Zfp954	0.73	2.33E-02	26.88	3.71	14.16	10.92	10.53	11.41	19.08	17.98	18.34	14.07	19.84	17.55	11.21	14.85	19.21
Zhx2	1.67	7.09E-03	1.66	1.20	1.35	1.08	1.39	0.85	5.31	2.83	4.98	7.20	6.23	10.81	8.23	8.08	8.81
Zhrf3	1.87	1.03E-03	5.87	2.69	3.55	1.27	1.06	1.83	7.63	3.55	7.92	14.44	17.97	9.43	12.51	11.72	10.70
Zscan26	0.83	1.49E-03	19.40	20.15	17.15	15.51	12.25	14.32	26.48	21.55	27.55	43.28	48.88	31.45	37.55	37.01	30.55
Zyx	-0.81	2.50E-02	91.54	112.54	108.74	106.20	147.73	127.80	89.56	75.31	52.88	52.15	44.72	51.04	55.15	54.71	69.41



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Chapter 6

Expression of the Notch ligands Jagged1 and Jagged2 on fibroblastic reticular cells, dendritic cells or T cells is not critical for house-dust mite-driven allergic airway inflammation *in vivo*

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Abstract

Background: Allergic asthma is typically characterized as T helper 2 (Th2) cell-mediated eosinophilic inflammation of the airways. Notch signaling is essential during *in vivo* Th2 cell-mediated responses.

In this context, Delta-like ligands and Jagged are thought to instruct Th1 and Th2 differentiation, respectively. We previously showed that mice with a dendritic cell (DC)-specific conditional deficiency of Jagged developed normal allergic airway inflammation (AAI) in response to house-dust mite (HDM). Therefore, we aimed to investigate whether Notch-mediated induction of Th2 responses in HDM-driven AAI is dependent on other Jagged-expressing cell types than DCs.

Objective: To investigate which cell types express Notch ligands during AAI in mice and whether Jagged expression is required for the induction of AAI.

Methods: Various cell types were isolated using FACS and their Notch ligand expression was quantified by qRT-PCR. In addition, we analyzed mice that specifically lack Jagged molecules on lymph node fibroblastic reticular cells (FRCs), on both FRCs and DCs, or on CD4⁺ and CD8⁺ T cells in a HDM-mediated model for AAI.

Results: HDM exposure induced expression of Jagged1 on FRCs but not on B cells. Th2 cells showed expression of Jagged2 and very low levels of other Notch ligands. However, mice lacking both Jagged1 and jagged2 molecules on FRCs, on both FRC and DCs, or on T cells still developed HDM-driven AAI. Hereby, the level of eosinophilic airway inflammation and Th2 cell activation was not different from that of control littermates following *in vivo* intranasal sensitization and challenges with HDM.

Conclusion: Expression of Jagged1 and Jagged2 on DCs, FRC, B cells and T cells is not required for Th2-mediated AAI in a HDM-driven asthma model.

Introduction

Asthma is a chronic heterogeneous disease in which patients present with episodes of bronchoconstriction, airway hyperreactivity, increased mucus secretion and airway inflammation. Allergic asthma is the most common type of asthma³⁻⁵ and ~85% of hospitalized patients with recurrent allergic asthma are allergic to house-dust mite (HDM)^{232, 456}. Allergic asthma is typically characterized as a T helper 2 (Th2) cell-mediated eosinophilic inflammation of the airways. Exposure to HDM, which contains protease activity, acts on epithelial cells and leads to decreased barrier function and TLR4 triggering⁴⁵⁷. As a result, epithelial cells secrete pro-inflammatory cytokines including IL-33, IL-25, Thymic stromal lymphopoietin (TSLP) and Granulocyte-macrophage colony-stimulating factor (GM-CSF), which activate dendritic cells (DCs) and type 2 innate lymphoid cells (ILC2)^{6, 11}. In addition, DCs may directly become activated by allergenic stimulation. Upon activation, DCs mature and migrate to the draining lymph nodes and this process is supported by IL-13, produced by ILC2¹⁴. To migrate to the lymph nodes, DCs and naïve T cells require C-C chemokine receptor type 7 (CCR7) which binds to

Chemokine (C-C motif) ligand 19 (CCL19), produced by fibroblastic reticular cells (FRCs)⁷. In the draining lymph nodes, activation of naïve T cells by DCs induces Th2 cell differentiation. Allergen-specific Th2 cells are attracted to the lungs by the chemokines CCL17 and CCL22, with DCs being their major source²⁰. Th2 cells are well known to produce the cytokines IL-4, IL-5 and IL-13 which cause asthma hallmarks including IgE class switching of B cells, maturation and activation of eosinophils and smooth muscle hyperreactivity and goblet cell hyperplasia, respectively⁸.

Cytokine production by Th2 cells is regulated by the key transcription factor Gata3 (**Chapter 2**). Gata3 can be upregulated by IL-4 signaling via STAT6. However, the origin of the IL-4 remains unclear, since IL-4 is required to generate the cell type that is its major producer. Although many immune cell types can secrete IL-4⁴⁵⁸, when only T cells can make IL-4 Th2 cell responses were still generated, suggesting that no external source of IL-4 is required for the induction of Th2 cell differentiation^{41, 42}. Moreover, Notch signaling can instruct Th2 cell differentiation by direct activation of (1) a 3' enhancer of the *Il4* gene, and (2) an upstream promoter of *Gata3*²⁰⁷. Importantly, we and others have demonstrated that Notch signaling is essential during *in vivo* Th2 cell-mediated responses^{132, 134, 207} (**Chapter 3-5**), as well as during differentiation of other CD4⁺ subsets including Th1, Th9, Th17, T follicular helper (Tfh) cells and T regulatory (Treg) cells²⁰⁷. The highly conserved Notch pathway mediates cell-cell contact-dependent signaling which drives cell proliferation, apoptosis and a broad array of cell fate decisions which include T cell development and differentiation^{116, 207}.

Four Notch receptors (Notch1-4) and five Notch ligands (Delta-like ligand (DLL) 1, 3, and 4 and Jagged 1 and 2) exist in human and mouse. Notch ligand-receptor interaction between neighboring cells leads to two consecutive proteolytic cleavages of the Notch extracellular domain (NECD) and the Notch intracellular domain (NICD) by metalloproteases from the A disintegrin and metalloproteinases (ADAM) family and a γ-secretase complex, respectively^{444, 459}. The cleaved NICD travels to the nucleus and recruits co-activators including the DNA binding transcription factor recombination-signal-binding protein for immunoglobulin Jκ region (RBPJκ) and proteins of the Mastermind-like family (MAML) which induces transcription of downstream target genes^{444, 459}. The Notch ligands Delta-like ligand (DLL) and Jagged were suggested to instruct Th1 and Th2 cell differentiation, respectively⁹³. These findings were supported by studies that show an instructive role for Jagged1, but not Jagged2, in the induction of Th2 cell differentiation and function using bone marrow-derived DCs (bmDCs)²⁰⁸⁻²¹⁰. However, we recently found that mice with a DC-specific conditional deficiency of both Jagged1 and Jagged2 developed normal allergic airway inflammation (AAI) in response to *in vivo* HDM-exposure (**Chapter 3**). Therefore, we hypothesized that Notch-mediated induction of Th2 responses in HDM-driven AAI is dependent on other Jagged-expressing cell types than DCs. It was previously shown that antigenic stimulation not only induces Notch ligand expression on DCs, but also on macrophages, B cells, T cells and FRCs^{148, 205, 460, 461}. CD46 and CD3-stimulation led to an upregulation of Jagged1 on human T cells⁴⁶², suggesting that T cells can provide Notch signals to each other. Moreover, the deletion of Notch receptors on T cells or the Notch ligand DLL4 on FRCs, resulted in reduced Tfh cell numbers^{147, 148}. This is of interest since Th2-mediated inflammation also involves IL-4-producing Tfh cells^{34, 68}. Therefore, in this study, we aimed to elucidate which cell types express Jagged during AAI in mice and whether this expression is essential for Th2 cell differentiation and induction of AAI upon HDM exposure, using mice that specifically lack Jagged molecules on FRCs, on both FRC and DCs, or on CD4⁺ and CD8⁺ T cells.

Methods

Mice

Wild-type (WT) mice were purchased from Harlan (Zeist, the Netherlands). *Jag1^{fl/fl}* mice³⁸⁹, *Jag2^{fl/fl}* mice³⁹⁰ and Rosa26-stop-EYFP reporter (ROSA^{EYFP}, Jackson Laboratories, Bar Harbor, ME, USA) mice were bred with CCL19-cre⁴⁶³, CD11c-cre³⁷⁷ and CD4-cre transgenic mice³⁹² and genotyped by PCR as described³⁷⁷.^{389-392, 463} In GATIR mice, an IRES-YFP sequence was introduced into the 3' untranslated region of the *Gata3* gene which results in concomitant protein production of both Gata3 and YFP^{432, 433} (kindly provided by HJ Fehling, Ulm). All mice were on a C57BL/6 background. For experiments, both male and female mice that were 6-16 weeks old were used. All experiments involving animals were performed under specific-pathogen free conditions in the Erasmus MC animal facility and were approved by the Erasmus MC Animal Ethics Committee.

In vivo HDM-driven allergic airway inflammation

To activate *in vivo* FRC and B cells, WT mice were treated intranasally (i.n.) with 50 µg HDM (Greer, Lenoir, NC, USA) in 40 µl PBS (Invitrogen, Carlsbad, CA, USA) or with 40 µl PBS under isoflurane anesthesia 72 hrs prior to sacrifice. HDM-mediated AAI was induced as described before (**Chapter 3**). To block DLL4, mice were treated i.n. with 140 µg murine anti-DLL4 (clone HMD4-2, BioXCell, West Lebanon, NH, USA) which was combined with the HDM suspension.

T helper cell cultures

T cells were isolated from spleen and lymph nodes of OTII mice⁴³¹ using a CD4⁺ T cell MACS isolation kit (Miltenyi Biotec) and were polarized in Th1 and Th2 conditions as described⁴³⁵.

Flow cytometry procedures

Single-cell suspensions were obtained as previously described (**Chapter 3**). For flow cytometric analysis or sorting of lymph node stromal cell subsets, lymph nodes were dissected as described⁴⁶⁴. To obtain sufficient numbers of FRCs, for each sample mediastinal lymph nodes (MedLN) from six mice were pooled. Prior to sorting of FRCs, MedLN cell suspensions were enriched for stromal cells by depleting leukocytes using streptavidin-labeled Dynabeads (Invitrogen Dynal AS, Oslo, Norway) after lymph node cell suspensions were stained with biotin-labeled antibodies for CD45, CD3 and CD19 (**Supplementary table 1**).

Cells 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. After sorting, cells were collected in RNA lysis (Qiagen, Venlo, the Netherlands) buffer containing 2-mercaptoethanol.

For flow cytometric analysis, single-cell suspensions were stained with a mixture of fluorochrome labeled antibodies in FACS buffer as described previously (**Chapter 3**). A list of all fluorochrome labeled antibodies that were used can be found in **Supplementary table 1**.

RNA extraction and quantitative real-time PCR

RNA was extracted using RNeasy Micro Kit (Qiagen) according to manufactures' protocol. RNA from B cells and cultured T helper cells (methods for T helper cultures are described in **chapter 5**) 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* FRC 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). Primers sequences for Notch ligands are previously described (**Chapter 3**). qRT-PCR reactions were performed in an Applied Biosystems Prism 7300 Sequence Detection System (Applied Biosystems). Gata3⁺ CD4⁺ T cells were sorted and RNA sequencing was performed as described in **chapter 5**.

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

Upon HDM exposure Jagged1 is upregulated on FRCs but not on B cells

We have recently shown that although DCs upregulate Jagged expression after HDM exposure, neither Jagged1 nor Jagged2 expression on DCs was relevant during the induction of HDM-mediated eosinophilic airway inflammation (**Chapter 3**). Therefore, we wondered which other cells in the lymph nodes would express Notch ligands. First, we investigated FRCs, since these cells are required for the attraction of T cells and DCs to the draining lymph nodes and are present in the paracortex where T cell activation is initiated⁴⁶⁵. We sorted gp38 (podoplanin)⁺CD31⁺ FRCs from MedLN from WT mice that were i.n. treated with HDM or PBS for 72 hrs (**Figure 1A**). In FRCs, both Jagged1 and DLL4 were expressed at baseline (PBS) and were upregulated upon exposure to HDM, whereas DLL1 was only detectable upon HDM stimulation (**Figure 1B**).

Because B cells can activate T cells via antigen presentation and cytokine secretion⁴⁶⁶, we measured Notch ligand expression in resting (CD86⁻) and activated (CD86⁺) B cells, sorted from MedLN from WT mice that were i.n. treated with HDM or PBS for 72 hrs (**Figure 1C**). In contrast to DCs and FRC, Notch ligand expression on B cells is very low and apparently unaltered upon activation of these cells by HDM exposure (**Figure 1D**).

In summary, i.n. HDM exposure resulted in upregulation of Notch ligand expression on FRC in MedLN, but not on B cells. Therefore, we hypothesized that Jagged expression on FRC is involved in the induction of Th2 cells in AAI.

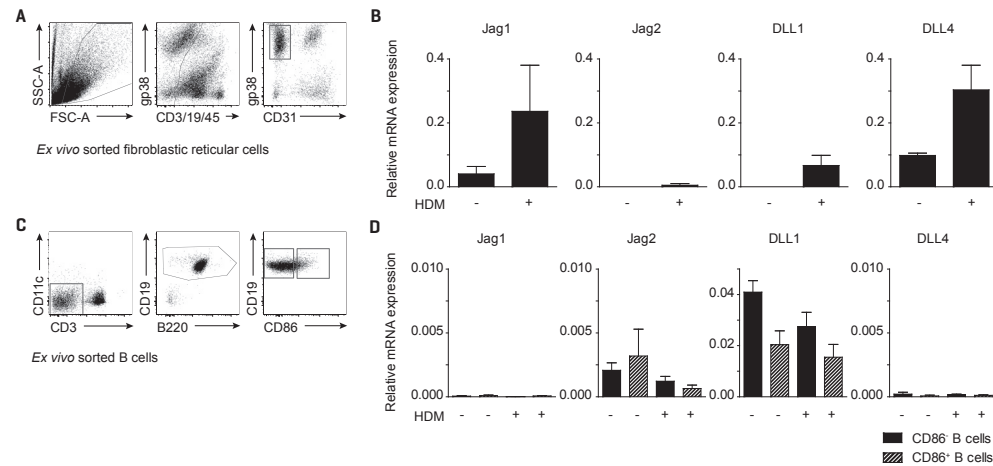


Figure 1. Upon HDM exposure, Jagged1 is upregulated on fibroblastic reticular cells but not on B cells

[A] Flow cytometric gating strategy of ex vivo sorted fibroblastic reticular cells (FRCs) from C57BL/6 mice that were i.n. treated with 50 µg HDM or PBS. Large live cells, negative for CD3, CD19 and CD45, were analyzed for gp38 and CD31 and gated as indicated.

[B] mRNA expression of the indicated Notch ligands, quantified by qRT-PCR, in FRCs from MedLN after 72 hrs of *in vivo* stimulation with house dust mite (HDM). 6 mice were pooled per sample. Data are shown as mean + SEM of 2-3 samples per group.

[C] Gating strategy of ex vivo sorted B cells from C57BL/6 mice that were i.n. treated with 50 µg HDM or PBS.

[D] mRNA expression of the indicated Notch ligands, quantified by qRT-PCR, in DAPI⁺CD3⁺CD11c⁺CD19⁺CD86⁺ B cells and CD86⁺ [activated] B cells from MedLN after 72 hrs of *in vivo* stimulation with HDM. Data are shown as mean + SEM of 3-4 samples per group.

Jagged1 and Jagged2 expression on FRCs is dispensable for the development of AAI *in vivo*

To be able to explore whether expression of Jagged ligands on FRC is essential for AAI induction, we employed CCL19-cre transgenic mice, expressing Cre recombinase under the control of the FRC-specific CCL19 promoter⁴⁶³. First, we crossed the CCL19-cre mice with ROSA^{EYFP} mice, enabling the identification of cells with Cre recombinase activity by EYFP expression due to cre-mediated excision of a loxP-flanked transcriptional STOP sequence⁴⁶³. We sensitized and challenged WT x ROSA^{EYFP} and CCL19-cre x ROSA^{EYFP} mice with HDM to induce AAI and mice were analyzed four days after the last challenge (Figure 2A). Following HDM exposure both WT x ROSA^{EYFP} and CCL19-cre x ROSA^{EYFP} mice developed similar AAI inflammation, which was characterized by increased eosinophils, other inflammatory cells and type 2 cytokines in broncho alveolar lavage (BAL) fluid, compared to PBS-sensitized mice (Figure 2B and data not shown). Various stromal cell populations from MedLN, including gp38⁺CD31⁺ FRC, gp38⁺CD31⁺ lymphatic endothelial cells (LEC) and gp38⁺CD31⁺ blood endothelial cells (BEC), as well as CD45⁺ leukocytes were analyzed for EYFP expression by flow cytometry. This analysis revealed that the majority of the FRCs (~63-73%) was EYFP⁺, indicative for Cre-mediated deletion in these cells (Figure 2C). Moreover, we could confirm that CCL19-cre is FRC-specific, as >90% of the EYFP expression was expressed by FRCs, in agreement with previous findings showing similar results in CCL19-cre x ROSA^{EYFP} mice⁴⁶³. These findings showed that (1) CCL19-cre transgenic mice developed AAI similar to non-transgenic mice and (2) that the CCL19-cre transgene is effective specifically in FRCs.

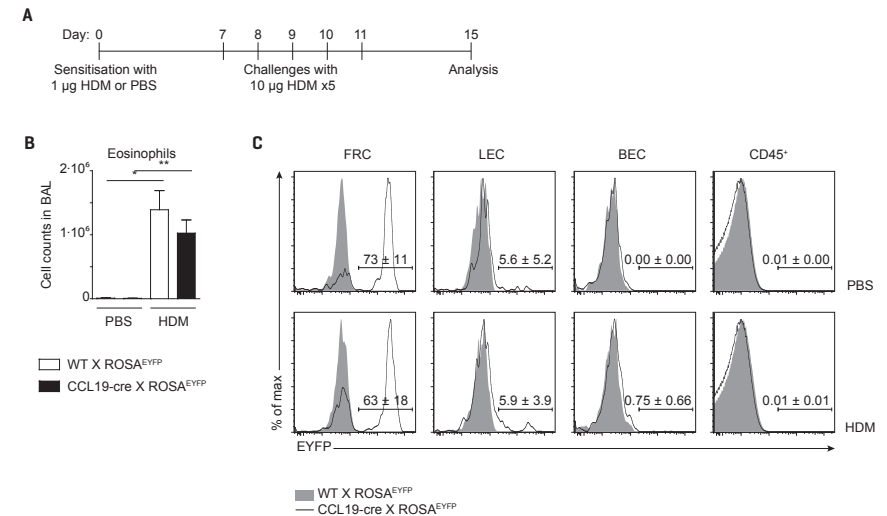


Figure 2. CCL19-cre mice develop normal AAI upon HDM exposure and CCL19-cre is effective specifically in FRCs

[A] Scheme of HDM-mediated AAI induction in mice.

[B] Total numbers of eosinophils in BAL from PBS- or HDM-sensitized HDM-challenged WT x ROSA^{EYFP} or CCL19-cre x ROSA^{EYFP} mice.

[C] EYFP expression in MedLN from PBS- or HDM-treated WT x ROSA^{EYFP} and CCL19-cre x ROSA^{EYFP} mice. Data are shown as histogram overlays of EYFP expression in the indicated mice. Samples were concatenated, data are shown as mean + SD of six mice (HDM treated) or 4 mice (PBS treated) per group, in one experiment.

Next, we created an FRC-specific Jag1/Jag2 conditional knockout mouse, by crossing *Jag1^{fl/fl}* and *Jag2^{fl/fl}* mice, in which the Jag loci contain loxP sites, with CCL19-cre transgenic mice. When we analyzed HDM-mediated AAI induction in CCL19-cre transgenic *Jag1^{fl/fl}Jag2^{fl/fl}* mice (*Jg1Jg2^{ΔCCL19/ΔCCL19}*) and *Jg1Jg2^{+/+}* control mice, we found that *Jg1Jg2^{ΔCCL19/ΔCCL19}* mice and *Jg1Jg2^{+/+}* mice developed a similar AAI phenotype. We did not detect differences in the numbers of alveolar macrophages, eosinophils, neutrophils, B cells and T cells in BAL of the two mouse groups (Figure 3A). Moreover, by intracellular flow cytometry we measured similar increases in percentages and absolute counts of IL-4, IL-5, IL-13, IL-9, IFN-γ and IL-17A expressing CD4⁺ T cells in *Jg1Jg2^{ΔCCL19/ΔCCL19}* and *Jg1Jg2^{+/+}* mice that were exposed to HDM (Figure 3B and 3C). In addition, the numbers of Gata3⁺, Rorγt⁺ and Foxp3⁺CD25⁺ CD4⁺ T cells in BAL fluid were also not different between the two groups (Figure 3D). In these analyses, T-bet⁺ CD4⁺ T cells were not detected (not shown). Likewise, when analyzing Jag single gene conditional knockouts, we found that both *Jg1^{ΔCCL19/ΔCCL19}* and *Jg2^{ΔCCL19/ΔCCL19}* mice developed similar AAI upon HDM exposure, when compared with WT littermates (data not shown).

In conclusion, we found no evidence for a critical role for Jagged1 or Jagged2 expression on FRCs in our HDM-driven asthma model.

Mice with a conditional deletion of Jagged1 and Jagged2 in both FRCs and DCs developed AAI similar to WT mice

Both DCs and FRCs can express high and similar levels of Notch ligands, which are both upregulated upon HDM exposure (Figure 1A and 3; and Chapter 3). We previously found that Jagged1 and Jagged2 on DC are not required for HDM-induced AAI (Chapter 3). However, it cannot be excluded that there was redundancy with other Jagged-expressing cells. Since DC-specific and FRC-specific Jagged knockout mice still develop AAI, we questioned whether these mice still had the capacity to develop HDM-driven AAI because of redundancy between Jagged-expressing DCs and FRCs.

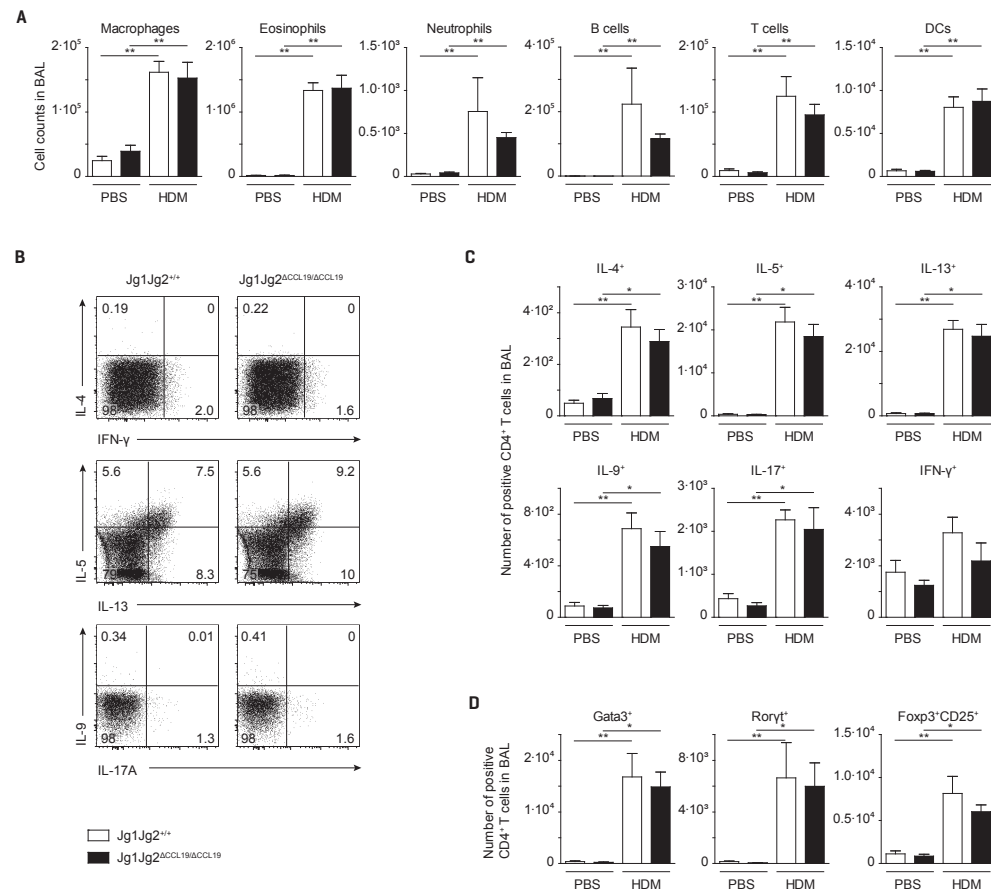


Figure 3. Jagged1 and Jagged2 expression on FRCs is dispensable for the development of AAI in vivo

(A) Numbers of macrophages [FSC^{high}SSC^{high}autofluorescent⁺CD11c⁺Siglec-F⁺], eosinophils [FSC^{high}SSC^{high}Siglec-F⁺], neutrophils [Ly-6G⁺], B cells [CD19⁺], T cells [CD3⁺], DCs [CD11c⁺MHCII^{hi}] in BAL in Jg1Jg2^{ΔCCL19/ΔCCL19} or Jg1Jg2^{+/+} mice exposed to HDM-mediated AAI induction (Figure 2A). (B) Intracellular flow cytometric analysis of cytokine production by CD3⁺CD4⁺ T cells in BAL from the indicated mice and (C) numbers of IL-4⁺, IL-5⁺, IL-13⁺, IL-9⁺, IL-17A⁺ and IFN-γ⁺ CD3⁺CD4⁺ T cells in BAL in mice treated with either PBS or HDM. (D) Total numbers of Gata3⁺, Rorγt⁺ and Foxp3⁺CD25⁺ CD3⁺CD4⁺ T cells in BAL from Jg1Jg2^{ΔCCL19/ΔCCL19} or Jg1Jg2^{+/+} mice treated PBS or HDM, as indicated.

Data are shown as mean + SEM of 5-7 mice per group and are representative of two independent experiments. * p < 0.05, ** < 0.01, using Mann-Whitney U-test.

Therefore, we crossed Jg1Jg2^{ΔCCL19/ΔCCL19} mice with Jg1Jg2^{ΔCD11c/ΔCD11c} mice to obtain mice that lacked both Jagged1 and/or Jagged2 on both FRC and DCs (termed Jg1Jg2^{ΔCCL19ΔCD11c/ΔCCL19ΔCD11c} mice). We studied HDM-mediated AAI induction in both Jag1 single knockout mice (Jg1^{ΔCCL19ΔCD11c/ΔCCL19ΔCD11c}) and Jag1/Jag2 double knockout mice (Jg1Jg2^{ΔCCL19ΔCD11c/ΔCCL19ΔCD11c}), which we compared with PBS- and HDM-treated Jg1/Jg2^{+/+} mice. In this comparison, we found that upon HDM exposure all three genotype groups displayed similar increases in inflammatory cell numbers (Figure 4A), as well as similar increases in CD4⁺ T cells that were positive for IL-4, IL-5, IL-13, IL-17A or IFN-γ by intracellular flow cytometry in BAL (Figure 4B). Finally, the numbers and percentages of Gata3⁺, Rorγt⁺ and Foxp3⁺CD25⁺ CD4⁺ T cells were not different between the three groups, indicating that induction of Th2 cells, Th17 cells and Tregs, respectively, was independent of the expression of Jagged1 or Jagged 2 on FRC and DCs (Figure 4C and 4D).

In conclusion, this shows that expression of Jagged1 and Jagged2 on both DCs and FRC stromal cells is dispensable for the induction of HDM-mediated AAI in vivo.

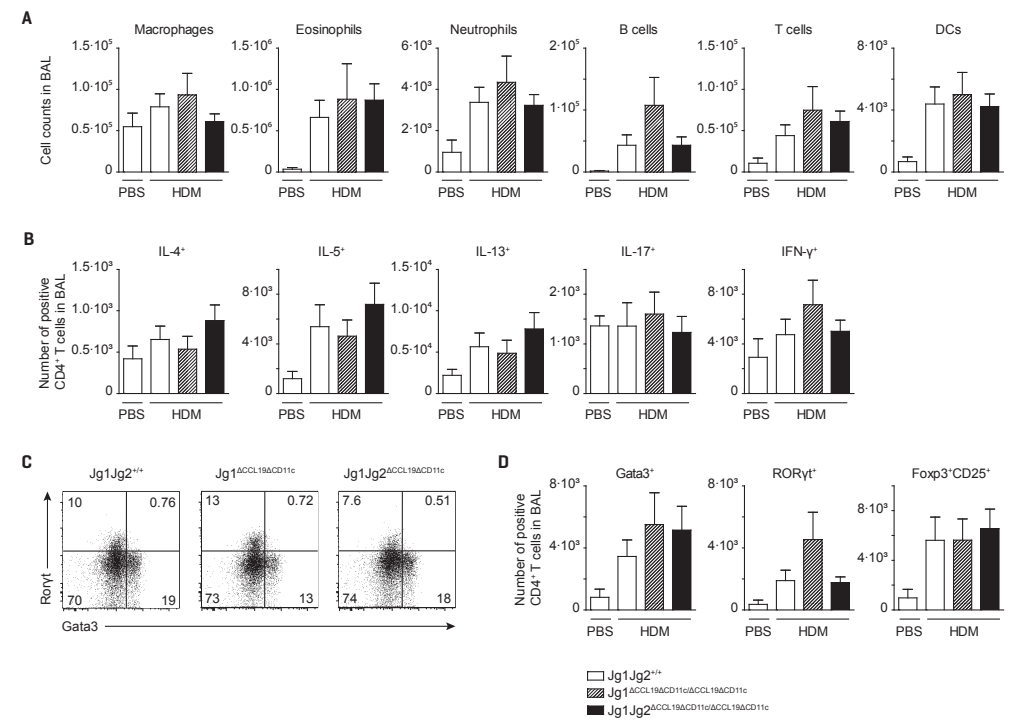


Figure 4. Mice with a conditional deletion of both Jagged1 and Jagged2 on both FRCs and dendritic cells development AAI which is similar to WT mice

(A) Total numbers of the indicated cell populations in BAL from Jg1/Jg2^{+/+}, Jg1^{ΔCCL19ΔCD11c/ΔCCL19ΔCD11c} or Jg1Jg2^{ΔCCL19ΔCD11c/ΔCCL19ΔCD11c} mice exposed to HDM-mediated AAI induction (Figure 2A). (B) Quantification of the total numbers of cytokine-positive CD3⁺CD4⁺ T cells in BAL by intracellular flow-cytometry. (C) Flow cytometry profile of transcription factor expression in BAL CD3⁺CD4⁺ T cells in mice treated with HDM. (D) Quantification of Gata3⁺, Rorγt⁺ and Foxp3⁺CD25⁺ CD4⁺ T cells in BAL.

Data are shown as mean + SEM of 4-11 mice per group and are representative of two independent experiments. Significance was calculated using Mann-Whitney U-test.

Expression of Jagged1 and Jagged2 on T cells is not required for the development of AAI *in vivo*

It was previously shown that TCR stimulation induces Notch ligand expression on CD4⁺ and CD8⁺ T cells^{461, 462}. Since we found that (i) expression of Jagged1 and Jagged2 on both DCs and FRC stromal cells is dispensable for the induction of HDM-mediated AAI *in vivo* and (ii) Notch signaling is specifically required during the challenge phase of HDM-mediated AAI (Chapter 4 and 5), we hypothesized that CD4⁺ T cells may provide Notch signals to each other. Therefore, we measured mRNA expression of Notch ligands on T cells that were *in vitro* polarized in Th1 and Th2 conditions using stimulating antibodies for CD3 and CD28 and polarizing cytokines⁴³⁵ (Figure 5A). We found that Jagged1 is specifically expressed on Th2 cells but not on Th1 cells, although the expression levels of Jagged1, DLL1 and DLL4 were extremely low. Jagged2 expression was higher compared to other Notch ligands and was expressed on both Th1 and Th2 cells. We found similar results when we performed genome-wide RNA-seq on sorted fractions of Gata3⁺CD62L⁺ naïve T cells and Gata3⁺ Th2 cells that we isolated from HDM-treated GATIR mice. Expression of all Notch ligands was lower than 1 Read Per Kilobase Million (RPKM). Jagged2 expression was higher compared to other Notch ligands and was highest on naïve T cells (Figure 5B).

To investigate whether this low-level expression of Jagged on T cells is required for HDM-mediated AAI, we created T cell-specific Jag1 and Jag2 conditional knockout mice, by crossing Jag1^{fl/fl} and Jag2^{fl/fl} mice, with CD4-cre transgenic mice. However, we found that mice with a T cell-specific deletion of Jag1, Jag2 or both (Jag1^{ΔCD4/ΔCD4}, Jag2^{ΔCD4/ΔCD4} and Jag1Jag2^{ΔCD4/ΔCD4} mice, respectively) and Jag1/Jag2^{+/+} control mice developed a similar HDM-induced AAI phenotype, compared to PBS-sensitized control mice. In particular, we did not detect significant differences in the numbers of alveolar macrophages, eosinophils, neutrophils, B cells and T cells in BAL between the four

HDM-exposed mouse groups (Figure 6A). Additionally, we measured slightly higher percentages and absolute counts of IL-4, IL-5, IL-13, IL-9, IFN-γ and IL-17A expressing CD4⁺ T cells in Jag1^{ΔCD4/ΔCD4}, Jag2^{ΔCD4/ΔCD4} and Jag1Jag2^{ΔCD4/ΔCD4} compared to Jag1Jag2^{+/+} mice (Figure 6B and 6C). Also, percentages and numbers of Gata3⁺, Rorγt⁺ and Foxp3⁺CD25⁺ CD4⁺ T cells in BAL were not different between the four groups (Figure 6D and 6E).

In conclusion, expression of Notch ligands on CD4⁺ T cells was low and not required for the induction of AAI.

Intranasal blockade of DLL4 did not alter AAI in WT mice nor in conditional Jagged deficient mice

Although the role of DLL in the induction of AAI remains controversial^{212, 216-218, 406}, our findings that we could not detect a critical role for Jagged ligands on multiple cell types suggests that there may be redundancy with DLL in the absence of Jagged ligands. To investigate this possibility, we exposed Jag1^{ΔCCL19ΔCD11c/ΔCCL19ΔCD11c}, Jag1Jag2^{ΔCCL19ΔCD11c/ΔCCL19ΔCD11c} and Jag1/Jag2^{+/+} mice *i.n.* to HDM in the presence of anti-DLL4 antibodies, both during the sensitization and during the challenge phase. However, in all three genotypes that were *i.n.* treated with anti-DLL4 during the HDM exposures, we found similar numbers of eosinophils and CD4⁺ T cells in BAL fluid, compared with mice that did not receive anti-DLL4 antibodies (Figure 7A). In addition, all mice had similar numbers of Gata3⁺, Rorγt⁺ and Foxp3⁺CD25⁺ CD4⁺ T cells in BAL (Figure 7B), as well as similar increases in CD4⁺ T cells that were positive for IL-4, IL-5, IL-13, IL-17A or IFN-γ, as determined by intracellular flow cytometry (Figure 7C). To confirm specificity of the anti-DLL4 antibodies employed, cytospin slides with OP9 stromal cells expressing DLL1 and DLL4⁴⁶⁷ were stained using the same antibody. Anti-DLL4 antibodies did not bind to OP9-DLL1, but bound specifically to OP9-DLL4 cells, indicating that anti-DLL4 was bioactive and specific for DLL4.

In summary, although we found high levels of Notch ligands on DCs and FRCs and low levels on T cells, Jagged expression on DCs, FRC and T cells was not required to induce Th2 cell-mediated AAI induced by HDM exposure. In addition, *i.n.* treatment with DLL4-specific antibodies did not affect Th2 inflammation and eosinophilia.

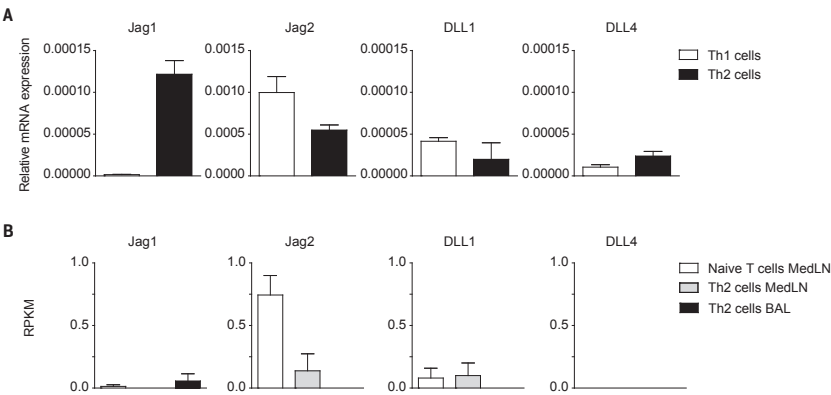


Figure 5. Notch ligand expression on naïve T cells, Th1 cells and Th2 cells
[A] mRNA expression of the indicated Notch ligands, quantified by qRT-PCR, in *in vitro* polarized Th1 and Th2 cells. Data are shown as mean + SEM of 2 mice.
[B] Comparison of Reads Per Kilobase Million [RPKM] values of the selected genes measured using genome-wide RNA sequencing in sorted Naïve T cells and Th2 cells [Gata3⁺] from MedLN and BAL from GATIR mice that were exposed to the HDM-driven protocol to induce AAI. Data are shown as mean + SEM of 3 pools of 2-3 mice.

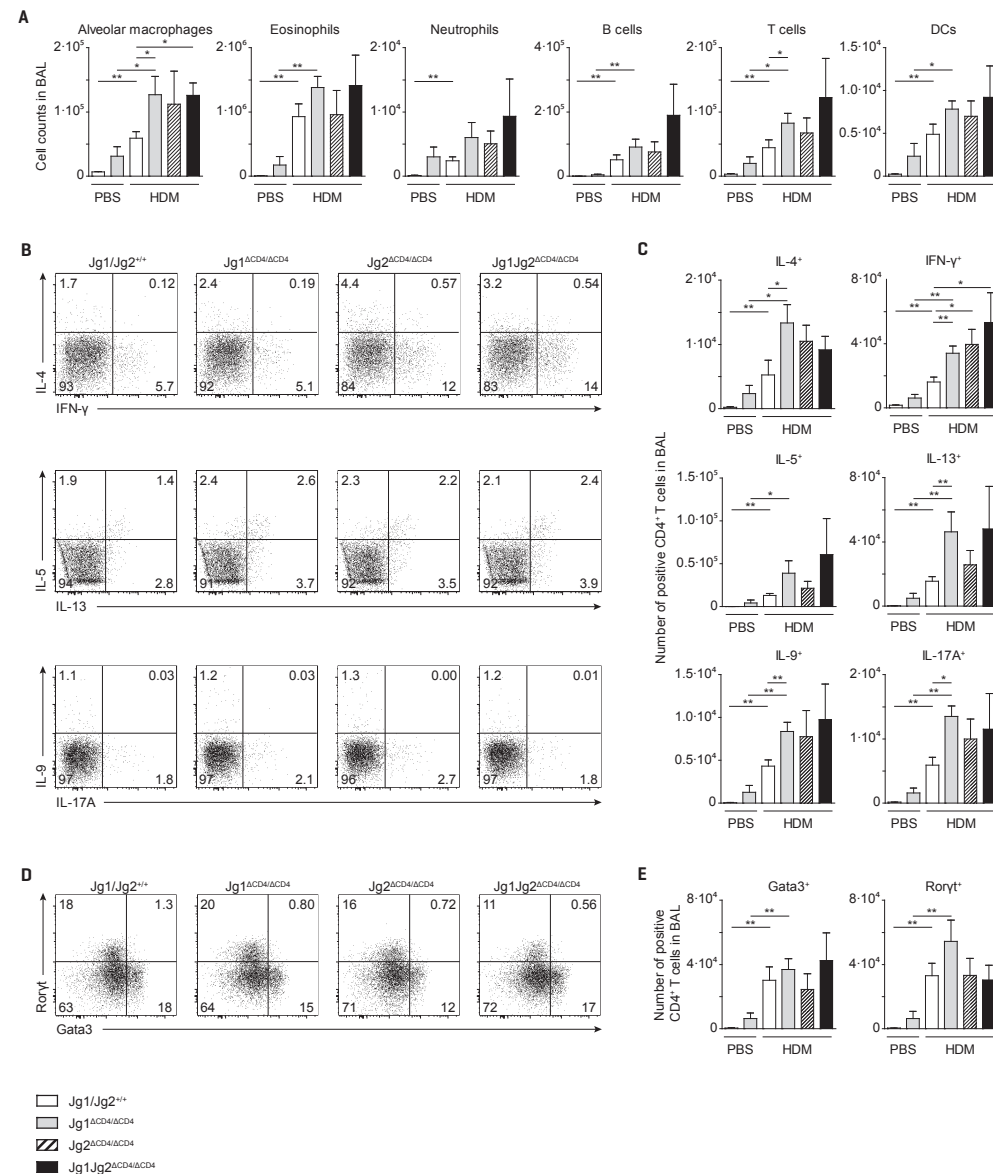


Figure 6. Expression of Jagged1 and Jagged2 on T cells is not required for the development of AAI *in vivo*

(A) Total numbers of the indicated cell populations in BAL from *Jg1/Jg2*^{+/+}, *Jg1*^{ΔCD4/ΔCD4}, *Jg2*^{ΔCD4/ΔCD4} or *Jg1Jg2*^{ΔCD4/ΔCD4} mice exposed to HDM-mediated AAI induction (Figure 2A). (B) Intracellular flow cytometric analysis of cytokine production by CD3⁺CD4⁺ T cells in BAL from the indicated HDM treated mice and (C) quantification of the total numbers of cytokine-positive CD3⁺CD4⁺ T cells in BAL in mice sensitized with either PBS or HDM. (D) Flow cytometry profile of transcription factor expression in BAL CD3⁺CD4⁺ T cells in mice treated with HDM and (E) Quantification of Gata3⁺ and Rorγt⁺ CD4⁺ T cells in BAL. Data are shown as mean + SEM of 4–7 mice per group and are representative of two independent experiments. Significance was calculated using Mann-Whitney U-test.

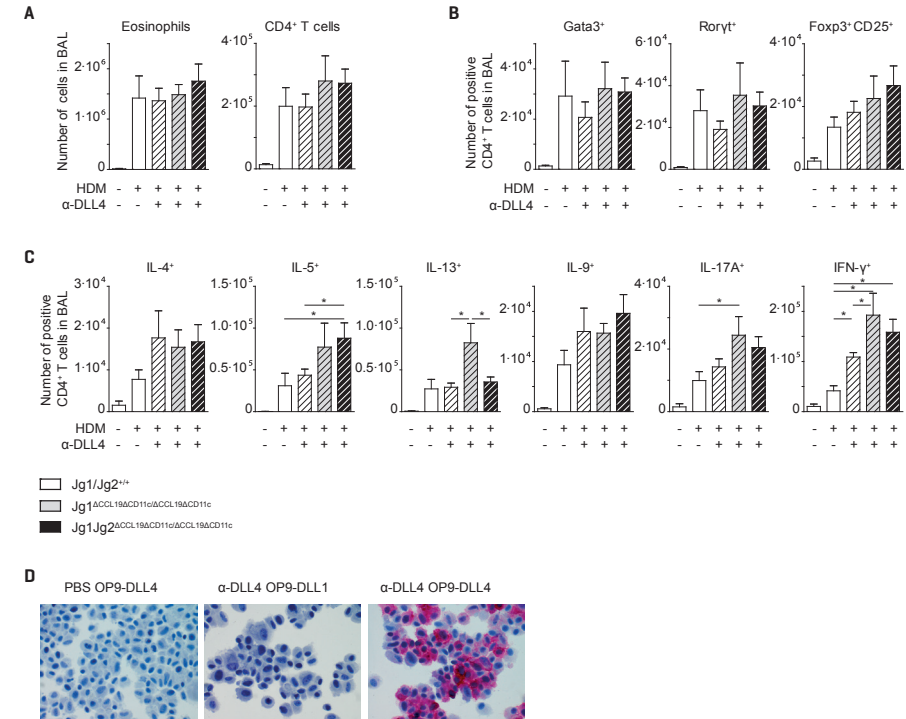


Figure 7. Intranasal blockade of DLL4 did not alter AAI in WT mice and in conditional Jagged deficient mice

(A) Total numbers of eosinophils and CD4⁺ T cells in BAL from *Jg1/Jg2*^{+/+}, *Jg1*^{ΔCCL19/ΔCCL19/ΔCCL19/ΔCCL19} or *Jg1Jg2*^{ΔCCL19/ΔCCL19/ΔCCL19/ΔCCL19} mice that were exposed to HDM-mediated AAI induction and treated with DLL4-blocking antibodies (Figure 2A). (B) Quantification of Gata3⁺, Rorγt⁺ and Foxp3⁺CD25⁺ CD4⁺ T cells in BAL quantified by intracellular flowcytometric staining. (C) Quantification of the total numbers of cytokine-positive CD3⁺CD4⁺ T cells in BAL using intracellular flowcytometry. (D) Immunohistochemical staining of DLL4 on cytopins from OP9-DLL1 and OP9-DLL4 cell lines. Data are shown as mean + SEM of 3–5 mice per group and are representative of two independent experiments. Significance was calculated using Mann-Whitney U-test.

Discussion

The Notch ligands DLL and Jagged were suggested to endow differentiation of Th1 and Th2 cell differentiation, respectively⁹³. Indeed, we and others were able to demonstrate that Notch signaling is essential to establish *in vivo* Th2 cell-mediated responses^{332, 334, 207} (Chapter 3-5). However, we recently showed that Jagged expression on DCs is not required for the induction of HDM-mediated allergic type 2 inflammation in the airways (Chapter 3). Therefore, we hypothesized that either Jagged on other cells would be required for Th2 cell mediated inflammation, or alternatively other Notch ligands such as DLL1 and DLL4 would have the capacity to support Th2 cell-mediated responses. Here, we demonstrate that although Notch ligand expression is induced in FRCs upon HDM stimulation, neither Jagged1 nor Jagged2 expressed on CCL19⁺ FRC is required for the induction of AAI. We did not exclude a role for Jagged expression on B cells and found that Notch ligand expression on B cells was low. Together with the previous observation that mice lacking B cells develop normal HDM-driven eosinophilic airway

inflammation⁴³⁴, we conclude that Notch ligand expression on B cells is not essential for the induction of Th2-mediated HDM-driven AAI. Although not all FRCs (~63-73%) showed CCL19-Cre mediated deletion of floxed genes, others have shown that this deletion is sufficient when investigating the function of DLL4 in the differentiation of Tfh cells¹⁴⁸. Since alveolar macrophages express CD11c, we also excluded a role for Jagged expression on alveolar macrophages by using CD11c-Cre mediated deletion of Jag1 and Jag2 (**Chapter 3**). Furthermore, expression of Notch ligands on CD4⁺ T cells was low and dispensable 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, i.n. blockade of DLL4 did not affect eosinophilia or Th2 cell-driven airway inflammation.

The question remains what Notch ligand on what cell type is required for the induction of a Th2 response in HDM-mediated AAI. We and others have shown that Notch signaling is especially required during the challenge phase of AAI^{132, 133} (**Chapter 4 and 5**). Therefore many inflammatory cell types could give Notch signals to T cells, including ILC2s, infiltrating granulocytes, monocytes and lung structural cells. We found that ILC2s sorted from mice that were exposed to a chronic HDM-mediated asthma protocol expressed Jag1, but not Jag2^{432, 433}. Expression of Jagged1 on ILC2 could be relevant since these cells can express MHC class II as well as a range of co-stimulatory molecules and were therefore suggested to interact with T cells³⁵⁵. In contrast to the notion that ILC2s are an early T-cell independent source of IL-5 and IL-13³⁵⁵, we showed that in HDM-driven AAI T cells are required for ILC2 activation³⁹⁴, which argues against a model where ILC2 provide necessary Notch signals to T cells. Granulocytic inflammation is an important hallmark of asthma. It is generally thought that granulocytes are attracted to the side of inflammation by cytokines produced by T cells but it could be worthwhile to investigate whether granulocytes express Notch ligands that could stimulate T cells. Monocytes can differentiate into macrophages and inflammatory DCs and changes in circulating monocytes in some asthma patients were found 24 hours after allergen challenge⁴⁶⁸. It is unclear whether monocytes express Notch ligands that could already induce Notch signaling in T cells before these monocytes differentiate and start to express CD11c. Jagged1 is crucial for the differentiation of alveolar epithelial cells⁴⁶⁹, but it is currently unclear what the role is of Jagged1 expression by lung epithelial cells during immune responses. Future studies should address which cell types express Jagged and whether Jagged expression is relevant during Th2 cell-mediated airway inflammation.

Given that Notch signaling is important for the differentiation of multiple T helper cell subsets, the bipotential instructional model could not be sufficient to clarify the function of Notch signaling in Th cell differentiation. Bailis *et al.* showed that Notch signaling could simultaneously induce differentiation of Th1, Th2 and Th17 cell programs¹³⁵ and hypothesized that Notch signaling acts as a general amplifier of Th cell responses rather than an direct instructor of specific T helper cell programs. Interestingly, Notch signaling was shown to promote antigen sensitivity of CD4⁺ T cells via promoting co-stimulatory signals in T cells and Notch is essential for T cell expansion, CD25 and IL-2 induction and promotes survival by increasing anti-apoptotic signals and glucose uptake²⁰⁷. The hypothesis that Notch act as an unbiased amplifier in addition also suggest that there is an overlap between the role of individual Notch ligands in the induction of T helper cell responses and it is therefore conceivable that DLL has a critical role in the induction of Th2 responses. In contrast, several studies showed a regulatory role for DLL4 during Th2 responses^{212, 216-218, 470}. However, decreased Th2

responses were found when DLL4 was blocked in a mouse model for RSV-mediated allergic asthma exacerbations⁴⁰⁶. Therefore, it is currently unclear what the role is of DLL4 during Th2 cell responses and whether Notch acts as a bipotential instructor or as an unbiased amplifier during T helper cell differentiation. In this context, it is interesting to mention that depletion of Notch receptors on T cells or DLL4 on FRC, resulted in a reduction of Tfh cells^{147, 148}. The findings that that Th2 inflammation also crucially involves IL-4-producing Tfh cells^{34, 68}, may point at a role for DLL4 in the lymph nodes, particularly in FRCs, during Th2 cell inflammation. Although in our hands, we observed no effects of i.n. treatment with DLL4-specific antibodies on HDM-driven AAI, it cannot formally be excluded that the blocking capacity of the antibodies that we used was not sufficient *in vivo*. Therefore, future studies should address the role of DLL4 in the lymph nodes, e.g. by specifically removing DLL4 from lymph node FRCs using a CCL19-cre or with intraperitoneal or intravenous treatment with (various concentrations of) DLL4-blocking antibodies in a HDM-mediated model for AAI.

Alternatively, several studies have implicated a ligand-independent pathway of Notch signaling⁴⁷¹. Spontaneous Notch cleavage was found upon TCR triggering^{107, 110, 114, 472}. Therefore, studies using Notch receptors incapable of binding ligands could provide much information regarding the mechanisms of Notch activation during Th2 cell inflammation.

Since a subgroup of patients do not achieve disease control with the standard treatment with inhaled corticosteroids, β_2 -adrenergic receptor agonists and oral leukotriene inhibitors^{1, 2}, it is essential to establish additional therapeutic approaches to treat asthma patients. The prominent role of Notch signaling in Th2 cell differentiation suggests that the Notch signaling pathway could serve as a potential therapeutic target in asthma patients. Most studies use γ -secretase inhibitors (GSI) to block Notch signaling. Unfortunately, effects of GSI are not limited to Notch signaling and potentially cause side-effects^{398, 234}. Specific antibodies targeting Notch receptors²⁰²⁻²⁰⁴ or Notch ligands^{205, 206} have been designed. However, in this context knowledge about which Notch ligands on what cell types are exactly required to induce Th2-mediated allergic asthma is crucial. Preferably, studies addressing the role of Notch ligands on different cell types should use cell-specific knockouts for different Notch ligands.

Acknowledgements

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Supplementary tables

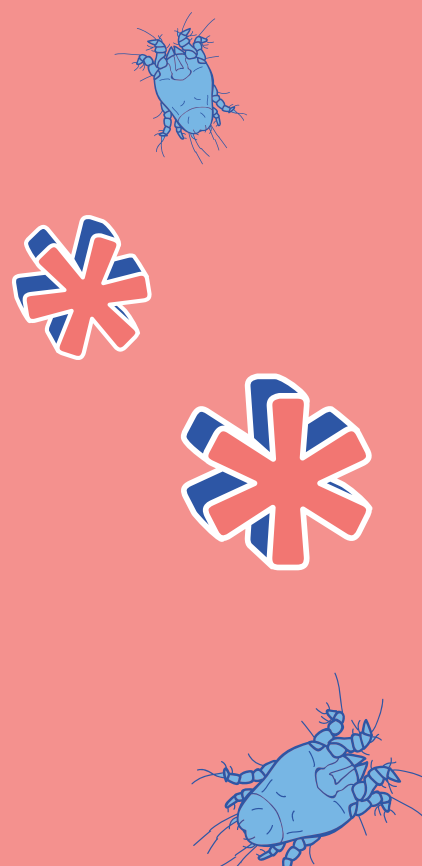
Supplementary Table 1. Antibodies used for flow cytometry

Target	Conjugate	Company	Clone
B220	PE	eBioscience	RA3-6B2
CD3	APC-ef780	eBioscience	17A2
CD3	Biotin	BD biosciences	145-2C11
CD3	FITC	BD biosciences	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
CD8a	FITC	eBioscience	Ly-2
CD11c	APC-ef780	eBioscience	N418
CD11c	BV786	BD biosciences	HL3
CD19	APC-ef780	eBioscience	1D3
CD19	Biotin	BD biosciences	1D3
CD19	PerCP-Cy5.5	eBioscience	eBio1D3
CD21/35	APC	eBioscience	7E9
CD25	PerCP-Cy5.5	eBioscience	PC61.5
CD31	PE-Cy7	eBioscience	390
CD31	FITC	eBioscience	390
CD45	Biotin	eBioscience	30-F11
CD45	BV711	BD biosciences	30-F11
CD45	PerCP-Cy5.5	eBioscience	30-F11
CD86	PE-Cy7	BD biosciences	GL1
Foxp3	PE-Cy7	eBioscience	FJK-16s
Gata3	ef660	eBioscience	TWAJ-14
IFN-γ	e450	eBioscience	XMG1.2
IFN-γ	PE-Cy7	eBioscience	XMG1.2
IL-4	BV711	BD biosciences	11B11
IL-5	Biotin	BD biosciences	TRFK4
IL-9	PE	BD biosciences	D9302C12
IL-13	ef660	eBioscience	eBio13A
IL-17A	AF700	BD biosciences	TC11-18H10.1
Live/Dead	Amcyan	Invitrogen	
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
Podoplanin	PE	Biolegend	eBio8.1.1
Roryt	PE	BD biosciences	Q31-378
Siglec-F	PE	BD biosciences	E50-2440
Streptavidin	APC-ef780	eBioscience	

Streptavidin	PerCP-Cy5.5	eBioscience	
T-bet	BV421	BD biosciences	Q4-46



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Chapter 7

Evidence for enhanced Notch signaling in Th2 cells in peripheral blood from asthma patients

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Abstract

Background: Asthma is a prevalent and heterogeneous inflammatory condition of the conducting airways. Many asthma patients display an exaggerated T helper type 2 (Th2) cell-mediated inflammation. Since Notch signaling in T cells is required for the induction of asthma in mice, we extensively characterized CD4⁺ T cell populations and Notch signaling in these cells in peripheral blood from asthma patients.

Objective: To identify quantitative differences in circulating CD4⁺ T cell subsets and surface expression of NOTCH1 and NOTCH2 on CD4⁺ T cell subsets in peripheral blood from healthy individuals and asthma patients.

Methods: We used multi-color flow cytometry to quantify Th1, Th2 and Th17 subsets in peripheral blood from patients with controlled (n=17), partially controlled (n=17) and uncontrolled (n=25) asthma, treated with inhaled steroids, as well as healthy individuals (n=28). In a selected group of healthy individuals, controlled and uncontrolled patients (n=8 in each group), we determined gene expression profiles of sorted peripheral blood Th2 cells by RNA-sequencing. We correlated our flow cytometry and RNA-sequencing profiles to clinical and immunological parameters.

Results: The frequency of total CD4⁺ T cells was increased in peripheral blood from asthma patients and correlated with disease severity, but the CD4⁺ T cell subset distribution was unaltered. Interestingly, we found higher percentages of surface NOTCH1 and NOTCH2 receptor positivity on memory T cells and Th2 cells from partially-controlled and uncontrolled asthma patients, compared to healthy controls. RNA sequencing of purified Th2 cells from controlled and uncontrolled asthma patients and healthy controls revealed 132 differentially expressed genes. In addition, pathway analysis showed that genes involved in lymphocyte activation were increased in Th2 cells from asthma patients.

Conclusion: Taken together, our findings provide evidence for enhanced Notch signaling and a unique expression signature of circulating Th2 cells in asthma patients.

Introduction

Asthma is the most prevalent chronic inflammatory lung disease in humans and comprises a range of disease subtypes that share similar clinical characteristics including episodes of bronchoconstriction, airway hyperreactivity, mucus hyperproduction and airway inflammation^{473, 474}. While many asthma patients respond well to standard treatment with glucocorticoids and β_2 -agonists^{1, 2}, a fraction of patients does not achieve disease control. The underlying mechanisms driving the disease in uncontrolled asthma patients requires further investigation, as these patients display the highest morbidity and symptom burden⁴⁷⁵. The most common type of asthma is allergic asthma, which often presents as eosinophilic inflammation³⁻⁵. The major immunological hallmark of allergic asthma is T cell-mediated inflammation. Most patients with allergic asthma manifest Th2 cell-mediated inflammation^{476, 477}. T helper 2 (Th2) cells typically produce the cytokines IL-4, IL-5 and IL-13, which play an important role in asthma immunopathogen-

esis by inducing increased serum IgE levels, eosinophilia and goblet cell hyperplasia⁸⁻¹⁰, respectively. In addition, IL-17 producing Th17 cells have been suggested to play a role in moderate to severe asthma⁴⁷.

GATA-3 is essential and sufficient to induce Th2 cytokine gene expression^{31, 32} (**Chapter 2**). Pathways that have been implicated in the initial transcription of GATA-3 include the IL-4-STAT6 axis, T-cell receptor triggering, IL-2 signaling via STAT5a and the Notch signaling pathway^{132, 133, 290-292}. The highly conserved Notch signaling pathway mediates cell-cell contact-dependent signaling and is essential for T cell development in the thymus and for CD4⁺ T cell differentiation. Notch expression is increased upon activation of CD4⁺ T cells^{107, 110}. Notch signaling is required for Th2 cell-mediated responses in mice^{93, 130-134} (**Chapter 3-5**) and can directly induce transcription of *Il4* and *Gata3*^{39, 93, 129, 130}. In addition, Notch signaling has been implicated in differentiation of multiple CD4⁺ T cell subsets including Th1, Th9, Th17 and T follicular helper (Tfh) cells²⁰⁷. Notch signaling is initiated upon ligand-receptor interaction between neighboring cells, which leads to two successive proteolytic cleavages of the receptor upon which the Notch intracellular domain (NICD) is liberated. The NICD heterodimerizes with the transcription factor recombination-signal-binding protein for immunoglobulin J κ region (RBPJ κ) in the nucleus. NICD binding to RBPJ κ leads to the assembly of a transcriptional activator complex and thereby to transcription of target genes¹⁰⁰. Since Notch signaling within T cells is required during Th2 cell-mediated allergic airway inflammation in mice (**Chapter 3-5**), blocking the Notch signaling pathway offers a potential therapeutic strategy for treating asthma patients¹³² (**Chapter 4**).

The observation that a subgroup of asthma patients fails to establish symptom control despite the use of corticosteroids and β_2 -agonists drives an international effort to characterize the immune mechanisms involved in these uncontrolled asthma patients. Several attempts have been made to group asthma subtypes based on key clinical and immunological characteristics^{478, 479}. However, most studies group patients into descriptive categories that provide information mainly regarding one single dimension of the disease, such as factors that trigger the disease, airway obstruction patterns, immunology or disease control^{475, 480-482}. The development of methods for tissue- or cell-type specific molecular phenotyping contributed to our insights in the heterogeneity of asthma and has the potential to identify biomarkers. Earlier reports that studied transcriptomic features of asthma patients used heterogeneous cell populations, such as bronchial biopsies or total CD4⁺ T cells from asthmatic patients. However, differences in the relative contribution of particular cell types to the total population analyzed can affect the obtained gene expression patterns⁴⁸³⁻⁴⁸⁶. Other studies that profiled cell samples enriched for Th2 cells, did not investigate clinical parameters such as asthma control⁴⁸⁷. Combining clinical parameters of asthma patients with extensive immunophenotyping by flow cytometry and gene expression profiling could be used to develop strategies for therapy management into a more personalized direction.

Based on our key findings in mice, in this report we focus on the analysis of the Notch signaling pathway in peripheral blood T cells from asthma patients. We specifically questioned whether there would be quantitative differences in Th1, Th2 and Th17 cells or the expression of NOTCH1 and NOTCH2 on these cells between healthy subjects and asthma patient subgroups with controlled, partially-controlled or uncontrolled asthma. In addition, we performed RNA-sequencing on sorted peripheral blood Th2 cells from healthy subjects and asthma patients with the aim to identify differences in the expression (1) of genes associated with the Notch signaling pathway, or (2) of asthma susceptibility genes that have previously been identified by genome-wide association studies (GWAS).

Methods

Participants

Patients were diagnosed with asthma when at least one of the following criteria was met in the 5 years before enrollment in the study: (1) Reversibility to β 2-agonists, as shown by an increase from baseline in Forced Expiratory Volume in the first second (FEV₁) $\geq$ 12% predicted and $\geq$ 200 ml after 400 μ g inhaled salbutamol or equivalent; (2) bronchial hyper-responsiveness (BHR) to metacholine (PD < 1.76) or histamine (PD < 2.59); (3) peak-flow variability of > 20% over a period of 14 days; (4) drop in FEV₁ > 12% and > 200 ml by tapering of treatment. To engage in the study, diagnosed asthma patients had to meet the following inclusion criteria: (1) recent (< 12 months) methacholine or histamine provocation test; (2) daily use of inhaled corticosteroids and β 2-agonists when required; (3) age 18-60. Exclusion criteria were: (1) use of systemic corticosteroids > 7.5 mg three months prior to inclusion; (2) treatment with other immunomodulatory medication; (3) concomitant pulmonary diseases (including chronic obstructive pulmonary disorders or interstitial lung diseases); (4) autoimmune diseases; (5) malignancies; (6) human immunodeficiency virus seropositivity; (7) smoking > 10 pack years; (8) current pregnancy. Asthma control was evaluated using the asthma control questionnaire (ACQ). All asthma patients required Global Initiative for Asthma (GINA) treatment step 3 or 4 medication⁴⁸⁸.

For this study, 59 asthma patients and 28 healthy controls (HC) donated peripheral blood. Asthma patients were included at the Franciscus Gasthuis and Vlietland (Rotterdam, the Netherlands). Healthy participants were recruited at the Franciscus Gasthuis and Vlietland and at the Erasmus MC Rotterdam, according to the above mentioned exclusion criteria and were in addition excluded if they had an FeNO > 50 ppb.

This study was approved by the Medical Ethical Committee of the Erasmus MC Rotterdam. Written informed consent was obtained from every participant prior to study inclusion. Further subject characteristics are shown in **Table 1** and **Table 2**.

Study materials

To be able to measure granulocytes in blood using flow cytometry, peripheral blood was lysed with osmotic lysis buffer. Peripheral blood mononuclear cells (PBMCs) were isolated, stored at -80°C and thawed as previously described⁴⁸⁹.

Flow cytometry

PBMCs were stained with a mixture of fluorochrome-labeled antibodies as described previously (**Chapter 3**). A list of all fluorochrome-labeled antibodies used can be found in **Supplementary Table 1**. Data were acquired with a LSR II flow cytometer operated using FACS Diva software 6.1 (BD biosciences) and analyzed using Flowjo 9.8.5 (tree Star Inc., Ashland, OR, USA).

Cell sorting

T cell subsets were sorted using a FACS Aria equipped with BD FACS Diva software (BD Biosciences, San Jose, CA, USA). Live 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 based on positivity for CD3 and CD4 and negativity for CD25. Naïve T cells were subsequently gated as CD45RO⁺, Th1 cells as CD45RO⁺CCR6⁻CXCR3⁺CCR4⁺, Th2 cells as CD45RO⁺CCR6⁺CXCR3⁺CCR4⁺ and Th17 cells as CD45RO⁺CCR6⁺. A list of all

Table 1. Characteristics of participants [FACS staining]

	HC	CA	PA	UA
n	28	17	17	25
Age	31.0 [22-50]	32.5 [19-48]	34.1 [18-56]	31.0 [18-51]
Sex [M/F]	8/20	4/13	3/14	1/24
BMI	23.4 [18.9-31.0]	24.6 [19.0-30.6]	26.5 [18.6-37.7]	27.7 [20.6-36.6]
FEV1		97.4 [66-122]	95.7 [73-119]	94.4 [60-120]
ACQ score		0.43 [0-0.67]	1.03 [0.75-1.42]	2.40 [1.5-4.17]
FeNO		19.6 [7-47]	25.5 [6-70]	37.2 [9-152]

Table 2. Characteristics of participants [RNA sequencing]

	HC	CA	UA
n	8	8	8
Age	32.4 [26-47]	33.9 [19-48]	27.0 [18-42]
Sex [M/F]	0/8	0/8	0/8
BMI	23.0 [18.9-26.6]	24.2 [20.2-26.9]	25.5 [20.6-30.8]
FEV1		101.1 [82-122]	91.6 [82-115]
ACQ score		0.6 [0.17-1.17]	1.9 [1.17-2.8]
FeNO		20.0 [6-47]	31.3 [9-70]

fluorochrome-labeled antibodies can be found in **Supplementary Table 1**. After sorting, cells were collected in RNA lysis buffer containing 2-mercaptoethanol.

RNA sequencing and gene expression analysis

RNA was extracted using the RNeasy Micro Kit (Qiagen, Venlo, the Netherlands) according to manufactures’ protocol. RNA sequencing was performed and analyzed as described before^{432, 433} (**Chapter 5**). Library preparation was performed using Smart-seq2 methodology⁴³⁷ and sequenced on an Illumina HiSeq2500 (single read mode, 51 bp read length) according to the Illumina TruSeq Rapid v2 protocol. HISAT2 was used to align reads to the human genome (mm10 build)⁴³⁸. Statistical analysis was executed using R; genes with >0.5 absolute log2 fold change and unadjusted P < 0.005 (Mann-Whitney U test) were considered differentially expressed (DE). Genes with an average tag per million (TPM) of >1 in >1 patient group were considered expressed. K-means clustering was performed in Past3 software (version 3.17). Pathway analyses on DE genes were performed using Metascape (<http://metascape.org>)⁴⁴³.

Statistical analyses

Comparisons between groups were performed using a Mann-Whitney U test. Correlations were analyzed using Pearson correlation test. P-values were two sided, and analyses were performed using Microsoft excel 2010, R (version 3.3.1) and GraphPad Prism software (version 5.01). P<0.05 was considered statistically significant; for genome-wide RNA Sequencing analysis a P<0.005 threshold was used.

Results

Proportions of CD4⁺ T cells are increased in peripheral blood from asthma patients and correlate with asthma severity

Asthma control level was based on the asthma control questionnaire (ACQ) score: controlled asthma (CA) was defined as ACQ < 0.75, partially-controlled asthma (PA) as ACQ 0.75-1.50 and uncontrolled asthma (UA) as ACQ > 1.5⁴⁹⁰ (Figure 1A). We found that exhaled NO, serum IgE levels, lung function (FEV1) and proportions

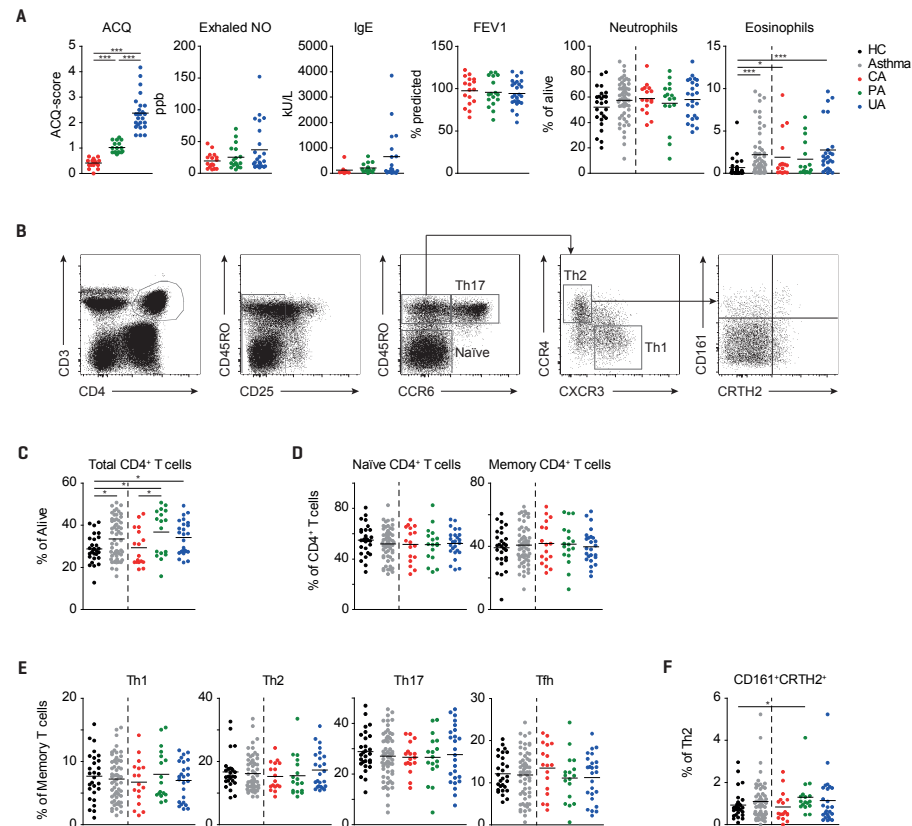


Figure 1. Total CD4⁺ T cells are increased in peripheral blood from asthma patients and correlate with asthma severity, while CD4⁺ T cell subset distribution is unaltered

(A) Measurements for exhaled NO, serum IgE, FEV1 and percentage of neutrophils [FSC^{high}SSC^{high}CD45⁺CD33⁺CD16^{high}Siglec-8⁻] and eosinophils [FSC^{high}SSC^{high}CD45⁺CD33⁺CD16^{high}Siglec-8⁻] in peripheral blood.
(B) Gating strategy for characterization of naïve T cells [CD45RO⁺], Th1 cells [CD45RO⁺CCR6⁺CXCR3⁺CCR4⁻], Th2 cells as [CD45RO⁺CCR6⁺CXCR3⁺CCR4⁻] and Th17 cells as [CD45RO⁺CCR6⁺] and the flow cytometric gating of CD161 and CRTH2 expression gated on Th2 cells.
(C) Percentage of CD3⁺CD4⁺ T cells as percentage of alive peripheral blood mononuclear cells [PBMCs].
(D) Percentages of naïve [CD45RA⁺] and memory [CD45RA⁻] T cells as percentage of CD4⁺ T cells after CD25⁺CD127⁻ Tregs were excluded.
(E) Percentages of Th1 cells [CD45RA⁺CXCR5⁺CCR6⁺CCR4⁻CXCR3⁻], Th2 cells [CD45RA⁺CXCR5⁺CCR6⁺CCR4⁻CXCR3⁻], Th17 cells [CD45RA⁺CXCR5⁺CCR6⁺] and Tfh cells [CD45RA⁺CXCR5⁺] as percentage of memory CD4⁺ T cells.
(F) Percentages of CRTH2⁺CD161⁺ double positive Th2 cells.

Data was obtained using flow cytometry and are shown as mean, * p < 0.05, ** p < 0.01, *** p < 0.001 which was calculated with a Mann-Whitney U-test. Abbreviations: HC, healthy control; CA, controlled asthma; PA, partially controlled asthma; UA, uncontrolled asthma.

of eosinophils or neutrophils were not different between the three asthma groups. As expected, eosinophil numbers were increased in peripheral blood of asthmatics compared to healthy controls (HC) (Figure 1A).

Next, we used flow cytometry to measure the frequencies of CD4⁺ T cells as well as CD4⁺ T cell subsets within PBMC fractions, based on differential chemokine receptor expression (Figure 1B). We found that proportions of CD3⁺CD4⁺ T cells within total alive PBMCs were significantly increased in PA and UA patients, compared with HC and CA (Figure 1C). However, within the CD4⁺ T cell population, the distribution of naïve T cells and memory T cells (Figure 1D) or the proportions of Th1, Th2, Th17 and Tfh cells within the memory compartment were not different between HC and asthma patients (Figure 1E). Also, CXCR3⁺CCR4⁺ Th17 cells and CXCR3⁺CCR4⁺ Th17.1 cells were not different when comparing HC with asthmatics (Supplementary figure 1A). It was recently reported that CRTH2⁺CD161⁺CD49d⁺ 'Th2A' cells were associated with allergic disorders⁴⁹¹. Therefore, we measured CRTH2 and CD161 expression on Th2 cells (Figure 1B, 1F and supplementary figure 1B), and found that CRTH2⁺ Th2 cells and CRTH2⁺CD161⁺ Th2 cells were only significantly increased in partially controlled asthma patients compared with HC. We did not detect differences in the percentages of total CD4⁺ T cells, Th2 cells, CRTH2⁺ Th2 cells and CRTH2⁺CD161⁺ Th2 cells between patients classified as allergic or non-allergic based on an inhaled-allergen test (Supplementary figure 1C).

In summary, whereas proportions of CD4⁺ T cells were increased in the blood of asthmatics, we did not observe differences in CD4⁺ T cell subset distribution when comparing asthma patient groups with healthy subjects.

Increased surface expression of NOTCH1 and NOTCH2 on memory T cells and Th2 cells from partially controlled and uncontrolled asthma patients

Next, we measured expression of surface NOTCH1 and NOTCH2 on peripheral blood CD4⁺ T cell subsets in our asthma patient cohort (Figure 2A). In general, frequencies of NOTCH1 or NOTCH2 expressing cells were low. The proportions of NOTCH1⁺ cells, but not the proportions of NOTCH2⁺ cells were increased within the total CD4⁺ T cell populations from PA and UA patients (Supplementary figure 2A). In addition, we found that the proportions of NOTCH1⁺ cells and NOTCH2⁺ cells within the fraction of CD4⁺ T cells (containing CD8⁺ T cells and $\gamma\delta$ T cells) were increased in asthma patients compared to HCs (Supplementary figure 2B). Within the CD4⁺ T cell compartment, we found that frequencies of NOTCH1⁺ cells were higher both in naïve and in memory CD4⁺ T cell fractions from PA and UA patients. Proportions of NOTCH2⁺ cells were only significantly increased in the memory T cell compartment from PA and UA patients (Figure 2B). Although within Th2 cells the proportions of NOTCH-expressing cells were low, for both NOTCH1⁺ and NOTCH2⁺ these were increased in UA patients (Figure 2B). We found low but significant positive correlations between NOTCH1 and NOTCH2 expression on naïve T cells and memory T cells, but not on Th2 cells (Figure 2C). Expression of NOTCH receptors was not different when comparing memory T cells from allergic and non-allergic asthmatics (Supplementary figure 2C).

Altogether, these data show that proportions of both NOTCH1 and NOTCH2 expressing cells were increased in total memory T cell and Th2 cell populations from patients, particularly in PA and UA patients.

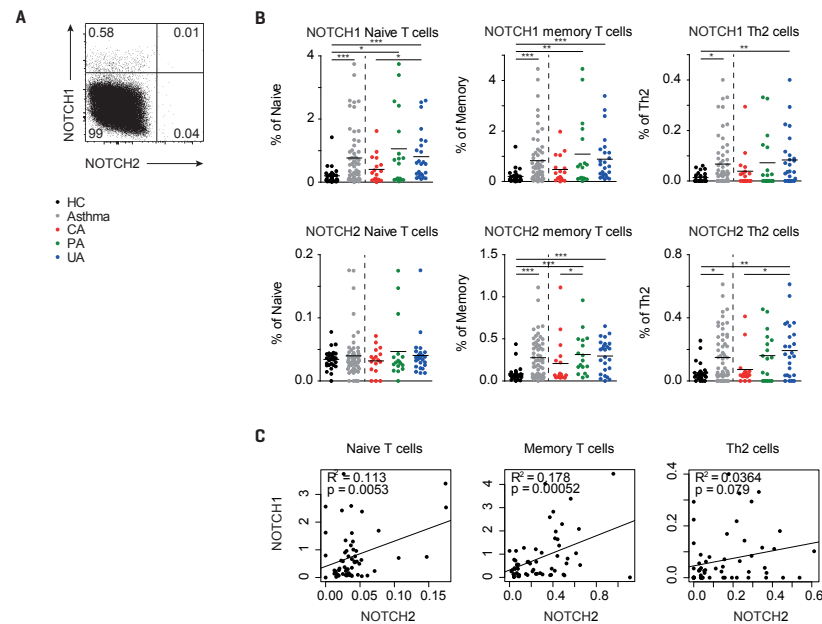


Figure 2. Proportions of NOTCH1⁺ and NOTCH2⁺ cells are increased within the memory T cell and Th2 cell compartments from partially controlled and uncontrolled asthma patients

(A) Flow cytometric gating of NOTCH1 and NOTCH2 expression on T cells.

(B) Percentages of NOTCH1 and NOTCH2 positive naive T cells, memory T cells and Th2 cells as gated as described in **Figure 1**.

(C) Correlations of proportions of NOTCH1⁺ and NOTCH2⁺ cells within naive T cells, memory T cells and Th2 cells measured in peripheral blood from asthma patients.

Data are shown as mean, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ using Mann-Whitney U-test. Correlation was calculated using a Pearson correlation test.

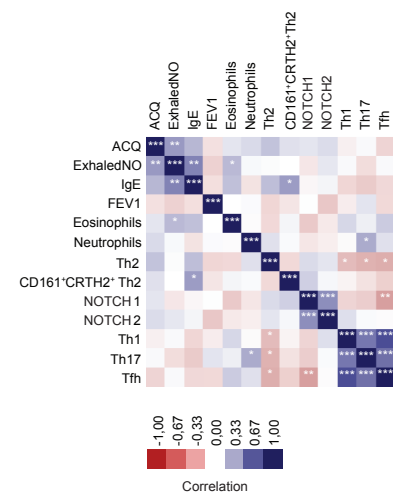


Figure 3. Correlation matrix of immunological and clinical parameters within the asthma patient cohort

Correlation matrix including asthma control questionnaire score (ACQ), Exhaled Nitric oxide (NO), serum IgE, FEV1, neutrophils and eosinophils as frequency of alive, Th2 cells as percentage of memory T cells, CRTH2⁺CD161⁺ Th2 cells as frequency of Th2 cells, NOTCH1 and NOTCH2 positive memory T cells and Th1, Th17 and Tfh cells as frequency of memory T cells.

Correlation was calculated using a Pearson correlation test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Proportions of NOTCH positive cells in the memory CD4⁺ T cell compartment do not correlate with CD4⁺ T cell subset distribution or clinical parameters

Whereas proportions of NOTCH-expressing cells were increased in the total PBMC memory CD4⁺ T cell population in asthmatics, we found no differences and a high spread in the CD4⁺ T cell subset distribution. Therefore, we questioned whether expression of Notch receptors would correlate with CD4⁺ T cell subsets, or other parameters including lung function, eosinophilia and IgE levels. Using a correlation matrix, we found that proportions of NOTCH1⁺ or NOTCH2⁺ memory CD4⁺ T cells did not significantly correlate with any of the Th subsets, except that proportions of NOTCH1⁺ memory CD4⁺ T cells negatively correlated with the percentages of Tfh cells (**Figure 3**). In contrast, we found that Th1 cells, Th17 cells and Tfh cells positively correlated with each other, negatively with Th2 cells, but not with lung function or eosinophilia. However, Th17 cells correlated with percentages of neutrophils in peripheral blood (**Figure 3**). We found that frequencies of CD161⁺CRTH2⁺ cells within the Th2 population weakly correlated with serum total IgE levels, whereas the proportions of Th2 cells within the memory T cell compartment did not correlate with IgE levels (**Figure 3**). Lastly, we did not find correlations with the proportions of lineage⁺CRTH2⁺GATA-3⁺ ILC2s in total live PBMCs (data not shown).

Taken together, we have shown that although proportions of NOTCH1⁺ and NOTCH2⁺ cells were increased in the memory CD4⁺ T cell compartment, NOTCH1 and NOTCH2 did not correlate with immunological or clinical parameters. In contrast, within our asthma patient cohort we found that CD161 and CRTH2 expression on Th2 cells correlated with serum IgE levels.

Notch gene expression analysis of Th2 cells from healthy subjects and asthma patients

To identify differences in the expression of (1) genes associated with the Notch signaling pathway or (2) asthma susceptibility genes that have previously been identified GWAS, we performed RNA-sequencing on sorted peripheral blood Th2 cells from 8 HC, 8 CA patients and 8 UA patients (**Table 2**). To verify our sorting strategy and to investigate which differentially expressed (DE) genes are Th2 cell-specific, we also performed RNA-sequencing on naive T cells, Th1 cells and Th17 cells from 2 HC, 2 CA patients and 2 UA patients. Unsupervised clustering using t-Distributed Stochastic Neighbor Embedding (t-SNE) revealed that naive T cells and Th1 cells were clearly transcriptionally distinct from Th2 and Th17 cells (**Figure 4A**). We verified this by investigating the expression of canonical naive T cell, Th2 cell, Th1 cell and Th17 cell markers (**Supplementary figure 3A-3D**), revealing a partial overlap of Th2 cells and Th17 cells (for example GATA3 or CCR4 expression). This analysis also showed that expression of CRTH2 on total Th2 cells was low. In addition, we observed low expression of the NOTCH receptors (except NOTCH3) and substantial expression of the nuclear effector RBPI, but these were not different across the CD4⁺ T cell subsets (**Supplementary figure 3E**).

Clustering analyses did not reveal a clear separation of HC, CA patients or UA patients by bulk transcriptional profiling (Figure 4B). When investigating the expression of genes associated with the Notch signaling pathway, we found that expression of NOTCH receptors was low and did not differ between HC and CA and UA patients. Surprisingly, NOTCH2 was decreased in CA and UA patients compared to HC in sharp contrast to our flow cytometry data (Figure 4C). We did not detect differences in expression of RBPJ and GATA3 and the Notch target HES1 was undetectable. Proportions of surface NOTCH1⁺ and NOTCH2⁺ cells within the Th2 cell populations, as measured by flow cytometry, did not correlate with tag per million (TPM) values of NOTCH1 and NOTCH2 in sorted Th2 cells (Figure 4D). Expression of Notch-associated genes (based on KEGG and REACTOME gene sets) with P-values <0.05 (when we performed comparisons of Th2 cells between asthma patient groups and healthy individuals) are shown in Figure 4E and included Deltex-3, which was increased in asthma patients compared to HC.

In summary, RNA expression of most NOTCH receptors was low and not different between HC, CA and UA patients. Expression of NOTCH2 was reduced in Th2 cells from asthma patients compared to HC. In contrast, expression of the Notch target DTX3 was increased in Th2 cells from asthma patients compared to HC.

An asthma-associated transcriptional signature in Th2 cells

To identify DE genes across Th2 cells from healthy people and asthma patients, we performed 4 comparisons: HC vs. all asthma patients, HC vs. CA, HC vs. UA, and CA vs. UA. Genes showing >0.5 log₂ fold change and P<0.005 in at least one of these comparisons were selected for subsequent analyses (Figure 5A), which resulted in 132 DE genes (Figure 5B, Supplementary Table 2). The 132 DE genes contained only a single Notch signaling-associated gene, which was NOTCH2. When comparing the expression profiles of these 132 genes in Th2 cells with other CD4⁺ T cell subsets (P<0.05), nine genes were different between Th2 and naïve T cells, 13 genes between Th2 and Th1 cells, two genes between Th2 and both naïve and Th1 cells, and only one gene between Th2 and Th17 cells (Figure 5C).

Pathway analysis on these 132 DE genes revealed enrichment for genes involved in positive regulation of fibroblast proliferation (BMI1, CD74, FNTB, PRKDC, ZEB1), negative regulation of lymphocyte apoptotic process (CD74, PRKCQ, CCL5, NOTCH2, PRKDC, SATB1, VAMP2, ZEB1, LST1, SKAP2, LTB, POLR2H, POLR3G, GBP5) and activation of JUN kinase activity (MAP3K9, MAP4K5, HACD3, CD74, PRKDC, UBE2G2, ZNF277, IER5, RINT1, CCL5, TGFBR1) (Figure 5D). Examples of DE genes are shown in Figure 5E and include CD74, which is involved in lymphocyte activation via association with class II major histocompatibility complex (MHC). CCL5 (RANTES), which was significantly upregulated in Th2 cells of UA patients, is involved in eosinophil and memory T cell attraction and has the capacity to activate eosinophils and cause histamine release from basophils⁴⁹². SATB1 expression is rapidly induced upon T cell activation to form a unique transcriptionally active chromatin structure at the Th2 cytokine locus³⁰⁹.

Altogether, DE genes that were increased in Th2 cells from asthma patients include genes involved in fibroblast proliferation, lymphocyte activation, Th2 cytokine expression and apoptotic processes.

Gene expression profiles correlating with disease outcome or type of inflammation

We next compared our asthma-associated transcriptional signature with genes previously reported to be linked to asthma via GWAS. We constructed a list of 665 asthma-associated genes by combining all genetic variants found in asthma GWAS to date and linking them to their nearest neighboring genes⁴³³. Five of our 132 DE genes overlapped with the 665 GWAS genes (Figure 6A). Of these genes, only IGFBP3 was increased in CA patients while the other genes were decreased in at least one of the patient subgroups. Finally, we questioned whether the DE genes could predict disease outcome by showing if they correlated with lung function and eosinophilia. Figure 6B shows a correlation matrix of the DE genes of which at least one of the correlations with ACQ, exhaled NO, FEV₁, eosinophils and neutrophils had a R² value > 0.5. The 5 genes that had the strongest negative correlation with FEV₁ were AAMDC,

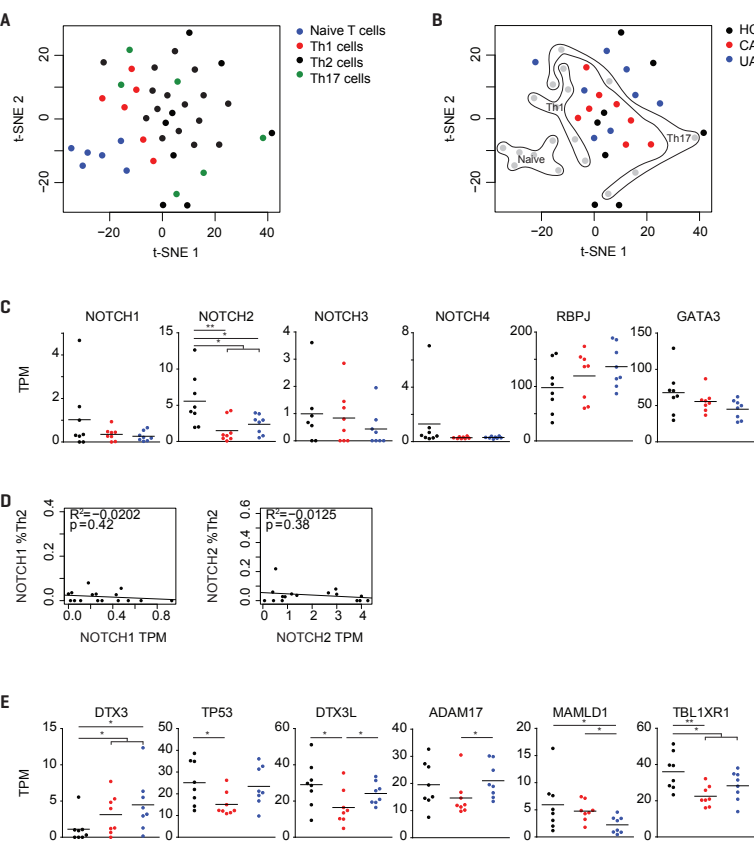


Figure 4. NOTCH gene expression analysis of Th2 cells from healthy subjects and asthma patients

[A] t-SNE plot of Log₂ transformed tag per TPM values from naïve T cells, Th1 cells, Th2 cells and Th17 cells.
[B] t-SNE plot indicating Th2 cells from HC, CA and UA patients.
[C] TPM values of Th2 cells from HC, CA and UA patients of NOTCH receptors, RBPJ and GATA3.
[D] Correlations of the proportions of NOTCH1⁺ and NOTCH2⁺ cells within the Th2 compartment with TPM values of NOTCH1 and NOTCH2.
[E] TPM values of Th2 cells from HC, CA and UA patients of Notch genes in which at least one of the comparisons p < 0.05. Correlation was calculated using a Pearson correlation test.
Data are shown as mean, * p < 0.05, ** p < 0.01, *** p < 0.001 which was calculated with a Mann-Whitney U-test.

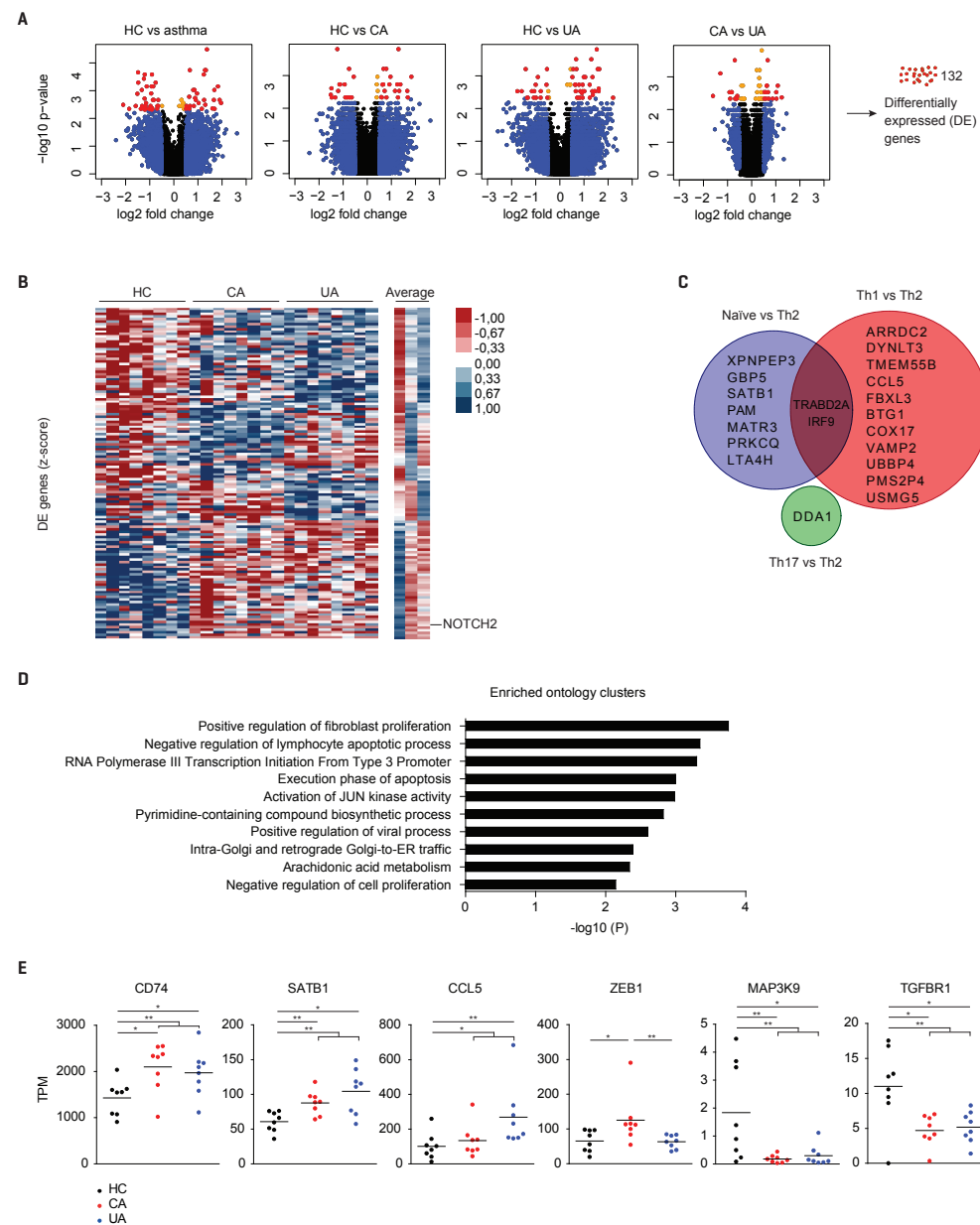


Figure 5. Gene expression analysis of Th2 cells from healthy subjects and asthma patients

[A] Volcano plot of all genes >1 TPM. Genes were identified as differentially expressed (DE) (red) when they met the following criteria; <0.005 unadjusted p-value and >0.5 $\log_2$ fold change in at least one of the four depicted comparisons.

[B] Heatmap of DE genes clustered using K-means. Average TPM values per gene per cluster per patient group are plotted.

[C] Venn diagram of genes significantly different when comparing TPM values of Th2 cells with naïve T cells, Th1 cells and Th17 cells calculated with a paired Mann-Whitney test.

[D] Pathway analysis on the 132 DE genes.

[E] TPM values of Th2 cells from HC, CA and UA patients of selected DE genes.

* p < 0.05, ** p < 0.01, *** p < 0.001 using a Mann-Whitney U test.

FNTB, HIBCH, LTB and TM4SF19–TCTEX1D2 (**Figure 6C**). These genes were surprising since the function of these genes in asthma and type 2 immunity have not yet been described. Together, we identified genes that positively or negatively reflect disease outcome by correlating with ACQ, exhaled NO, FEV1, eosinophils or neutrophils.

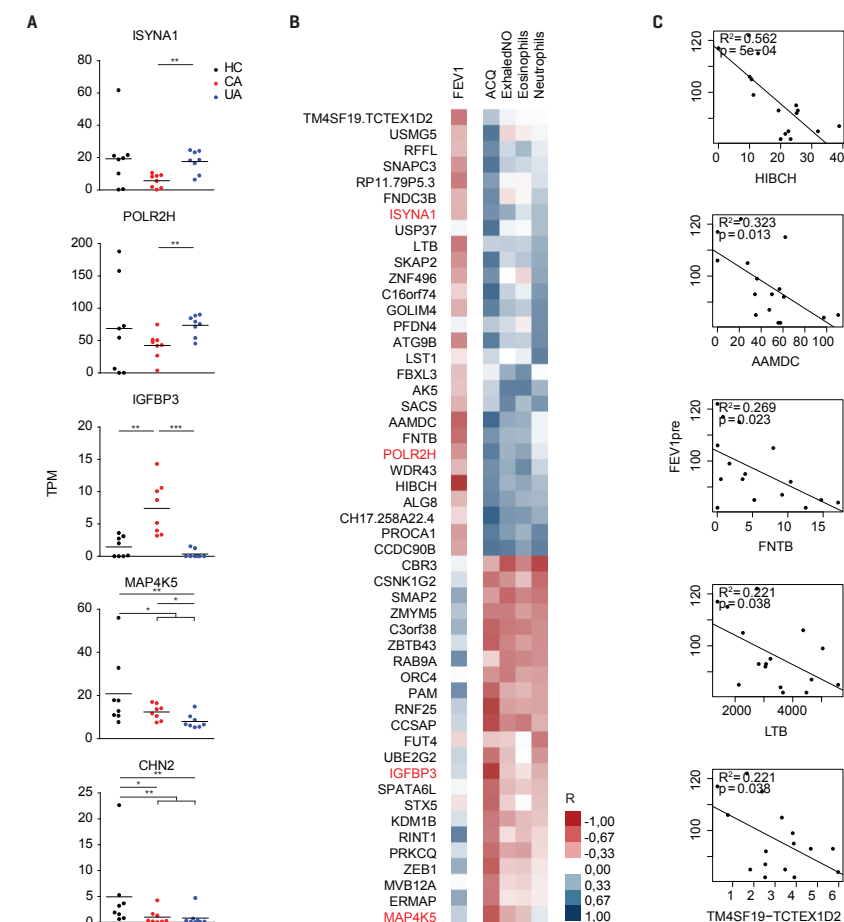


Figure 6. Gene expression profiles correlating with disease outcome or type of inflammation

[A] TPM values of Th2 cells from HC, CA and UA patients of DE genes from GWAS. * p < 0.05, ** p < 0.01, *** p < 0.001 using a Mann-Whitney U test.

[B] Correlation matrix including asthma control questionnaire score [ACQ], Exhaled Nitric oxide [NO], serum IgE, FEV1, neutrophils and eosinophils as frequency of alive against TPM values of DE genes of which at least one correlation exceeded a $\log_2$ fold change of 0.5. GWAS genes are shown in red.

[C] Correlations of the top 5 genes correlating with FEV1.

Correlation was calculated using a Pearson correlation test.

Discussion

In this chapter, we showed that the proportions of total CD4⁺ T cells are increased in peripheral blood from PA and UA patients, whereas we did not find differences in CD4⁺ T cell distribution between asthma patients and HC. We found that proportions of NOTCH1⁺ and NOTCH2⁺ cells were increased in the memory CD4⁺ T cell compartment and in Th2 cells, particularly in patients with poorly controlled asthma. As proportions of Th2 cells did not differ between HC and asthma patients, we set out to investigate whether Th2 cells from asthma patients have different characteristics compared to Th2 cells from HC. Ultimately, such differences could be used to predict disease outcome, including lung function and the level of asthma control achieved upon steroid treatment. RNA sequencing of purified Th2 cells from CA and UA patients and HC revealed a unique gene signature comprising 132 DE genes, including NOTCH2 which was decreased in sharp contrast to our flow cytometry data. While we found increased levels of NOTCH1 and NOTCH2 using flow cytometry, RNA expression of NOTCH1 was very low and NOTCH2 RNA was decreased in asthma patients. Also, proportions of surface NOTCH1⁺ and NOTCH2⁺ cells within the Th2 cell populations, as measured by flow cytometry, did not correlate with TPM values of NOTCH1 and NOTCH2 in sorted Th2 cells. These differences could be a result of complex post-transcriptional mechanisms or feedback loops that control NOTCH protein levels. To be expressed on the cell membrane, newly generated Notch receptors are cleaved and glycosylated in the endoplasmic reticulum and Golgi apparatus, leading to the generation of a heterodimer composed of the Notch extracellular domain non-covalently attached to the Notch intracellular domain inserted in the membrane⁴⁴⁴. Therefore, RNA expression is possibly not a good reflection of Notch receptor expression on the membrane.

We were interested in studying Notch expression on CD4⁺ T cells and Th2 cells, as we recently found that Notch expression on T cells is critical during Th2 cell-mediated allergic airway inflammation in mice (**Chapter 3-5**), implicating Notch inhibitors as potential therapeutic agents for asthma treatment (**Chapter 4**). To our knowledge, this is the first report showing that proportions of NOTCH1⁺ and NOTCH2⁺ cells were increased in the memory CD4⁺ T cell compartment and in Th2 cells from PA and UA patients compared to CA patients or HC. This observation suggests that inhibiting Notch signaling could be especially helpful in patients with poor symptom control using corticosteroids. More T cells were positive for NOTCH1, compared to NOTCH2, which is interesting since Notch1 is more important during allergic airway inflammation in mice than Notch2, perhaps indicating that Notch1 blockade would be most beneficial to asthma patients (**Chapter 5**). Nevertheless, proportions of NOTCH positive cells that we detected were very low within the T cell compartment in peripheral blood. It would therefore be interesting to study NOTCH expression on T cells in the lungs of asthma patients using T cells isolated from BAL, lung draining lymph nodes or induced sputum. Alternatively, Notch receptor expression on T cells could be analyzed using lung biopsies. Since we recently found evidence that Notch expression supports Th2 cells to exit the lymph nodes (**Chapter 5**), it will be particularly interesting to study Notch receptor expression on T cells in lymph node biopsies.

CRTH2 is a receptor for prostaglandin D2 (PGD2), a signaling molecule that is released from mast cells, Th2 cells, and dendritic cells⁴⁹³. Others have reported increased expression of PGD2 in patients with severe asthma⁴⁹⁴ and the existence of a subpopulation of CRTH2⁺CD161⁺CD49d⁺ Th2 cells that was uniquely present in patients with allergic disorders⁴⁹¹. We did not detect increased CRTH2 expression on Th2 cells from asthma patients, although we did find that CD161 and CRTH2 expression on Th2 cells correlated with serum IgE levels. A previous study using a CRTH2 antagonist achieved no improvement in lung function

or reduction in sputum eosinophilia, but a small reduction in symptoms compared with placebo-treated patients was observed⁴⁹⁵. We detected significant variation in CRTH2 expression on Th2 cells amongst asthmatics, providing a possible explanation for the poor efficacy of CRTH2 antagonist therapy. Selective treatment of patients with high CRTH2 expression with CRTH2 antagonists or combining CRTH2 antagonists with anti-IgE therapy or Notch inhibitors might be a next step towards personalized medicine in treating asthma patients.

To our knowledge, this is the first study to compare RNA expression in Th2 cells from healthy subjects and asthma patients that have been stratified according to their level of symptom control using inhaled steroids. Upregulated genes involved in immune cell activation include the gene encoding the HLA class II histocompatibility antigen gamma chain (CD74), which can be targeted by milatuzumab⁴⁹⁶. Lymphotoxin-beta (LTB), which was also a DE gene between murine Notch sufficient and Notch deficient Th2 cells (**Chapter 5**), correlated negatively with FEV1. CCL5 (RANTES) was specifically upregulated in UA patients and is involved in eosinophil and memory T cells recruitment during inflammation. Lastly, ZEB1 - a transcriptional repressor of IL-2 - was specifically upregulated in CA patients suggesting that IL-2 signaling might contribute to lower responsiveness to inhaled corticosteroids. Interestingly, the gene encoding Phosphodiesterase 4D Interacting Protein (PDE4DIP) was decreased in asthma patients. This protein was described to bind phosphodiesterase 4D and mutations in this gene may cause a myeloproliferative disorder associated with eosinophilia⁴⁹⁷. The PDE4 pathway is a promising therapeutic target, since PDE4 inhibitors have a broad spectrum of inhibiting effects on inflammatory cells including T cells, eosinophils, neutrophils, mast cells, airway smooth muscle and epithelial cells. Moreover, PDE4 inhibitors were shown to be highly effective in *in vivo* models of allergic airway inflammation^{498, 499}.

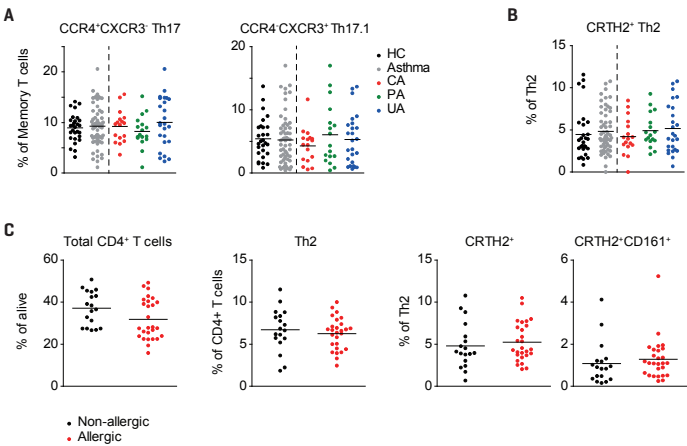
In summary, through detailed characterizing of Th2 cells from asthma patients we found increased Notch expression and defined a set of genes that is differentially expressed between Th2 cells from asthma patients and HC. The activity of part of this gene signature correlates with clinical parameters including ACQ scores, exhaled NO, lung function and inflammation type. Thereby, we have set a framework of potential biomarkers for disease severity, including putative therapeutic targets. Future research using larger cohorts of patients, preferably followed longitudinally, should enable validation of these markers and to established key candidates for follow-up studies. Technological advances in characterizing immune cells and molecules will provide promising tools to better characterize the different immunological endotypes within the asthma patient population. Our identified DE genes, together with the clinical phenotype of patients, may be helpful to classify patients and to predict the type of therapy they might respond to.

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Supplementary figures and tables



Supplementary figure 1. CD4⁺ T cell subset distribution is unaltered in peripheral blood from asthma patients
[A] Percentages of Th17 cells [CD45RA⁺CXCR3⁺CCR6⁺CCR4⁺CXCR3⁺] and Th17.1 cells [CD45RA⁺CXCR3⁺CCR6⁺CCR4⁺CXCR3⁺] as percentage of memory CD4⁺ T cells.
[B] Percentages of CRTH2⁺ single positive Th2 cells.
[C] Percentages of CD3⁺CD4⁺ T cells as percentage of alive PBMCs, Th2 cells as percentage of memory CD4⁺ T cells and CRTH2⁺ and CRTH2⁺CD161⁺ positive Th2 cells in allergic or non-allergic asthma patients.
Data are shown as mean, significance was calculated with a Mann-Whitney U-test.

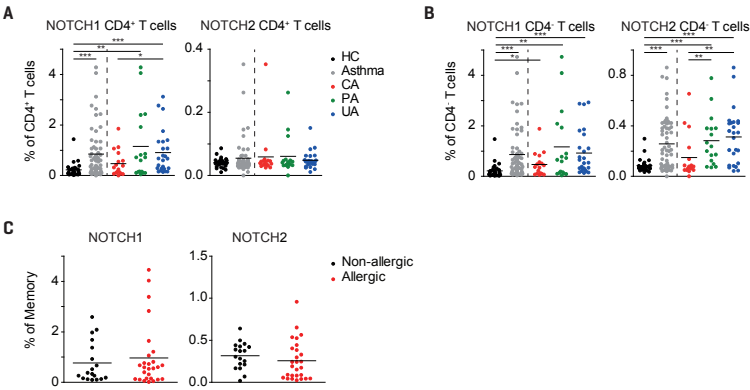
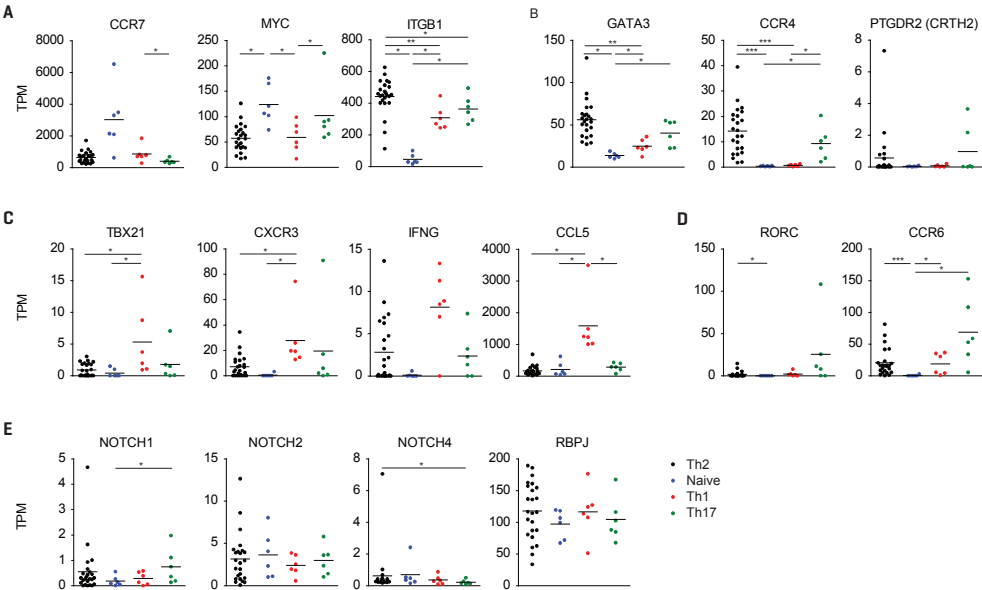


Figure 2. Proportions of NOTCH1⁺ and NOTCH2⁺ cells within the CD4⁺ T cell and CD4⁺ T cell compartments
[A-B] Percentages of NOTCH1 and NOTCH2 positive CD4⁺ T cells [A] and CD4⁺ T cells [B].
[C] Percentages of NOTCH1 and NOTCH2 positive memory CD4⁺ T cells in allergic or non-allergic asthma patients.
Data are shown as mean, * p < 0.05, ** p < 0.01, *** p < 0.001 which was calculated with a Mann-Whitney U-test.



Supplementary figure 3. Gene expression in naive T cells, Th1 cells, Th2 cells and Th17 cells
[A-E] TPM values of naive T cells, Th1 cells, Th2 cells and Th17 cells from HC, CA and UA patients combined of genes associated with T cell activation [A], Th2 cells [B], Th1 cells [C], Th17 cells [D] and Notch signaling [E]. Data are shown as mean, * p < 0.05, ** p < 0.01, *** p < 0.001 which was calculated with a Mann-Whitney U-test for comparisons with Th2 cells and a paired Mann-Whitney test to compare Th0 cells, Th1 cells and Th17 cells.

Supplementary table 1. Antibodies used for flow cytometry and cell sorting

Target	Conjugate	Company	Clone
CCR4	FITC	R&D systems, Minneapolis, MN, USA	205410
CCR6	APC	BD Biosciences	11A9
CD2	FITC	eBioscience	RPA-2.10
CD3	AF700	eBioscience	UCHT1
CD3	APC-ef780	eBioscience	UCHT1
CD4	AF700	eBioscience	OKT4
CD4	BV786	BD Biosciences	SK3
CD4	PE-Cy7	BD Biosciences	SK3
CD8a	FITC	BD Biosciences	SK1
CD11b	FITC	eBioscience	ICRF44
CD14	FITC	eBioscience	61D3
CD16	FITC	BD Biosciences	3G8
CD16	PerCP-Cy5.5	BD Biosciences	3G8
CD19	FITC	BD Biosciences	H1B19
CD20	FITC	eBioscience	2H7
CD25	BV605	Biolegend, San Diego, CA, USA	BC96

Target	Conjugate	Company	Clone
CD25	PerCP-Cy5.5	eBioscience, San Diego, CA, USA	BC96
CD33	PE-Cy7	eBioscience	VI M505
CD45	APC-ef780	BD Biosciences	HI30
CD45	V450	BD Biosciences	HI30
CD45RA	PE-Texas Red	Invitrogen, Carlsbad, CA, USA	MEM-56
CD45RO	PE	BD Biosciences, San Jose, CA, USA	UCHI-1
CD56	AF488	BD Biosciences	B159
CD127	BV650	BD Biosciences	hIL7R-M21
CD161	PE-Cy7	eBioscience	HP-3G10
CD235a	FITC	eBioscience	HIR2 [GA-R2
CD278	PerCP-Cy5.5	Biolegend	C398,4A
CD294 [CRTH2]	Biotin	Miltenyi Biotec B.V., Leiden, the Netherlands	BM16
CD294 [CRTH2]	PE	Miltenyi Biotec B.V., Leiden, the Netherlands	BM16
CXCR3	BV421	BD Biosciences	G-025H7
CXCR3	BV711	BD Biosciences	1C6/CXCR3
CXCR5	PerCP-Cy5.5	BD Biosciences	RF8B2
FcεRI	FITC	eBioscience	AER-37
GATA-3	EF660	eBioscience	TWAJ-14
HLA-DR	BV711	BD Biosciences	G46-6
Live/Dead	Amcyan	Invitrogen	
NKp44	ef450	eBioscience	44,189
NOTCH1	BV421	BD Biosciences	MHN1-519
NOTCH2	Biotin	Miltenyi Biotec B.V.	MHN2-25
RORγt	PE	eBioscience	Q21-559
Siglec-8	APC	Biolegend	7C9
Streptavidin	BV650	BD biosciences	
Streptavidin	BV786	BD biosciences	
TCRγδ	FITC	eBioscience	B1.1

Supplementary table 2. Average TPM values of DE genes

Annotation	Th2 HC	Th2 asthma	Th2 CA	Th2 UA
AAMDC	35.525	47.053	26.539	67.566
ACY1	4.943	16.126	16.521	15.732
AK5	7.543	2.073	1.662	2.483
ALG8	154.905	84.730	67.710	101.750
ANGEL2	24.644	52.851	53.405	52.296
APOL3	62.929	36.305	34.134	38.476
AREL1	14.100	8.304	9.715	6.894
ARL4C	70.274	110.477	113.595	107.359
ARRDC2	33.722	82.529	106.420	58.639
ASB15	0.467	3.225	3.808	2.641
ATG9B	11.446	9.568	5.979	13.158

BMI1	9.641	13.017	9.498	16.536
BTG1	55.455	92.416	101.460	83.372
BTN3A2	274.268	181.334	186.923	175.744
C16orf74	4.076	4.894	1.450	8.339
C1orf194	4.268	0.204	0.000	0.408
C3orf38	25.221	26.707	37.023	16.391
CBR3	105.730	37.837	42.765	32.908
CCDC25	51.687	79.048	65.352	92.743
CCDC90B	131.449	91.287	69.765	112.810
CCL5	100.921	201.549	133.844	269.254
CCSAP	7.326	3.067	4.075	2.059
CD74	1428.303	2034.769	2098.651	1970.886
CEP290	9.762	4.837	5.121	4.554
CEP57L1	23.700	8.040	7.747	8.333
CETN2	9.513	22.007	20.136	23.878
CH17-258A22.4	3.870	3.277	1.680	4.874
CHN2	4.940	0.905	1.009	0.800
COMP	0.231	0.952	0.571	1.334
COPB2	84.856	48.886	43.178	54.594
COX17	61.051	85.742	78.937	92.547
CSNK1G2	12.810	12.614	19.119	6.110
CYP20A1	26.450	48.837	50.725	46.948
DDA1	12.487	21.593	20.326	22.859
DDX59	30.530	12.687	12.223	13.151
DENND2D	195.720	127.397	105.189	149.605
DHFR2	4.109	1.958	1.025	2.891
DLST	4.560	11.179	12.502	9.855
DYNLT3	41.034	72.915	71.684	74.145
ERMAP	1.799	1.189	2.189	0.189
FAM221A	4.373	18.540	18.883	18.196
FBXL3	8.616	15.410	16.438	14.382
FNDC3B	2.034	5.721	4.211	7.230
FNTB	3.180	5.688	2.044	9.332
FUT4	1.524	0.499	0.955	0.043
GBP5	29.058	71.719	64.306	79.131
GFPT1	2.455	8.719	8.066	9.372
GOLIM4	0.010	2.561	0.130	4.992
HACD3	55.738	32.998	34.507	31.489
HEBP2	9.818	19.967	21.215	18.719
HIBCH	15.231	19.218	12.942	25.494
IER5	1.417	4.254	4.291	4.216
IGFBP3	1.444	3.879	7.405	0.352
IGSF9B	2.298	0.067	0.132	0.002
IRF9	120.346	63.008	55.752	70.264
ISYNA1	19.213	11.608	5.648	17.568

Annotation	Th2 HC	Th2 asthma	Th2 CA	Th2 UA
JMY	1.555	4.061	4.854	3.267
KDM1B	2.069	0.938	1.373	0.503
LOXL2	8.917	3.453	4.014	2.892
LST1	25.926	48.340	46.347	50.332
LTA4H	86.164	37.986	35.771	40.200
LTB	2168.614	3361.285	3052.867	3669.702
MAP3K9	1.839	0.241	0.180	0.301
MAP4K5	20.778	10.149	12.345	7.953
MATR3	255.033	226.417	281.326	171.507
MMRN2	0.706	1.364	2.554	0.173
MRPL1	32.452	52.010	53.700	50.320
MVB12A	26.971	52.008	58.300	45.715
NCBP2	71.929	38.664	39.498	37.831
NOTCH2	5.573	1.943	1.504	2.383
OARD1	46.721	24.321	19.039	29.603
OR7A5	0.000	0.640	1.023	0.258
ORC4	55.729	33.166	36.139	30.192
OTUD4	1.838	4.907	4.588	5.225
PAM	12.529	15.974	23.244	8.705
PDE4DIP	81.033	47.964	45.353	50.574
PFDN4	41.170	55.632	52.025	59.240
PLA2G4B	5.432	3.265	5.511	1.018
PMS2P4	2.524	3.910	2.761	5.058
POLDIP2	41.196	50.269	62.257	38.282
POLR2H	68.461	57.961	42.356	73.565
POLR3G	3.185	0.045	0.015	0.074
PRKCQ	65.097	33.306	38.318	28.293
PRKDC	72.702	22.384	19.412	25.356
PROCA1	12.938	14.578	9.068	20.088
RAB9A	9.756	18.030	21.317	14.743
RFFL	31.492	19.959	17.842	22.077
RINT1	23.079	17.020	24.590	9.450
RNF25	17.951	24.116	37.956	10.275
RP11-392E22.9	10.921	2.166	3.013	1.318
RP11-79P5.3	7.493	10.636	8.862	12.410
SACS	2.828	3.375	1.499	5.251
SATB1	60.861	96.088	87.635	104.541
SKAP2	5.713	5.991	3.110	8.872
SLC25A14	3.450	13.057	12.858	13.256
SMAD2	25.597	53.648	41.265	66.031
SMAP2	20.802	39.572	43.826	35.317
SNAPC3	10.913	9.215	5.659	12.771
SNTB2	1.364	3.678	3.882	3.473
SNX5	154.784	86.325	95.184	77.466

SPATA6L	6.461	1.197	1.620	0.774
SPIN2A	2.835	6.685	5.804	7.566
STX5	4.706	7.407	11.061	3.752
SUCO	9.710	18.707	15.690	21.723
SUGT1P3	2.778	0.804	0.612	0.997
TARDBP	209.934	134.026	127.898	140.155
TBPL1	36.424	66.801	67.590	66.011
TCTEX1D2	14.486	26.481	22.433	30.529
TGFBR1	11.016	4.923	4.684	5.162
TIPRL	28.647	52.212	55.232	49.191
TM4SF19-TCTEX1D2	1.257	3.087	2.573	3.602
TMEM55B	4.193	12.053	12.572	11.535
TRABD2A	139.821	88.454	83.390	93.518
UBBP4	147.039	206.860	177.988	235.733
UBE2G2	45.856	64.182	78.936	49.428
USMG5	133.312	171.579	148.667	194.491
USP37	4.788	5.781	1.946	9.617
VAMP2	173.460	249.142	227.513	270.771
WDR11	35.469	15.187	12.879	17.496
WDR43	50.762	104.679	94.367	114.991
XPNPEP3	3.723	12.069	11.167	12.972
YPEL5	157.323	249.542	280.829	218.256
ZBTB43	7.969	7.762	11.301	4.222
ZEB1	65.491	94.169	125.208	63.130
ZMYM5	34.567	54.808	65.282	44.334
ZNF134	2.831	10.070	15.452	4.687
ZNF208	1.682	0.276	0.421	0.130
ZNF277	12.036	23.851	20.650	27.052
ZNF284	14.674	4.943	5.111	4.775
ZNF300	2.116	0.083	0.128	0.039
ZNF426	19.588	8.460	10.042	6.878
ZNF496	0.870	1.196	0.220	2.172



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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 signals²³, 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 cells⁴⁰⁹. 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, respectively⁹³. Alternatively, the ‘unbiased amplifier’ model proposes that Notch ligands are not instructive but rather function to generally amplify Th cell responses¹³⁵. 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 required⁵⁰⁰. 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 uptake¹⁰⁷⁻¹¹⁰. 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 PKC θ , which is crucial for TCR and CD28 signaling and regulation of the actin cytoskeleton¹¹³. Moreover, upon TCR stimulation NICD interacts with other proteins in the cell in a non-canonical, RBPJk-independent pathway that leads to NF κ B 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 TLR9 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 unknown⁵⁰²⁻⁵⁰⁴. 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 increased¹³²⁻¹³⁴. Moreover, upon gene ablation of Notch1/Notch2 or RBPJk, IL-4 production was abrogated and functional responses against parasitological pathogens were reduced⁹³. 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) model²¹². 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 response¹³⁵. Moreover, decreased Th2 responses were found when DLL4 was blocked in a mouse model for RSV-mediated allergic asthma exacerbations⁴⁰⁶. 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.

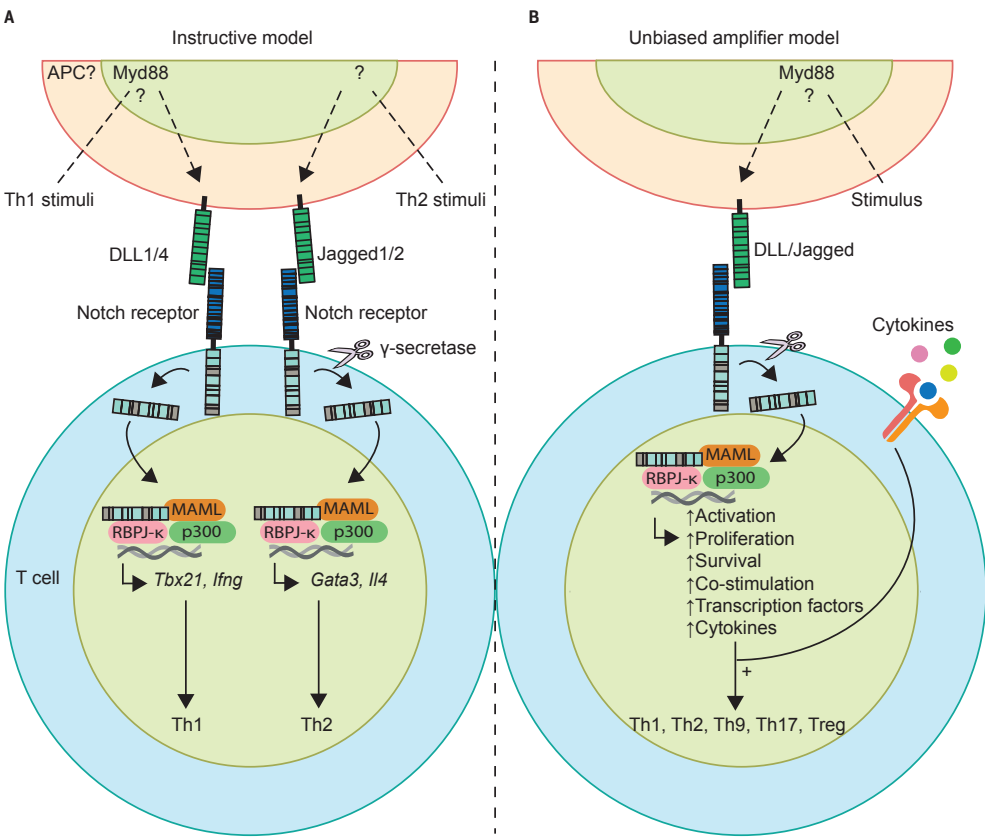


Figure 1: Schematic overview of the two models describing the role of Notch signaling in Th cell differentiation. [A] According to the instructive model, Th1-stimuli and Th2-stimuli induce DLL and Jagged ligand expression on APCs, respectively. Upon receptor-ligand binding, Th1 differentiation is induced by Notch intracellular domain (NICD) binding and activating transcription of the Th1 transcription factor gene *Tbx21* and signature cytokine *Ilfng*. 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							Reference
APC	Stimulant	Notch Ligand		Additional findings			
		Jag1	Jag2	DLL1	DLL4		
GM-CSF bmDCs ¹⁾	PGE2 or	0 ²⁾	↑	0	0	Jag1-expressing APC induce Th2 cytokines in CD4 ⁺ T cells	93
	Cholera toxin						
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	-	↑	-	0	c-kit deficient DCs lack Jag2 (but not DLL4) and induce reduced Th2 inflammation and AAI	215
	CpG	-	0	-	↑		
GM-CSF bmDCs	LPS [†] OVA/GP peptide	-	-	-	-	DCs lacking Mib1 show impaired Th2 but not Th1 differentiation <i>in vitro</i>	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	0	0	0	↑	DCs, pretreated with DLL4, induce reduced AHR and AAI	218
	OVA + DLL4	↑	0	↑	↑		
GM-CSF bmDCs	Derp7	↑↑	-	-	-	Derp7 induces IL-4 secretion by CD4 ⁺ T cells	219
	LPS	↑	-	-	-		
Human B0CA1 ⁺ mDCs	-	-	-	-	-	Jag1 expression correlated with IL-4 expressing T cells	228
Human B0CA1 ⁺ 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 mDCs	APE	0	↑	↑	0	In a DC-CD4 ⁺ T cell co-culture, APE increases IL-5 and IL-10 secretion	221
	APE/PGE2 [†] LPS	0	↑	↓	↓		

Instruction of Th1 differentiation						Reference	
APC	Stimulant	Notch Ligand		Additional findings			
		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 <i>in vitro</i>	225
CD11c ⁺ DCs, CD19 ⁺ B cells	MOG35-55 peptide in CFA	↑	-	↑	-	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 mDCs	LPS	↑	L	0	↑	Expression of DLL4 correlated with IFN-γ inducing capabilities of DCs	227
Human B0CA1 ⁺ mDCs	R-848	↓	L	L	↑	Jag1 expression negatively correlated with IFN-γ expressing T cells	228
Human monocytes, macrophages, GM-CSF mDCs	Dengue-virus	L	L	↑	0	DLL1 induces IFN-γ but not IL-4 production by CD4 ⁺ T cells <i>in vitro</i>	229
Human CD11c ⁺ DCs and pDCs	R-848	-	-	-	↑	DLL4-blockade decreases IFN-γ and IL-17 expressing CD4 ⁺ T cells <i>in vitro</i>	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 RBPJ κ knockout mice, demonstrated that Th1 cell function was unaffected^{129-131, 137, 419}. Therefore, these findings suggest that signals that regulate Th1 differentiation involve RBPJ κ -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 model¹³³. 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 cadherins⁹⁸.

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 model¹⁵². 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 ligation¹³⁸. The *Rorc*, *Il17* and *Il23r* gene promoters are direct Notch targets and, accordingly, Th17 cell differentiation is impaired when Notch signaling is blocked¹³⁹⁻¹⁴³. Hereby, DLL1, DLL3 and DLL4 ligands were found to be essential^{212, 213, 230, 406, 505}, but a role for Jagged1 remains controversial⁵⁰⁶⁻⁵⁰⁸. 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 receptor⁵¹⁰. Differentiation and function of Tregs requires Notch signaling in T cells^{105, 144-146}, whereby both DLL and Jagged ligands can promote Treg expansion⁵¹¹⁻⁵¹⁸. Although the key Treg transcription factor Foxp3 is a direct Notch target⁵¹⁹, 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. RBPJ κ -dependent or independent, or signals that differ in strength or kinetics⁵²⁴, the molecular mechanisms involved are currently unknown.

It has been shown that DLL4 induces a stronger Notch signal than DLL1 or Jagged1⁵¹⁶. 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 inhibited⁹⁹. 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 preferences⁵²⁵, distinct ligand-receptor combinations may produce quantitatively or qualitatively distinct signals⁵²⁶. 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 kinetics⁵²⁷. 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 cytokines¹⁵⁰. 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 outcomes¹³⁵. In addition, Notch signaling via DLL4 was shown to boost antigen sensitivity of CD4⁺ T cells via promoting co-stimulatory signals in T cells¹⁰⁸. 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 macrophages⁴⁰⁰. 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 cells⁴⁶², 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 pathway⁵²⁹ which would hamper concerted Notch-mediated differentiation and polarization of adjacent T helper cells. Finally, Notch receptors can become activated independent of ligand binding⁴⁷¹. 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 PKC θ -dependent mechanism that enhances Notch activation¹¹³. 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 subsets²⁴ 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 complex¹⁹⁹⁻²⁰¹, as well as specific antibodies that target Notch receptors²⁰²⁻²⁰⁴ or Notch ligands^{205, 206} have been designed. Promising results were obtained with Notch pathway blocking antibodies in cancer patients⁵³⁴ 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-4³³⁻⁴⁰, 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 RBPjk 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 subsets⁴⁵⁵, 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 kinetics⁵²⁴. Also, ligands might preferentially bind to Notch receptors depending on the glycosylation status of the extracellular domain of the Notch receptor⁹⁹. In addition, different Notch intracellular domains (NICDs) might have different target gene preferences⁵²⁵. It is therefore not unlikely that distinct ligand-receptor combinations can produce quantitatively or qualitatively distinct signals⁵²⁶ 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?

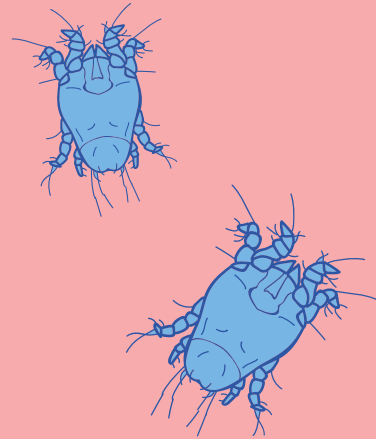
Notch signaling was not only described to induce differentiation of Th2 cells, but also for the differentiation of multiple T helper cell subsets (as described in detail in **Chapters 1 and 8**). These include Th1 cells, since the Th1 genes *Ifng* and *Tbx21* were identified as direct Notch targets^{135, 136}. Moreover, Notch signaling cooperates with TGF- β to induce Th9 cell differentiation and IL-9 expression¹³⁸ and the promoter regions of the Th17 cell-associated *Rorc*, *Il17* and *Il23r* genes are identified as direct Notch targets¹³⁹⁻¹⁴³. 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-RBPJ κ 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.

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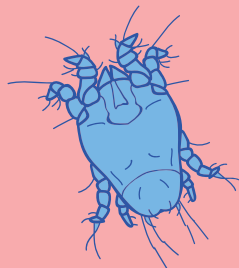
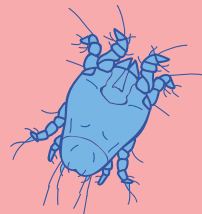
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English | summary



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 J κ region (RBPJ κ). 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 RBPJ κ 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, RBPJ κ -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 RBPJK 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.

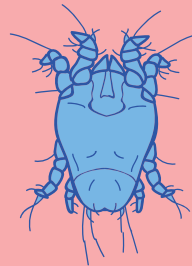
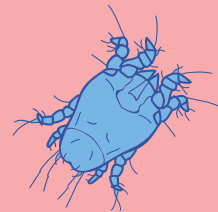
We have shown that mice lacking RBPJK or the Notch1 and Notch2 receptors or mice that were treated with the inhibitory peptide SAHM1 to target the Notch transactivation complex failed to generate Th2 responses or eosinophilia in acute and chronic HDM-driven AAI mouse models [AAI] [Chapter 3-5]. In contrast, 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 RBPJK. Wij hebben onderzocht wat de functie is van Notch signalering tijdens het ontstaan van astma door muizen te genereren die specifiek de RBPJK 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 RBPJK 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 verschillende 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 celoppervlak 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 RBPJK, 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 RBPJK 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**).

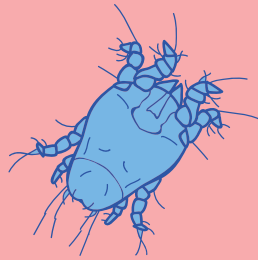
In hoofdstuk 3 tot en met 5 hebben we aangetoond dat muizen die Notch receptoren of de Notch-geassocieerde transcriptiefactor RBPJK missen in hun T cellen of muizen die behandeld worden met

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

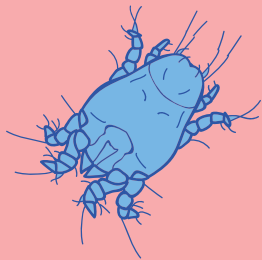


AAI	Allergic airway inflammation
ACQ	Asthma control questionnaire
ADAM	A disintegrin and metalloproteinases
AHR	Airway hyperreactivity
AGM	Aorta-gonad-mesonephros
APC	Antigen presenting cell
BAL	Bronchoalveolar lavage
BATF3	Basic Leucine Zipper ATF-Like Transcription Factor 3
BCL	B-cell lymphoma protein
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 acetyltransferase
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

Nfi3	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
RBPjk	Recombination-signal-binding protein for immunoglobulin JK region
RELB	V-rel avian reticuloendotheliosis viral oncogene homolog B
RNA	Ribonucleic acid
Roryt	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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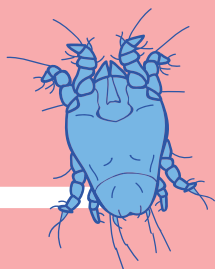
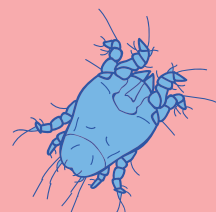
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Dankwoord



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Beste Alex, ik wil je bedanken voor alles wat je hebt opgezet als basis voor dit project. Onze samenwerking heeft tot nu toe al geleid tot 2 ontzettend mooie papers met hopelijk nog eentje onderweg. Bedankt voor een hoop wijze woorden en ga het vooral missen om even een "bakkie" te doen. Lieve Marjolein, ik vind het fantastisch hoe jij altijd voor iedereen klaar staat. Bedankt voor je hulp tijdens experimenten, sorts, PCRs,

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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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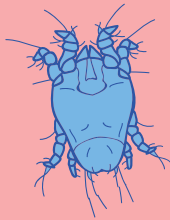
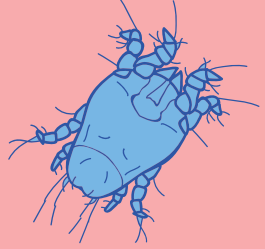
Beste Saskia, bedankt voor de layout en cover van mijn proefschrift, ik ben heel blij met het resultaat!

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]**

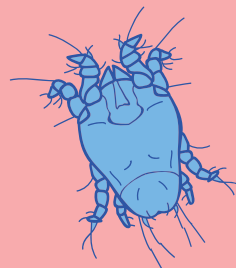
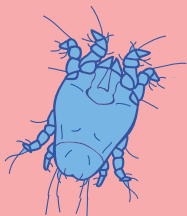
Teaching:

- 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

Scholarships, grants and prizes:

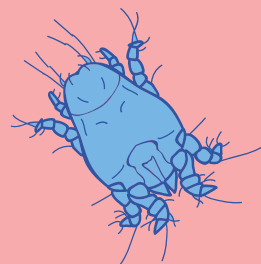
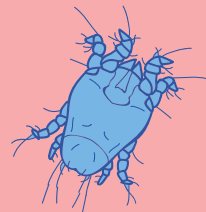
- 2014 Travelgrant EAACI Winter School
- 2014 NRS travelgrant to attend the EMBO Conference on Lymphocyte Signalling, Bertinoro, Italy, €1250
- 2016 Travelgrant IFRc-SIgN Winter School
- 2016 NVVI travelgrant to attend the BSI / NVVI annual congress, Liverpool, UK, €200
- 2016 BSI's third-placed 'PhD 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 scholarship 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.



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T helper 2

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