Stage-dependent functions of GATA-3 in lymphocyte lineage determination and in type-2 immunity

Roeland Gerrit Joseph Klein Wolterink

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Erasmus MC Rotterdam, the Netherlands and the Innate Immunity Unit, Institut Pasteur, Paris, France.

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tijdens lymfoïde differentiatie en type-2 immuniteit

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Caractérisation des fonctions spécifiques de GATA-3 suivant les étapes de la différenciation des lignages lymphoïdes et dans l'immunité de type 2

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Immunologie

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Thèse dirigée par
M. James DI SANTO / M. Rudolf HENDRIKS

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M. Shigeo KOYASU, Rapporteur
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Chapter I

Introduction

A part this chapter was published as a review entitled "The intrathymic crossroads of T and NK cell differentiation" in Immunological Reviews (Klein Wolterink et al., 2010).

Another part of this chapter was published as a review entitled "Type two innate lymphocytes in allergic airway inflammation" in Current Allergy and Asthma Reports (Klein Wolterink and Hendriks, 2013).

Transcription factors control cell development and differentiation

All eukaryotic cells of the body carry the same DNA. By using specific sets of genes, cells are able to develop into a wide variety of cell types with specific functions (e.g. muscle, neuronal or immune cells) using the same genetic information. Transcription factors regulate which genes are turned 'on', and which genes are 'off', allowing the controlled expression of specific genes at specific times and in a specific cell type. A transcription factor (TF) is a protein that binds to a specific DNA-sequence to control the transcription of DNA to mRNA. TFs typically function in a complex with other TFs and accessory proteins that may not have DNA-binding capacity. Some TFs mainly act as activators (e.g. by recruiting RNA polymerase), while others block gene transcription. One TF can carry out both functions, depending on the context: it may be a repressor at one locus and have activating functions at another. The function of a TF can also depend on the cell type in which it acts and on the developmental stage of a cell; e.g. one TF can have a different function in a stem cell than in a fully differentiated cell.

GATA transcription factors in the hematopoietic system

Every day, the human body generates 1011-1012 new blood cells in order to maintain the normal hemostatic functioning of the hematopoietic system. The generation of blood cells is referred to as hematopoiesis and relies on hematopoietic stem cells (HSC) that reside in the bone marrow. When HSC divide, they give rise to one daughter cell that can give rise to all hematopoietic cells (e.g. red blood cells or erythrocytes, lymphocytes and granulocytes) while the other daughter cell remains as HSC. This asymmetric self-renewal division is crucial to maintain a life-long pool of stem cells.

While primitive hematopoiesis starts in the mesoderm of the visceral yolk sac, definitive hematopoiesis commences in the para-aortic splancho-pleura (P-Sp) and aorta-gonad-mesonephros (AGM) (1). Later in fetal development, hematopoiesis continues in the fetal liver and eventually moves to the bone marrow where it will be maintained life-long. Hematopoiesis is tightly controlled and a hierarchical network directs the development of specific hematopoietic lineages.

GATA is one of the TF families involved in hematopoiesis and the expression patterns of GATA TFs are highly conserved among species. The GATA family of zinc-finger TFs consists of 6 members that all bind the consensus WGATAR sequence. This family can be divided in two subfamilies, based on expression profile: GATA-1, GATA-2 and GATA-3 are primarily expressed in the hematopoietic and neural system, while GATA-4, GATA-5 and GATA-6 are primarily used by the cardiac, pulmonary and digestive system [reviewed by Shimizu and Yamamoto, (2)]. The 'hematopoietic GATA factors' (GATA-1, GATA-2 and

GATA-3) contain two first exons that are employed differentially by hematopoietic and non-hematopoietic tissues, and encode a 5' untranslated region (UTR). These two exons are followed by five translated exons (3–5) that encode the functional domains of the GATA proteins. Only the role of the hematopoietic GATA factors in hematopoiesis will be considered here **(Figure 1)**.

GATA-1 and GATA-2

The gene encoding GATA-1 lies on the X chromosome and is involved in terminal maturation of erythrocytes and megakaryocytes (6,7). Furthermore, GATA-1 is required for the early stages of eosinophil differentiation (8) and late stage mast cell development (9). GATA-1 expression is largely restricted to the hematopoietic lineage: erythrocytes, megakaryocytes, eosinophils and mast cells are known GATA-1 expressing cells. The only cell type outside the hematopoietic system reported to express GATA-1 are Sertoli cells (3).

GATA-2 is indispensable for efficient hematopoiesis. It is expressed by stem cells and fulfills at least two functions. First, in embryonic hematopoiesis, GATA-2 is required for the production and expansion of the first HSC in the AGM region. Secondly, competitive transplantation assays using haplo-sufficient stem cells showed that GATA-2 is important for the proliferation of HSC (10).

Several groups have demonstrated that levels of GATA-1 and GATA-2 need to be narrowly regulated for efficient hematopoiesis. Overexpression of GATA-1 in myeloid progenitors diverts cells to the megakaryocyte lineage (6,7). In another study, GATA-1 overexpression under control of the globin locus completely blocked erythropoiesis, leading to lethal anemia (11). Forced expression of GATA-2 in erythroid precursors stimulates proliferation, but completely blocks development (12,13).

GATA-1 and GATA-2 often inhibit each other's function and are known to be able to recognize their own binding sites, but also those of family members (14,15). The phenomenon of one GATA factor influencing the expression of another family member by direct interaction with it partner's binding site has been called 'GATA switch' (16,17). In erythrocytes, GATA-2 is expressed at early stages until the erythroid colony-forming stage (CFU-E) stage. Until that stage, GATA-2 occupies multiple regulatory regions at its own locus. Upon GATA-1 induction, GATA-1 displaces GATA-2 at those binding sites and induces the repression of GATA-2 (17).

GATA-3

The third hematopoietic GATA factor, GATA-3, is expressed by HSC, T lymphocytes and NK cells. It is also expressed during embryonic and adult stages by the nervous system, adrenal glands, kidney, inner ear, hair follicles, skin and in mammary gland (18–25). Several functions of GATA-3 in T cell development have been studied in detail. It was shown to play an essential role at several stages of thymocyte development and in T helper 2 (Th2) differentiation [reviewed by Ho et al., (26)].

T cell development is a tightly-controlled stepwise program

T lymphocytes are part of the mammalian immune system and are crucial for the defense against hazardous pathogens, such as bacteria, viruses, parasites and fungi. These cells recognize antigens via the T cell receptor (TCR) and respond by clonal expansion and cellular differentiation, thereby providing lifelong protection against reinfection by the same pathogen.

In contrast to other hematopoietic lineages that are generated in the bone marrow, T cell production primarily takes place in the thymus as a dedicated organ for their development. Extrathymic T cell development has also been described (27–29). After mature T cells egress from the thymus, they retain the capacity to further specialize in response to the environment (e.g. through antigen recognition) or to ligand/receptor interactions (e.g. via cytokines stimulation). These interactions trigger differentiation programs that are tightly controlled by key regulators. In this Introduction, we will summarize our current knowledge about T lymphocyte lineage generation with a focus on GATA-3, which is essential at multiple stages of T cell development, during commitment, β-selection, $CD4$ development and Th2 differentiation **(Figure 2)**. As little is known about the role of GATA-3 in T cell commitment, we will discuss the early steps of T cell development and the pathways involved in more detail.

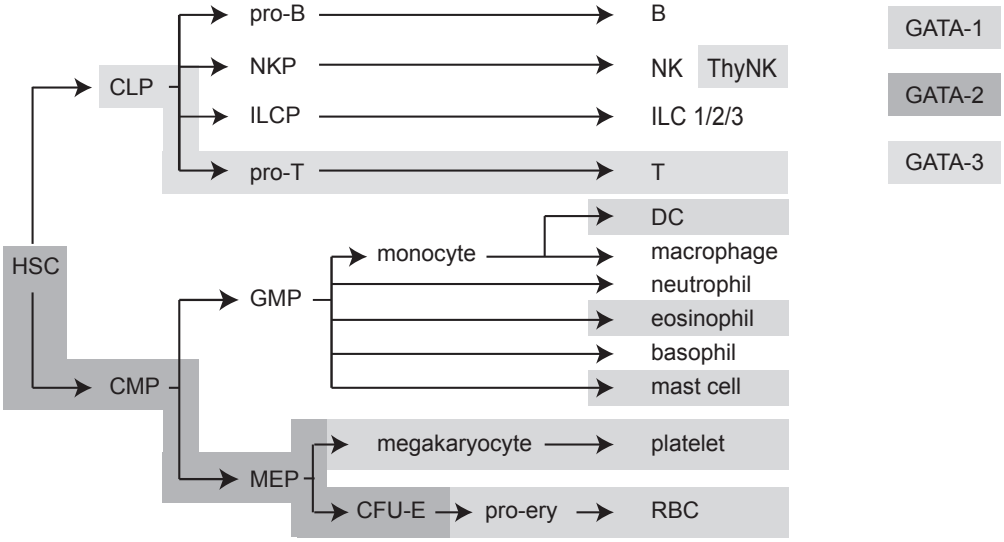


Figure 1. GATA transcription factors in hematopoiesis *(full color version: page 229)*
This schematic overview shows the role of GATA transcription factors at various stages of hematopoiesis. Hematopoietic stem cells (HSC) are multipotent cells that can give rise to all lymphoid lineages via the common lymphoid precursor (CLP) and all myeloid lineages via the common myeloid precursor (CMP) (31,32,360). Other models that propose intermediate precursors between the HSC and lymphoid/myeloid-restricted precursor have also been reported (38,361). CLPs give rise to B and T lymphocytes and NK killer cells (NK), including a subset of thymic NK cells (ThyNK) that are GATA-3 dependent. Recently, CLP were also found to be precursors for innate lymphoid cells (ILC) that possibly share a common ILC precursor (ILCP). The role of GATA factors in ILC development is currently under investigation. Erythrocytes (or red blood cells; RBC) and platelets develop from a megakaryocytic-erythroid precursor (MEP) via colony forming units (CFU-E) and megakaryocytes respectively. Granulocytes, mast cells and monocytes stem from granulocyte/monocyte precursor (GMP). Monocytes can further differentiate into macrophages and dendritic cells (DC). Shaded areas show stage-dependent requirement of the indicated GATA factors.

Early T cell development in the thymus

Based on 'The intrathymic crossroads of T and NK cell differentiation', *Immunol Rev* 2010

Thymopoiesis relies on stem cells that initiate and commit to the T cell lineage in a step-wise program

T lymphocytes are a unique type of blood cells, as they require the thymus as a dedicated organ for their development. Although T cell development has been described outside the thymus (28–30), it is the thymic microenvironment that primarily supports early T cell development and guides the process of thymocyte positive and negative selection.

Thymopoiesis relies on a constant influx of hematopoietic stem cell (HSC)-derived progenitors from the bone marrow (BM). It is generally agreed that BM-resident HSC (defined as $Lin^-Thy1.1^{lo}Sca\text{-}1^+c\text{-}Kit^+Flt3^-$) give rise to multipotent progenitors (MPPs) that have lost their self-renewal capacity before differentiating into more largely restricted common myeloid progenitors (CMP; defined as $Lin^-Sca\text{-}1^-c\text{-}Kit^+IL\text{-}7R\alpha^-$) (31) and common lymphoid progenitors (CLP; defined as $Lin^-Sca\text{-}1^{lo}c\text{-}Kit^{lo}IL\text{-}7R\alpha^+$) (32). Earlier work has shown that early thymic progenitors (ETP; $Lin^-Sca\text{-}1^+IL\text{-}7R\alpha^-CD25^-c\text{-}Kit^+$), in addition to harboring T and NK cell potential, possess some B lymphocyte potential (33). This led to early models proposing that BM-derived CLP can seed the thymus and subsequently generate ETP (32,34,35). More recent studies, however, revealed that thymic immigrants still possess myeloid potential (36,37), leading to a new model in which the thymus is seeded by lymphoid-myeloid progenitors (LMPs; $Lin^-Sca\text{-}1^+c\text{-}Kit^+Flt3^+$) with developmental potentials for myeloid, B, T and NK cell lineages (38–40). Given that more than one precursor may seed the thymus, the precise identity of the HSC-derived progenitor(s) that generate ETPs and their downstream progeny remains a subject of debate, but clearly involves cells with developmental potentials for other (non-T cell) lineages.

The phenotype and developmental potential of intrathymic ETPs has been studied extensively in human and mouse. Obviously, T cell differentiation potential is dominant in different subpopulations studied in the early thymic compartment, although potential for myeloid and other lymphoid lineages (B, NK) is also present (reviewed in (40)). Concerning B cell potential, the murine thymus exports approximately 2×10^4 mature B cells to the periphery each day, whereas more than 1.2×10^6 αβ T cells egress from the thymus daily (41). As the majority of ETPs lack B potential (42–44), it is unclear how (or where) these B cell are generated. Early studies identified a bi-potent T/NK-committed progenitor in the murine thymus, characterized by expression of the natural killer marker NK1.1 (45–47). The corresponding human T/NK bi-potent thymic progenitor was also described (48,49). These T/NK precursors lacked appreciable B cell and myeloid cell potential. While it is generally accepted that the BM is the primary site for NK cell development (50,51), the discovery of bi-potent T/NK precursors suggests the possibility of a distinct developmental pathway

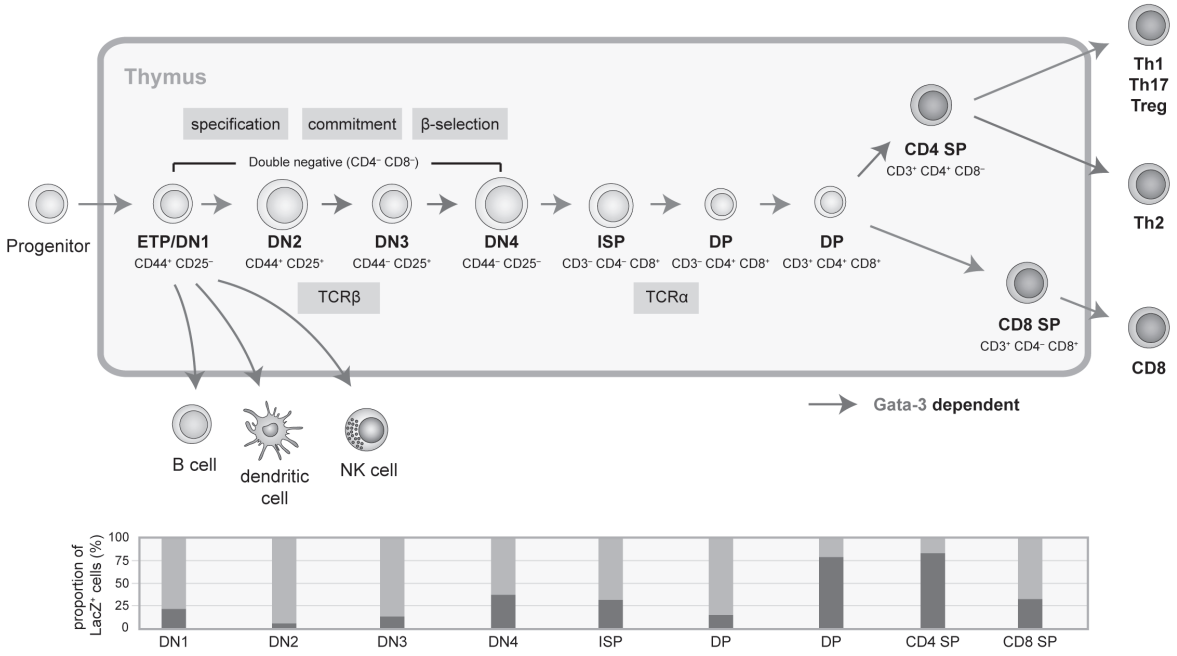


Figure 2. Overview of T cell development and GATA-3 expression in developing thymocytes. *(full color version: page 230)*
T cell development starts with a multipotent progenitor that enters the thymus. During four $CD4^-$ $CD8^-$ double negative (DN) phases, early thymocytes subsequently initiate the T cell program (specification), exclude all other fates (commitment) and test their TCRβ chain for functionality after rearrangement (β-selection). DN cells then acquire CD8 to become immature single positive (ISP) cells and will start re-arrangement of the TCRβ chain. This process is completed at the double positive (DP) stage at which cells now express both CD4 and CD8. CD4- or CD8-committed cells lose the expression of the other co-receptor and become single positive (SP) cells that egress from the thymus. CD4 T cells can further differentiate into one of the T helper (Th) lineages, characterized by production of a specific set of cytokines. GATA-3 is one of the many transcription factors that steer T cell development. The various stages at which GATA-3 is crucially involved are shown with red arrows. Using a Gata3-lacZ knock-in mouse ($Gata3^{lacZ/+}$) and FDG substrate (146), the expression levels of GATA-3 in various T cell subsets were analyzed and are shown in the lower panel.

for NK cells in the thymus that could share properties with T cell development (52). Along these lines, a recent report demonstrated some T cell potential in the previously described population of BM-resident NK cell precursors (53,54).

The existence of an evolutionarily conserved bi-potent T/NK progenitor in the thymus demonstrates that the thymic microenvironment is permissive for and can selectively foster the development of T lymphocytes and thymic NK cells. Lineage choice depends on two crucial mechanisms: the initiation of a developmental program for a certain lineage ("specification") and the final exclusion of any other fate ("commitment"). These processes involve a cascade of irreversible checkpoints that are required to shape the identity of the resultant lymphoid cell subset. Changes in gene expression, developmental potential and proliferation levels mark these phases, and this process is well-described in the thymus. During the initial stages of development, T cell progenitors lack expression of CD4 and CD8 co-receptors and are therefore termed 'double negative (DN)'. DN thymocytes differentially express CD44 and CD25 surface makers (55). ETP have the $CD44^+$ $CD25^-$ phenotype

(DN1 stage) and acquire CD25 as they mature to become $CD44^+$ $CD25^+$ DN2 cells. Both of these early thymocyte precursor stages show high levels of proliferation that are driven by external signals derived from growth factors, including stem cell factor and interleukin (IL)-7. Efficient T cell progenitors are found among DN1 cells bearing the corresponding receptor (c-Kit^+); this fraction is a very minor population (0.01%) of the young adult mouse thymus (44,56). The DN2 population is further enriched with T cell progenitors (57) and can be subdivided into a c-Kit^{high} DN2a population that maintains a potential to develop into DCs, mast cells, monocytes and NK cells (42,58–60), while the c-Kit^{low} DN2b population has extinguished DC and mast cell potential (61). Early thymocytes that reach the DN3 stage have down-regulated CD44 expression and are fully committed to the T cell lineage. While TCRβ D-J rearrangements may start before this stage (62), DN3 cells arrest their growth to allow for TCR rearrangement. Successful in-frame TCRβ rearrangements lead to expression of a functional pre-TCR, composed of the invariant pre-Tα chain, the TCRβ chain and the associated CD3 complex. Signals from the pre-TCR (β-selection) drive further thymocyte differentiation to the double-positive stage (19).

The cell surface phenotype of the aforementioned bi-potent T/NK progenitor ($NK1.1^+CD117^+CD44^+CD25^+$) strongly suggested that this population was contained amongst the DN2 subset of early thymocytes (57). DN2 cells uniformly express IL-7Rα (63) and a subset of the bi-potent T/NK precursors express IL-2Rβ (57). It is not clear whether IL-2Rβ expression varies amongst DN2a versus DN2b thymocyte subsets (64) or whether the T, NK or bi-potent T/NK potential of cells bearing IL-2Rβ differs from those lacking this cytokine receptor chain.

To acquire a T cell identity, thymocyte progenitors must first switch off other cell fates. The early stages of thymocyte development are marked by dramatic changes in gene

Table 1. Influence of selected cytokine and transcription factors on intrathymic T and NK cell development

	T	NK	Thymic NK	References
IL-7	essential	not required	essential	(64,65,72,80,126,358)
IL-15	not required	essential	essential	(80,83,84,91,126)
Notch	essential	not required	not required	(94,103,104,107)
Gata3	essential	not required	essential	(80,105,110,111,116)
Id2	not required, redundancy with other Id factors?	essential	essential	(121,124,125,127,128)
Nfil3/E4bp4	not required	essential	ND	(129,130)
Bcl11b	essential	limits development ?	ND	(135–137)

ND = not determined

expression and include key regulators of lymphocyte development. The complex interplay between external signals (cell-cell interactions, soluble factors of the microenvironment) and intrinsic signals (via transcription factors) determines lineage choice. The thymus creates a unique environment that strongly supports T cell development, but is also permissive for development of other cell types. In the next section, we will consider some of the extrinsic and intrinsic regulators that condition the choice between T cell and NK cell development in the thymus from T/NK bi-potent precursors **(Table 1)**.

Regulators of the T and NK cell fate determination in the thymus

Interleukin-7

Interleukin-7 (IL-7) is an essential cytokine for T cell development in both mice and man [(65,66) and **Figure 3**]. IL-7 is produced by epithelial stromal cells in the thymus and bone marrow (67,68) and by fibroblastic reticular cells in the lymph node (69).

IL-7 signals through the heterodimeric IL-7 receptor (IL-7R) composed of the IL-7Rα chain and the associated common cytokine receptor gamma chain (γc) (reviewed in (70)). The IL-7R is expressed by early lymphoid precursors (including CLP, pro-B, pro-T and most naïve mature T cells) and therefore plays a critical role in the homeostasis of developing as well as mature T lymphocytes. IL-7 signaling impacts via several intracellular biochemical pathways. Activation of Janus kinases (JAK)-1 and -3 lead to subsequent phosphorylation and dimerization of signal transducer and activator of transcription 5 (STAT5) members that control gene expression. Parallel stimulation of phosphoinoside-3 kinases (PI3Ks), as well as Ras and mitogen-activated protein kinase (MAPK) pathways also impinge on gene transcription and survival (reviewed in (71)).

Loss of IL-7 function in mice leads to a strong decrease in thymic cellularity, although TCRαβ cells (but not TCRγδ cells) are produced and enter the peripheral lymphoid tissues (45,46). Interestingly, *Il7*- or *Il7ra*-deficient mice show a relative increase of DN1 T cell precursors (65,72), suggesting that homeostasis of post-DN1 thymocytes depend on IL-7 signals that mediate both survival and proliferative effects (66,73,74). IL-7Rα is expressed during the DN1 and DN2 stage of T cell development, but is sharply down-regulated when cells progress to the DN3 stage (63). This expression pattern is consistent with a model whereby IL-7-driven DN1/DN2 proliferation generates the cellular substrate for TCR rearrangements that take place at the non-proliferating DN3 stage. This state of IL-7 non-responsiveness persists at the double positive stage but then IL-7 re-emerges as an important factor during the cell-fate decision at the later CD4 or CD8 single positive stage, as signals through IL-7Rα have been proposed to promote CD8 T cell differentiation (75–77). Finally, IL-7 maintains naïve T cells in the periphery and can contribute to 'homeostatic' expansion and proliferation of peripheral T cells following insults that cause lymphopenia

(78), reviewed in (79).

BM NK cell development proceeds normally in the absence of IL-7 (80) and numbers and function of peripheral NK cell numbers in IL-7-deficient mice are unperturbed (72). In contrast, NK cells in the thymus appear highly dependent on IL-7 and this is correlated with a predominant expression of IL-7Rα on this subset of NK cells (80). As such, thymic NK cells, but not BM-derived NK cells, resemble T cells in their homeostatic requirement for IL-7. Additional similarities between thymic NK cells and T cells include their developmental requirement for GATA-3 (see below).

Considering their IL-7 dependency, one would predict that DN2 (and to a lesser extent DN1 cells) and thymic NK cells would localize in proximity to IL-7-producing thymic stromal cells. Using an IL-7 reporter mouse, IL-7-expressing MHC class II+ thymic epithelial cells (TECs) could be identified (81). The majority of these IL-7$^+$ TECs expressed cortical markers and were localized as a band of cells at the cortico-medullary junction (CMJ). Petrie and Zúñiga-Pflücker reviewed the distinct microenvironments in the thymus, correlating the location of different thymocyte populations throughout development (82). ETPs enter the thymus at the CMJ, where DN1 and DN2 cells proliferate before migrating through the cortex. When T cell commitment is completed at the DN3 stage, the thymocytes are found in the sub-capsular zone where IL-7 expression is lowest. Although ETPs do not yet express IL-7Rα, its expression is increased as cells progress to the DN2 stage and gradually decreases as cells progress further during development. Interestingly, thymic NK cells have been found primarily at the CMJ (J. Di Santo, unpublished observations). Taken together, these findings suggest that IL-7 availability conditions not only early thymocyte survival and proliferation, but also plays a role in development and/or maintenance of NK cells within the thymus.

Interleukin-15

Interleukin-15 (IL-15) was first discovered as an IL-2-like cytokine that promotes T cell activation in vitro (83,84). The molecular basis underlying the biological similarities between IL-2 and IL-15 can be explained by fact that the receptors for these cytokines share multiple components, including the IL-2Rβ and γc chains (70,85). Nevertheless, these structurally related cytokines have unique biological roles in vivo, in part due to the expression and function of their corresponding IL-2Rα and IL-15Rα chains (86,87). Studies using mice deficient for IL-2Rα and IL-2Rβ suggested that IL-2 is required for regulation of peripheral T lymphocyte activation (via regulatory T cells), whereas IL-15 is a critically required for development and survival of NK cells (88,89), as well as other innate T lymphocytes **(Figure 3** and reviewed in (90)).

The essential role for IL-15 in BM NK cell development begins with the triggering of IL-2Rβ chains expressed by NK cell progenitors (NKP) (53). NKP are Lin$^-$ progenitors that lack most cell surface receptors expressed by peripheral NK cells (including NK1.1 and

DX5) and upon IL-15 stimulation, proliferate and differentiate to generate phenotypically mature, and fully functional NK cells. IL-15 is therefore important for the development and maintenance of immature and mature NK cells, but not for the generation of NKPs (80). This last point is important as it indicates that other non-γc-dependent signals control NK cell lineage commitment.

IL-15 also plays an essential role in the development of thymic NK cells (80,91). As thymic NK cell development also requires IL-7 (80), unique and/or redundant roles for IL-7 and IL-15 in the development, differentiation and maintenance of this unusual NK cell subset can be envisaged. The observation that both IL-7 and IL-15 are essential for NK thymopoiesis, indicates that the signals delivered by these cytokines cannot compensate for each other. Both IL-7 and IL-15 can promote cell survival by enhancing expression of anti-apoptotic members of the Bcl2 family [**Figure 3** and (73,89)]. Perhaps thymic NK cells receive limiting survival signals from each of these two cytokines and survival can only be ensured if both cytokines are present. An alternative explanation is that IL-7 and IL-15 act at distinct but sequential stages in thymic NK cell development. One probable scenario would include an important role for IL-7 in the development and/or maintenance of ETPs or T/NK bi-potent cells (that contain NK cell precursors), while IL-15 would act in a fashion similar to that of BM NK cell development, promoting survival, proliferation and differentiation of committed NKP once they have been fully specified. It is interesting to recall that before the identification of the bi-potent T/NK progenitor, it was shown that cytokines influenced cell fate decisions in this precursor population with the levels of IL-2 and IL-15 controlling whether IL-2Rβ$^+$ TCR$^-$ cells develop into T or NK cells (86). As such, intrathymic IL-15 availability would dominantly promote (and control) thymic NK cell development, but might not impact on the initial T versus NK cell fate decision at the level of the T/NK bi-potent progenitor. This notion is also consistent with the lack of any obvious defects in early thymopoiesis in IL-15-deficient mice.

Notch1

Notch1 is a transmembrane signaling receptor required at multiple stages of T cell development. Notch1 signaling is essential for the initiation of T cell development in the thymus, as Notch1-deficiency completely ablates further T cell development from thymic ETPs (92,93). Moreover, Notch1 is critical for suppression of B cell fate from thymic progenitors, as Notch1 deficiency leads to enhanced thymic B cell development. Ectopic activation of Notch1 signaling in hematopoietic progenitors resulted in emergence of T lymphocyte development at the expense of B cell development in the bone marrow and was independent of the thymic microenvironment (94). Furthermore, Notch1 signaling influences later stages of T cell development, including the choice between αβ and γδ T cell fate (95), between CD4 and CD8 T cell fate (96) and between Th1 versus Th2 cell fates (97).

In mammals, Notch signaling is activated by binding to one of four known ligands:

Jagged1 (98), Jagged2 (99), Delta-like-1 (Dll1) or Delta-like-4 (Dll4) (99,100). Ligand binding leads to a series of proteolytic cleavages of the Notch molecule, resulting in the membrane release and transport of the intracellular Notch domain to the nucleus where it behaves as a transcriptional activator (reviewed in (101)). While in vitro studies indicated that both Dll1 and Dll4 promoted Notch1-dependent T cell development (102), thymic epithelial cells (TECs) only express Dll4 (103,104). Furthermore, inactivation of Dll4 expression in TECs leads to a complete block in T cell development (105) indicating that Dll4 is the essential Notch1 ligand provided by the thymic microenvironment for initiation of T cell development. This strengthens the view that the thymus creates a special niche that provides Notch ligands, guiding hematopoietic progenitors towards the initiation of the T cell developmental program.

Does Notch1 signaling impact on NK cell development in the thymus? Notch1 is expressed by ETP and triggering of this receptor plays a critical role in extinguishing B cell fate in this precursor population. Given the presence of thymic NK cells and the abundance of Dll4 expressed by cortical TEC (including those at the CMJ; (103)), Notch1 signals must either be neutral for development of thymic NK cells or alternatively a fraction of ETPs must develop into thymic NK cells before encountering a Dll4-expressing TEC. Bhandoola and colleagues showed that Notch signals are required for the generation of ETPs, whereas the population of phenotypically identical $Lin^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$ cells in the BM and in the circulation was not affected (106). Notch1 deletion in HSC does not significantly impact subsequent BM NK cell development or the overall numbers, phenotype or function of splenic NK cells (107). Recently, the effect of Notch1 deletion on thymic NK cell development was investigated (108). Whereas *Notch1*-deficiency completed abrogated ETP and subsequent T cell development as expected (92–94), the number of thymic NK cells that developed in the absence of Notch1 was normal. Collectively, these results indicate that Notch1 signals neither inhibit nor promote NK cell development in the thymus.

GATA-3

Germ-line *Gata3*-deficiency in mice leads to lethality by embryonic day 11.5 (109). Hematopoietic chimeric mouse models generated using *Gata3*-deficient FL precursors provided important insights in the role of GATA-3 in lymphocyte development. GATA-3 plays critical roles in the development of T and thymic NK cells, as *Gata3*-deficient hematopoietic progenitors fail to generate these cells. Concerning T cell development, few cells progress past the DN2 stage (105,110,111), and using conditional gene ablation techniques, GATA-3 was also shown to be required at later stages of T cell development, including β-selection (112), the generation of $CD4^{+}$ SP thymocytes (112) and for $CD4^{+}$ T-helper-2 cell (Th2) differentiation (113,114). GATA-3 dependency correlates with expression levels that are highest in DN2 and DN3, diminish in DP and rises again during generation of CD4 SPs and Th2 cells [(110) and **Figure 2**].

Although GATA-3 is essential for T cell development, overexpression of GATA-3 in T cell progenitors completely blocks T cell development and diverts their development towards the mast cell lineage (115). In this case, Notch acts as an antagonist of GATA-3 instead of as a collaborator in T cell development, demonstrating that transcription factors can have multiple effects, based on tightly regulated expression levels. GATA-3 is essential for development for thymic NK cells that express IL-7Rα and seed peripheral lymph nodes (80), while GATA-3 facilitates NK cell maturation (promoting IFN-γ production) and homing of liver-resident NK cells (116).

Both Notch1 and GATA-3 are involved in T cell specification, although it remains unclear whether they play specific, redundant or synergistic roles in this process. Initial work by Hoflinger and colleagues using *Pax5*-deficient B cell progenitors cultured on Dll1-expressing stroma, showed that GATA-3 expression was up-regulated after 24 hours of culture in a Notch1-dependant fashion (117). Flavell and colleagues demonstrated that Notch1 could directly regulate the expression of *Gata3* in developing Th2 cells through direct binding of RBP-Jκ to the *Gata3* promoter (97). Together, these studies suggest that Notch1 signals might directly control *Gata3* expression in the murine T cell lineage. Interestingly, studies using human thymocyte precursors did not find *GATA3* as a Notch1 target (118,119).

Id2

Inhibitor of DNA binding 2 (Id2) is one of four related helix-loop-helix (HLH) proteins that inhibit transcriptional activity of basic HLH E-box transcription factors, such as E2A, E2-2 and HEB (reviewed in (120)). The balance between E-box and Id proteins clearly influences cell fate decisions within the hematopoietic system: for example, *Id2* or *Id3* overexpression efficiently inhibits B and T cell developmental programs from hematopoietic precursors (121), whereas E2A-deficiency results in a B cell developmental arrest (122). In contrast, Id proteins favor generation of NK cells under these conditions and Id2 is essential for BM NK cell development in vivo (123).

The role of Id2 in the generation of thymic NK cells is not fully defined. That Id2 plays an important role in NK cell lineage commitment from thymic bi-potent T/NK cell precursors is evidenced in the mouse by the fact that early thymocytes can be directed to the NK lineage by ectopic expression of *Id2* and that *Id2*-deficiency appears to eliminate thymic NK cell precursors (124). Nevertheless, the residual population of NK cells that has been documented in the spleens of *Id2*-deficient mice bears IL-7Rα (125). As thymic NK cells express IL-7R (80), this led Boos and colleagues to propose that these few NK cells in *Id2*-deficient mice were the product of a thymic pathway of NK cell development that was Id2-independent (108). While this remains possible, we have observed that thymic NK cells are strongly decreased in the absence of *Id2* and that the athymic (*Foxn1*-deficient) nude mice reconstituted with *Id2*-deficient BM HSC can still generate a a similar small population of IL-7Rα$^+$ splenic NK cells (Hasan et al, unpublished observations). Interestingly, immature BM

NK precursors (that normally express low levels of IL-7Rα; (126)) appear increased in the absence of *Id2* (Hasan et al., unpublished observations) and may contribute to this unusual population found in the spleens of *Id2*-deficient mice.

Recent observations in human NK cells demonstrated that Id2 expression in $CD34^+$ $CD1a^-$ T/NK cell precursors inhibited T cell development, and dramatically increased NK cell development 45-fold. The Id2-induced NK developmental potential could be further enhanced by addition of IL-15 (127). These results suggest a model whereby Id2 plays a critical role in the cell fate decision that generates NK cells from bi-potent T/NK precursors that is then sustained and promoted by intrathymic IL-15.

Nfil3

Nfil3 (nuclear factor interleukin-3 regulated), also known as E4bp4 (E4-binding protein 4) is a basic leucine zipper (bZIP) transcriptional activator and was initially described for its ability to limit viral transcription and for promoting IL-3 mediated survival of pro-B cells (128). The involvement of bZIP proteins in the control of developmental processes, both in and outside the hematopoietic system, suggested that Nfil3 might also serve a role in lymphoid development. Although Nfil3-deficiency did not lead to gross hematopoietic abnormalities (erythroid, myeloid and T, B and NKT cell development appeared unaffected), three independent laboratories showed that Nfil3-deficient mice almost completely lacked mature NK cells (129–131). These observations identify Nfil3 as an essential transcription factor required for NK cell lineage development **(Figure 3)**. The NK cell defects caused by Nfil3 deficiency are cell-intrinsic, as they are recapitulated following transfer of Nfil-3-deficient HSC. In addition, retroviral transduction of Nfil3 into HSC promoted NK cell (129).

How does Nfil3 fit among the network of transcription factors and cytokines that have been implicated in NK cell development? As stated earlier, IL-15 is a crucial factor for NK cell development and survival (88), and the expression patterns of Nfil3 correlates with the dependency on IL-15 at the different stages of NK cell development (126): NFIL3 is detected in NKPs, is up-regulated in immature NK cells and is maintained in mature NK cells. Stimulation of progenitor cells with IL-15 did not induce NK cell development in the absence of Nfil3, however, ectopic expression of Nfil3 in *Il15ra*-deficient HSCs or in the presence of IL-15 blocking antibodies improved NK cell development, indicating that Nfil3 may function downstream of IL-15 (129). Gene expression analysis revealed that *Id2* and *Gata3* levels were reduced in the absence of Nfil3, while over-expression of *Nfil3* in *Nfil3*-deficient HSCs enhanced *Id2* and *Gata3* levels compared to non-transfected control cells. Moreover, retroviral transduction of *Id2* into *Nfil3*-deficient cells improved NK cell generation. Together these data suggest that Id2 and GATA-3 function downstream of Nfil3 (129).

A role for Nfil3 in the development of thymic NK cells is unknown. It is possible that the consequences *Nfil3*- and *Id2*-deficiency for thymic NK cell generation should largely overlap, as Nfil3 can influence *Id2* levels in the NK cell lineage (129). Still, development of

lymph nodes and Peyer's patches (that require Id2) appears normal in the absence of *Nfil3* (H. Brady, personal communication), indicating that the control of *Id2* expression by *Nfil3* might be cell-specific, or that other transcription factors can compensate for the loss of Nfil3 in the development of lymphoid tissues. Analysis of *Nfil3* expression in early thymocyte precursors and an assessment of thymic NK cell development in *Nfil3*-deficient mice should provide important information in this regard.

Bcl11b

Bcl11b and the related Bcl11a are Krüppel-like transcription repressors that play essential and opposing roles in lymphocyte development. Whereas Bcl11a has mainly been implicated in B cell development (132), Bcl11b plays a major role in early T cell development [(133) and **Figure 3**]. Bcl11b acts as a stimulator of TCRβ gene rearrangement, necessary for αβ but not γδ T cell development, and also plays an important role in early thymocyte survival and expansion via regulation of Bcl-X_L (133,134). Recent studies further characterized the role of Bcl11b in the stepwise differentiation of early thymocyte precursors (135–137). In the absence of *Bcl11b*, a profound developmental block at the $CD44^+CD25^+$ DN2 stage could be documented. Bcl11b-deficient DN2 cells appeared T-lineage specified as they expressed normal levels of *Gata3*, *Tcf7* and *Ets1* (137). In contrast, stem cell-like genes (*Tal1*, *Sfpi1*, *Lyl1*, *Gfi1b* and *Erg*) and genes that are normally associated with a NK cell identity (*Id2*, *Il2rb*, *Ncr1* and *Nfil3*) were de-repressed in *Bcl11b*-deficient DN2 cells. These results place Bcl11b as a major regulator of T cell identity in multipotent thymocyte progenitors that acts to extinguish alternative cell fates (including the NK cell lineage).

What signals regulate Bcl11b expression at the DN2 stage? DN2 cells can be maintained in vitro using immobilized Dll4 in the presence of IL-7, SCF and Flt3L (135) and retain potentials to develop into αβ T cells, NK cells, dendritic cells and macrophages. By simply reducing IL-7 levels, these DN2-dominated cultures demonstrate brisk and robust T cell differentiation. This observation suggested that IL-7 acts as a rheostat to regulate further T cell development. Ikawa and colleagues further showed that *Bcl11b* expression was inversely related to IL-7 levels, thereby linking this cytokine/transcription factor pair in control of T cell commitment (135).

The Liu laboratory reported that induced ablation of Bcl11b in either immature or mature T cells leads to the adoption of a NK cell phenotype (136). *Bcl11b* loss led to a decrease in the expression of T cell-related genes (*Gata3*, *Ets1*, *Tcf1*, *Hes1*) and a corresponding up-regulation of NK-associated genes (*Id2*, *Il2ra*, *Nfil3*). The resultant Induced T-to-Natural Killer (ITNK) cells expressed the NK cell marker NKp46 and lysed tumor cells in vivo and in vitro. Even mature $CD8^+$ T cells could be 'reprogrammed' to ITNKs by deletion of *Bcl11b*, suggesting that Bcl11b is represses 'NK cell-like' qualities, long after commitment to the T cell lineage.

These studies identify Bcl11b as a crucial factor for induction and maintenance of T

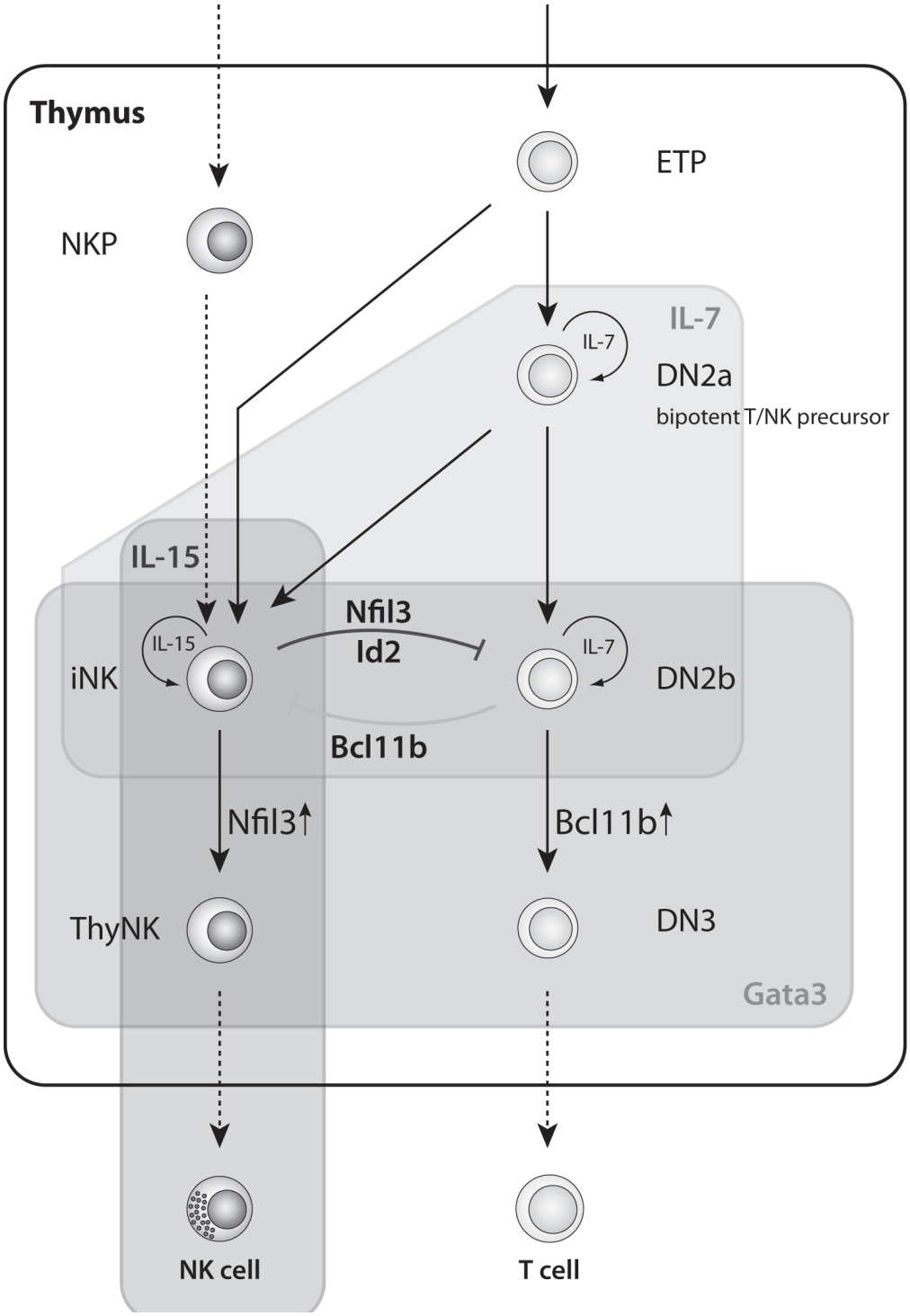
cell identity, and are an important contribution to our understanding of the T cell commitment. Although Notch1 signals are important for initiation of T cell specification, these signals alone cannot suppress B, NK or myeloid cell fates. Additional transcription factors are thus required to advance (and maintain) the T cell instruction program. These recent studies demonstrate that Bcl11b is essential to repress myeloid and NK cell potentials in DN2 cells. A sequential cascade of T cell specification and commitment emerges, whereby Notch1, GATA-3 and Bcl11b act in concert to extinguish stem cell, B cell, myeloid and NK cell potentials. The inter-relationships between these three transcription factors are not fully defined, although Notch signaling has been suggested to regulate both *GATA3* and *BCL11B* expression (136,138). Critical targets of GATA-3 (that are either activated or repressed) are not known, although *Bcl11b* now emerges as a potential candidate.

Integrating T/NK cell fate decisions during early thymopoiesis

The thymus is considered as the "cradle" for T cell development, since this organ provides the unique microenvironment that simultaneously promotes T cell development and suppresses many residual cell fates in the developing hematopoeitic precursors that enter into it. How is it then possible that NK cells (and to a lesser extent DC, myeloid cells and B cells) develop within this "hostile" environment? One simple model would propose that these non-T cell fates would result only upon failure to successfully complete the T cell program. Nevertheless, experimental evidence for signs of "T cell failure" (i.e. out of frame TCRβ chain VDJ rearrangements) is generally lacking in non-T cells, in the thymus or outside of it. We are therefore left with the model whereby early thymocyte progenitors may have an equally good chance of becoming a T cell or any one of the non-T cell fates. Let's examine such a model in more detail.

With the assumption that immature thymic progenitors have equal potential to develop into cells of any lineage, cell-cell interactions within the thymic microenvironment, together with the directed or stochastic expression of cytokine receptors, might provide the signals that then lead to changes in the expression of specific transcription factors (139,140). These transcription factors (acting alone or in concert) control gene expression and subsequent lineage specification, and at the same time the repression of genes from alternative cells fates. Ultimately, the successful commitment and differentiation to the T

Figure 3. T and NK cell development from early thymocyte progenitors (ETPs) (*right page; full color version: p231*) ETPs that seed the thymus differentiate progressively towards the T-cell lineage via phenotypically defined intermediates. DN2a cells retain T and NK cell potential and expand in response to IL-7 (IL-7 responsive cells are indicated by an orange region). The DN2b subset is also IL-7 responsive, but has lost NK potential in a process including the upregulation of Gata3 (indicated by a green region). Upregulation of *Bcl11b* coincides with loss of IL-7 responsiveness and marks the progression to the DN3 stage where progenitors are fully T-cell committed. Immature NK cells (iNK) can derive from several pathways, including direct differentiation from ETP, via DN2a or from NK cell precursors (NKP) that seed the thymus. Thymic NK (ThyNK) cell differentiation depends on Nfil3 and requires IL-15 (shown by a blue region). Bcl11b and Nfil3/Id2 mutually inhibit the NK cell and T-cell pathways, respectively.


Thymus
ETP
NKP
IL-7
IL-7
DN2a
bipotent T/NK precursor
IL-15
Nfil3
Id2
IL-15
IL-7
iNK
DN2b
Bcl11b
Nfil3↑
Bcl11b↑
ThyNK
DN3
Gata3
NK cell
T cell

cell lineage will depend on the interplay of several different transcription factors (including Notch1, GATA-3, Bcl11b and others) that are expressed at early stages of T cell development. Recent data demonstrate the necessity for continued expression of some of these factors to maintain "T cell identity", suggesting that permanent suppression of other cell fates (at least for the NK cell lineage) may operate even in mature T cells (136).

Initiation of T cell differentiation depends on Notch1 signaling. Without Notch1 signals, T cell precursors fail to become specified, and instead give rise to B cells (92). This function of Notch1 is mediated by the conversion of RBPSuh, a repressor by default, into an activator as a result of translocation of the intracellular domain of Notch to the nucleus (141,142). However, DN1 cells that have experienced Notch signals (for even extended periods) have not fully excluded their B cell potential (143), indicating that there is (at least) one additional factor necessary that acts in concert with Notch1 to extinguish B cell potential as cells progress to the DN2 stage (where B cell potential is completely suppressed, (64)).

The fact that DN2 cells also possess NK potential in various experimental systems further strengthens the heterogeneity within this developmental stage. Recent work has shed some light on this heterogeneity, as it was possible to associate the NK cell potential of this subset with a particular cell surface phenotype. Yui et al. demonstrated that the NK potential of DN2 cells selectively resides in the population of DN2 cells expressing high levels of CD117 (termed DN2a), while CD117lo cells (denoted DN2b) lack NK potential and are committed to the T cell lineage (64). Intuitively one would predict that DN2a cells are the precursors of DN2b cells, although this has yet to be experimentally demonstrated, leaving open the possibility that both subpopulations derive independently. Again, Bcl11b is likely playing a central role in this transition, as Bcl11b acts to repress NK cell lineage traits (136) and can also modulate CD117 (c-Kit) expression (along with other cytokine receptors including CD122 and CD127; (136)). Sustained Notch1 signaling at the DN2 stage may have a role in the transition to the DN2b stage as *Bcl11b* has been proposed as a direct Notch target (115).

All of the above-mentioned signals are clearly focusing the DN2 cells towards the T cell lineage. Despite this overwhelming T-cell-orienting influence, a subset of cells apparently manages to develop along the NK cell pathway. Here again, modulation in cytokine receptor and transcription factor expression likely play determinant roles. The transcription factor Runx3 is highly expressed in the DN1 subset and has been shown to control IL-2Rα expression in developing NK cells (144). A fraction of DN2 cells can apparently up-regulate IL-2Rβ expression, possibly due to sustained *Runx3* expression. Accordingly, these cells would become sensitive to locally produced IL-15 in the thymus. How could IL-15 signaling help specify the NK cell lineage program? *Nfil3* can be induced in hematopoietic precursors following exposure to IL-15 (129). Still, additional intrathymic signals would be necessary for commitment to the NK cell lineage, as Nfil3 is also expressed by non-NK cells (including DC and myeloid cells) and can be induced upon stimulation in B cells (130,131).

One possibility would involve Nfil3-induced Id2 expression (129). Moreover, Nfil3 may also be involved in regulating GATA-3 expression (145). As such up-regulation of Nfil3 might go a long way in selectively promoting intrathymic NK cell development through inhibiting B cell potential (via GATA-3 in concert with Notch1), and blockade of T (and B) cell potential through Id2-mediated squelching of E-box protein activity. In this model, Nfil3, Id2, and GATA-3 might act in concert to allow intrathymic NK cell development to occur.

Interestingly, *Bcl11b* deficiency leads to increased levels of Nfil3 in early thymocyte precursors, indicating that a cross-inhibitory regulatory loop might exist between these two factors **(Figure 3)**. Up-regulation of Bcl11b at the DN2b stage might inhibit Nfil3 and Id2 expression, resulting in the suppression of the NK fate, while up-regulation of Nfil3 (and subsequently Id2) might counter Bcl11b-induced T cell potential by suppressing E-box activity (136) and thereby promoting NK fate. The relative levels of Bcl11b versus Nfil3 in DN2a cells would therefore be major determinants of the T versus NK cell fate at this critical juncture. Interestingly, DN2a and DN2b cells express similar amounts of Bcl11b mRNA (64). While this might not necessarily translate into similar levels of Bcl11b protein in these populations, it could indicate that the promotion of the T cell fate and the suppression the NK cell fate might necessitate additional signals. However, once the T cell fate is established Bcl11b appears to be sufficient to assure this, as deletion of only Bcl11b in mature, peripheral T cells results in the conversion of these cells into NK cells (ITNK; (136)).

In line with this model in which relative expression levels of *Nfil3*/*Bcl11b* determine DN2 NK/T cell fate decisions, we found that Nfil3 was also expressed by all DN subsets (Vosshenrich et al., unpublished results). Although *Nfil3* levels are lower than in NK cells (including thymic NK cells), highest expression levels are found among the DN2 subset and decline during subsequent stages of T cell differentiation. A similar phenomenon may explain the expression of Bcl11b by a fraction of thymic NK (136) that might hint at their 'DN2 origins' and would be extinguished as these cells mature further.

In conclusion, much of the recent data appear to indicate a decisive split between T cell and thymic NK cell fate at the DN2 stage **(Figure 3)**. Does this imply that all thymic NK cells transit via the DN2 intermediates? We think that this is not likely to be the case, as thymic NK cells can develop normally in absence of Notch signaling that lack ETP and DN2 cells (108). Thus, the DN2 stage probably does not represent an obligatory stage in the development of thymic NK cells. Alternative sources for DN2-independent thymic NK cells include other BM-derived multi-potent hematopoietic precursors or NKP themselves. Elevated Nfil3 expression in any of these precursors would promote their NK potential given a sufficient amount of IL-15 in the tissue microenvironment. The proportion of thymic NK cells that express *Bcl11b* (as a possible indictor of passage through the DN2 stage) could provide an indicator of the relative contribution of these different pathways.

The role of GATA-3 in T cell development beyond lineage commitment

GATA-3 in T cell receptor β-selection

In the $CD44^{-}$ $CD25^{+}$ DN3 stage, committed T cell precursors test their re-arranged TCRβ chain in a process called β-selection. Signaling through the pre-TCR (TCRβ chain joined with an invariant pre-Tα chain) induces downregulation of CD25, preluding the last DN stage. DN4 cells rapidly proliferate before they upregulate the CD4 and CD8 co-receptors and start re-arrangement of the TCRα chain.

GATA-3 protein expression is upregulated between the DN3 and DN4 stage (146), even though transcriptional *Gata3* levels do not change much and even go down after the DN4 stage (147,148). In a study in which *Gata3* was specifically deleted at the DN3 stage using a *Lck*-Cre inducible system, DN3 cells accumulated and DN4, DP and SP cell numbers were reduced (112). Furthermore, the *Gata3*-deficient DN3 cells were found not to increase in cell size and most DN4 cells lacked the expression were TCRβ-negative. Interestingly, our laboratory previously used *Cd4*Cre $Gata3^{fl/fl}$ mice to show that $TCR\beta^{+}$ DN3 cells fail to increase the expression of CD69 and fail to increase in cell size, suggesting that GATA-3 is necessary for proper control of cell cycle or survival (149). However, we found no indications that GATA-3 controls TCRβ expression.

Thus, the exact mechanism by which GATA-3 controls β-selection remains unknown. In several studies a similar phenotype was observed when Notch signaling was impaired. These studied employed conditional knockouts of *Notch1* (150), RBPJκ, the Notch signaling molecule (142) or the transcription factor MYB. Whereas a similar partial block in development at the point of β-selection was observed for all three conditional knockout models (DN3 increased, DN4 and later stages decreased), cells were defective in their re-arrangement of the *Tcrb* locus (150,151). This was not observed in *Gata3*-deficient DN3/DN4 cells (112) and suggest that Notch and GATA-3 play different roles in β-selection. Whereas Notch controls *Tcrb* re-arrangement, the role of GATA-3 in β-selection is less clear. GATA-3 may be involved in regulation of cell cycle and survival, and may also play a role in the regulation of TCRβ expression.

A study by Maillard et al. (152) indicates that the observed GATA-3-mediated defects in β-selection leading to a partial block may in fact be more profound and reflect limitations of the Cre-loxp system. In this study, a dominant-negative form of the Notch signaling molecule Mastermind-like 1 (MAML1) coupled to green-fluorescent protein (GFP) under the control of the Lck promotor was used. Therefore, cells that express the dominant-negative form of MAML1 and have impaired Notch signaling can be detected by GFP expression. To determine the effects of blocked Notch signaling, GFP^{+} DN3 cells were isolated and injected into the thymus of irradiated recipient mice. Analysis showed a complete block in the generation of DP cells. Furthermore, inhibition of Notch caused downregu-

lation of CD25, indicating that the TCRβ$^-$ DN4 cells that were found in previous studies (142,150,151), may instead be DN3 cells that downregulated CD25. Interestingly, the block at the DN3 level caused by absence of GATA-3 or Notch signals can be rescued by forced expression of a *Tcrab* transgene (112,152). These results suggest that GATA-3 either modulates the effects of pre-TCR signaling or functions in parallel with pre-TCR signaling, but it remains unclear how GATA-3 mediates β-selection.

GATA-3 in CD4 commitment

β-selection is followed by rearrangement of the *Tcra* locus, and positive and negative selection. At the same DP stage, developing T cells make a choice between the CD4 and CD8 lineage. This complicated process has been studied extensively and has been clearly summarized in various reviews (153,154). Here, we will focus on CD4 commitment and the role of GATA-3 and its co-operation with other factors that are important in this process.

The transcription factor ThPOK (encoded by the *Ztbtb7* gene) was identified by two groups in a naturally occurring mouse strain that lacks CD4$^+$ (155,156), but has normal CD8$^+$ T cell development (157). It was shown that ThPOK is necessary and sufficient for commitment to the CD4$^+$ T helper cell lineage (155,156). Analyses that followed showed that ThPOK also functions to prevent downregulation of CD4, to stabilize its own expression and to inhibit the CD8-promoting factor Runx3 (158,159).

Several groups have investigated the function of GATA-3 in CD4 commitment using different approaches. Our group has previously shown that CD2-driven *Gata3* overexpression leads to decreased mature CD8 T cells, but does not impact on CD4 development (160). These results are in conflict with studies in which retroviral expression and knockdown of *Gata3* in fetal thymocytes showed that GATA-3 promotes development of CD4 T cells at the expense of CD8 development (161,162). Later, we generated *Gata3*-deficient animals in which *Gata3* expression was rescued by a *Gata3* transgene under the CD2 promotor, thus generating a system in which both CD4- and CD8- precursors express equal amounts of *Gata3* (149). In these mice, we observed both CD4 and CD8 development, showing that GATA-3 does not determine CD4/CD8 fate decisions. Another group conditionally ablated *Gata3* in CD4 T cells using a *Cd4*Cre mouse model to show that GATA-3 is dispensable for CD8 maturation, but is required for normal CD4 development (112).

In a publication by the Rajewsky group (150), it was shown that conditional deletion of the TF Myb causes a similar phenotype as *Gata3* deletion in CD4 T cells: a reduction of the CD4/CD8 ratio and a decrease in absolute CD4 numbers. In line with previous observations made in GATA-3 overexpression models (161,162), forced overexpression of MYB was shown to block CD8 development (163). In ChIP and reporter assays, MYB was shown to directly bind to the *Gata3* promotor (163). Together, these results indicate that TFs such as GATA-3 and MYB probably acts after CD4 commitment, while ThPOK is important for lineage commitment.

Yet, later results showed that ThPOK and GATA-3 do not act independently in a simple sequential model. The group of Bosselut observed that GATA-3 directly binds to the ThPOK promotor and that *Gata3*-deficient positively selected CD69$^+$ thymocytes have decreased levels of ThPOK protein (164). This raises the possibility that GATA-3 also acts to control ThPOK, possibly by direct activation of this TF. However, introduction of a *Zbtb7*-transgene into *Gata3*-deficient thymocytes did not restore CD4 development, but CD8 development was still inhibited. Together, these results suggest that GATA-3 fulfills two functions during CD4 commitment. GATA-3 is required for regulation ThPOK that functions to inhibit CD8 development. Secondly, GATA-3 is required at the post-commitment phase, probably for regulation of survival of CD4 cells.

T helper cell subsets & type-2 immunity in allergic asthma

Based on 'Type 2 Innate Lymphocytes in Allergic Airway Inflammation', *Current Allergy and Asthma Reports 2013*

T helper cell differentiation

The combination of TCR triggering and cytokine-mediated signaling instructs the further differentiation of naïve CD4$^+$ T cells. Already in 1986, Mosmann and Coffman observed that CD4 T cell can be divided in at least 2 groups based on their cytokine profile, but also based on the expression of surface molecules (165). Th1 cells are potent producers of IFN-γ, while Th2 cells produce IL-4, IL-5 and IL-13 as their signature cytokines. Later, two additional subsets were identified: Th17 cells (produce IL-17A, IL-17F and IL-22) (166–168) and regulatory T cells (Treg; produce TGF-β; (169–173) **(Figure 4)**.

Cytokine-instructed Th cell differentiation

Soon after the discovery of distinct Th subsets, methods were developed to induce cell subset differentiation in vitro. This differentiation requires T cell activation (initially using anti-CD3 and anti-CD28 antibodies) and triggering using a specific set of cytokines. Th1 differentiation was shown to be achieved using IL-12 stimulation and IL-4 neutralization (174), whereas Th2 cells were obtained using IL-2 and IL-4 (175–178). In a similar fashion, it was shown that Th17 cells could be derived from CD4 cells in vitro by TGF-β combined with IL-6 or IL-21 (179–181), while Treg cells can be induced using TGF-β combined with IL-2 (169–172).

Interestingly, for all Th subsets, one of the signature cytokines produced after differentiation is also required for the induction of these cells: IFN-γ for Th1, IL-4 for Th2, IL-21 for Th17 and TGF-β for Tregs. This powerful positive feedback loop has been studied extensively and was most characterized extensively for Th2 cell differentiation.

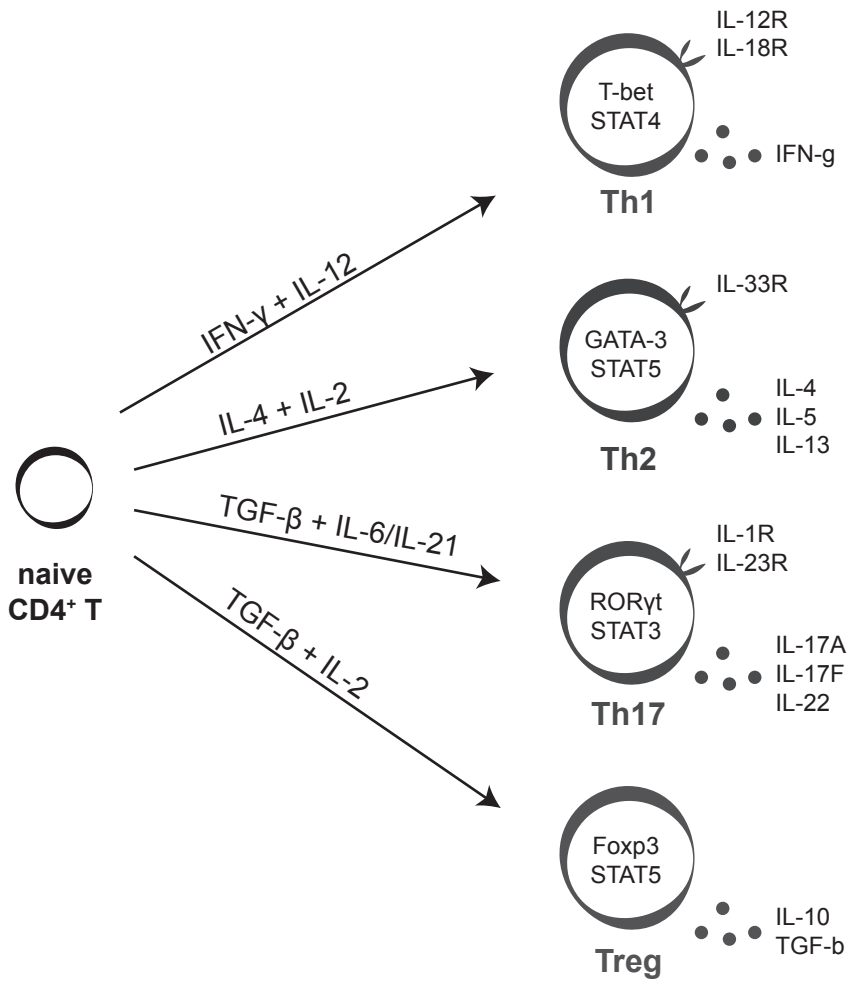


Figure 4. T helper cell differentiation *(full color version: page 232)*
Schematic overview showing cytokine-induced differentiation of naïve T helper (Th) cells into various lineages. Naïve $CD4^+$ T cells are activated via TCR triggering by an antigen presenting cell (APC) such as a dendritic cell. Together with signals delivered by the APC, the cytokine milieu will steer differentiation into one of the Th lineages. Each lineage is characterized by a master transcription factor and an associated STAT protein. These master TFs are also involved in the secretion of a characteristic set of effector cytokines. All Th subsets are associated with an IL-1 family receptor that, if triggered, induced rapid secretion of cytokines independently of TCR activation.

For every subset, key transcription factors have been identified. In addition, each Th subset is characterized by the expression of a distinct member of the signaling transducer and activator (STAT) family. Whereas the levels of activity of the main transcription factors are mainly determined by their expression levels, the activation statuses of STAT proteins are regulated by cytokine-mediated posttranslational modifications.

Th2 differentiation: GATA-3 and STAT5/6

GATA-3 has been widely accepted as the key regulator of the Th2 lineage and was the first key TF to be identified for one of the Th lineages (113,182). While Th2 cells upregulate GATA-3 levels during development, Th1 cells importantly downregulate GATA-3 levels (182–184). The introduction of exogenous GATA-3 in Th1 cells induces IL-4 production and endogenous GATA-3 production (183,185), whereas removal of GATA-3 from established Th2 cells reduced IL-4 production and completely abolishes IL-5 and IL-13 production (186,187). This is the most likely explanation for the observed block in airway hyperreactivity (AHR) induction in vivo (186). Th2 differentiation is completely blocked in the absence of GATA-3 (187,188).

STAT6 is activated by IL-4 and is the major signal transducing protein for the Th2 lineage. The activation of STAT6 is necessary and sufficient for induction of high levels of GATA-3 in vitro (189,190). STAT6 regulates the activity of the *Il4*/*Il13* locus control region (LCR), but does not directly regulate *Il4* expression (191). Although STAT6 signaling is strictly required in vitro, Th2 differentiation can be induced in vivo without STAT6 but still depends on GATA-3 (192–196).

STAT5 appears in the two isoforms (STAT5a and STAT5b) and is necessary for most cytokines that use the common gamma chain, such as IL-2 and IL-7 (197). Low levels of STAT5 signaling are required for regulation of cell survival and proliferation (198), but high STAT5 levels are required for Th2 differentiation (199,200). In Th2 cells, but not in Th1 cells, STAT5 directly binds to the DNase I hypersensitive sites (HS) II and III in the *Il4* locus. Furthermore, STAT5 has been implicated in the regulation of IL-12 signaling, as *Stat5a*-deficient cells are hyperresponsive to IL-12, thereby favoring Th1 differentiation.

Th1 differentiation: T-bet and STAT4

The main transcriptional regulator for Th1differentiation is T-bet (encoded by the *Tbx21* gene). This TF drives the expression of IFN-γ and is also able to do so in fully differentiated Th2 cells (201). T-bet exerts its function by remodeling of the Ifng locus and upregulating the expression of IL-12Rβ2, thus further enhancing the positive feedback loop induced by IL-12 (202,203). The IFN-γ response in *Tbx21*-deficient (T-bet knockout) mice infected with Leishmania major is reduced, but not completely abolished (204). Interestingly, infection with L. major induced increased IL-4 and IL-5 production in $Tbx21^{-/-}$ mice (204). In addition, $Tbx21^{-/-}$ mice spontaneously develop AHR (205). However, $Tbx21^{-/-}$ cells can be differentiated in vitro to produce IFN-γ if IL-4 is neutralized (184), suggesting that T-bet may be involved in repression of GATA-3 expression.

IL-12 and most likely IFN-γ activate STAT4 (206,207), while GATA-3 and IL-4 negatively regulate STAT4 activation (208,209). STAT4 directly induces expression of IFN-γ, IL-12Rβ2 and T-bet. Furthermore, IFN-γ-mediated STAT1 activation induces T-bet during Th1 differentiation (210,211).

Th17 differentiation: RORγt and STAT3

RORγt is the master regulator of the Th17 lineage (212). Stimulation of naïve CD4 T cells with IL-6 and TGF-β rapidly induces expression of RORγt. Overexpression of RORγt in activated T cells induces IL-17 production and mice deficient for *Rorc* (the gene encoding RORγ and RORγt) make very little IL-17. Removal of RORγt from differentiated cells significantly reduces IL-17 production, but some residual IL-17 is produced under the control of family member RORα (213). IL-17 production is completely abolished in mice deficient for both RORγt and RORα (213).

IL-6, IL-21 and IL-23 can activate the STAT3 pathway (166–168,179–181). It binds directly to Il17 (214) and Il21 (215) and induces expression of RORγt and IL-23R (166,167,216). At the same time, IL-6-mediated STAT3 activation downregulates Foxp3 expression (168,216–218), demonstrating the critical role of IL-6 in the fate determination of Th17 and Treg cells.

Treg differentation: Foxp3 and STAT5

The development of natural and inducible Treg cells is regulated by the TF Foxp3 (180,219,220). Continuous expression of Foxp3 is required to maintain a suppressive phenotype. Likewise, transduction of conventional T cells with Foxp3 induces the production of cytokines of TGF-β and IL-10 that have suppressive capacity (220).

Like Th17 cell differentiation, Treg cell differentiation in vitro is also stimulated by TGF-β. Treg development requires co-stimulation with IL-2 to induce the STAT5 pathway (221–223). STAT5 directly binds to the *Foxp3* promotor (223,224).

Regulation of the Th2 locus

The so-called 'Th2 locus' comprises the genes and control elements of the type 2 cytokines IL-4, IL-5 and IL-13 (reviewed in (225) and **Figure 5)**. Th2 cell development involves histone modifications in this locus that change the chromatin structure, so that the cytokine genes become accessible to the transcription machinery (225,226). Most of these changes occur around DNase I hypersensitive sites (HS). Indeed, GATA-3 has been found to bind at several regulatory elements of the Th2 locus, such as the conserved non-coding sequence (CNS)-1, the 3' *Il4* enhancer HSVa, the conserved GATA response element (CGRE), the promotor for *Il5* and HSII in the fourth intron of the *Il4* gene [(227–231) and **Figure 5)**. GATA-3 also binds to three sites in the locus control region (LCR) that lies within the *Rad50* gene and plays an important role in regulation of expression of Th2 cytokines (191,232,233). Expression of *Il5* and *Il13* is directly induced by GATA-3 (182,234–236) and removal of GATA-3 from established Th2 cells completely abolished IL-5 and IL-13, but not IL-4 production (187). Furthermore, GATA-3 has been implicated in the upregulation of Th2-related genes, such as *Dec2* which via JunB induction acts on the *Il4* promotor. GATA-3

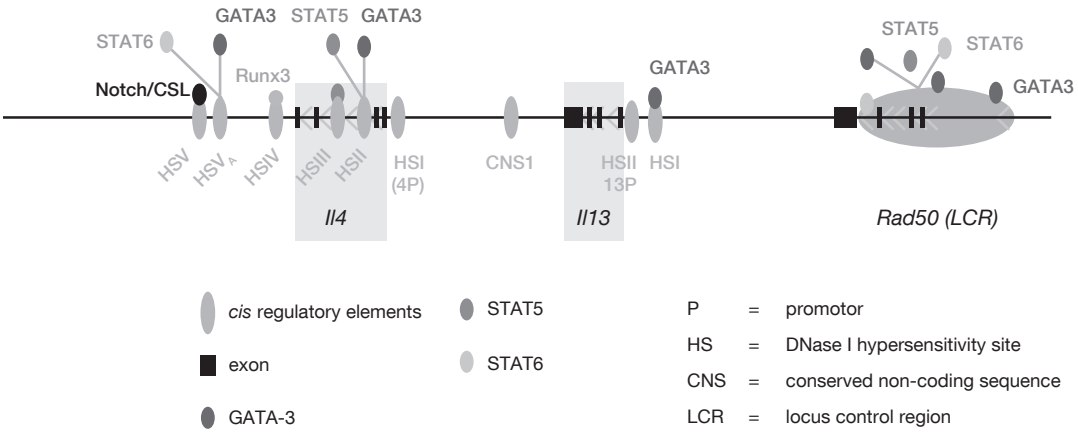


Figure 5. Cis-regulatory elements and binding transcription factors in the Th2 locus *(full color version: page 233)* The well-studied Th2 locus located on chromosome 5q23.3/5q31 (human) and 11qB1.3 (mouse) contains several regulatory elements, including the locus control region (LCR), CNS and various HS sites associated with the *Il4* gene. Many transcription factors directly bind in the Th2 locus, including GATA-3, Notch/CSL, Runx3, STAT5, STAT6, c-Maf and NFAT (the latter two are not depicted). The figures represents a ~7okb region. The GATA-3 binding sites were recently confirmed in a genome-wide analysis of GATA-3 binding sites (362). Figure adapted from a review by Zhu and Paul (363).

cannot exert all these functions alone, but acts in concert with STAT5 for some of these functions. In the absence of STAT5, GATA-3 is not sufficient for induction of IL-4 (199,200). STAT5 is also required for regulation of IL-4α expression (237) and to maintain adequate levels of GATA-3 in mature cells (238). In addition, several other TF have been shown to be essential for Th2 differentiation, including *Irf4*, *Satb1* and *c-Maf*. Interestingly, c-Maf was reported to control *Il4* expression but not the production of other Th2 cytokines (239).

GATA-3 is also known as an important suppressor of Th1 cell differentiation via suppression of IL-12/STAT4 signaling. Ectopic expression of GATA-3 in developing cells that have been activated with antigen, inhibits expression of Il12rb2 at the mRNA level (183). In addition, GATA-3 directly suppresses STAT4, thereby further limiting Th1 cell development and impairing production of IFN-γ (208). Thirdly, GATA-3 has been implicated in the inhibition of Runx3-mediated (IL-12/STAT4-independent) production of IFN-γ (183).

Taken together, these results suggest that GATA-3 fulfills at least two functions in the regulation of type 2 cytokine expression: direct activation (*Il5*, *Il13*) and chromatin remodeling (*Il4*). In addition, GATA-3 silences the Th1 program via multiple mechanisms.

Notch signaling pathway in Th2 cell differentiation

Notch signals are crucial for the first steps of T cell development (see above), but are also implicated in the differentiation of naïve CD4 T cells directed by dendritic cells (DCs).

Delta-ligands have been associated with Th1 cell differentiation, while Jagged-ligands are connected to Th2 cell differentiation. For instance, CD8α- DCs that have been pulsed with LPS express Dll4, which directs Th1 differentiation independent of IL-12 (240). Likewise, enforced expression of Dll1 and Dll4 on IL-12-deficient DCs also promotes Th1 cell differentiation and suppresses Th2 cell development (241). The intra-cellular domain of Notch (ICD) can form a complex with NF-κB and c-Rel that activates the Ifng promotor and induces T-bet expression (242–244). In contrast, conditional deletion of *Rbpj* using *Cd4*Cre transgenic mice abolishes Th2 differentiation (142,245). Amsen et al. identified a CSL-binding site in the *Il4* gene and found that the Notch ICD upregulates *Il4* expression (245). The same complex can also directly regulate *Gata3* independently of STAT6 by binding to an alternative *Gata3* promotor (97).

Th2 cells are key players in type-2 immunity

Almost 50% of the world population is infected by parasitic worms (helminths) or suffers from an allergic disorder, such as asthma, atopic dermatitis, allergic rhinitis or food allergies (246,247). A common feature of this sort of infection and inflammation is the so-called 'type-2 immune response'. Type-2 responses are characterized by the induction of Th2 cells that can secrete IL-4, IL-5, IL-9 and IL-13.

Type-2 immunity is necessary for the expulsion of helminths, but also underlies pathology caused by inflammatory responses to allergens or to microbial products. Common allergens are derived from nature (e.g. pollen), the interior (e.g. house dust mite) or food (e.g. shellfish and peanuts). Venoms, for instance from bees and adjuvants used in vaccines (e.g. alum) can also induce a type-2 response. Some viruses and bacteria can also induce a type-2 response, for example via lipopolysaccharides (LPS) or peptidoglycans, although this is less common.

Type-2 responses are very diverse, both in the wide collection of stimuli that trigger a type-2 response, as well as in the cellular and molecular pathways that are activated in response to these stimuli. Much has been learned from animal models that mimic human type-2 responses. The availability of several mouse models of allergic asthma has greatly advanced our understanding of the pathogenesis of allergic asthma. Here, we will discuss allergic asthma as a prototypic type-2 condition and will focus on the key cellular players and molecules involved.

Asthma is a heterogeneous disease and an important cause of morbidity worldwide

Asthma is disorder of the conducting airways and affects over 300 million people world-

wide. Its prevalence has increased considerably over the last three decades, especially in the western world (248). The disease is characterized by chronic airway inflammation and airway hyperreactivity (AHR) leading to shortness of breath, coughing and recurrent wheezing and these symptoms often present at childhood. Multiple pathogenetic environmental factors and more than 100 major and minor susceptibility genes have been identified (249,250). Many forms of asthma have been described, with allergic asthma being the most prevalent type of asthma. In addition, asthma can be induced by virus infection, obesity, exercise, cold air or exposure to air pollution, ozone, cigarette smoke and diesel exhaust particles (251–256).

Introduction to the immune system in asthma

Over the years, most asthma research focused on allergic asthma. Many groups have contributed to our current understanding of the pathogenesis of asthma and the development of new therapeutic strategies. Allergen-specific T helper type 2 (Th2) cells are present in almost all asthmatic patients, especially in those with allergic asthma (257), and have been recognized as key players in type 2 immune responses **(Figure 6)**. These potent cytokine producing cells are thought to be key players in the establishment and maintenance of disease, as they regulate the allergen-specific production of the key type 2 cytokines IL-4, IL-5 and IL-13 that fulfill specific roles in asthma: IL-4 induces IgG1 and IgE class switching in allergen-specific B cells, IL-5 recruits eosinophils and IL-13 triggers mucus production and AHR. In addition to this trio of classic Th2 cytokines, IL-9 and GM-CSF play important roles in asthma, as they regulate differentiation and maturation of mast cells (IL-9) and maturation and survival of eosinophils and recruitment of basophils (GM-CSF) (reviewed by Holgate et al.; (258)).

IL-25, IL-33 and TSLP: initiator cytokines in asthma

Most antigens and allergens do not directly act on T cells. Therefore, Th2 cell differentiation requires intermediate cells that sample the airway lumen, process antigens and produce cytokines that instruct other immune cells.

IL-4 has widely been recognized as the key instructing cytokine for Th2 cell differentiation. However, there is increasing evidence that other cytokines, often produced by non-immune cells, also have important roles in allergic asthma and may be essential for the initiation of disease. Here, we will discuss TSLP, IL-25 and IL-33 as three important instructing cytokines in type 2 immunity.

Thymic stromal lymphopoietin (TSLP)

TSLP was originally discovered in media of a thymic stromal cell line that supported B development and proliferation of unfractionated thymocytes (259). While its name suggests

otherwise, TSLP is mainly expressed by epithelial cells lining the skin and mucosal surfaces. The expression of TSLP is regulated by pro-inflammatory cytokines, such as IL-1β and tumor necrosis factor (TNF)-α, and involves NF-κB signaling (260). Not surprisingly, many Toll-like receptor (TLR) agonists induce TSLP expression by epithelial cells (reviewed by Zhang and Zhou; (261)).

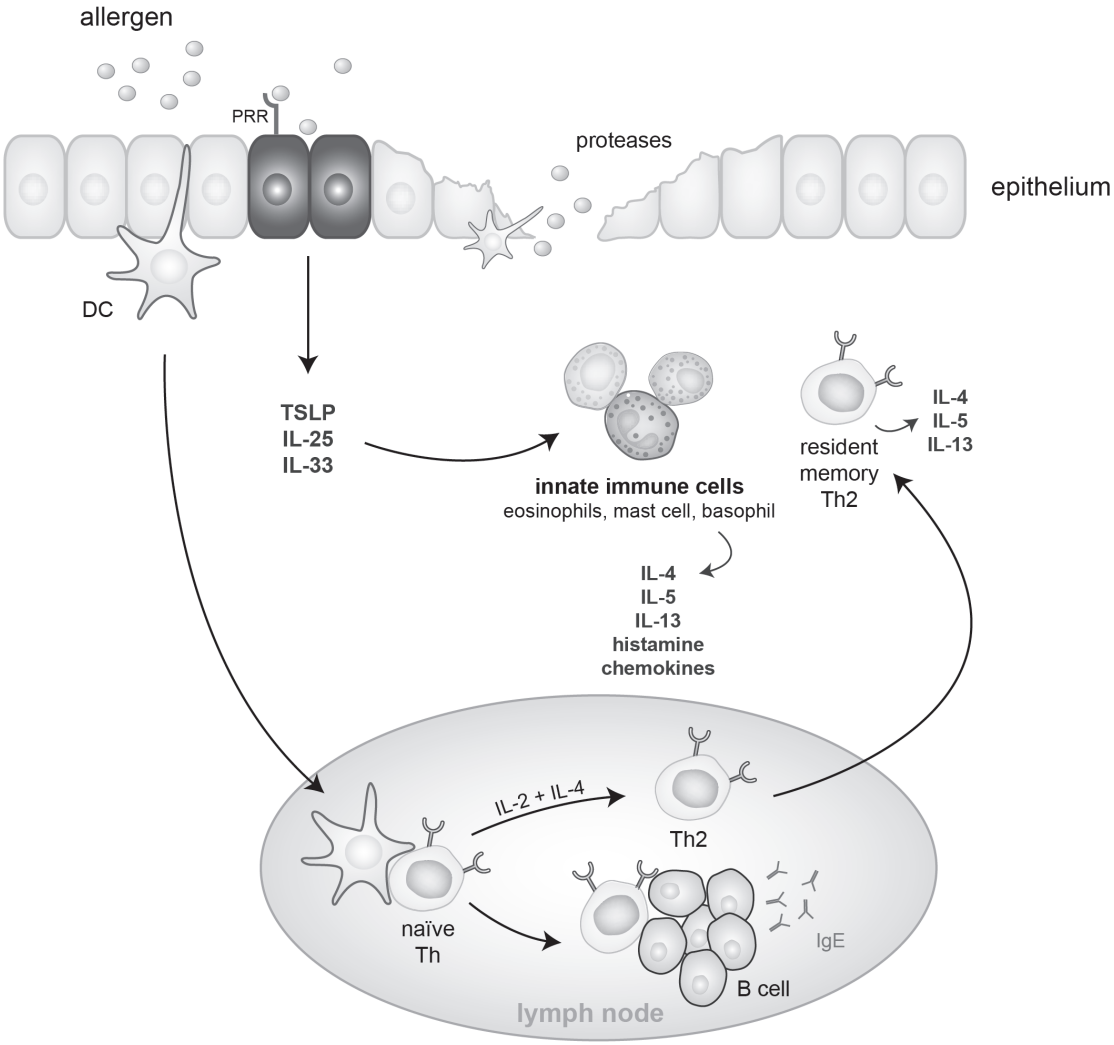


Figure 6. Dendritic cells link the adaptive and immune system in allergic inflammation. *(full color version: p234)* Inhaled allergens, such as house dust mite, enter the airways and can activate epithelial cells and dendritic cells (DC) via pattern recognition receptors (PRR). Furthermore, DC constantly sample the airway lumen for peptides. Activated DCs migrate from the lung to the local draining lymph node where they interact with naïve T helper (Th) lymphocytes and present antigens. The combination of the cytokine milieu, namely the presence of IL-2 and IL-4, and factors expressed by DCs, such as OX40L and Notch ligand Jagged2, induces Th2 cell differentiation. Furthermore, follicular T helper cells (Tfh) promote B cell differentiation and IgE production. Differentiated Th2 cells will migrate to the lung, where they reside as memory cells and rapidly can produce important amounts of IL-4, IL-5 and IL-13. Innate immune cells are important for the initial response and produce type 2 cytokines and chemokines that attract other immune cells.

The functional TSLP receptor consists of a dimer of the TSLP-specific TSLPR chain and the IL-7Rα chain (shared with the IL-7 receptor) (262). The common gamma chain (γc), which is required for the receptors for IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21 is thus not required for TSLP binding and function (262). TSLP was shown to activate STAT5 via JAK1 (IL-7Rα chain) and JAK2 (TSLPR chain) in human $CD4^+$ T cells (262–265), but induced phosphorylation and activation of all STAT members except STAT2 in human myeloid DCs (266). In addition to JAK/STAT signaling, TSLP also induced activation of the NF-κB molecules p50 and RelB, which bound to the OX40L promotor in myeloid DCs (266). Further evidence for a role of TSLP in promoting type 2 responses was found in a study in which TSLP stimulation of murine $CD4^+$ T cells induced IL-2-independent IL-4 production via STAT5 (267).

TSLP has often been associated with allergic asthma. Several genome-wide association studies (GWAS) have associated TSLP with an increased asthma risk (268,269). In mouse models of allergic asthma, TSLP was found expressed at increased levels (270–272). Conversely, type 2 responses in ovalbumin-induced asthma are diminished in $TSLPR^{-/-}$ mice (273) and administration of fusion proteins (272–274) or blocking antibodies (272) reduced allergic airway inflammation.

Interleukin-25

IL-25, also known as IL-17E, was originally discovered as a cytokine secreted by Th2 cells (275). Epithelial cells, basophils, eosinophils and mast cells have also been reported as sources of IL-25 (276–280). The receptor for IL-25 is constituted by IL-17RA (shared with the receptor of IL-17A) and IL-17RB (shared with the receptor of IL-17B). IL-25 activates the NF-κB signaling pathway, MAPK-AP-1 and C/EBP via recruitment of Act1 and TRAF6 (reviewed in by Iwakura et al.; (281)).

The initial description of IL-25 included a demonstration of the potency of IL-25 as a cytokine that induces type-2 immunity. Intra-peritoneal injection of IL-25 leads to eosinophilia, increased mucus production and epithelial cell hyperplasia, probably mediated by increased levels of IL-4, IL-5 and IL-13 and accompanied by increased serum IgE, IgG1 and IgA (275). Several studies have linked IL-25 to airway inflammation, showing that overexpression of IL-25 in airway epithelium induces a type 2 response (276) and that intranasal administration of IL-25 induces AHR (282). Likewise, administration of antibodies against IL-25, and Il25-deficiency in mice, reduced airway inflammation (282–284). Furthermore, polymorphisms in the human *IL17RB* gene have been associated with asthma (285).

Interleukin-33

In an attempt to discover distinct markers for Th1 and Th2 cells, two receptors for IL-1 family members were discovered: Th1 selectively express IL-18R, Th2 selectively express

ST2 (286). By then an orphan receptor, ST2 was later recognized as the unique chain of the IL-33R (287). The IL-1 receptor accessory protein (IL-1RAcP) is the other chain of the functional IL-33R (288,289). Like other IL-1 family members, the IL-33 signaling pathway signals via the MyD88 pathway that recruits IRAK1 and IRAK4, leading to downstream phosphorylation of several signaling molecules, including ERK1/2, p38 and NFκB, ultimately inducing target gene expression (287). ST2 is expressed both by innate (e.g. macrophages, DCs) and adaptive (e.g. T cells) immune cells. Interestingly, IL-33 signaling in DCs induces the expression of the co-stimulatory molecule OX40L that has been associated with Th2 cell polarization (290). Mast cells, eosinophils and basophils all increase cytokine and chemokine production upon IL-33 stimulation (291–294). IL-33 also impacts directly on naïve Th cells and polarizes to an typical Th2 phenotype, characterized by IL-5 and IL-13 production, but not IL-4 (295–297). In polarized Th2 cells, IL-33 stimulation can induce cytokine expression without TCR triggering (238). B1 cells, characterized by rapid production of natural antibodies upon B cell activation by cytokines or TLR stimulation, without the need for B cell receptor-mediated antigen-specific signals (298), produce increased amounts of cytokines and IgM in response to IL-33 (299) .

Numerous studies in humans and mice show that IL-33 is widely expressed in stromal cells of most organ systems (including lung and intestine) and immune cells (e.g. macrophages, DCs and mast cells) as well as epithelial cells, fibroblasts and smooth muscle cells (reviewed by Mirchandani et al.; (300)). Importantly, the expression of IL-33 increases in inflammatory conditions. In macrophages, DCs and fibroblasts, stimulation of TLRs increases the expression of Il33 mRNA (301). There is increasing evidence that IL-33 is not secreted as a classical cytokine, but rather acts as an 'alarmin' that is released in necrotic cells. In contrast, apoptotic cells do not release IL-33, as caspase-1 activity in these cells inactivates IL-33 (302–304).

Interestingly, two publications indicate that IL-33 levels correlate with asthma severity (305,306) and genetic variants of IL-33 have been associated with susceptibility to hay fever (307) and the risk of asthma development (308). Various groups have assessed the potential of IL-33 as a trigger for allergic disease, and demonstrated activation of eosinophils (309), macrophages (295), DCs (290) and Th2 cells (295). Studies using IL-33-deficient animals showed that the development of AHR was abolished compared to control animals (310). Blocking of the IL-33 receptor using T1/ST2 neutralizing antibodies or fusion proteins decreased allergic airway inflammation (311).

Innate cell responses in asthma

Macrophages are present in high numbers in the airways and contribute to lung homeostasis through phagocytosis and secretion of cytokines. Macrophages can produce both anti-

and pro-inflammatory mediators, including most Th1, Th2 and Th17 cytokines (312,313). Furthermore, alveolar macrophages have been reported as an important source of IL-33 (314). It is clear that macrophages participate in allergic airway inflammation, but their role in asthma is still poorly characterized. More research, especially in humans, is necessary to assess their relevance as target for therapeutic interventions.

Basophils, eosinophils and mast cells are granulocytes that are often found in human asthma and animals models of asthma **(Figure 6)**. Basophils are circulating cells that express the high-affinity IgE receptor (FcεRI) and CD49b (DX5), but not the receptor for stem cell factor, c-Kit (CD117). These cells have been implicated in amplification of hypersensitivity responses by releasing histamine and by production of large quantities of IL-4. Furthermore, it has been suggested that basophils may participate in antigen presentation, as they express MHC class II and co-stimulatory molecules (315).

Eosinophils can produce a wide variety of cytokines, include the type 1 cytokines IFN-γ and IL-2, as well as the type 2 cytokines IL-4, IL-5, IL-10 and IL-13 (316). Like in basophils, expression of MHC class II by eosinophils in response to GM-CSF stimulation has been reported (317). In most experimental models of asthma, eosinophils are crucial for the development of AHR (318,319).

Mast cells, like basophils, express FcεRI and are positive for c-Kit. Unlike basophils and eosinophils that circulate, mast cells are found in tissue near mucosal surfaces. IL-3 was identified as an essential cytokine for mast cell proliferation, while IL-4 and GM-CSF up-regulate MHC class II expression in mast cells (320). Like basophils and eosinophils, mast cells can, via a poorly understood mechanism, also function as antigen pPresenting cell (APC). Upon activation by crosslinking of antigen-specific IgE bound to FcεRI, in response to TLR stimulation or in response to IL-33 triggering, mast cells release histamine granules. In addition, they can release a very wide range of cytokines (IL-1, IL-3, IL-4, IL-5, IL-6, IL-10, IL-13, IL-16, TNF-α and TGF-β) and chemokines (IL-8, lymphotactin, CCL1, CCL5, CCL2, MCP-1 and CCL3) (reviewed by Barrett et al.; (321)). In mast-cell deficient animals allergic airway inflammation and serum IgE levels are diminished (322,323).

The adaptive immune response in asthma

While the innate immune system induces a response to inhaled allergens or irritants, one of the immunological hallmarks of allergic asthma is the presence of antigen-specific Th2 cells. These cells constitute the memory that, upon successive exposure to the allergen, quickly mount a powerful immune response that leads to symptoms of chronic allergic disease.

The induction of an adaptive immunity response against a specific allergen starts with APCs that sample the airways. Once activated, these cells migrate to the lymph node, where they present the antigen to T and B lymphocytes that then differentiate into effector

and memory cells. We will first discuss dendritic cells as the prototypic APC and will then focus on Th2 cells that produce effector cytokines.

Dendritic cells link the innate and adaptive immune system

The most important APC involved in allergic asthma are dendritic cells (DC). These highly specialized antigen processors are found throughout the respiratory tract: in the conducting airways, interstitium, vasculature and pleura. Pulmonary DC express a variety of receptors, including TLRs, Nod-like receptors and C-type lectin receptors and will, upon recognition of an antigen, migrate to the draining lymph where they mature and activate other immune cells.

Although resident lung DC can directly recognize foreign antigens, recent studies have demonstrated that recognition of pathogen-associated molecular patterns (PAMPs) by epithelial cells is at least as important for activation of the various types of pulmonary DCs (reviewed by Lambrecht et al.; (324)). A recent study demonstrated that house dust mite allergen induces asthma via TLR4 triggering of epithelial cells. Thus, TLR4 expression by epithelial cells, but not by DC, is essential for the lung DC network (325). In addition, several cytokines produced by the epithelium, such as CCL20, β-defensin and IL-12p40 homodimers, recruit immature conventional DC (cDC) and inflammatory monocytes to the site of antigen exposure. Very recently, it was found that TLR4 signals in bronchial epithelial cells induces the release of IL-1α, which then acted in an autocrine manner to trigger the release of DC-attracting chemokines, GM-CSF, and IL-33 (326), whereas TSLP only became important if high doses of antigen where used for challenges.

Other cytokines (e.g. IL-1, GM-CSF and TSLP) and danger signals (e.g. uric acid) that are also produced by activated epithelial cells will instruct the maturation of DCs. Upon activation, DC will migrate to the draining lymph node where they will induce Th2 responses through the co-stimulatory molecules, such as OX40L-OX40 interaction, through Notch signaling via the Notch-ligand Jagged1 and through secretion of leukotriene C4 (LTC4) and IL-6 (in the absence of the Th1-priming cytokine IL-12p70) [(324) and **Figure 6**]. A recent study showed that transfer of 30 x 103 DC of an allergen-exposed mouse to a naïve animal is enough to transfer Th2 sensitization (327). The importance of DC is further underlined by mouse studies that show that depletion of $CD11c^+$ DC from the lung during sensitization using *Cd11c*-DTR transgenic mice abolishes asthma symptoms (328). In addition, these mice fail to induce Th2 cells that produce IL-4, IL-5 and IL-13.

Antigen-specific Th2 cells constitute allergen memory and mediate asthma exacerbations

As already described in this Introduction, naïve CD4+ T cells that egress from the thymus can differentiate into one of the several Th cell lineages **(Figure 4)**. IL-4 is widely accepted

as the key instructor that via JAK/STAT5 signaling, induces GATA-3 expression and Th2 cell differentiation.

Even though we understand that other T cell subsets are also important and fulfill specific roles in asthma, it is without doubt that Th2 cells are central to the pathogenesis of allergic asthma. Murine asthma models of allergic airway inflammation have demonstrated that without Th2 cells and Th2-secreted cytokines, eosinophilia and AHR are ablated (329–332). In addition, adoptive transfer of Th2 cells into naïve mice followed by exposure to antigen induces an asthma phenotype, including eosinophilia, increased mucus production and AHR (333–335).

Antigen-specific Th2 cells are first generated during the sensitization phase of asthma, in which DCs present sampled antigens to naïve T cells together with co-stimulatory molecules that induce Th2 differentiation. Primed and differentiated Th2 cells then become long-lived memory cells that migrate to the airways, where they reside even during asymptomatic periods (336–339). Antigen presentation in a Th2-prone system in which IL-4 is present, also induces IgE and IgG1 class switching in antigen-specific activated B lymphocytes.

After this sensitization phase, re-exposure to the same antigen can rapidly induce an exacerbation of airway inflammation. Binding of antigens to IgE already bound by the FcεR on the surface of basophils and mast cells causes cross-linking of the bound IgE, thereby inducing rapid degranulation of these cells. The antigen is also sampled by DC that will present the antigen to existing Th2 cells and additional naïve T cells in the lymph node. Activated Th2 cells will secrete important amounts of IL-4, IL-5 and IL-13, triggering other immune cells and thereby further aggravating asthma symptoms.

Table 2. Initial identification of group 2 innate lymphoid cells (ILC2)

Name	Tissue distribution	Function	Ref.
Natural helper cells (NHC)	Mouse: fat associated lymphoid clusters (FALC)	Worm expulsion (IL-13), B1 cell stimulation (IL-5/IL-6)	(352)
Nuocytes	Mouse: intestine, mesenteric lymph nodes, lung	Worm expulsion	(353)
Innate helper (Ih2) cells	Mouse: various tissues, including spleen, liver, intestine, peripheral blood	Worm expulsion	(359)
MPP type 2	Mouse: GALT, mediastinal lymph node, Peyer's patches	Worm expulsion	(354)
Type/group 2 innate lymphoid cell (ILC2)	Human: fetal and adult gut and lung, peripheral blood	Increased in chronic rhinitis patients	(357)

Innate-like immune cells in asthma

Immune cells are classically divided into an innate arm, comprising granulocytes, macrophages and other cells that recognize common pathogen-associated patterns, and an adaptive arm that recognizes specific epitopes. Relatively recent research indicates that there is an additional cell population that shares properties of both systems; so-called innate-like lymphocytes. NKT cells and the more recently identified populations of innate lymphoid cells (ILC) have also been found to play a role in asthma.

NKT cells

NKT cells constitute a unique subset of lymphocytes that share properties of T and NK cells and express a characteristic, limited repertoire of T cell receptors (TCR) consisting of Vα14 and Jα14 (mouse) or Vα24 and Jα18 (human) (340). Their invariant TCR functions as a pattern recognition receptor that recognizes both foreign and endogenous glycolipid antigens. While research continues to identify NKT ligands, the best analyzed ligand for the NKT antigen receptor is α-galactosylceramide (α-GalCer), a glycolipid that is exclusively presented by CD1d, a MHC class I-like molecule. Upon activation, NKT cells can produce both type 1 (IFN-γ), as well as type 2 cytokines (IL-4 and IL-13). Interestingly, NKT cells can be directly stimulated by TSLP, IL-25 and IL-33 (reviewed by Kim et al.; (341)).

In an ovalbumin-induced asthma model, NKT cells induced an allergic airway response independent of Th2 cells, which was ablated in NKT-deficient *Ja281*$^{-/-}$ and *Cd1d*$^{-/-}$ mice (342). Furthermore, administration of antibodies against CD1d or CD1d antagonists also diminished ovalbumin-induced inflammation and AHR (343,344), while administration of α-GalCer quickly induces AHR and airway inflammation, even in the absence of $CD4^+$ T cells (345).

Even though the role of NKT cells in murine models of asthma is relatively well-established, less is clear about the role of NKT in humans. In one report NKT cells in BAL were quantified and NKT cells were reported to constitute about 60% of $CD4^+$ T cells in BAL (346), but four similar studies could not confirm this result and found similar numbers of NKT cells in BAL fluid of patients and healthy controls (347–350). Additional functional studies of NKT cells in humans should provide insight into the role of NKT cells in allergic asthma.

Innate lymphoid cells (ILC)

The major source of type 2 cytokines in most models of murine asthma is the population of allergen-specific Th2 cells. Interestingly, *Rag*$^{-/-}$ mice that are deficient for B and T cells can still produce important amounts of type 2 cytokines and induce an asthma phenotype. In 2001, the first observation of a non-B non-T cell population that responded to IL-25 treatment by production of IL-5 and IL-13 was made by Fort et al. (275). Subsequent reports have shown a similar population that responded to IL-33 treatment (295,351). These

publications preluded the discovery of a novel family of cytokine-producing lymphocytes that lack classic lineage markers, but that, among other surface markers, express the lymphocyte-associated receptors for IL-2 and IL-7. Multiple groups identified so-called innate lymphoid cells (ILC) that produce important amounts of IL-5 and IL-13, but little IL-4. These type 2 cytokine-producing ILC were studied in the context of helminth immunity in the intestine, and were termed natural helper cells (352), nuocytes (353) and type 2 multipotent progenitor [MPP_{type2}; (354)] (summarized in **Table 2**). Phenotypically, the different populations show important similarities **(Table 3)**. Therefore, in this thesis, we will comply with a recent suggestion to refer to this family of lineage-negative cells as innate lymphoid cells (ILC) (355). The set of cytokines produced by ILC is indicated by a number that follows the abbreviation: e.g. ILC2 for group 2 ILC that produce type 2 cytokines. Other reports show that also for most other T helper cell subsets (Th1, Th17 and Th22) there are analogous lineage-negative populations that produces a similar set of cytokines (further described in **Chapter VI**).

Interestingly, studies that quickly followed the initial descriptions of ILC2 in the intestine described a similar population of type 2 cytokine-producing innate lymphoid cells in the pulmonary tract of man and mice (314,356,357). We and others asked what role ILC2 play in allergic asthma. In this thesis, we will describe our findings and will discuss the other studies investigating the role of ILC2 in murine models of allergic asthma and their role in human disease.

Table 3. Phenotype of group 2 innate lymphoid cells

	NHC	Nuocytes	Ih2	MPPtype2	ILC2 (Hu)
Lineage (CD3/4/8a/11b/11c/19, B220, NK1.1, DX5, Ter-119, Gr1, FcεRIα)	–	–	–	–	–
CD127 (IL–7Rα)	+	+	NT	+/–	+
Sca1	+	+	–	+	NA
CD117 (c–Kit)	+/–	+/–	+/–	+	+/–
CD25 (IL–2Rα)	+	NT	+	NT	+
IL–17RB (IL–25R)	NT	+	NT	+	+
ST2 (IL–33R)	+	+	NT	–	+
CD278 (ICOS)	NT	+	NT	NT	NT
CD90 (Thy1)	+	+	+	+	NT
CD294 (CRTH2)	NT	NT	NT	NT	+
MHCII	–	+	NT	NT	HLA–DR^{low}
GATA–3	+	+	+	–	+
RORα	+	+	NT	NT	+

NT = not tested, NA = not applicable

Aims and outline of the thesis

T lymphocytes are essential for effective immunity against infections causes by bacteria, viruses, fungi and parasites. Additionally, T lymphocytes are implicated in the development of autoimmune diseases, leukemia and allergies. Therefore, knowledge about the molecular mechanisms that underlie the development and function of T lymphocytes is essential for understanding the etiology and the development of novel therapeutic strategies of a wide variety of diseases.

Development of T lymphocytes from hematopoietic stem cells is crucially dependent on the regulation of gene expression by key transcription factors. GATA-3 is an essential transcription factor that fulfills specific functions at various stages of T cell development and Th2 differentiation. This factor has been widely recognized as the prototypic regulator of expression of the type 2 cytokines IL-4, IL-5 and IL-13 that underlie the pathogenesis of allergic asthma.

Allergic asthma is a chronic disease that affects over 300 million people worldwide. Allergic asthma is a complex, multi-trait disease, but type 2 cytokines can induce all cardinal features of asthma: AHR and reversible obstruction of airflow, leading to symptoms of wheezing, coughing and chest tightness.

A specific subset of the recently identified innate lymphoid cells (ILC) was shown to produce substantial amounts of type 2 cytokines in response to worm infections. Similar to T cells, cytokine production in group 2 ILC (ILC2) depends on GATA-3. In contrast to T cells, ILCs are not activated via an antigen-specific receptor. Rather, they rapidly respond to environmental cues, such as cytokines, which are also readily produced in the airways of asthma patients.

This thesis describes the various role of GATA-3 in development and function of T cells and ILC2. Even though it is known that GATA-3 is essential during the first steps of T cell development, its exact role at this stage is unknown. In **Chapter II**, we aim to decipher the role of GATA-3 in T cell commitment and exclusion of other fates at a molecular level. We use both in vivo and in vitro approaches to determine the function of GATA-3 in T cell commitment and development.

ILC2 are recently discovered members of the family of innate lymphocytes. Many parallels between T cell and ILC function have been described, such as the shared dependency on GATA-3 for type 2 cytokine production. In **Chapter III**, we determine the role of transcription factor GATA-3 during development of ILC2. We employed in vivo models using chimeras and mixed bone marrow chimeras and in vitro models using fetal liver stem cells to study the role of GATA-3 in ILC2 development.

Th2 cells are classically recognized as an important source of type 2 cytokines in allergic asthma. In **Chapter IV**, we use various mouse models of allergic airway inflammation to untangle the contribution of Th2 and ILC2 to the production of type 2 cytokines.

GATA-3 is required for expression of IL-5 and IL-13 in both Th2 cells and ILC2. In **Chapter V**, we studied the effects of varying GATA-3 levels on the cytokine production by Th2 cells and ILC2 in two classical murine models of allergic airway inflammation.

The implications of these studies and directions for further research are presented in the General discussion in **Chapter VI**. Here, we focus on the stage-specific functions of GATA-3 in various cell types and ask the question how GATA-3 can perform these various roles.

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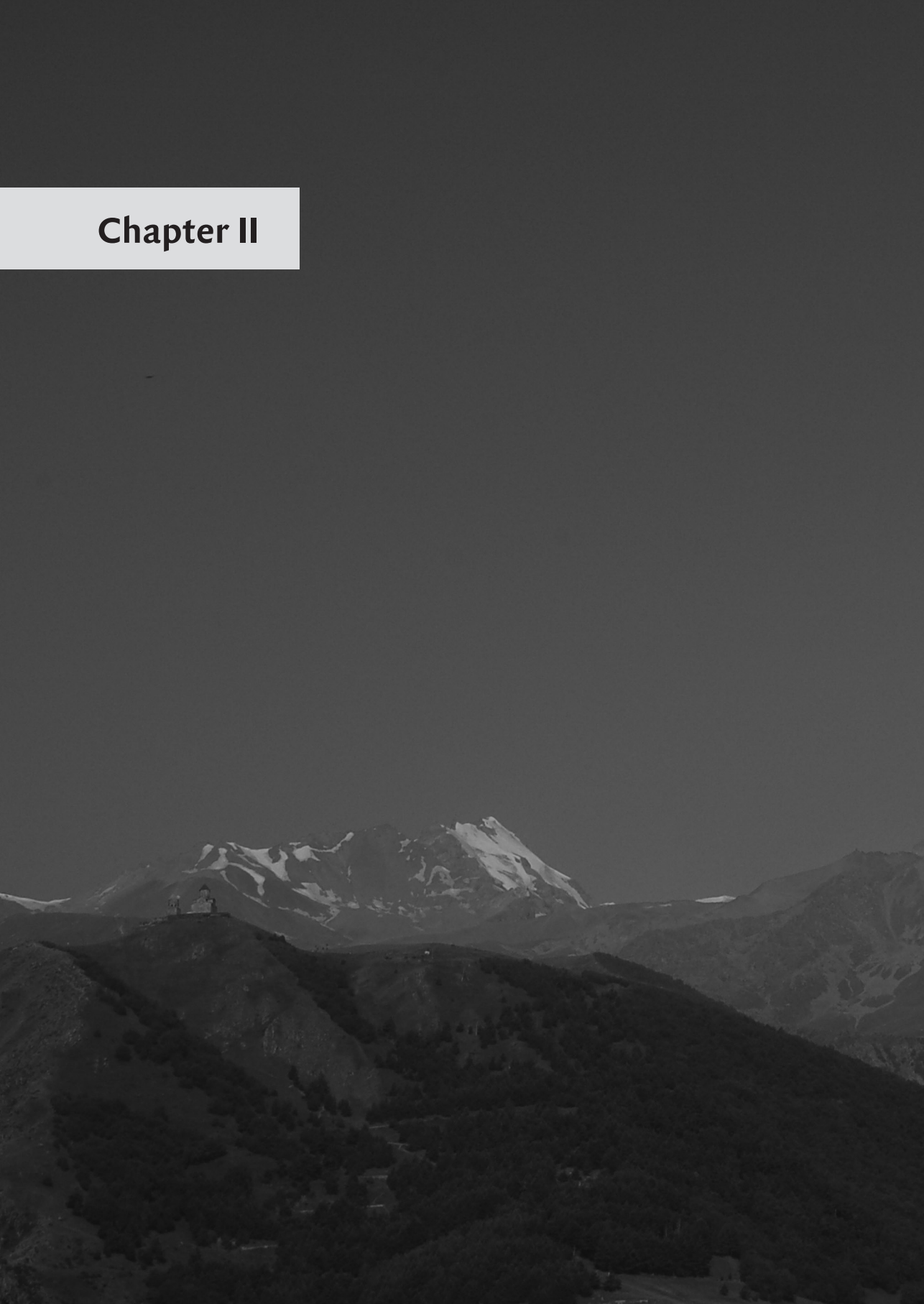
Chapter II



GATA-3 promotes T cell specification by repressing B cell potential in pro-T cells

Transcription factors orchestrate T-lineage differentiation in the thymus. One critical checkpoint involves Notch1 signaling that instructs T-cell commitment at the expense of the B-lineage program. While GATA-3 is required for T cell specification, its mechanism of action is poorly understood. Here we show that GATA-3 works in concert with Notch1 to commit thymic progenitors to the T-cell lineage via two distinct pathways. First, GATA-3 orchestrates a transcriptional repertoire that is required for thymocyte maturation up to and beyond the pro-T-cell stage. Second, GATA-3 critically suppresses a latent B-cell potential in pro-T-cells. As such, GATA-3 is essential to seal Notch-induced T-cell fate in early thymocyte precursors by promoting T cell identity through repression of alternative developmental options.

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Introduction

T-cell commitment takes place in the thymus, where multi-potent hematopoietic precursors sequentially shed their potential to differentiate into non-T-cell lineages while being focused towards becoming a T cell (a process termed specification). Thymopoiesis requires continuous replenishment by bone marrow-derived thymus-seeding progenitors (TSP) that generate early thymic progenitors (ETP) enriched for T cell potential (1). Several regulatory pathways are involved in early thymocyte differentiation, including Notch signaling and the transcription factors (TF) GATA-3, Bcl11b, Runx1, Ikaros, c-Myb, Tcf-1 and the basic helix-loop-helix factors E2A and HEB (for a review, see (1,2)). Nevertheless, the molecular targets for these TF in developing thymocytes are only poorly characterized, and our knowledge about the potential functional interplay between these different regulatory pathways during the T cell specification is limited.

Notch signals play a decisive role in the T-cell commitment process. T cell development from hematopoietic precursors requires expression of Notch-1, interactions with the Notch ligand Delta-like 4 (Dll4, expressed by thymic epithelial cells; (3,4)) and activation of the Notch canonical signaling cascade (mediated through CSL/RPB-Jk) (5,6). During T cell commitment, developing immature $CD4^{-}CD8^{-}$ 'double-negative' (DN) thymocytes proceed through phenotypically distinct stages that can be defined using the cell surface antigens CD25, CD44 and CD117 (7,8). Essentially all ETP reside in the DN1a,b subset that expresses CD117 (7,9) and requires Notch1 signals to develop (10). While Notch-1/Dll4 interactions are required for T cell commitment, it is not clear whether Notch-1 signals directly specify T-lineage cells, act to maintain early T cell progenitors so that their transcriptional program would unfold, and/or suppress alternative cell fates in lymphoid progenitors that are not fully committed. With respect to the latter, there has been much debate about mechanisms leading to exaggerated B cell development in the thymus of chimeras generated with Notch-1-deficient HSC (6). Since ETP do not develop in this context (10), it is likely that a subset of TSP not including ETP (7) can generate thymic B cells in the absence of Notch-1 signals. More recent studies using Carboxypeptidase A-Cre transgenic mice (that is active in pro-T cells just downstream of the CLP to ETP transition) also demonstrated that Notch-1 ablation in already generated ETP can results in fate conversion of ETP to the B cell lineage (11).

There is compelling evidence that a transcriptional program resulting from sustained ligation of Notch-1 is required for ETP to differentiate to the DN2 stage and to exclude B cell and DC potential from these early lymphoid progenitors (12,13). T-cell factor 1 (Tcf-1 encoded by *Tcf7*) is a direct Notch target required for T cell specification, and over-expression of Tcf-1 can drive T cell development from progenitors lacking Notch-1 by inducing T cell signature genes such as GATA-3 and Bcl11b (14,15). Notch signaling is likewise required for Bcl11b induction at the DN2 stage (16,17), suggesting that Notch-1 triggers

synergistic activation of key T cell identity factors. Bcl11b is a known transcriptional repressor that targets proto-oncogenes (*Tal1*, *Sfpi1*, *Lyl1* and *Erg*), cytokine receptors (*Kit*, *Flt3*, *Il2rb* and *Il7ra*) and NK lineage regulators (*Id2* and *Nfil3*) (16,18,19). In the absence of Bcl11b, DN2 cells do not develop into T cells but self-renew or are diverted towards the NK cell lineage (16,17,20). Curiously, both *Tcf7*- and *Bcl11b*-deficient progenitors normally extinguish B cell potential upon Notch-1 ligation demonstrating that other mechanisms are involved in this process.

GATA-3 expression is essential for normal T-cell development (21–23). Studies using mice with a GATA-3–driven LacZ or GFP reporters enabled developmental mapping of the dynamics of GATA-3 transcriptional activity (23,24). Conditional ablation of GATA-3 expression using mice bearing GATA-3 'floxed' alleles and harboring *Lck*-Cre or *Cd4*-Cre transgenes demonstrated that GATA-3 was critical for TCRβ-selection at the DN->DP transition and for promoting CD4 lineage choice (25). Less is known about the role for GATA-3 at the earliest stages of T cell development, as germline GATA-3-deficiency compromises fetal development and Cre-mediated GATA-3 deletion at the DN1 or DN2 stages has not been performed. Studies using hypomorphic GATA-3 alleles suggest a cell-autonomous role for GATA-3 in ETP (24), while over-expression of GATA-3 in T lineage precursors diverts these cells towards the mast cell lineage (26). As such, regulation of GATA-3 expression in early T cell progenitors is critical.

Both Notch-1 and GATA-3 are required for T cell specification, but it remains unclear whether they play specific, redundant or synergistic roles in this process. Here we decipher the role of GATA-3 in the network of transcription factors that program thymopoiesis. We found that GATA-3 controls T cell development up to and beyond the DN2 stage by regulating a transcription factors that promote T cell identity. In the absence of GATA-3, Notch-triggered T cell progenitors retain B cell potential, identifying an unappreciated role for GATA-3 in the suppression of B cell fate in DN2 thymocytes. These results indicate that GATA-3 promotes T cell development via complementary feed-forward and alternative fate suppression pathways.

Material & Methods

Gata3$^{-/-}$ fetal liver hematopoietic progenitor cells.
C57BL/6 mice carrying one *Gata3*^{nlslacZ} allele (23) were mated and E14.5 embryos were obtained as described (27). Mutant embryos were identified by PCR. Hematopoietic chimeras in *Rag2*$^{-/-}$ or *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice on the Ly5.1 background were generated as described (28). Mice were bred at the laboratory animal facility at the Institut Pasteur, Paris, France and were provided with food and water ad libitum. All animal experiments were approved by the Animal Care and Use Committee of the Institut Pasteur and were performed in accordance with French law.

Antibodies and flow cytometric analysis.
Single cell suspensions from thymus, spleen, bone marrow and lymph nodes were obtained, stained using fluorochrome-conjugated antibodies and analyzed by FACS as described (28). FACS analysis was performed on LSR, Canto, CantoII and LSRFortessa flow cytometers (Becton Dickinson, Mountain View, CA). Data was analyzed using Flowjo software (TreeStar Inc., San Carlos, CA). Fluorchrome-labeled monoclonal antibodies used for analysis are described in Supplemental **Table 1**. LIVE/DEAD stain (Invitrogen), DAPI or PI was used to identify viable cells.

Magnetic-activated and flow cytometric cell sorting.
Lineage-positive cells were depleted using magnetic-activated cell sorting (MACS, Miltenyi Biotec, Paris, France) or using flow cytometric sorting as indicated. Lineage antibodies are indicated in Supplemental **Table 1**. For MACS, lineage-positive cells were depleted using PE- or biotin-conjugated Microbeads and LS columns (both Miltenyi). DAPI-negative cells were sorted using Moflo (Cytomation Inc., Fort Collins, CO), FACSAria or FACSAria II (Becton Dickinson) machines.

Viability assays
For Bcl2 detection, Lin^- $CD44^+$ c-Kit^+ DN1 and DN2 cells were flow sorted, fixed and permeabilized using the BD Fix&Perm kit (Becton Dickinson) and stained according to the manufacturer's recommendations. Apoptotic cell were detected using Annexin V (Pharmingen) and mitochondrial potential was analyzed using 40 nM 3,3'-dihexyloxacarbocyanine iodide ($DiOC_6$, Invitrogen) for 30 minutes at 37°C.

Lymphocyte differentiation in vitro.
Unfractionated FL from *Gata3*$^{+/-}$ or *Gata3*$^{-/-}$ embryos was cultured on OP9, OP9Δ1 or OP9Δ4 monolayers supplemented with recombinant mouse IL-7 and Flt3L as described (29). For limiting dilution analysis, Lin^- $CD117^+$ $CD44^+$ $CD25^+$ (DN2) cells were FACS sorted and directly dispensed at 1, 3, and 9 (*Gata3*$^{-/-}$) or 10, 30 and 30 (*Gata3*$^{+/-}$) cells/well onto OP9 monolayers with indicated combinations of cytokines. Wells were scored for growth after 1 week and single cell clones were phenotyped by FACS. Clonal frequencies were calculated using Extreme Limiting Dilution Analysis (ELDA) (30).

Transcriptional analysis and TCRβ rearrangements.
Molecular profiling was performed on sorted cell populations. RNA was purified (RNeasy kit, Qiagen, Carlsbad, CA), oligo (dT)-primed cDNAs were synthesized using SuperScript II reverse transcriptase (Invitrogen Life Technologies, Carlsbad, CA), and qRT-PCR was performed using TaqMan primers with detection by an ABI Prism 7000 (Applied Biosystems) or Bio-Rad MiniOpticon (Bio-Rad, Marnes-la-Cognette, France). Primers sequences are available upon request. Genomic DNA was assessed for $D_\beta1$-$J_\beta1$, $D_\beta2$-$J_\beta2$ and $V_\beta8$-$J_\beta2$ rearrangements as described (31).

Retroviral production and transduction.
The pMXI-EGFP and the pMXI-GATA3-EGFP vectors have been previously described (32). Plasmid DNA was used to transfect Plat-E cells and viral supernatants were collected after 48 hours, 0.45μm filtered and frozen at –80°C. *Gata3*$^{-/-}$ OP9Δ1 cultures were depleted of lineage+ cells using MACS, mixed with half-diluted retroviral supernatant in complete media supplemented with IL-7, Flt3L, and 8 μg/mL polybrene (Sigma-Aldrich) and centrifuged onto OP9Δ1 monolayers for 2 hours at 2000 rpm

at 32°C. Following overnight culture at 32°C, a second cycle of infection was performed before adding cytokine-containing complete media.

Tat-Cre-mediated *Gata3* deletion.
Thymocytes from mice bearing floxed *Gata3* alleles (*Gata3*$^{flx/flx}$; (33)) were depleted of CD4, CD8 and CD19 expressing cells using directly conjugated MACS Microbeads (Miltenyi) and incubated with 2 μM Tat fusion proteins at 37°C for 15 minutes. DN2 thymocytes were sorted and cultured on OP9 stroma as above. Resultant colonies were phenotyped at day 10 and expanded for DNA analysis. Tat-Cre and Tat-GFP expression plasmids were provided by S. Dowdy and fusion proteins were prepared as described (34).

Results

GATA-3-deficient hematopoeitic precursors seed the thymus

Previous studies of embryonic chimeras made through complementation of WT or *Rag2*$^{-/-}$ blastocysts with *Gata3*$^{-/-}$ ES cells revealed that *Gata3*$^{-/-}$ hematopoietic precursors (HP) did not contribute to thymus reconstitution, thereby explaining the lack of T cell development, while B cells and other myeloid lineages were generated normally (21,23). In more recent studies, *Gata3*$^{-/-}$ HP showed a variable degree of thymic repopulation, but ETP homeostasis in the thymus was strongly reduced (24). These results suggest that GATA-3 could play a role in thymic ETP migration, similar to the role for GATA-3 in the migration of lymphoid cells to the liver (28).

An alternative hypothesis is that resident host elements limit the access of donor-derived *Gata3*$^{-/-}$ thymus-settling progenitors for thymopoietic 'niches'. We compared hematopoietic chimeras made using fetal liver HP (E14-E16) bearing heterozygous or homozygous mutant *Gata3*^{lacZ} alleles (23) in *Rag2*$^{-/-}$ recipients (lacking mature B and T cells) and *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice (also lacking NK cells and their lymphoid precursors). Residual early thymocytes in *Rag2*$^{-/-}$ mice are 100-fold reduced in the thymus of *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice thereby providing a more favorable thymic environment (35). Although thymic seeding was observed in *Gata3*$^{-/-}$ HP chimeras made in *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice **(Figure 1a)**, *Gata3*$^{-/-}$ precursors were rare in *Rag2*$^{-/-}$ chimeras **(Supplemental Figure 1)** consistent with previous results (3) and suggesting that residual *Rag2*$^{-/-}$ thymic lymphoid precursors could compete with *Gata3*$^{-/-}$ thymus-settling progenitors. In *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ recipient thymi, an abnormal accumulation of early CD4^{-}CD8^{-} thymocyte precursors with the CD44^{+}CD25^{-} phenotype (DN1 cells) and a smaller subset of CD44^{+}CD25^{+} cells (DN2) were observed **(Figure 1a)**. DN1 cells in both *Gata3*$^{+/-}$ and *Gata3*$^{-/-}$ chimeras expressed equivalent levels of CD117 (c-kit; **Supplemental Figure 2**). These data indicate that GATA-3 is not essential for thymic colonization by HP, but is rather essential for proper development of early thymocytes up to and beyond the DN2 stage. Interestingly, we observed that donor-derived B cells were increased in the thymi of mice engrafted with *Gata3*$^{-/-}$ HP **(Figure 1a)**.

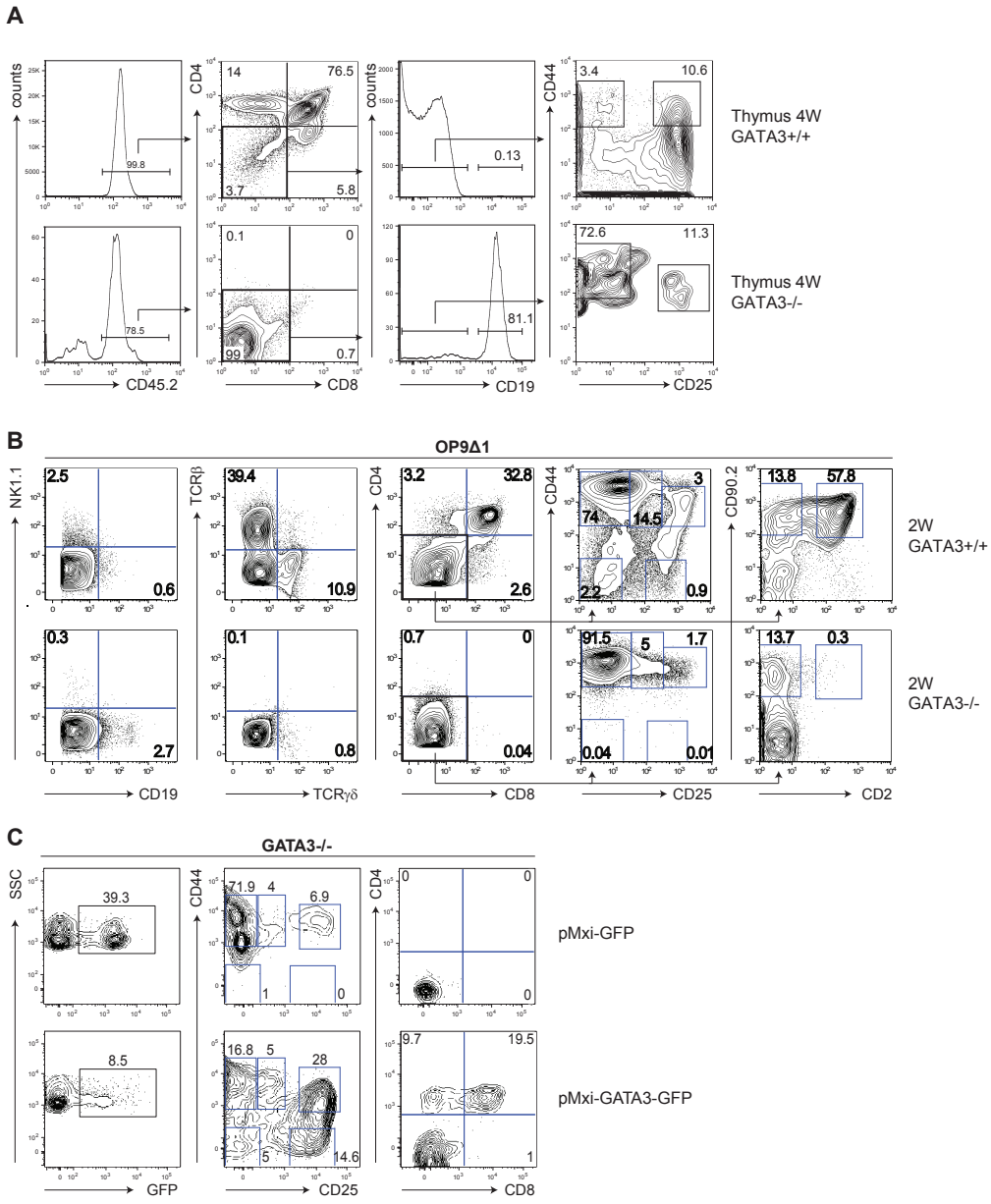

Figure 1. Notch-stimulated *Gata3*$^{-/-}$ hematopoietic progenitors fail to generate T cells
A) Flow cytometric analysis of the thymus of chimeras generated by injection of CD45.2^{+} *Gata3*$^{+/-}$ and *Gata3*$^{-/-}$ fetal liver cells into sublethally irradiated CD45.1^{+} Rag2$^{-/-}$Il2rg$^{-/-}$ recipient. Animals were analyzed 4 weeks after transplantation. All plots are gated on live lymphocytes. **B)** *Gata3*$^{+/-}$ and *Gata3*$^{-/-}$ fetal liver cells were cultured on OP9 stromal cells expressing the Notch-ligand Delta-like 1 (OP9Δ1) or Delta-like 4 (OP9Δ4) in the presence of IL-7 and Flt3L for 2 weeks. Plots show the expression of indicated surface markers on live CD45.2^{+} lymphocytes. **C)** Retroviral transduction of Lin^{-}CD117^{+} precursors from 2-week *Gata3*$^{-/-}$ OP9Δ1 cultures with pMxi-GFP or pMxi-GATA3-GFP retroviral particles. Two weeks after transfection, transduced cells were analyzed for expression of GFP (left panels). CD45.2^{+} GFP^{+} Lin^{-} cells were analyzed for expression of CD44 versus CD25 (middle panels) and CD4 versus CD8 (right panels) respectively.

The in vivo transfer of *Gata3*$^{+/-}$ and *Gata3*$^{-/-}$ HP allowed long-term reconstitution of *Rag2*$^{-/-}$ and *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice to a similar degree (as assessed by bone marrow and splenic cellularity), and these chimeric mice harbored donor-derived B cells, myeloid cells and splenic NK cells, while GATA-3-competent HP also generated T cells **(Supplemental Figure 3)** in accordance with previous results (18,21,23,28). These results are consistent with earlier reports demonstrating that GATA-3 is largely redundant for HSC maintenance and self-renewal (36).

Notch-stimulated T lineage precursors arrest at the DN2 stage without GATA-3

As the yield of early thymocyte precursors from thymi of *Gata3*$^{-/-}$ chimeras was limited, we further characterize these cells using a stromal cell based system (OP9Δ1/OP9Δ4; (29)) that can efficiently recapitulate T cell development from HP in vitro (reviewed in (37)). We found that both *Gata3*$^{+/-}$ and *Gata3*$^{-/-}$ HP proliferated extensively when cultured on OP9Δ1 or OP9Δ4 stromal cells in the presence of cytokines (including IL-7, SCF and Flt3L; similar results were obtained on both OP9Δ1 and OP9Δ4 stroma, data not shown) confirming previous reports (3,24). While *Gata3*$^{+/-}$ precursors develop into mature CD3$^+$ T cells (via CD4$^+$CD8$^+$ T cell intermediates) on OP9Δ cells, the vast majority of cells in *Gata3*$^{-/-}$ cultures remain at the CD4$^-$CD8$^-$ DN stage **(Figure 1b)**. Neither TCRαβ nor TCRγδ cells were present in *Gata3*$^{-/-}$ OP9Δ cultures, in contrast to WT cultures **(Figure 1b)**. Generation of B cells was inhibited in OP9Δ cultures of *Gata3*$^{+/-}$ and *Gata3*$^{-/-}$ HP progenitors **(Figure 1a)**, although significantly more CD19$^+$ B cells were observed in cultures derived from *Gata3*$^{-/-}$ HP **(Figure 1b)**. As expected, B cells were readily generated from both WT and *Gata3*$^{-/-}$ HP following culture on OP9 stroma **(Supplemental Figure 4)**.

We further characterized the DN thymocytes present in these OP9Δ cultures. As expected, *Gata3*$^{+/-}$ OP9Δ cultures contained immature T cells from all DN stages **(Figure 1b)** consistent with their capacity to generate TCRαβ and TCRγδ T cells and thymic NK cells (18,29). In contrast, *Gata3*$^{-/-}$ HP generated a high proportion of DN1 cells and a clearly defined but less prominent population of CD25$^+$ DN2 cells **(Figure 1b)**. *Gata3*$^{-/-}$ OP9Δ cultures did not progress beyond the DN2 stage, and despite prolonged culture (> 5 weeks), *Gata3*-deficient DN2 cells failed to accumulate to any appreciable degree **(Supplemental Figure 5a)**. Nevertheless, ectopic expression of GATA-3 (using retroviral particles transferring a bi-cistronic transcript encoding GATA-3 and GFP) was able to rescue the arrested *Gata3*$^{-/-}$ T-cell precursors present in 2-week OP9Δ cultures and allow their differentiation to the DN3-DN4 stage and beyond (CD4$^+$CD8$^+$ DP cell stage; **Figure 1c)**. Therefore, the complete developmental arrest in the absence of GATA-3 occurs at the DN2 stage where T-cell progenitors normally become committed can be corrected by Gata3 overexpression. Still, our results clearly demonstrate a role for Gata3 in the generation of early thymocyte progenitors and in the DN1 to DN2 transition.

Characterization of GATA-3-deficient DN2 cells

We found that the growth factor receptors CD117 (c-Kit) and CD127 (IL-7Rα) were normally expressed on DN2 cells in the absence of GATA-3 **(Figure 2a)**. Previous reports demonstrated normal Annexin V staining and higher proliferation in c-Kit$^+$DN1 cells from day 4 *Gata3*$^{-/-}$ OP9Δ cultures (24), a result that we confirmed **(Supplemental Figure 6)**. We found that *Gata3*$^{-/-}$ DN2 cells exhibited normal Annexin V binding and $DiOC_6$ staining compared to WT cells **(Figure 2b)**, and expression levels of the anti-apoptotic protein Bcl2 were comparable between GATA-3-competent and GATA-3-deficient precursors **(Figure 2c)**. Taken together, the apparently normal survival of *Gata3*$^{-/-}$ DN2 cells suggests that GATA-3 plays a role in the DN1 to DN2 transition that generates and expands early thymocyte precursors.

We next analyzed the molecular profiles of *Gata3*$^{+/-}$ and *Gata3*$^{-/-}$ DN2 cells **(Figure 3)**. Both types of DN2 cells were clearly differentiated towards the T cell lineage and expressed transcripts for *Ptcra*, *Cd3e* and importantly, *Tcf7* (encoding TCF-1) that is critical for T-lineage specification and differentiation (15). Notch-stimulated targets that are expressed in DN2 cells include *Tcf7*, *Ptcra*, *Hes1* and *Deltex1* (13); all are clearly expressed in DN2 cells in the absence of GATA-3, further confirming the T-cell profile of *Gata3*$^{-/-}$ DN2 cells.

Numerous transcription factors are dynamically regulated during early thymocyte differentiation (reviewed in (38)). Although the expression of most transcription factor chang-

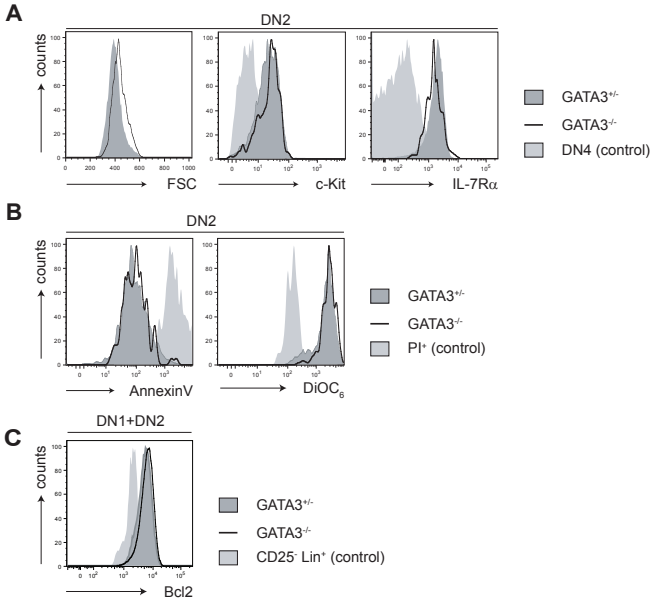

Figure 2. GATA-3-deficiency does not influence survival of DN2 T-cell precursors
A) Flow cytometric analysis of forward scatter (FSC), c-Kit and IL-7Rα expression on CD44$^+$CD25$^+$ CD4$^-$CD8$^-$ "double negative 2" (DN2) subsets of *Gata3*$^{+/-}$ and *Gata3*$^{-/-}$ OP9Δ1 co-cultures after two weeks. Filled histograms indicate *Gata3*$^{+/-}$ cells and solid lines indicate *Gata3*$^{-/-}$ cells. DN4 cells served as controls (light grey histograms). **B)** DN2 cells were analyzed for Annexin V binding (apoptosis) and $DiOC_6$ staining. Filled histograms indicate *Gata3*$^{+/-}$ cells and solid lines indicate *Gata3*$^{-/-}$ cells. PI$^+$ cells were used as controls (light grey histograms). **C)** DN1 and DN2 cells were analyzed for intracellular Bcl2 expression. Filled histograms indicate *Gata3*$^{+/-}$ cells and solid lines indicate *Gata3*$^{-/-}$ cells. Lin$^-$ CD25$^+$ cells were used as negative control (light grey histogram).

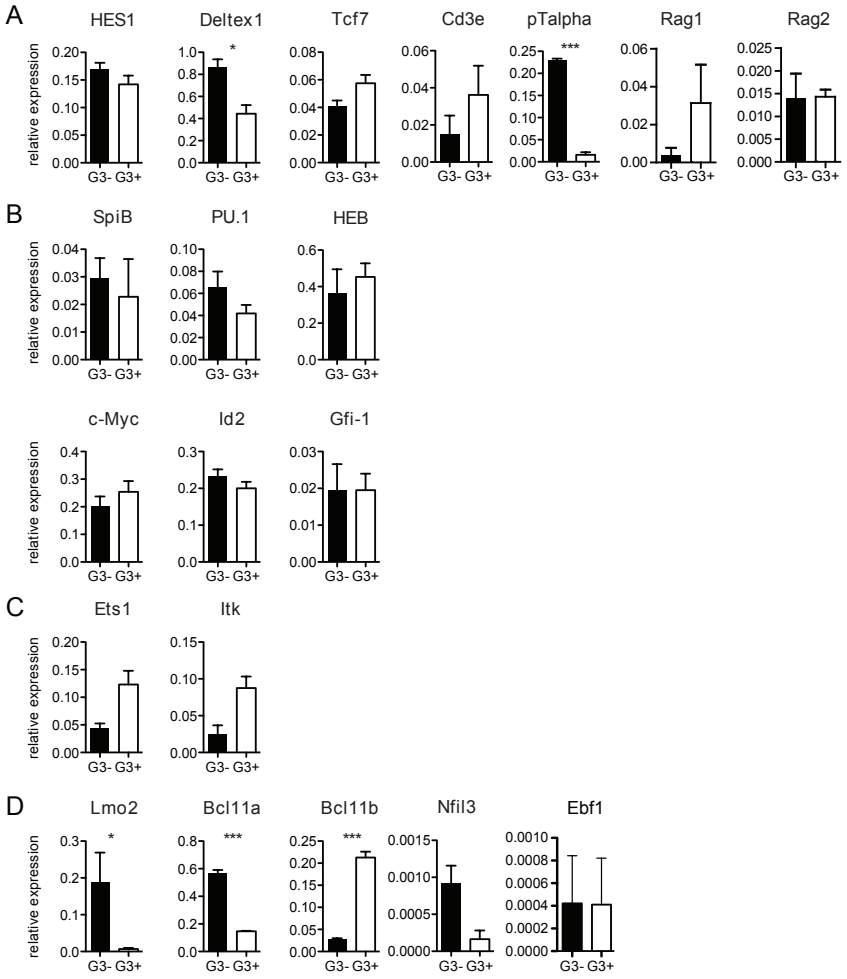

Figure 3. Transcriptional analysis of Gata3$^{-/-}$ DN2 T cell progenitors
A–D) Quantitative RT-PCR for the indicated genes in sorted DN2 populations from *Gata3*$^{+/-}$ and *Gata3*$^{-/-}$ OP9Δ1 cultures shown as expression levels relative to *Gapdh* expression levels. * indicates $p < 0.05$, *** $p < 0.001$.

es gradually during development from ETP to DN2 (such as Notch1, GATA-3, Tcf-1 and Gfi1), *Bcl11b*, *Lef1* and *Hebalt* levels are dramatically up-regulated and *PU.1*, *Tal1*, *Lyl1* and *Bcl11a* are down-regulated during this transition (reviewed in (38)). We found that GATA-3 was apparently not involved in the regulation of many of these TFs in DN2 cells, including PU.1, HEB, Gfi1, or Tcf7 **(Figure 3b**,c). We also found that levels of *Spib* (critical for DC development) and the transcriptional repressor *Id2* (critical for NK cell development) were unaltered in the absence of *Gata3* **(Figure 3b,c)**. While c-Myc expression is up-regulated in mice over-expressing GATA-3 at the DP stage (39), we did not observe changes in *Myc* expression in *Gata3*$^{-/-}$ DN2 cells. In contrast, *Ets1* and *Bcl11b* expression levels were clearly

reduced in the absence of GATA-3. While Ets-1 targets are poorly defined, Bcl11b has been shown to play an essential role in T-cell commitment by repressing alternative (myeloid and NK cell) fates in DN1 and DN2 cells (16,17,20). Along these lines, we found that *Nfil3* expression (required for NK cell development and normally repressed by Bcl11b; (17)) was up-regulated in *Gata3*$^{-/-}$ DN2 cells. Expression levels of B cell factors in DN2 cells were at (*Ebf1*) or below (*Pax5*) detection levels and were not altered in *Gata3*$^{-/-}$ DN2 cells **(Figure 3d**; **Supplemental Figure 7)**. Interestingly, two additional TF (*Lmo2* and *Bcl11a*) were clearly up-regulated in the absence of GATA-3 **(Figure 3d)**. Both *Bcl11a* and *Lmo2* are highly expressed in multi-lineage progenitors and their over-expression can lead to lymphoid cell malignancies (40,41). These results demonstrate that GATA-3 is essential for Notch-1-induced T-cell differentiation up to and beyond the DN2 stage that in a process that involves multiple TFs (*Ets1*, *Bcl11b*).

While *Gata3*$^{-/-}$ DN2 cells showed evidence of Notch-mediated transcriptional activation, Ptcra and Deltex1 over-expression **(Figure 3a)** led us to consider that excessive Notch stimulation might paradoxically block T cell development in *Gata3*$^{-/-}$ OP9Δ cultures. We tested this hypothesis by culturing *Gata3*$^{+/-}$ and *Gata3*$^{-/-}$ HP on OP9 stromal cells bearing the Notch ligands Jagged 1 (OP9J1) or Jagged 2 (OP9J2) compared with OP9Δ1 or OP9Δ4. Jagged proteins can interact with Notch receptors on early lymphoid precursors, but their capacity to promote T cell differentiation (and suppress B cell differentiation) in vitro is reduced compared to Dll1 or Dll4 (42). OP9J1 and OP9J2 cultures were permissive for B cell development from both *Gata3*$^{+/-}$ and *Gata3*$^{-/-}$ HP (albeit less efficient than parental OP9 cells), but only OP9J2 cells allowed generation of CD3^{+} T cells and this only occurred from *Gata3*$^{+/-}$ HP **(Supplemental Figure 8)**. These results are consistent with a hierarchy in potency of Notch ligands for promoting T cell development (Dll1 & Dll4 > Jagged 2 > Jagged 1) that corresponds to their efficiency in suppressing B cell development in this culture system as previously reported (43). Nevertheless, none of these OP9 stromal lines supported the development of CD3+ T cells from *Gata3*$^{-/-}$ HP, suggesting that excessive Notch signaling was not toxic for early thymocytes in the absence of GATA-3.

GATA-3-deficient DN2 cells possess an abnormal B cell potential

Thymic DN2 cells are highly enriched in T-cell progenitors, completely lack B cell potential, and maintain a latent NK cell potential that is lost after productive TCRβ gene rearrangement at the DN3 stage (7–9,14). Since *Gata3*$^{-/-}$ OP9Δ cultures did not generate T cells, we assessed whether mutant DN2 cells harbored NK cell precursors. To test their developmental potential, we isolated *Gata3*$^{+/-}$ and *Gata3*$^{-/-}$ DN2 cells and cultured them on OP9 stroma (thereby releasing them from enforced Notch stimulation) with or without IL-2. Robust growth was observed from both *Gata3*$^{+/-}$ and *Gata3*$^{-/-}$ DN2 cells with indistinguishable colony burst sizes and limiting dilution analysis indicated a clonal growth frequency of about 1 in 3.5 cells **(Figure 4a)**. Analysis of the single cell colonies showed that *Gata3*$^{+/-}$

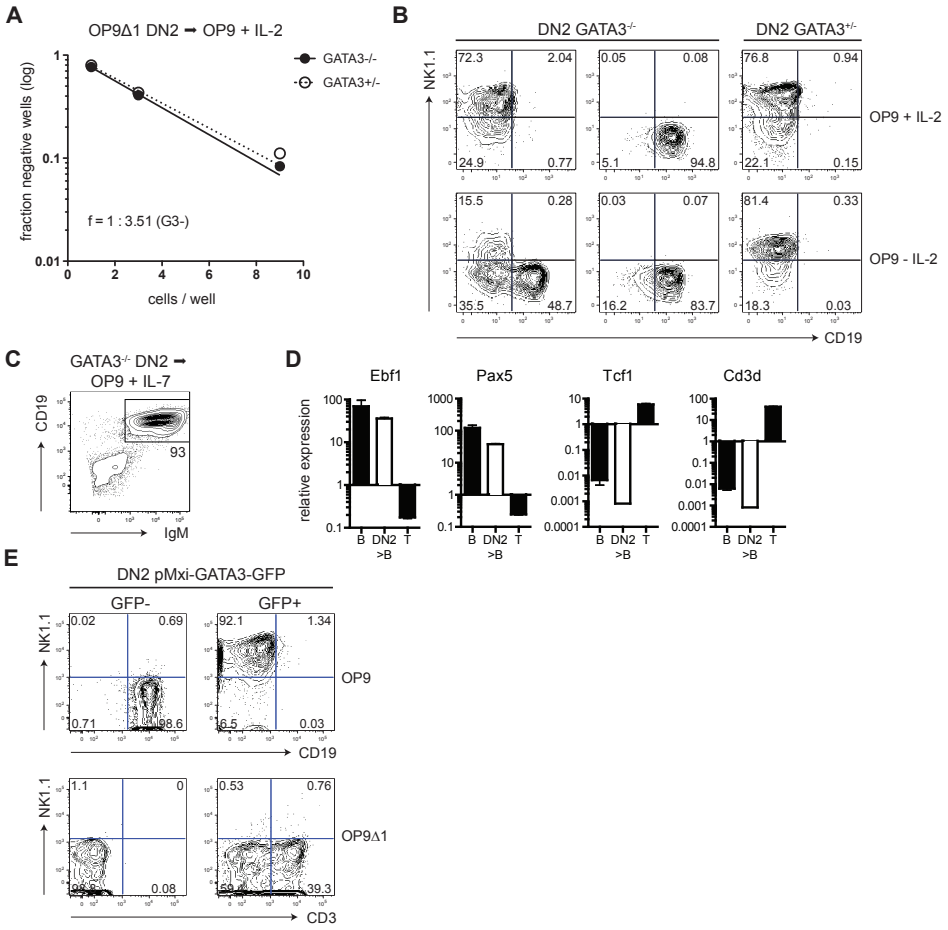

Figure 4. Lymphoid potential of *Gata3*$^{-/-}$ DN2 T cell progenitors
A) Limiting dilution and clonal frequency analysis of *Gata3*$^{+/-}$ and *Gata3*$^{-/-}$ DN2 cells (sorted from OP9Δ1 cultures) following replating on OP9 stroma in the presence of IL-7, Flt3L and IL-2. Wells were scored for growth after one week. **B)** Clones derived from single *Gata3*$^{+/-}$ and *Gata3*$^{-/-}$ DN2 cells after re-culture on OP9 stroma with IL-2 (upper panels) or without IL-2 (lower panels) were analyzed for expression of NK1.1 and CD19. **C)** *Gata3*$^{-/-}$ DN2 cells were re-cultured on OP9 stroma with IL-7 and NK1.1^{-} lymphocytes were analyzed for expression of CD19 and IgM. The gate indicates the population that was sorted for analysis of transcripts (panel D). **D)** RT-PCR analysis showing expression of B and T cell transcription factors by B cells grown from sorted DN2 cells on OP9 stroma (DN2>B; as shown in panel C) compared with sorted splenic B (B) and T (T) lymphocytes from a C57BL/6 control mouse. **E)** *Gata3*$^{-/-}$ T cell precursors were retrovirally transduced with pMxi-GATA3-GFP, and single GFP^{+} or GFP^{-} DN2 cells were cultures on either OP9 (upper panels) or OP9Δ1 (lower panels) stroma with IL-7 and Flt3L. One week later, plates were scored for growth and colonies derived from a single DN2 and were stained for NK1.1, CD3 or CD19 expression as indicated.

DN2 cells generated (as expected) exclusively NK cell clones, irrespective of the presence of IL-2 (108 single cell wells seeded giving rise to 11 colonies). Surprisingly, about 50% of the *Gata3*$^{-/-}$ DN2 cells generated B cell clones (144 single cell wells seeded giving rise to 19 colonies) in the presence of IL-2 **(Figure 4b)**. The B-cell potential of *Gata3*$^{-/-}$ T-cell precursors was more apparent in the absence of IL-2, where single *Gata3*$^{-/-}$ DN2 cells generated

almost exclusively B cells (144 single cell wells seeded giving rise to 23 colonies) with rare precursors giving rise to both B and NK cells **(Figure 4b**, lower left panel). The B cell potential of *Gata3*$^{-/-}$ DN2 cells was not due to a subset of cells that were already committed to the B cell lineage, since mutant DN2 precursors did not express transcripts for the B cell specific genes EBF or Pax-5 **(Figure 3d**; **Supplemental Figure 7)**. In contrast, CD19^{+}IgM^{+} B cells derived from *Gata3*$^{-/-}$ DN2 cultures **(Figure 4c)** expressed *Ebf1* and *Pax5* similar to normal splenic B cells **(Figure 4d)**. Therefore, a latent B cell potential in DN2 cells was only revealed when the cells were removed from Notch signaling, and only in the absence of GATA-3.

In order to test if the repression of a latent B cell potential in DN2 cells relied on GATA-3 expression, *Gata3*$^{-/-}$ DN2 cells were infected with the GATA-3-containing retrovirus and B, T and NK cell potential was assessed from GFP^{+} (GATA-3-transduced) and GFP^{-} (control) cells. GATA-3 expression in *Gata3*$^{-/-}$ DN2 cells restored T-cell potential upon culture on OP9Δ and allowed NK cell development on OP9 stromal cells **(Figure 4d)**. The latent B cell potential in *Gata3*$^{-/-}$ DN2 cells was therefore inhibited by GATA-3.

Inducible GATA-3 deletion in WT DN2 thymocytes unmasks a latent B cell potential

The unexpected B cell potential observed in *Gata3*$^{-/-}$ DN2 cells might result from their prolonged in vitro culture. We therefore isolated bone fide DN2 thymocytes from mice bearing a conditional GATA-3 allele (*Gata3*$^{flx/flx}$; (33)), induced GATA-3 deletion in these cells using a soluble form of the Cre recombinase (Tat-Cre) and cultured them on OP9 stroma. As expected (7–9)(14), DN2 cells **(Figure 5a**,b) from control thymi or from Tat-GFP-treated *Gata3*$^{flx/flx}$ thymi gave rise to few colonies under these conditions (the clonal frequency < 1 in 7,000; **Figure 5b)** consisting mostly of NK1.1^{+} NK cells (data not shown). In contrast, conditional ablation of GATA-3 expression in DN2 thymocytes using Tat-Cre completely abolished T cell development (0/144 clones tested; data not shown) and resulted in dra-

Table 1. Clonal frequency analysis of DN2 cells
Gata3$^{+/-}$ and *Gata3*$^{-/-}$ DN2 precursors were co-cultured with OP9 stroma under the indicated conditions and analyzed after 2 weeks. Values represent frequencies (1/x) with 95% confidence intervals. Frequencies were calculated according to (30). ND = not detected.

Gata3$^{+/-}$ **DN2 (OP9)**	**B**	**NK**	**myeloid**
IL-2	ND	2.85 (2.31 - 3.53)	ND
IL-7 + Flt3L	ND	5.33 (4.24 - 6.70)	ND
GM-CSF + IL-4	ND	ND	ND
Gata3$^{-/-}$ **DN2 (OP9)**	B	NK	myeloid
IL-2	11.24 (8.47 - 14.91)	12.79 (9.51 - 17.21)	ND
IL-7 + Flt3L	6.57 (5.17 - 8.35)	77.46 (40.18 - 149.32)	ND
GM-CSF + IL-4	9.56 (6.25 - 14.6)	11.49 (5.48 - 24.1)	4915 (691-34948)

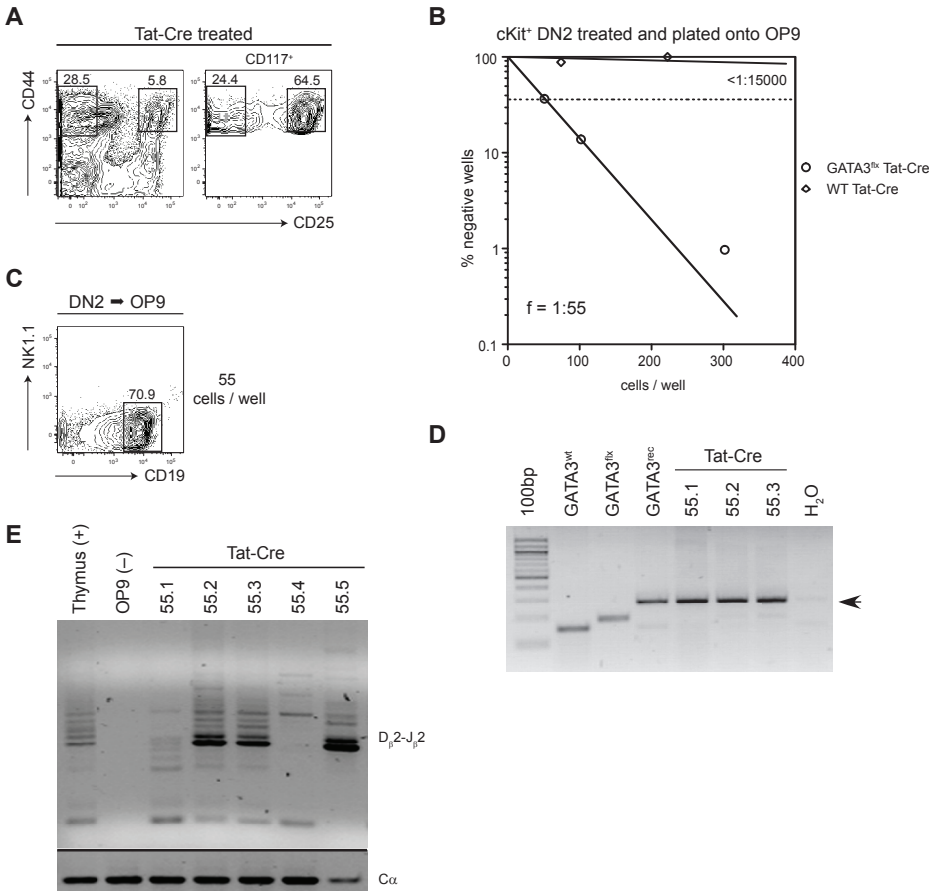

Figure 5. Conditional ablation of *Gata3* in DN2 cells reveals their B cell potential.
A) CD44 versus CD25 expression on Tat-Cre-treated CD117$^+$ thymocytes derived from *Gata3*$^{flx/flx}$ mice. Sort gates used to isolate CD44$^+$CD25$^+$ DN2 cells are indicated. **B)** Limiting dilution and clonal frequency analysis of CD117$^+$ DN2 cells from wild type and Gata3flx/flx thymocytes after treatment with Tat-Cre and plating on OP9 stroma in the presence of IL-7 and Flt3L. Colony growth was scored at one week. **C)** Representative B cell colony resulting from culture of Tat-Cre-treated *Gata3*$^{flx/flx}$ DN2 thymocytes (55 cells/well plated). **D)** Molecular analysis of the *Gata3* locus in CD19$^+$ B cell colonies derived following conditional Gata3 ablation in DN2 thymocytes. **E)** Analysis of TCR-D_β2-J_β2 rearrangements in B cell colonies after culture of Tat-Cre-treated *Gata3*$^{flx/flx}$ DN2 thymocytes. DNA from total thymus was used as a positive control and OP9 stroma DNA was used as negative control.

matically increased B cell clonal frequencies (f = 1/55; **Figure 5b)**. DNA from these DN2-derived B cell colonies **(Figure 5c)** bore recombined (deleted) GATA-3 alleles **(Figure 5d)**. Furthermore, these *Gata3*$^{-/-}$ DN2-derived B cell colonies harbored distinct TCR-D_β2-J_β2 rearrangements **(Figure 5e)**, which are molecular markers that characterize T-cell progenitors, and which are not observed in B cells under normal conditions. These results using *Gata3*$^{flx/flx}$ mice confirm the essential role of GATA-3 in early committed T cell progenitors to limit B cell potential.

Discussion

Here we analyze the role for the transcription factor GATA-3 in T cell specification and lineage commitment. Using a combination of in vivo reconstitution and in vitro differentiation from *Gata3*$^{-/-}$ hematopoietic precursors, we demonstrate that GATA-3 plays a critical role in the generation of CD25^{+} pro-T cells by regulating the TF 'repertoire' required for further T cell differentiation. Beyond its role as a catalyst of T cell lineage development, we also found that GATA-3 plays a critical role as a negative regulator of B cell fate in early thymic progenitors. In the absence of GATA-3, pro-T cells retained B cell potential that could be demonstrated using in vitro derived *Gata3*$^{-/-}$ cells or in vivo by conditional deletion of *Gata3* in CD25^{+} DN2 thymocytes. Taken together, these results suggest that GATA-3 promotes T cell fate in two ways: by synergizing with the Notch-initiated T cell program and by extinguishing alternative lymphoid cell fates.

Germ-line GATA-3 ablation results in prenatal mortality around embryonic day 11 (E11) due to the lack of noradrenalin synthesis (22). Administration of α- and β-adrenergic receptor agonists can rescue *Gata3*$^{-/-}$ embryos (up to E18; (27)) and we and others have used this approach to study the role for GATA-3 in early thymopoiesis (18,24,44). These studies provided clear evidence for an essential role of GATA-3 in the generation of early T cell progenitors. Thymus seeding by *Gata3*$^{-/-}$ precursors was demonstrable in vivo, although greatly reduced in irradiated congenic hosts (24) and most thymic progenitors were blocked at the DN1/ETP stage (24). Use of conditional *Gata3* deletion in hematopoietic cells (*Vav1*^{Cre} x *Gata3*$^{flx/flx}$ mice) generated similar results and transfer of Gata-3 KO precursors to FTOC or in vitro culture on OP9 stromal cells expressing Dll1 recapitulated the in vivo results (24,44). While *Gata3*$^{-/-}$ ETP did not appear to have defective survival characteristics, their transcriptional profiles were not studied. Here we have performed an extensive molecular and function analysis of *Gata3*$^{-/-}$ CD25^{+} DN2 cells.

The DN2 stage of thymocyte development represents a critical step in T cell specification and commitment. DN2 cells are highly enriched in T cell potential, but are not fully restricted to the T cell lineage, since they may still give rise to NK cell and DC given appropriate conditions. In contrast, DN2 cells lack demonstrable B cell potential (7–9). The DN2 cell fate is associated with a transcription factor 'repertoire' that reflects the ongoing specification and commitment to the T cell lineage. This includes increasing expression of *Tcf-1*, *HEB*, *Gfi-1*, *Bcl11b* and *Gata3*, while *PU.1*, *Lmo2*, *Bcl11a*, *Tal1* and *Lyl1* are extinguished (reviewed in (38)). DN2 cells also express T cell-specific genes (*Ptcra* and CD3 components) and engage the recombination machinery required for generation of pre-TCR and γδ T cell receptors.

Recently a detailed analysis of GATA-3 binding sites present during early thymocyte precursor differentiation was reported (45). GATA-3 sites were identified that were not associated with histone methylation or acetylation marks, but were clearly dependent on

the stage of T cell differentiation (45). Comparing our results of *Gata3*$^{-/-}$ DN2 cells to this genome-wide GATA-3 binding site analysis reveals interesting points. First, *Gata3*$^{-/-}$ DN2 cells showed clear evidence of T cell specification with expression of 'signature' genes (*Ptcra*, *Lck*, *Zap70* and *Cd3e*). While Notch1 targets *Ptcra* and *Deltex1* were over-expressed without GATA-3, these genes do not bind GATA-3 (45); how Notch signaling is released from GATA-3 inhibition therefore remains unclear. Second, absence of GATA-3 did not alter the expression of *HEB*, *Gfi1*, *c-kit* and *Tcf7* that have previously been associated with defects in the DN1->DN2 transition. Since *Gfi1*, *HEB* and *Tcf7* bear GATA-3 binding sites (45), a GATA-3-independent mechanism for their activation must exist. Third, a normal unfolding of the T-lineage program, including up-regulation of Tcf-1 and down-regulation of PU.1 (46) occurs normally in *Gata3*$^{-/-}$ DN2 cells. Finally, expression levels of several critical T cell factors (*Bcl11b*, *Ets1* and *Rag1*) were GATA-3-dependent, consistent with GATA-3 binding to these targets (45). While the role of Bcl11b in repressing alternative cell fates (i.e.: NK cell) in pro-T cells is now well recognized (16,17,20), the molecular mechanism by which Bcl11b promotes the completion of T cell specification is not understood. One hypothesis is that Bcl11b works by ensuring high E protein activity that sustains the DN2->DN3 transition (38). In this way, GATA-3 may indirectly control E protein levels that are required for successful T lymphopoiesis in addition to a possible direct regulation of E2A and HEB via GATA-3 binding (45). Ets-1 and Rag1 are generally involved at the DN3 stage, but Ets-1 may have earlier roles in DN thymocytes. GATA-3 is therefore an essential factor for further differentiation of DN2 cells.

We discovered an unexpected role for GATA-3 development in repressing development of other hematopoietic cell fates, especially B cells, in early thymocytes. We found that significantly more B cells developed from *Gata3*$^{-/-}$ precursors in vivo and even in vitro in the presence of the Notch ligands Dll1 or Dll4. *Gata3*$^{-/-}$ DN2 cells, that showed ample evidence of sustained Notch signaling, were still capable of 'reverting' to the B cell lineage, while this B cell potential was extinguished in *Gata3*$^{+/-}$ DN2 cells. Moreover, inducible GATA-3 deletion in DN2 thymocytes unleashed their B cell potential. Taken together, these different results strongly implicate GATA-3 in the suppression of a latent B cell potential in CD25^{+} pro-T cells.

While *Gata3*$^{-/-}$ DN2 cells showed robust B cell development, NK cell or myeloid cell development from these cells was demonstrable, but not appreciably augmented. The paucity of NK cell development was somewhat surprising given the reduction in Bcl11b that can repress NK cell lineage commitment (16,17). Still, the ability of *Bcl11b* deletion to promote NK cell 'conversion' has only been demonstrated in a GATA-3-competent context. This may be critical as GATA-3 has a documented role in the development of bone marrow and thymic NK cells (18,28). Some myeloid development was observed from *Gata3*$^{-/-}$ DN2 cells (4 of 78 clones tested) that mainly comprised CD11c^{+} DC. As *PU1* (and *Spib*) expression levels were not elevated in *Gata3*$^{-/-}$ DN2 cells, GATA-3 does not play a major role in antagonizing PU.1.

Collectively, our results demonstrate that *Gata3* deficiency selectively promotes a latent B cell potential in DN2 cells.

Signatures of B lineage specification, such as *Ebf* and *Pax5* are not normally expressed in DN2 cells and we found no evidence of their up-regulation in *Gata3*$^{-/-}$ DN2 cells. Tcf-1 appears redundant for repression of B cell potential from early thymocyte progenitors (15), and while Bcl11b acts to suppress alternative cell fates, there is no evidence for B cell 'conversion' from *Bcl11b*$^{-/-}$ DN2 cells (16). Interestingly, GATA-3 expression is preserved in *Tcf7*$^{-/-}$ or *Bcl11b*$^{-/-}$ thymic progenitors, consistent with the role for GATA-3 in B cell fate repression. We observed a clear up-regulation of *Bcl11a* in *Gata3*$^{-/-}$ DN2 cells. Bcl11a has multiple roles in the hematopoietic system; its over-expression can confer a myeloid phenotype while its absence is associated with defective B cell development (41). *Bcl11a* is normally down-regulated by the DN2 stage (38) and its loss is coincident with the onset of T cell specification. As absence of *Notch1* (11) or *Gata3* (this work) promote B cell fate conversion, it will be interesting to know if *Bcl11a* levels remain elevated in the absence of Notch-1 signals.

Previous studies suggested that *Gata3* expression in early T cell progenitors in mice might be regulated by Notch1 signals (47), although not in humans (48,49). Tcf-1 may regulate GATA-3 expression as levels are reduced in *Tcf7*$^{-/-}$ T cell progenitors (15). The combined actions of Notch1, Tcf-1 and GATA-3 result in a synergistic activation of Bcl11b that is required for T cell commitment **(Supplemental Figure 9)**. Our analysis of *Gata3*$^{-/-}$ DN2 cells additionally suggests that GATA-3 represses 'stem cell-like' genes (including *Lmo2*) that are highly expressed in immature lymphoid progenitors, associated with T cell transformation (40) and normally down-regulated by the DN2 stage. The ability of GATA-3 to promote T cell lineage development via extinction of stem cell qualities and repression of B cell fate parallels that of Bcl11b that inhibits the NK cell fate. In this way, these two critical T cell transcription factors have the ability to extinguish 'stemness' and to protect DN2 cells from diversion into alternative hematopoietic lineages **(Supplemental Figure 9)**.

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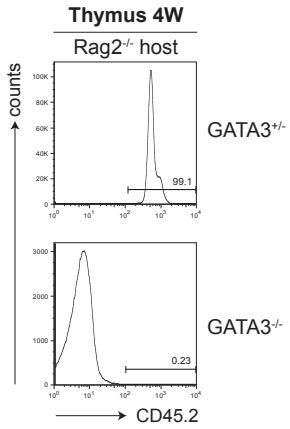
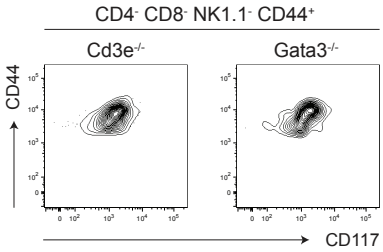
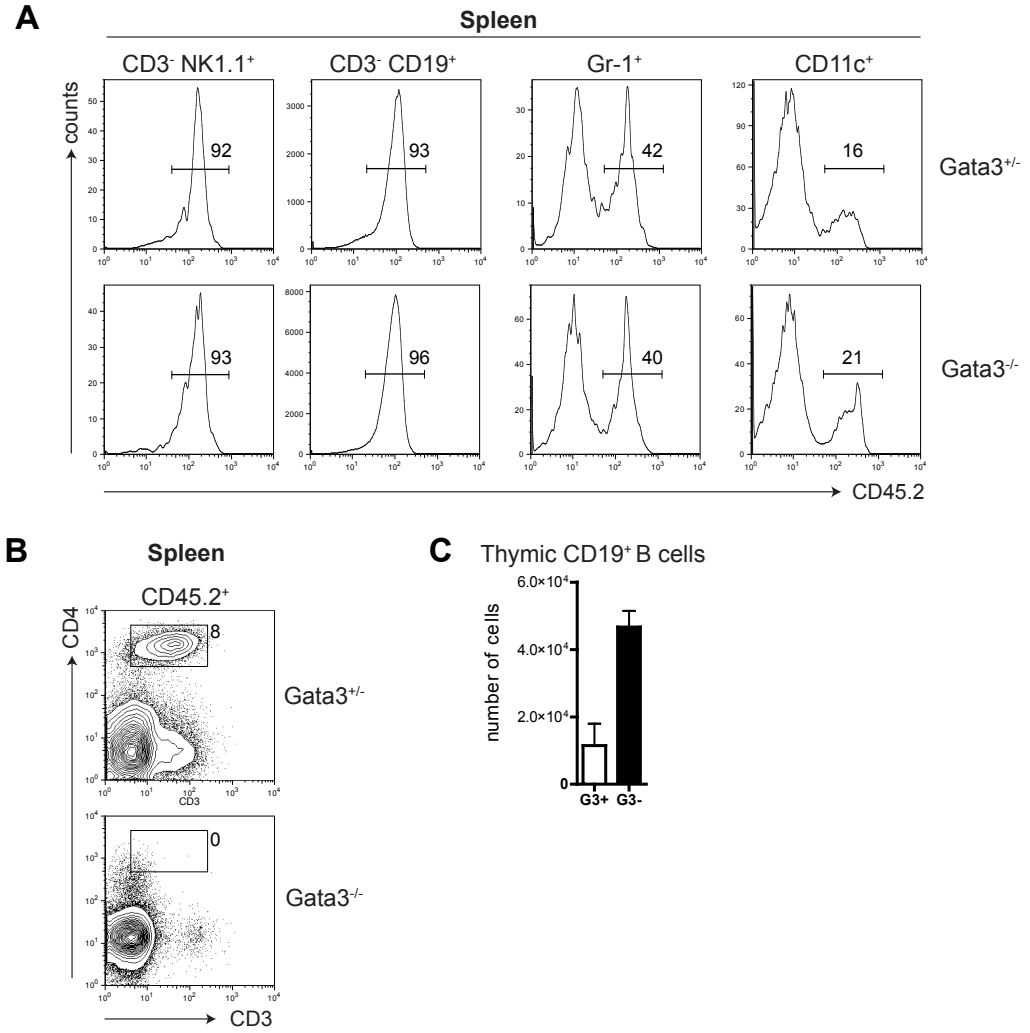
Supplemental information



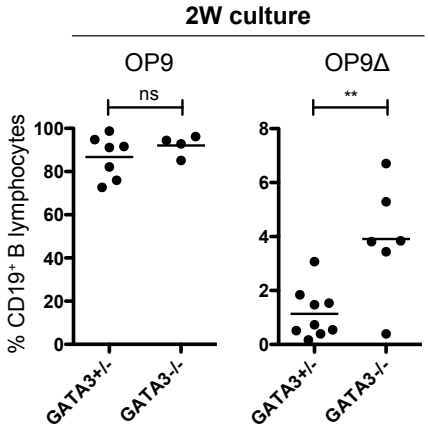
Supplementary Figure 1. *Gata3*$^{-/-}$ precursors more efficiently seed the thymus of *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ hosts compared to *Rag2*$^{-/-}$ recipients
Flow cytometric analysis of the thymus of chimeras generated by injection of CD45.2^{+} Gata3$^{+/-}$ and Gata3$^{-/-}$ FL precursors into irradiated CD45.1^{+} *Rag2*$^{-/-}$ or *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ hosts. Animals were analyzed 4 weeks after transplantation. Plots are gated on live lymphocytes.



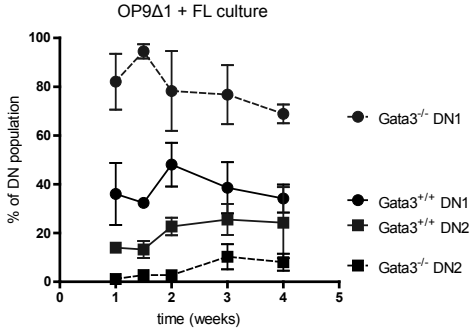
Supplementary Figure 2. ETPs develop normally in the absence of GATA-3.
Flow cytometric analysis of CD4^{-} CD8^{-} NK1.1^{-} CD44^{+} DN1 thymocytes from chimeras generated by injection of H2Db^{+} *Gata3*$^{-/-}$ FL and *Cd3e*$^{-/-}$ BM precursors into irradiated H2Db^{+} (BALB/c) *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ hosts. Animals were analyzed 5 weeks after transplantation.



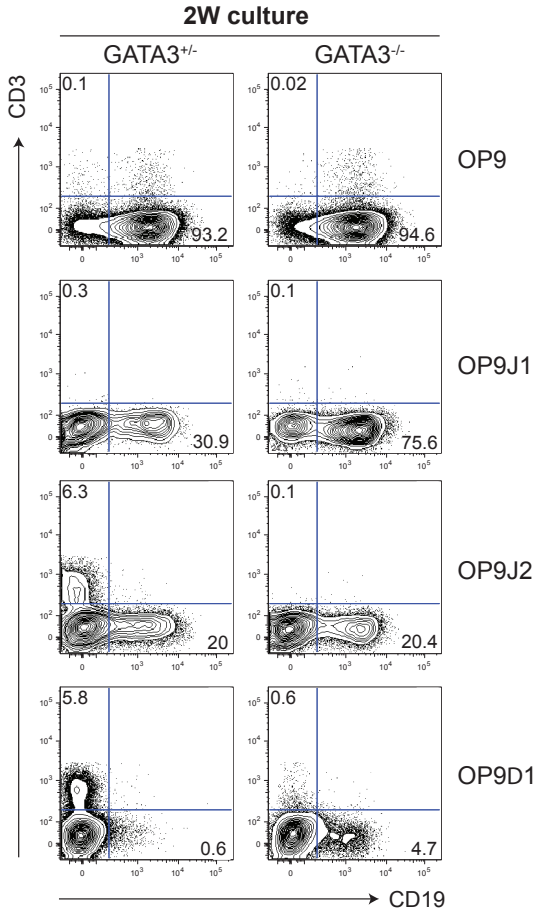
Supplementary Figure 3. Gata3-deficient precursors allow for long-term reconstitution of B, NK and myeloid cells.
Flow cytometric analysis of the thymus of chimeras generated by injection of CD45.2$^+$ *Gata3*$^{+/-}$ and *Gata3*$^{-/-}$ FL precursors into irradiated CD45.1$^+$ *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ hosts. **A)** Analysis of the indicated population in spleen of chimeras transplanted with the indicated precursors. **B)** Analysis of the splenic T cell population in donor-derived (CD45.2$^+$) lymphocytes.



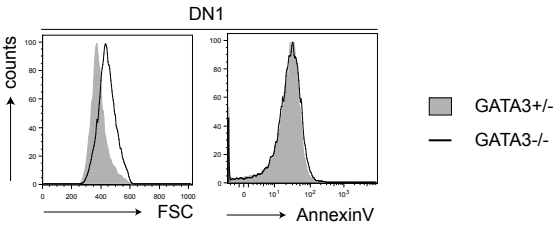
Supplementary Figure 4. B lymphocyte development in the OP9 and OP9Delta cultures from Gata3$^{+/-}$ versus Gata3$^{-/-}$ HP.
Gata3$^{+/-}$ and *Gata3*$^{-/-}$ FL precursors were cultured for 2 weeks on OP9 stroma transfected with Notch ligands Delta-like-1 (OP9Δ1) or Delta-like-4 (OP9Δ4) or not expressing Notch ligands (OP9). Dots indicate the frequency of CD19^{+} B lymphocytes in cultures. Pooled data from 7 independent experiments.



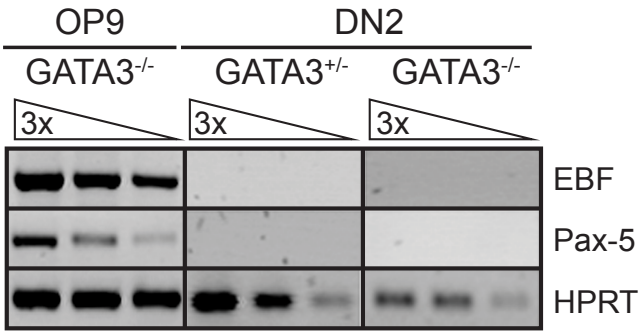
Supplementary Figure 5. Kinetics of DN subset development from Gata3$^{+/+}$ versus Gata3$^{-/-}$ HP.
A) *Gata3*$^{+/+}$ and *Gata3*$^{-/-}$ FL precursors were cultured on OP9Δ1 stroma and Lin^{-} (CD11b, CD11c, CD19, B220, NK1.1, Gr-1) CD4^{-} CD8^{-} DN lymphocytes were analyzed at the indicated time points. Shown is the percentage of DN1 and DN2 cells among the total DN population as means ± SEM with 3-5 independent cultures analyzed per time point. **B)** *Gata3*$^{+/-}$ and *Gata3*$^{-/-}$ FL precursors were cultured on OP9Δ1 stroma for 2 weeks and Lin^{-} CD4^{-} CD8^{-} CD11c^{-} CD19^{-} NK1.1^{-} CD44^{+} CD25^{-} DN1 cells were analyzed for expression of CD117.



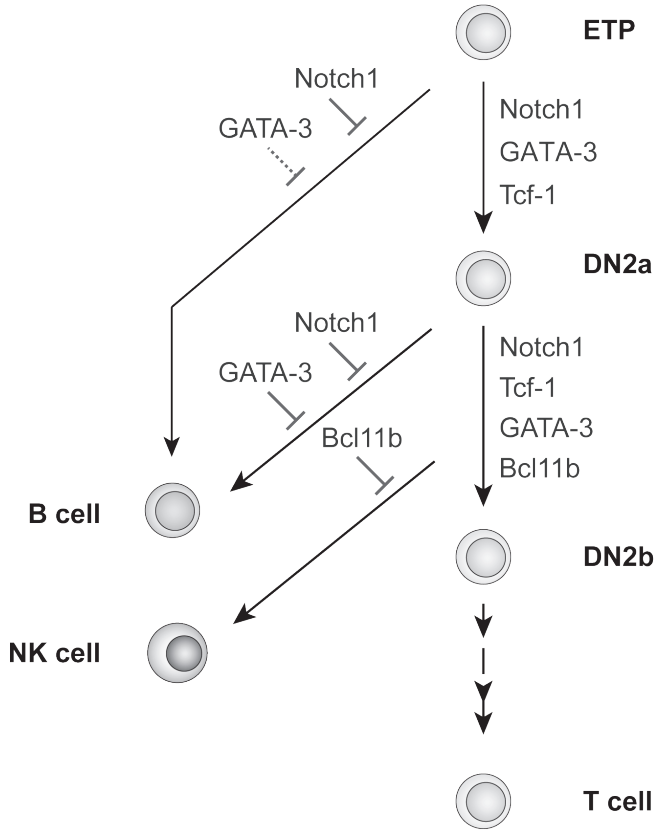
Supplementary Figure 6. Ability of different Notch ligands expressed on OP9 cells to promote T and B cell development from fetal liver progenitors in vitro
Gata3$^{+/-}$ and *Gata3*$^{-/-}$ FL precursors were cultured for 2 weeks on OP9 stroma transfected with Notch ligands Jagged1 (OP9J1), Jagged2 (OP9J2) or Delta-like-1 (OP9Δ1) or non-transfected (OP9). CD45.2^{+} live lymphocytes were analyzed for expression of CD3 and CD19. Numbers shows percentages of gated cells.



Supplementary Figure 7. DN1 analysis for cell size and apoptosis
Flow cytometric analysis of forward scatter (FSC) and AnnexinV binding (apoptosis) of CD44^{+}CD25^{-} CD4^{-} CD8^{-} DN1 subsets of *Gata3*$^{+/-}$ and *Gata3*$^{-/-}$ OP9Δ1 co-cultures after two weeks. Filled histograms indicate *Gata3*$^{+/-}$ cells and solid lines indicate *Gata3*$^{-/-}$ cells.



Supplementary Figure 8. Analysis B cell factor expression by DN2 cells from OP9Δ culture.
Semi-quantitative RT-PCR for *Ebf* and *Pax5* expression from CD117^{+} DN2 cells from *Gata3*$^{+/-}$ and *Gata3*$^{-/-}$ OP9Δ1 cultures. Bulk B cell cultures derived from *Gata3*$^{-/-}$ fetal liver hematopoietic progenitors cultured on OP9 stroma were used as control.



Supplementary Figure 9. Schematic overview of the role of GATA-3 in T cell development by suppression of a latent B cell potential in DN2a thymocytes. *(full color version: page 235)*
Solid red lines indicate demonstrated effects and dashed lines indicate putative roles of the indicated transcription factor.

Supplemental Table 1. Fluorochromes used for flow cytometry.

Target	Conjugate	Company	Clone
AnnexinV	APC	BD Pharmingen	
B220	PE	BD Pharmingen	RA3-6B2
B220	PECy7	eBioscience	RA3-6B2
Bcl-2	FITC	BD Pharmingen	
CD117 (c-Kit)	APC	eBioscience	2B8
CD11b	APCCy7	BD Pharmingen	M1/70
CD11c	PE	eBioscience	N418
CD11c	PECy7	BioLegend	N418
CD127 (IL-7Rα)	Alexa eFluor700	eBioscience	A7R34
CD127 (IL-7Rα)	PE	eBioscience	A7R34
CD127 (IL-7Rα)	PECy7	eBioscience	A7R34
CD19	APCCy7	BD Pharmingen	1D3
CD19	FITC	BD Pharmingen	1D3
CD19	PE	BD Pharmingen	1D3
CD19	PECy7	BD Pharmingen	1D3
CD19	PerCPCy5.5	BD Pharmingen	1D3
CD25	PECy7	eBioscience	PC61
CD25.4	PECy5.5	eBioscience	104
CD3	Alexa eFluor450	eBioscience	eBio500A2
CD3	PE	BD Pharmingen	145-2C11
CD3	PECy7	eBioscience	145-2C11
CD4	PE	BD Pharmingen	RM4-5
CD4	PECy7	eBioscience	GK1.5
CD4	PerCP	BD Pharmingen	RM4-5
CD44	AeFl780	eBioscience	IM7
CD45.1	Alexa eFluor780	eBioscience	A20
CD45.1	FITC	eBioscience	A20
CD45.2	PacificBlue	BioLegend	104
CD8α	APC	Southern Biotech	53-6.7
CD8α	APCCy7	BD Pharmingen	53-6.7
CD8α	PE	BD Biosciences	53-6.7
CD8α	PECy7	eBioscience	53-6.7
Gr-1	PE	BD Pharmingen	RB6-8C5
Gr-1	PECy7	eBioscience	RB6-8C5
HSA/CD24	Biotin	eBioscience	M1/69
LiveDead	AmCyan	Invitrogen	
NK1.1	PE	BD Pharmingen	PK136

Supplemental Table 1 *(continued)*. **Fluorochromes used for flow cytometry.**

NK1.1	PECy7	BD Pharmingen	PK136
NK1.1	PerCPCy5.5	BD Pharmingen	PK136
Streptavidin	APCCy7	BD Pharmingen	
Streptavidin	FITC	BD Pharmingen	
Streptavidin	PE	BD Pharmingen	
Siglec-H	Alexa eFluor647	eBioscience	eBio440c
STAT-5	PE	BD Biosciences	pY696
TCRαβ	Alexa eFluor647	Caltag	H57-597
TCRβ	FITC	BD Pharmingen	H57-597
TCRγδ	Biotin	BD Pharmingen	GL3
TER-119	PE	BD Pharmingen	TER-119
TER-119	PECy7	eBioscience	TER-119

Chapter III

Pulmonary innate lymphoid cells are major producers of IL-5 and IL-13 in murine models of allergic asthma

Allergic asthma is characterized by chronic inflammation and hyperreactivity of the airways and is thought to be mediated by an adaptive T helper 2 (Th2) cell-type immune response. Here, we demonstrate that type 2 pulmonary innate lymphoid cells (ILC2s) significantly contribute to production of the key cytokines IL-5 and IL-13 in experimental asthma. In naive mice, lineage marker negative ILC2s that express IL-7Rα, CD25, Sca-1, and T1/ST2 (IL-33R) were present in lungs and mediastinal lymph nodes (MedLNs), but not in bronchoalveolar lavage (BAL) fluid. Upon intranasal administration of IL-25 or IL-33 an asthma phenotype was induced, whereby ILC2s accumulated in lungs, MedLNs and BAL fluid. After IL-25 and IL-33 administration, ILC2s constituted ~50% and ~80% of IL-5$^+$/IL-13$^+$ cells in lung and BAL respectively. Also in house dust mite-induced or ovalbumin-induced allergic asthma, the ILC2 population in lung and BAL fluid increased significantly in size and ILC2s were a major source of IL-5 or IL-13. Particularly, in ovalbumin-induced asthma the contribution of ILC2s to the total population of intracellular IL-5$^+$ and IL-13$^+$ cells in the lung was in the same range as found for Th2 cells. We conclude that both ILC2s and Th2 cells produce large amounts of IL-5 and IL-13 that contribute to allergic airway inflammation.

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Introduction

Allergic asthma is characterized by a predominant eosinophilic airway inflammation, airway hyperreactivity and a chronic Th2 cell-type of immune response to various allergens, such as house-dust mites (HDMs), molds or animal dander [1, 2]. Available experimental models for asthma indicate that allergen-specific T helper type 2 (Th2) cells are key players in induction and maintenance of allergic asthma [3]. These cells produce vast amounts of cytokines that induce IgE synthesis (IL-4), recruit eosinophils and mast cells (via IL-5 and IL-9, respectively) and cause smooth muscle hyperreactivity via IL-13 (reviewed in [4]).

Although adaptive Th2 cells have been identified as important sources of IL-4, IL-5 and IL-13 [5], many recent studies emphasize the importance of innate cells in cytokine production, including but not limited to mast cells, eosinophils and basophils. Next to the canonical NK and lymphoid-tissue inducer (LTi) cells, a growing family of cytokine-producing 'helper' innate lymphoid cells (ILCs) has been identified [6]. Interestingly, a lineage-negative ILC population that produces high amounts of IL-5 and IL-13 was identified in fat-associated lymphoid clusters (FALCs) and mesenteric lymph nodes (MesLNs) [7-9]. These cells were named natural helper cells, nuocytes or multipotent progenitors and, because of their Th2 cytokine production, they have been dubbed type 2 ILCs [6]. ILC2s play a role in defense mechanisms and (re)shaping immune and non-immune tissues and can be stimulated with IL-25 and IL-33. In an *N. brasiliensis* infection model, ILC2s were essential and sufficient for clearance of this helminth from the mucosa, even in the absence of the adaptive immune system [7, 9].

Recently, various groups demonstrated the presence of ILC2s in the respiratory system of mice and humans [10-12]. ILC2 were shown to accumulate in the lungs of mice after infection with influenza virus, via an IL-33 dependent mechanism. These ILC2 induced airway hyperreactivity through IL-13 secretion [10] and also restored airway epithelial integrity and lung function and were contributed to airway remodeling by the production of amphiregulin [12]. In addition, ILC2s were found in nasal polyps of patients with chronic rhinitis, a classical Th2 disease [11].

IL-25 is a member of the IL-17 cytokine family that is expressed in human and mouse in response to allergens, particles and helminth infection [13-15]. Administration of IL-25 was shown to induce IL-4, IL-5 and IL-13 production, even in the absence of B and T lymphocyte in recombination activating gene (*Rag*)-deficient mice [15]. The main sources of IL-25 in asthma are the lung epithelium, eosinophils, mast cells and basophils [16]. Moreover, IL-25 amplifies Th2 responses and causes increased eosinophilic infiltration in mice [17]. IL-33 belongs to the IL-1 family and binds a receptor complex consisting of the IL-1R accessory protein and ST2 [18]. The membrane bound form of ST2 is highly expressed on mast cells and Th2 cells [19, 20], associating the IL-33 pathway with Th2 conditions. IL-33 is produced by epithelial cells in the lung [18] and epithelial expression of IL-33 increases

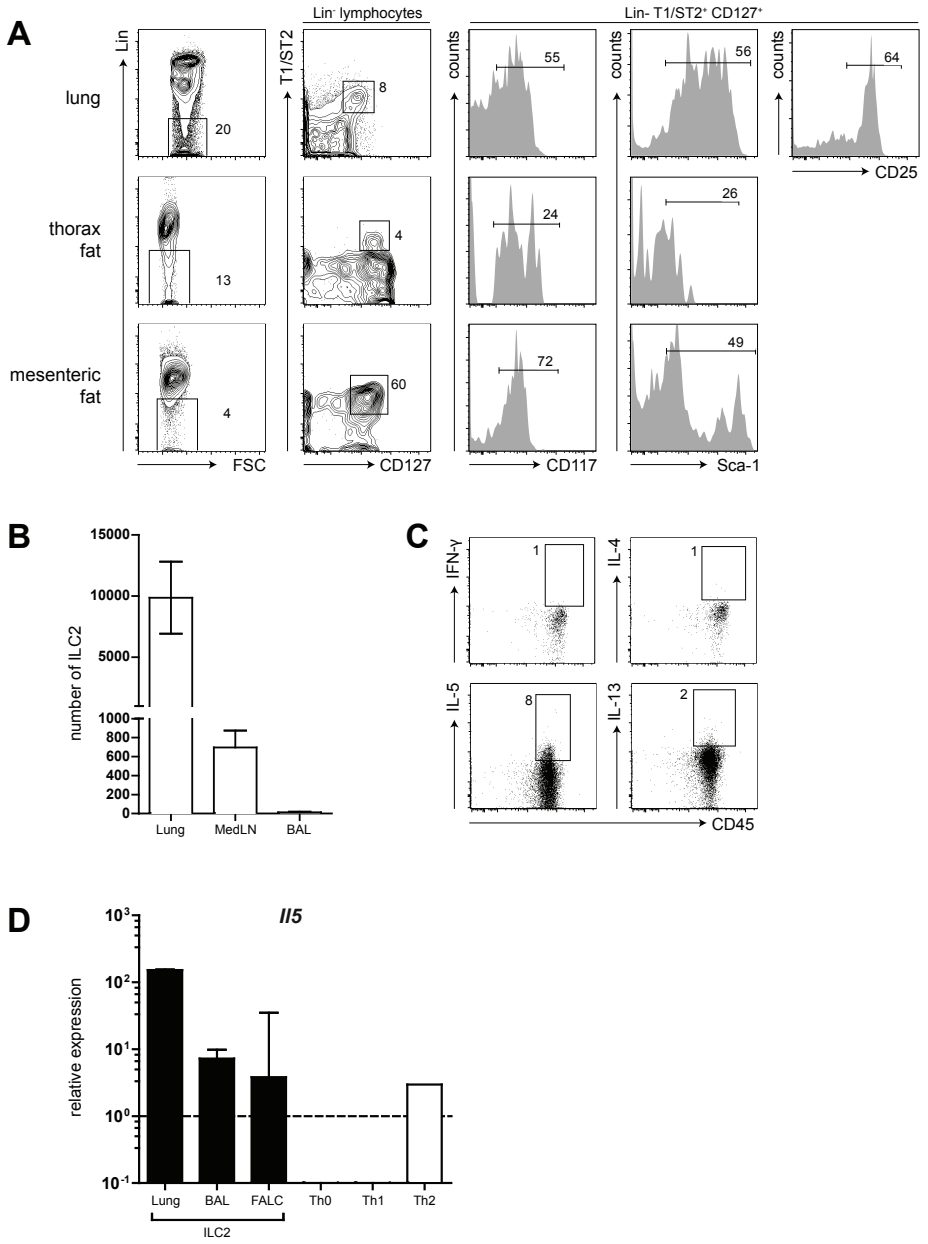


Figure 1. Pulmonary ILC2s are present in the respiratory tract of naïve mice.
A) Flow cytometric identification of pulmonary ILC2s is shown. Single-cell suspensions of collagen-treated lungs, thorax fat and mesenteric fat of naïve BALB/c mice were analyzed by flow cytometry. Cells negative for lineage markers were analyzed for expression of IL-33R (T1/ST2) and CD127 (IL-7Rα). T1/ST2⁺IL-7Rα⁺ cells were gated and analyzed for c-Kit (CD117), Sca-1 and IL-2Rα(CD25), shown as histograms. Proportions of cells within the gates are given. FSC = Forward Scatter. **B)** The quantification of ILC2s ($Lin^{-}CD45^{+}CD127^{+}T1/ST2^{+}CD25^{+}$ cells) in the pulmonary tract is shown. MedLN = mediastinal lymph node; BAL = broncho-alveolar lavage. Data are shown as mean + SEM of n≥2 animals per group and all data are representative of four independent experiments. **C)** Intracellular cytokine content of ILC2s is shown. Lin^{-} $CD127^{+}$ $T1/ST2^{+}$ lymphocytes were gated and analyzed for CD45 versus the indicated cytokines. Results are shown as dot plots. Single-cell suspensions were stimulated with PMA/ionomycin in the presence of monensin for 4h. **D)** RT-PCR quantification of *Il5* expression of the indicated FACS-sorted ILC2 cell suspensions, as well as polarized T-cell cultured under Tho, Th1 and Th2 conditions is shown. *Il5* levels were normalized to Actb levels. FALC = fat-associated lymphoid cluster. Data are shown as mean + SEM of n≥3 samples per group for ILC2 populations and one sample per group for Th populations and data are representative of two independent experiments.

in asthmatics [21]. The importance of IL-25 and IL-33 for asthma is underlined by the finding that disruption of the IL-25 or IL-33 pathway prevents induction of allergic airway inflammation in mice [22, 23]. Several studies have identified lineage-negative cells in the lung that manifest IL-5 and IL-13 production in response to IL-25 [14, 24] or IL-33 [25, 26] administration in mice.

In this report, we investigated the presence of ILC2s in the respiratory tract and hypothesized that pulmonary ILC2s are involved in allergic asthma by production of Th2 cytokines. We found that ILC2s produced substantial amounts of IL-5 and IL-13 in various murine models of allergic lung inflammation, both upon intranasal administration of IL-25 or IL-33 and in HDM or ovalbumin (OVA)-induced asthma. We conclude that both ILC2s and Th2 cells produce large amounts of IL-5 and IL-13 that contribute to allergic inflammation in asthma.

Results

Identification and characterization of pulmonary ILC2s

To explore the presence of ILC2s in the respiratory tract, we used flow cytometry and analyzed lung, mediastinal lymph node (MedLN), broncho-alveolar lavage (BAL) and thoracic fat of naive wild-type mice. We identified a lineage-negative, low side scatter population expressing IL-7Rα (CD127), T1/ST2 (IL-1RL1, a subunit of IL-33R), partially positive for c-Kit (CD117), stem cell antigen Sca-1 (Ly6A/E) and IL-2Rα (CD25) in the lung **(Figure 1A)**. This population resembled the recently identified population of ILC2s in gut-associated tissue and influenza infected lungs [6, 7, 9, 10, 12]. Sca-1 expression levels were higher in B6 mice than in BALB/c mice (data not shown). As ILC2s were initially found in fat-associated lymphoid tissue, we analyzed the thoracic fat attached to the thorax wall and around the aorta, and found varying but steadily limited numbers (<200) of $Lin^{-}T1/ST2^{+}CD127^{+}c\text{-}Kit^{+}$ cells. These ILC2s were present in the lung and MedLN of naive mice, but virtually absent in BAL **(Figure 1B)**.

Pulmonary ILC2s depend on common gamma chain cytokine signals

Pulmonary ILC2s were present in the lungs of C57BL/6 and Balb/c mice (Supporting Information **Figure 1)**. Intestinal ILC2s are found in *Rag2*$^{-/-}$ mice, but their development or survival depends on the common gamma (γc) chain, as *Rag2*/*Il2rg* double-deficient mice lack ILC2 [7]. Accordingly, we found pulmonary ILC2s in comparable numbers in the lungs and MedLN of *Rag1*$^{-/-}$ mice, but not in *Rag2*/*Il2rg* double-deficient mice **(Supplemental Figure 1)**. We noticed that the absence of Myd88, an adapter molecule that functions downstream of TLRs and IL-33R [27], had no significant effect on ILC2 numbers in the lung. IL-22 is an important cytokine in the lung and in the intestine for protection of epithelial surfaces [28] and is rapidly produced in conjunction with IL-17 by LTi cells. We detected no significant

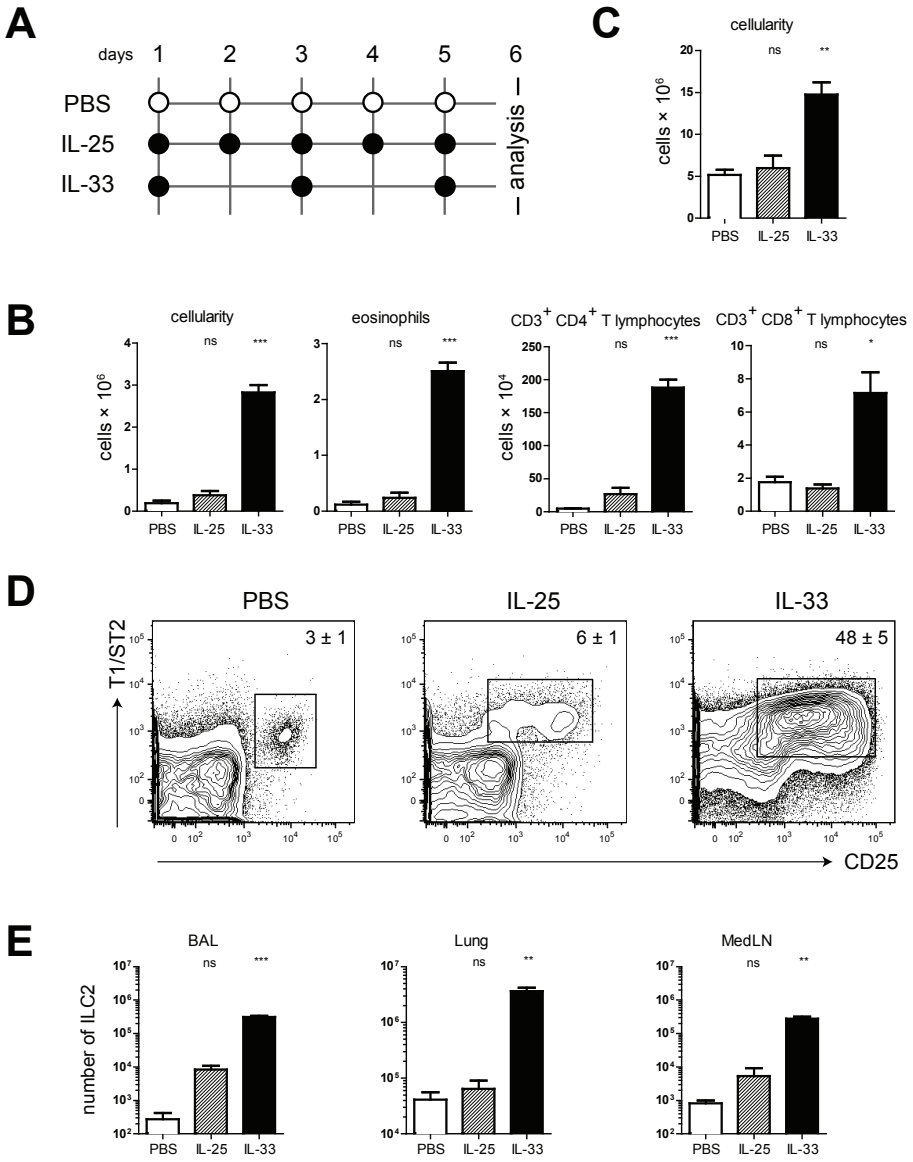


Figure 2. Administration of IL-25 and IL-33 induces eosinophilic airway inflammation and increases ILC2 numbers.

A) Scheme for IL-25 and IL-33 treatment of BALB/c mice. Isoflurane-anesthesized animals received 1µg intra-nasal administrations of the indicated cytokines (or PBS as a control) on the days marked with a circle and were analyzed 24 hours later. **B)** The quantification of indicated cell populations in the BAL fluid after cytokine treatment is shown. **C)** The total number of cells in the lungs of treated animals is shown. **D)** Flow cytometric identification of pulmonary ILC2s (gated) in single cell suspensions of the lungs of treated mice. Plots show expression of the indicated markers on cells negative for lineage markers. Numbers indicate the frequency of ILC2s in the total lymphocyte population ± SEM using the gates indicated. **E)** Quantification of ILC2s in different compartments of the pulmonary tract is shown. **B,C,E)** Data are shown as mean + SEM of n=3-4 mice per group and all panels are representative of two independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, one-way ANOVA analysis followed by Turkey post-tests to analyze statistical significance between PBS control versus treated animals. ns = not significant.

alterations in lung ILC2 numbers in *Il22*$^{-/-}$ mice **(Supplemental Figure 1)**. Closely related ILC populations, including ILC17 [29] and ILC22 [30-33] depend on the RORγt transcription factor for their development, while intestinal ILC2s do not express RORγt, but RORα and RORβ instead [7]. In agreement with this finding, pulmonary ILC2s were present in mice deficient for the *Rorc* gene, encoding RORγt **(Supplemental Figure 1)**.

In summary, we found that development of pulmonary ILC2s requires signaling via a γc-dependent cytokine pathway. Because of the high levels of IL-7Rα (CD127), it is conceivable that IL-7 signals are important for ILC2 development.

Pulmonary ILC2s produce IL-5 and IL-13 Th2 cytokines

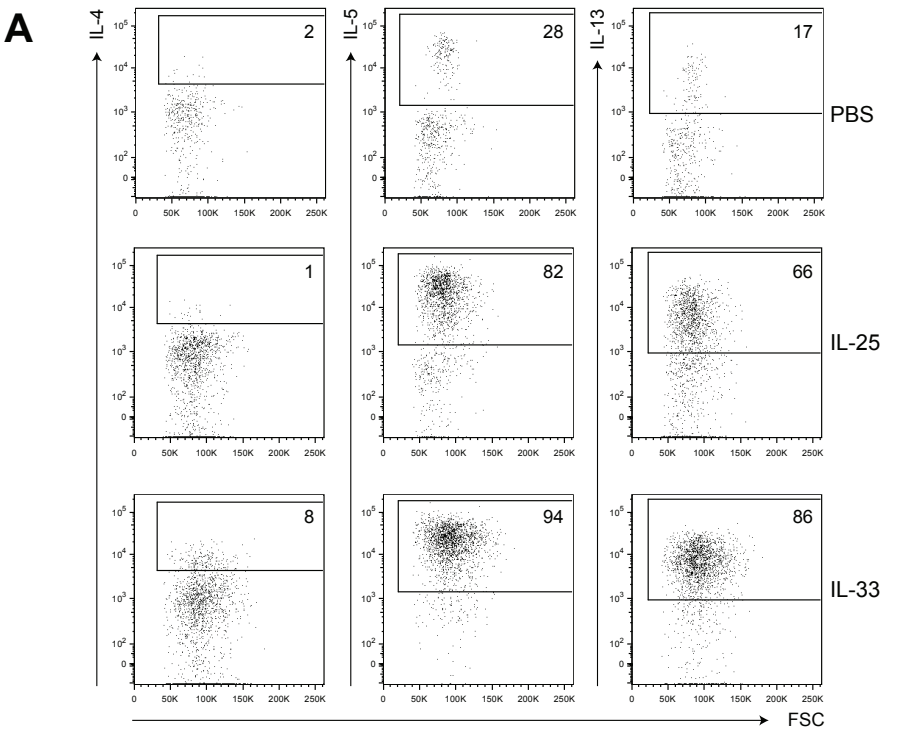
ILC2s produce large amounts of the Th2 cytokines IL-5 and IL-13 upon phorbol myristate acetate (PMA) and ionomycin stimulation [7, 9]. When we stimulated lung cell suspensions with PMA/ionomycin for 4h and probed for production of the Th2 cytokines by intracellular flow cytometry, we detected fractions of ILC2s positive for IL-5 and IL-13, while IFN-γ$^+$ and IL-4$^+$ ILC2s were hardly detectable **(Figure 1C)**. Quantitative RT-PCR analysis of sorted Lin$^-$CD127$^+$ T1/ST2$^+$ cells from naive mice stimulated in vitro with IL-2, IL-25 and IL-33 showed that lung ILC2 expressed higher levels of Il5 than BAL ILC2, FALC ILC2 or in vitro cultured polarized Th2 cells **(Figure 1D)**.

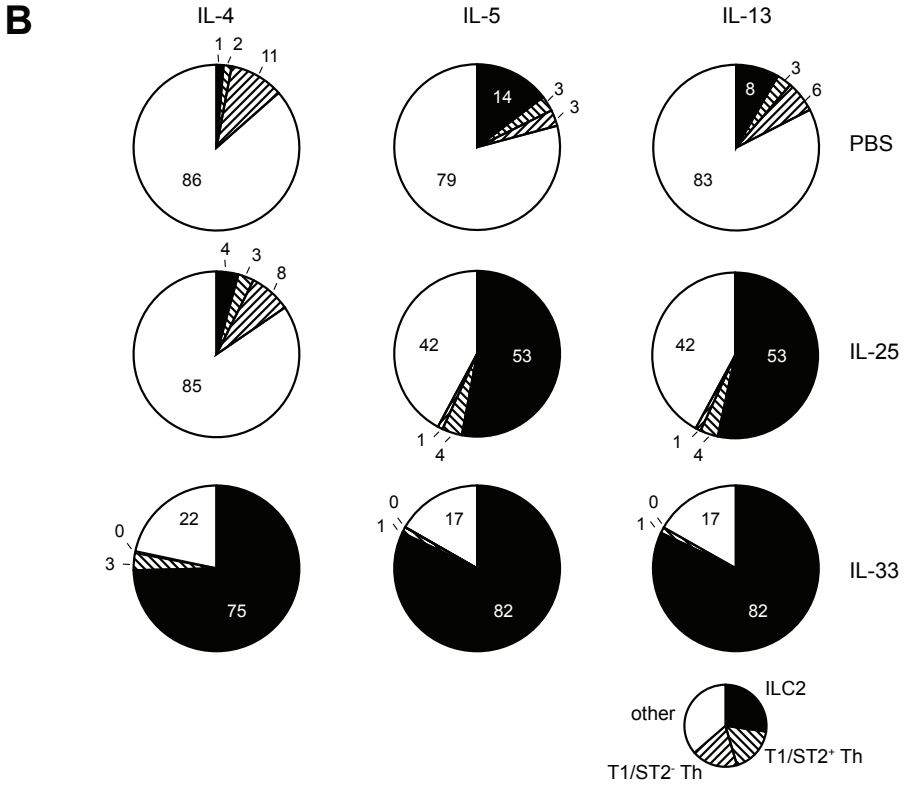
Pulmonary ILC2s are induced in IL-25- and IL-33-induced asthma

Although classic animal models of experimental asthma depend on sensitized Th2 cells, intra-nasal administration of IL-25 or IL-33 in mice have also been reported to induce an asthma phenotype with airway hyperresponsiveness, eosinophilic inflammation, mucus hypersecretion and Th2 cytokine production in the lung [14, 25, 34]. Since ILC2s are reported to release cytokines in response to IL-25 or IL-33 stimulation [7, 9], we hypothesized that pulmonary ILC2s contribute to airway inflammation in cytokine-induced asthma. Mice were intra-nasally treated with 5 doses of IL-25 or 3 doses of IL-33 and analyzed 24h after the last instillation **(Figure 2A)**. While we observed no significant increase in BAL cellularity in IL-25 treated mice, IL-33 induced a strong increase in total cellularity, eosinophils and T cells in BAL fluid **(Figure 2B)**, indicating the onset of airway inflammation. Likewise, lung cellularity also increased upon IL-33 administration **(Figure 2C)**. Administration of IL-25 and IL-33 induced upregulation of T1/ST2 on ILC2s **(Figure 2D)**. The number of ILC2s strongly increased in mice treated with IL-33 **(Figure 2E)**.

Pulmonary ILC2s are the major cytokine producing lymphocytes in IL-25- and IL-33-induced asthma

Next, we determined the contribution of ILC2s to production of IL-4, IL-5 and IL-13 in cytokine-induced airway inflammation by intracellular flow cytometry. We observed that intra-nasal treatment with PBS alone induced increased production of IL-5 and IL-13


A
IL-4
IL-5
IL-13
2
28
17
PBS
1
82
66
IL-25
8
94
86
IL-33
FSC


B
IL-4
IL-5
IL-13
1 2 11
86
14 3 3
79
8 3 6
83
PBS
4 3 8
85
42 53 1 4
42 53 1 4
IL-25
0 22 3 75
0 17 1 82
0 17 1 82
IL-33
ILC2
other
T1/ST2- Th
T1/ST2+ Th

(Figure 3A, compared with naive mice, **Figure 1C)**. Treatment with IL-25 or IL-33 further increased the fraction of IL-5 and IL-13 positive ILC2s to more than ~80% and ~60% respectively, while IL-4 production remained low **(Figure 3A)**. Comparisons with cytokine-content of T1/ST2$^+$ CD4$^+$ T cell fractions, which are enriched for Th2 polarized cells [20], and T1/ST2$^-$ CD4$^+$ T cell fractions, revealed that the ILC2 population comprised most of the IL-5$^+$ and IL-13$^+$ lymphocytes upon treatment with IL-25 and IL-33 (up to 3.4×10^6 IL-5$^+$ and 3.1×10^6 IL-13$^+$ cells/lung after IL-33 treatment, respectively) **(Figure 3B)** . The contribution of ILC2s to IL-4 production strongly increased upon IL-33 but not upon IL-25 administration. Likewise, IL-25 and IL-33 activated IL-5 and IL-13 production in ILC2s in BAL fluid **(Supplemental Figure 2A)**. After treatment with IL-25 and IL-33, ILC2 became the most important producers of Th2 cytokines in the BAL **(Supplemental Figure 2B)**.

Taken together, these results show that treatment with IL-25 induced a limited increase in BAL cellularity, whereby pulmonary ILC2s were potent producers of IL-5 and IL-13. In contrast, treatment with IL-33 induced a strong increase in total cellularity, and an increase of the numbers of eosinophils and ILC2s in BAL. These ILC2 manifested a high cytokine-content for IL-5, IL-13, and to a lesser extent IL-4, all of which contribute to allergic lung inflammation.

The ILC2 population is an important target of IL-25 and IL-33, independent of the adaptive immune system

Previous studies have shown that IL-25 and IL-33 can induce an asthma phenotype, even in the absence of B and T lymphocytes [14, 25, 34]. Consistent with reported findings [7, 8], we found that ILC2s develop in the absence of *Rag1* or *Rag2*, but are virtually absent from lungs in mice that lack the common gamma chain (Supporting Information **Figure 1** and **Figure 4A)**. To investigate whether ILC2s are activated in the absence of an adaptive immune system, we employed the cytokine-induced asthma models in *Rag2*-deficient (ILC2$^+$) and *Rag2*/*Il2rg* double deficient (ILC2$^-$) mice. Administration of IL-25 and IL-33 resulted in an increase in cellularity in lung and BAL and eosinophilic inflammation in *Rag2*-deficient mice, but not in *Rag2*/*Il2rg* double deficient mice **(Figure 4B)**. These findings indicate that a common gamma chain-dependent population is responsible for inducing airway inflammation upon IL-25/IL-33 administration. Administration of IL-33 induced increased ILC2 numbers in BAL and lung in *Rag2*-deficient mice, while we observed no increase in

Figure 3 (*left page*)**. ILC2s produce important amounts of IL-5 and IL-13 in cytokine-induced airway inflammation. A)** Intracellular cytokine content of pulmonary Lin$^-$CD25$^+$CD127$^+$T1/ST2$^+$ ILC2s in mice, treated as specified. Plots show forward scatter (FSC) versus the indicated cytokines. Single-cell suspensions of lungs were stimulated with PMA/ionomycin in the presence of monensin for 4h. Numbers indicate the proportions of cytokine-positive ILC2s. **B)** Contribution of various cell populations to Th2 cytokine production in the lungs after administration of PBS, IL-25 or IL-33. Proportions of cytokine expressing cells were determined by flow cytometry (as in panel A) in ILC2, T1/ST2$^+$ and T1/ST2$^-$ CD3$^+$CD4$^+$ T helper cells. Pie charts and percentages represent the contribution of various populations to the total number of cytokine-producing cells. Data are representative of two independent experiments with 3-4 mice per group.

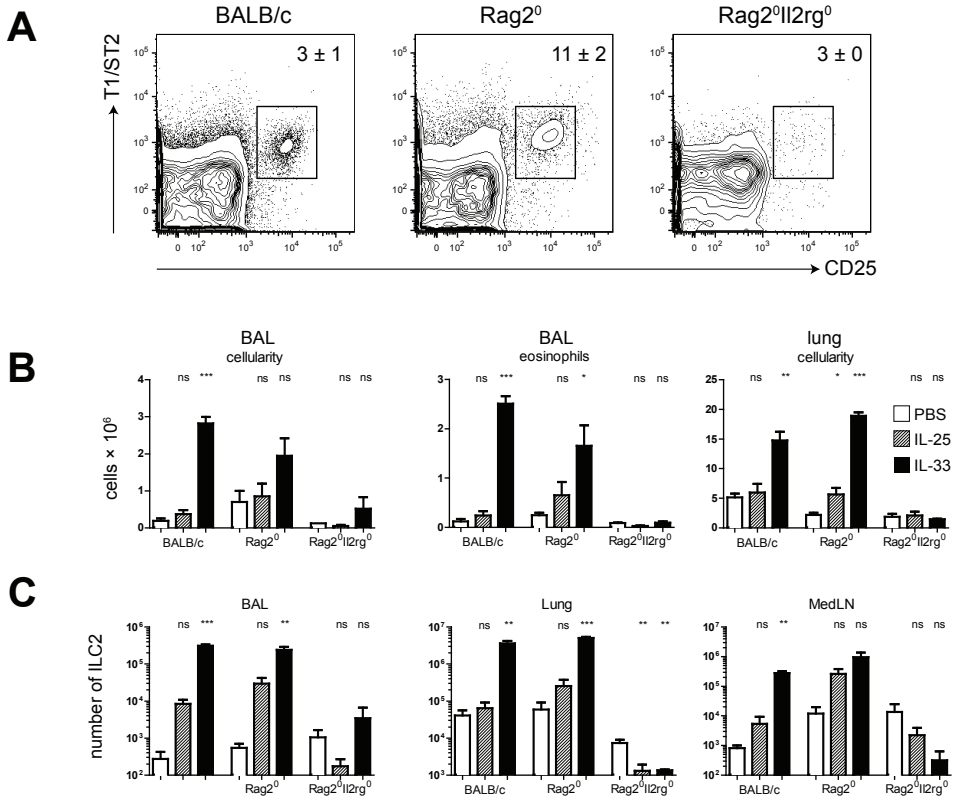


Figure 4. Induction of airway inflammation by IL-25 and IL-33 depends on a common gamma chain-dependent cell population.
A) Identification of ILC2s in the lungs of mice of the indicated strains is shown. Plots show expression of CD25 and T1/ST2 by Lin⁻ CD127⁺ lymphocytes. Values indicate the percentage of ILC2 of Lin-negative lymphocytes ± SEM. **B)** Quantification of indicated cell population in the BAL fluid of the indicated mice treated with PBS, IL-25 or IL-33 in BAL and lung is shown. **C)** Quantification of Lin⁻CD25⁺CD127⁺T1/ST2⁺ ILC2s in the pulmonary tract of the indicated mice after treatment is shown. **B,C)** Data are shown as mean + SEM of n=2-4 mice per group and are representative of two independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, one-way ANOVA analysis followed by Turkey post-tests to analyze statistical significance between PBS control versus treated animals. ns = not significant.

ILC2 numbers in *Rag/Il2rg*-deficient mice **(Figure 4C)**. Treatment of mice with PBS, IL-25 and IL-33 induced production of IL-5 and IL-13 but not IL-4 in *Rag2*-deficient lung ILC2 **(Supplemental Figure 3)**, demonstrating that ILC2 are activated in the absence of adaptive immunity.

In summary, we found that airway inflammation induced by IL-25 and IL-33 is mediated by a *Rag2*-independent, common gamma chain-dependent population. Moreover, a strong increase in the number of ILC2s, more than ~80% of which were intracellular IL-5 and IL-13 positive, was induced. Therefore, we conclude that ILC2s are the most important inducers of eosinophilic inflammation upon intra-nasal IL-25 or IL-33 administration.

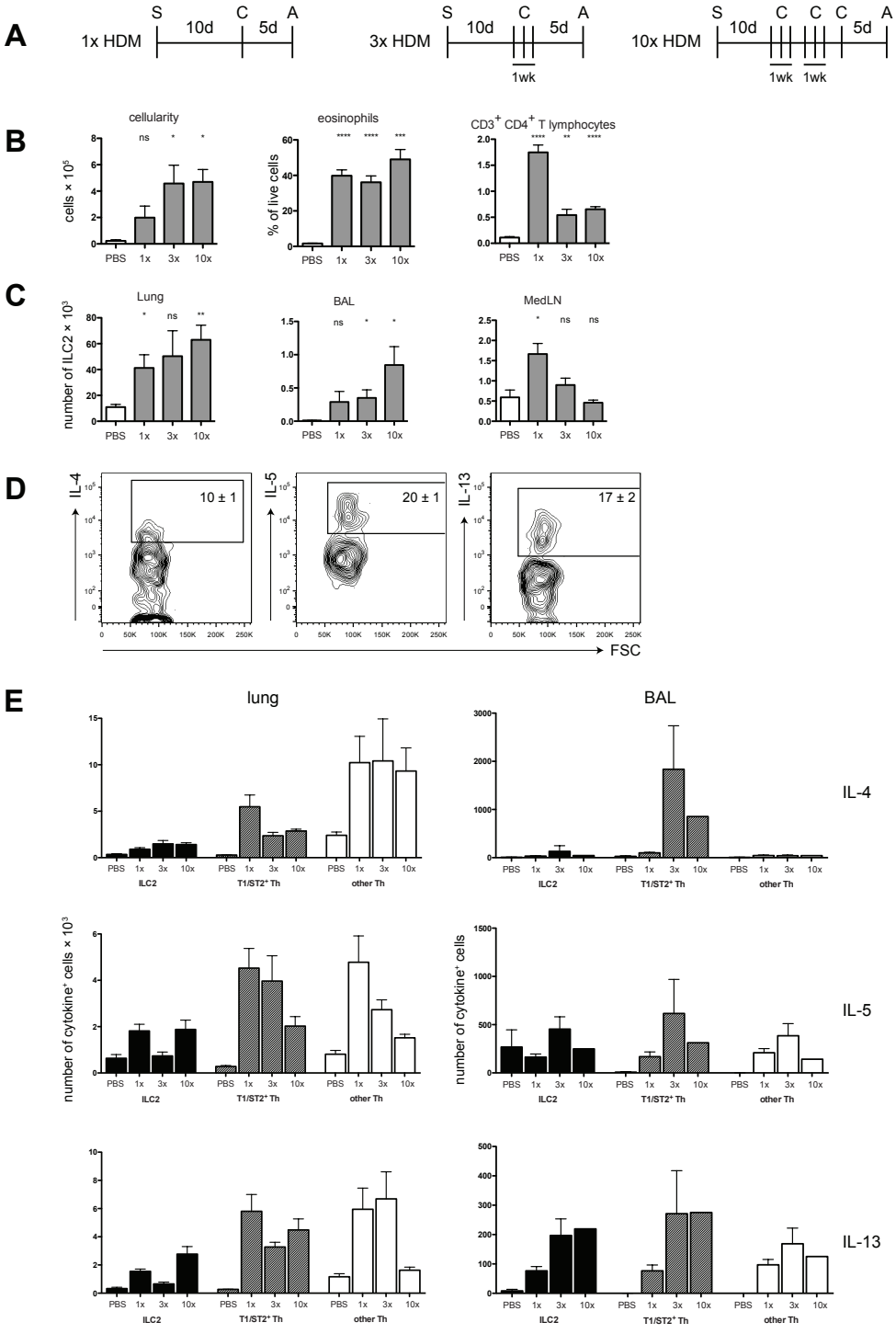
Pulmonary ILC2s are important cytokine producers in HDM-induced allergic asthma

Next, we induced allergic asthma in mice with HDM to determine to which extent ILC2s synergize with an adaptive inflammatory Th2 response in the respiratory tract. Mice were sensitized with HDM and challenged either once, three or ten times with HDM **(Figure 5A)**. Mice that were sensitized and challenged with PBS served as controls. As expected, HDM-challenged mice exhibited airway inflammation marked by increased cellularity, eosinophilia and T cell influx and BAL fluid, when compared with PBS controls **(Figure 5B)**. HDM-induced asthma was also accompanied by increased numbers of ILC2s in lung and BAL, but not in MedLN **(Figure 5C)**, indicating that during the Th2 inflammation ILC2s undergo proliferation or are attracted, possibly by cytokines.

As observed in cytokine-induced airway inflammation, in HDM-induced allergic asthma pulmonary ILC2s did not only increase in number, but concomitantly also the proportions of cytokine-containing cells increased. Intracellular flow cytometry demonstrated that in HDM-induced allergic asthma ILC2s in the lung produced IL-5, IL-13 and also IL-4 **(Figure 5D)**. To determine to what extent pulmonary ILC2s contribute to cytokine production in relation to conventional Th2 cells and other Th cells, we calculated absolute numbers of cytokine-expressing cells in lungs and BAL. The contribution of ILC2s to IL-4 was limited, compared with T1/ST2$^+$ or T1/ST2$^-$ T helper cells, and only detectable in the lung, where up to ~1,400 ILC2 produced IL-4. In the lung up to ~1,900 of total IL-5$^+$ cells were ILC2s, which is substantial, as compared to values between 2,000 and 4,500 for T1/ST2$^+$ or between 1,500 and 4,800 for T1/ST2$^-$ CD4$^+$ T cell fractions **(Figure 5E)**. Likewise, up to 2,800 ILC2s produced IL-13, while 3,200-5,800 T1/ST2$^+$ Th2 cells produced IL-13. Despite the modest numbers of ILC2s detected in BAL fluid, the number of IL-5 and IL-13 producing ILC2 and T1/ST2$^+$ Th2 cells were comparable. Collectively, these results illustrate that pulmonary ILC2s comprised a significant proportion of IL-5$^+$ and IL-13$^+$ cells in lung and BAL in HDM-induced allergic asthma, whereby the contribution of ILC2 cells to the total population of IL-5$^+$ and IL-13$^+$ cells in the BAL was in the same range as found for Th2 cells.

ILC2 are major IL-5 and IL-13 producers in OVA-induced asthma

Finally, we determined the contribution of ILC2 to cytokine production in an OVA-induced asthma model. After i.v. injection of Th2-polarized T cells bearing the DO11.10 OVA-specific T cell receptor [35], mice were challenged five times with OVA aerosols and analyzed 2 days after the last challenge. In this asthma model, total BAL cellularity increased to $3.0 \pm 0.5 \times 10^6$ cells and flow cytometric analysis revealed that $68 \pm 2\%$ of BAL cells were eosinophils (data not shown), confirming induction of eosinophilic airway inflammation. Flow cytometric analyses of the lungs revealed that ILC2s contained substantial proportions IL-5$^+$ and IL-13$^+$ cells, while only small proportions of the cells were IL-4+ **(Figure 6A)**.


A
1x HDM
3x HDM
10x HDM
S
C
A
10d
5d
1wk
B
cellularity
eosinophils
CD3+ CD4+ T lymphocytes
cells × 10⁶
% of live cells
PBS
1x
3x
10x
C
Lung
BAL
MedLN
number of ILC2 × 10³
D
IL-4
IL-5
IL-13
10 ± 1
20 ± 1
17 ± 2
FSC
E
lung
BAL
IL-4
IL-5
IL-13
number of cytokine⁺ cells × 10³
number of cytokine⁺ cells
ILC2
T1/ST2⁺ Th
other Th

When we determined the contribution of ILC2s to Th2 cytokine production, we noticed that ILC2s were poor producers of IL-4 in both lung and BAL, but were major producers of IL-5. In the lung ~21,000 ILC2s produced IL-5 (compared with ~32,000 T1/ST2$^+$ Th2 cells), while in BAL ILC2 was the main IL-5-producing cell population: ~7,500 ILC2s, compared with ~1,900 T1/ST2$^+$ Th2 cells **(Figure 6B)**. In addition, we found ~12,000 IL-13 producing ILC2 in the lung, compared to ~16, 000 IL-13 producing T1/ST2$^+$ Th2 cells. In contrast, the contribution of ILC2s to IL-13 production in the BAL was limited.

In summary, these results indicate an important role for pulmonary ILC2s in the production of IL-5 and IL-13 in OVA-induced asthma. In particular, pulmonary ILC2s are potent producers of IL-5 and make up the largest IL-5-producing population in the BAL fluid in OVA-induced asthma.

Discussion

ILC2 were shown to mediate airway hyperreactivity, independent of the adaptive immune system, in a non-allergic form of asthma induced by influenza virus infection. However, whether ILC2 have the capacity to synergize with allergen-specific Th2 cells in allergic asthma remained unexplored. Our studies in naive mice as well as in cytokine-, HDM- and OVA-induced asthma demonstrate the presence of ILC2s in the pulmonary tract and their substantial contribution to allergic inflammation. We conclude that ILC2s, defined as non-B/non-T lymphocytes expressing IL-7Rα, CD25, Sca-1, c-Kit and T1/ST2/IL-33R are major producers of IL-5 and IL-13, even in allergen-induced asthma, where Th2 cells are thought to be the most important sources of IL-5 and IL-13

It was previously reported that the lineage-negative cells that produce IL-5 and IL-13 in response to intranasal administration of IL-25 were T-cell independent, because they were also detected in *Rag2*$^{-/-}$ mice [14]. We have now shown that these cells are ILC2s and we additionally found that they were efficiently induced upon IL-25 or IL-33 administration in *Rag2*$^{-/-}$ mice, but not in *Rag2*/*Il2gc* double-deficient mice. While our studies were in progress, two other studies have demonstrated the activation of ILC2s in response to IL-25 and IL-33 administration [36] or in response to glycolipid antigens, which activate natural

Figure 5 (*left page*)**. Pulmonary ILC2s are major Th2 cytokine producers in HDM asthma.**
A) Asthma protocols showing sensitization (S) and challenges (C) with house dust mite (HDM) in BALB/c mice, followed by analysis (A). Control mice were treated with PBS instead of HDM. **B)** Flow cytometric quantification of the indicated cell populations in the BAL fluid after various HDM-asthma treatments is shown. **C)** Quantification of Lin$^-$CD127$^+$T1/ST2$^+$ ILC2 numbers in lung, BAL and mediastinal lymph node (MedLN) of HDM- or PBS-treated animals is shown. **D)** The intracellular cytokine content in HDM-challenged mice is shown. Total lung cell suspensions were stimulated with PMA/ionomycin for 4h. CD3$^-$CD4$^-$T1ST2$^+$CD127$^+$ cells were gated and analyzed for the indicated cytokines. Values indicate the proportions of cytokine positive cells ± SEM. **E)** Cytokine production by lymphocyte populations in lung and BAL fluid is shown. Bars show the absolute numbers of intracellular cytokine-positive cells within the indicated lymphocyte populations. **B,C,E)** Data are shown as mean + SEM of n>4 mice per group and are representative of 3 experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, unpaired t test. ns = not significant.

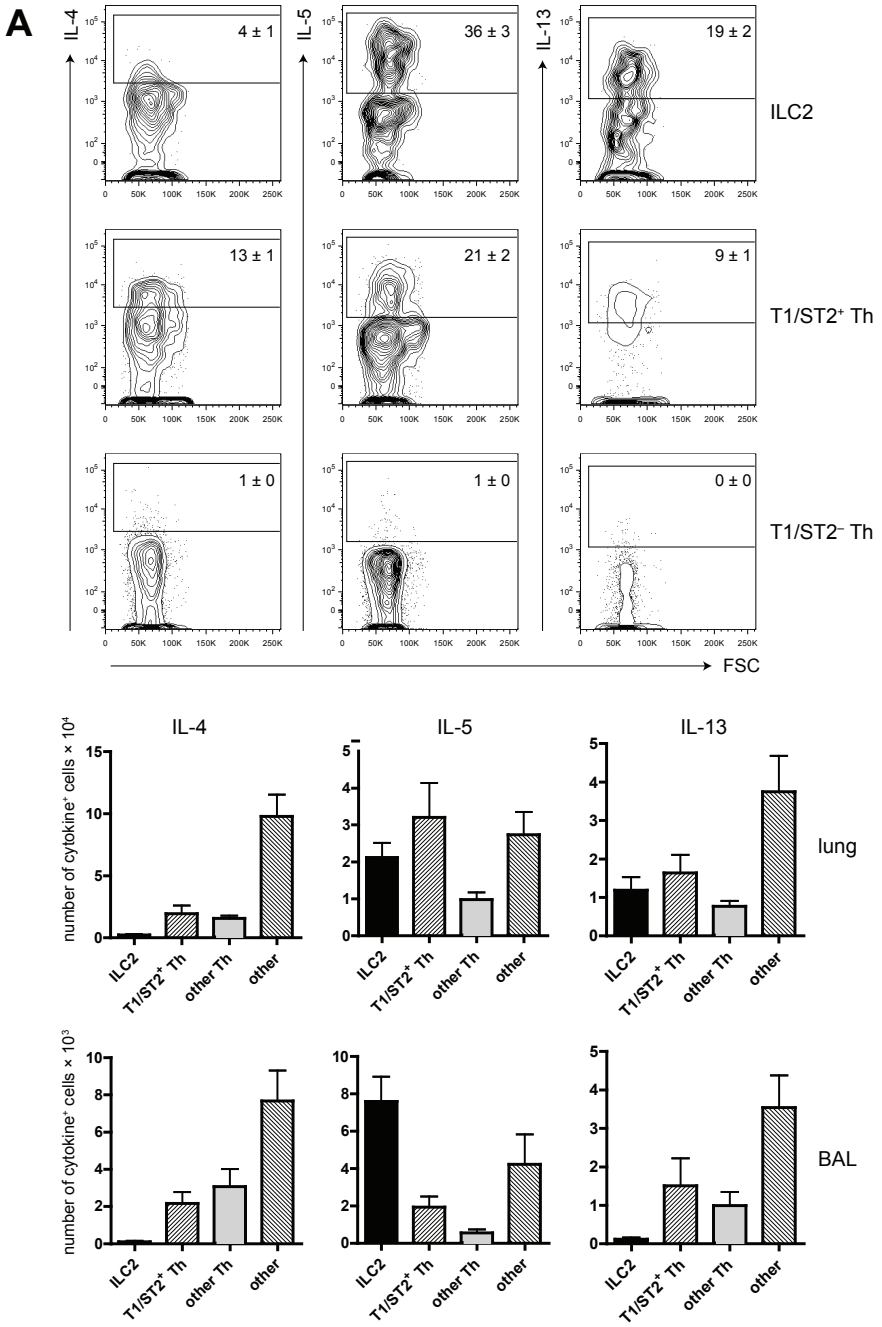


Figure 6. Pulmonary ILC2s are major Th2 cytokine producers in OVA-induced asthma.
A) Flow-cytometric analysis of intracellular cytokine content in OVA-challenged mice is shown. Total lung cell suspensions were stimulated with PMA/ionomycin for 4h. ILC2s were gated as $Lin^{-}CD25^{+}CD127^{+}T1/ST2^{+}$. T helper (Th) cells were gated as $T1/ST2^{+}$ or $T1/ST2^{-}$ $CD3^{+}CD4^{+}$ lymphocytes. Values indicate the proportions cells ± SEM positive for the indicated cytokines. Plots are from representative samples of 7 animals per group. **B)** Contribution of lymphocyte populations indicated in panel A to production of IL-4 and IL-5, IL-13 in lungs and BAL fluid, upon OVA treatment in vivo. Data are shown as mean + SEM of n=7 animals. Experiment was performed once.

killer T cells and thereby induce IL-33 production by alveolar macrophages [37], although these papers focus on the production of IL-13 by ILC2s. From our findings, we can conclude that ILC2s are an important source of both IL-5 and IL-13, not only in IL-25 or IL-33 induced allergic lung inflammation, but also in models of allergic asthma based on OVA and HDM allergens. In addition, we noticed that upon IL-33 stimulation in vivo **(Figure 3A)** or in OVA- or HDM-induced airway inflammation **(Figure 5D** and **Figure 6A)** low but detectable proportions of ILC2 had the capacity to produce IL-4. We have compared the expression levels of CD127/IL-7Rα, IL-2Rα , T1/ST2, and Sca-1 by flow cytometry in IL-4, IL-5 and IL-13 expressing ILC2, but did not detect significant differences.

ILC2s express c-Kit, IL-2Rα and IL-7Rα, suggesting a lymphoid origin. Although the exact nature of ILC2 precursors remains unknown, recent data confirm that bone marrow lymphoid-primed multipotent progenitors (LMPPs) and common lymphoid progenitors (CLPs) but not myeloid-erythryoid precursors can give rise to ILC2s [38]. Like other ILC subtypes that have recently been described (reviewed in [6]) and NK cells, ILC2s depend on the transcription factor Id2 [7, 39, 40]. The absence of ILC2s in *Rag2/Il2rg* double-deficient mice demonstrates dependency on a γc-chain cytokine. More specifically, ILC2s found in fat-associated lymphoid clusters were found to be dependent on IL-7 for their development [7]. We observed pulmonary ILC2s in mice lacking *Il2rb* (the β-chain of the IL-2R/IL-15R), showing that IL-2 and IL-15 are not crucial for their development and maintenance, or that their function can be compensated for by other cytokines (R.K.W., Vosshenrich et al., unpublished findings). It is possible that the ILC2 populations that have been recently described share a common precursor and that the local environment plays a decisive role in their terminal differentiation.

In summary, we have shown that the novel population of ILC2 surprisingly constitutes an important fraction of IL-5 and IL-13 cytokine producing cells, next to the classical Th2 cells, in HDM or OVA allergen-induced allergic asthma in mice. It is conceivable that ILC2s also play a crucial role in "classical" allergic asthma in human, because recent genome-wide association studies (GWAS) have demonstrated associations with variations in genes coding for IL-33 and its receptor ST2 [41]. ILC2s could therefore be an important novel target for development of future asthma therapies.

Materials & Methods

Mice and asthma induction

C57BL/6 and BALB/c mice were purchased from Harlan. All other mice were bred and kept at the Erasmus MC Laboratory Animal Center. Mice were kept under specific pathogen free conditions and provided with food and water ad libitum. Mice were 6-15 weeks at time of analysis.

In the cytokine-induced asthma models, 6-week-old anesthetized BALB/c mice were intranasally treated three (IL-25) or five (IL-33 and PBS) times, with 1μg recombinant mouse IL-25 or IL-33 (both R&D Systems) in 50μL PBS or PBS alone. Mice were sacrificed and analyzed 24 hours later.

For the HDM asthma protocol, 6-week-old BALB/c mice were anesthetized using isoflurane and sensitized intratracheally using 100µg HDM (Greer, Lenoir USA) or PBS (GIBCO, Invitrogen). Ten days later, mice were challenged once, three or ten times (3 times per week) with 10µg HDM or PBS intra-nasally. Five days after the last challenge, mice were sacrificed for analysis.

Ovalbumin asthma was induced by transfer of polarized Th2 cells bearing an ovalbumin-specific TCR as described previously [42]. In brief, DO11.10 cells obtained from lymph nodes of naïve mice were stimulated in vitro for 6 d in the presence of IL-4 (10ng/mL, PeproTech), anti-IFN-γ (10µg/mL, Bioceros B.V., The Netherlands), anti-IL-12 (1 µg/mL, Bioceros B.V., The Netherlands) and ovalbumin peptide (5 µg/mL, (Worthington Biochemical Corp). Th2 differentiation was confirmed by flow cytometric analysis of intra-cellular contents for IFN-γ, IL-4, IL-5, IL-13 and IL-17 production. After washing, 3×10^6 cells were injected into naïve BALB/c mice. Mice were challenged with daily ovalbumin aerosols (Sigma-Aldrich) for the subsequent 5 d and sacrificed 48h after the last challenge.

BAL fluid was obtained by flushing the lungs three times with 1mL PBS containing 0.5mM EDTA. All experiments were approved by the Erasmus MC Animal Ethics Committee (DEC).

Preparation of single cell suspensions

Single-cell suspensions were prepared from the spleen, lungs, mediastinal lymph nodes and BAL fluid. Spleen and lymph nodes were mechanically disrupted using a 100µm cell strainer (BD Falcon). Lungs were mechanically disrupted using a cell strainer or digested using collagenase type I (Invitrogen) or collagenase IV (Worthington), according to the manufacturer's protocol. Mesenteric and thoracic fat was digested using 2mg mL^{-1} collagenase I (Invitrogen) in DMEM (Invitrogen) supplemented with 4% FCS (Lonza) rocking at 37°C for 1h. Red blood cells from lung and spleen were lysed using osmotic lysis buffer ($NH_4Cl + KHCO_3 + Na_2EDTA$ in H_2O, Sigma-Aldrich).

T helper cell cultures

For in vitro T cell stimulations and Th1/Th2 polarization cultures, naive $CD62L^+CD4^+$ T cells were purified by cell sorting using a FACSAria equipped with BD FACSDiva software (BD Biosciences) and polarized in vitro as described previously [43]. The purity of obtained fractions was >98%.

Cell sorting, RNA extraction and PCR

Fat-associated lymphoid cluster cells, BAL and lung ILC2 were purified by cell sorting using a FACSAria (BD Biosciences). 50 cells per well were dispensed directly into 96-well plates and were stimulated for 4 hours using IL-2, IL-25 and IL-33 (all R&D Systems) in the presence of GolgiStop (BD Biosciences). Polarized Th subsets were also dispensed in 96-wells plates using a FACSAria. After stimulation, cells were directly lysed, resuspended and frozen until analysis in SuperScript III One-Step RT-PCR reaction buffer (Invitrogen) containing Igepal CA-630 (Sigma) and RNase Out (Invitrogen). Multiplex PCR was performed to directly amplify mRNA using primers for *Il4*, *Il5*, *Il6*, *Il13* and *Actb*. mRNA levels were quantified in a nested RT-PCR reaction using Maxima™ SYBR Green/ROX qPCR Master Mix (Fermentas) and gene-specific primers. Quantitative real-time PCR was performed using an Applied Biosystems Prism 7700 sequence detection system. The expression of *Il5* was calculated by substraction of Actb levels from the test mRNA, followed by calculation of the inverse $\log^2$ of the difference. Primers were ordered from Invitrogen and sequences were as follows for external primers: Il5-extF GTGGGGGTACTGTGGAAATG; Il5-extR ACCAAGGAACTCTTGCAGGT; Il4-extF ACGGATGCGACAAAAATCAC; Il4-extR TTGGAAGCCCTACAGACGAG; Il6-extF

GATGGATGCTACCAAACTGGA; Il6-extR TGAAGGACTCTGGCTTTGTCT; Il13-extF CCATCTACAGGACCCAGAGG; Il13-extR GCGAAACAGTTGCTTTGTGT; Actb-extF CCAACCGTGAAAAGATGACC; Actb-extR TCTCCGGAGTCCATCACAAT. Internal primers: ActbF TCAACACCCCAGCCATGTA; ActbR GTGGTACGACCAGAGGCATAC; Il5-forward ACATTGACCGCCAAAAAGAG; Il5-reverse ATCCAGGAACTGCCTCGTC.

Flow cytometric analysis

Cells were stained with a mix of fluorescently tagged antibodies in FACS buffer containing BSA. For measurements of cytokine production by flow cytometry, cells were re-stimulated for 4 hours at 37°C using PMA and ionomycin (both Sigma-Aldrich) supplemented with GolgiStop (BD Biosciences). After extracellular staining, cells were fixed using 4% PFA and permeabilized using 0.5% saponin (Sigma-Aldrich) in FACS buffer. Lineage-negative cells were gated as cells not expressing CD3, CD4, CD8α, CD11b, CD11c, CD19, B220, NK1.1, FcεRIα, Ter-119 and Gr-1. Purchased antibodies are described in **Supplemental Table 1**. Biotin-conjugated antibodies were detected using Pacific Blue (Invitrogen) or allophycocyanin-Cy7-conjugated (BD) streptavidin. Data acquisition was done using a LSR II flow cytometer equipped with 3 lasers and FACSDiva™ software (both BD Biosciences). Data analysis was done using FlowJo 8.8.7 (Tree Star Inc., Ashland, USA).

Statistical analysis

For comparisons between two groups either unpaired t tests (normal distribution) or Mann-Whitney U tests (if no normal distribution was expected) were used. Statistical significance of differences between more than two groups were calculated using one-way ANOVA analysis followed by Turkey post-tests. All analyses were performed using Prism (GraphPad software, La Jolla, USA).

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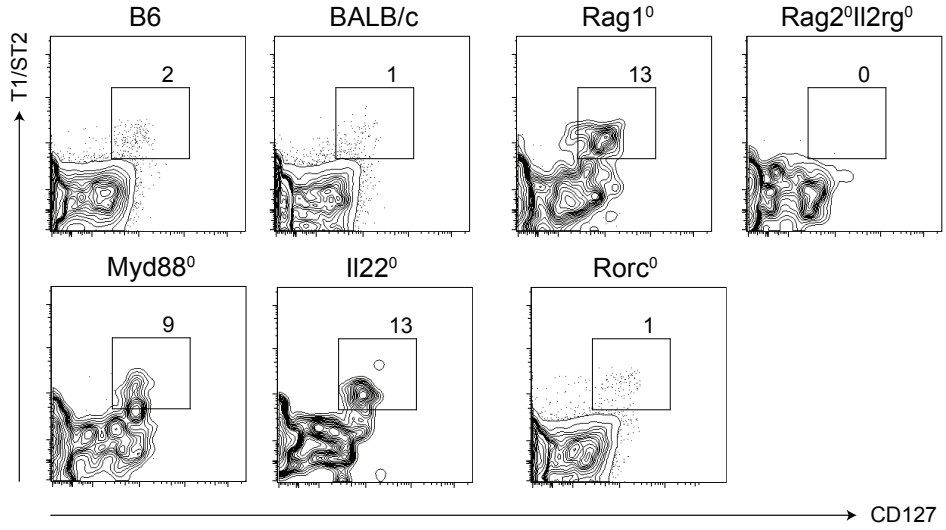
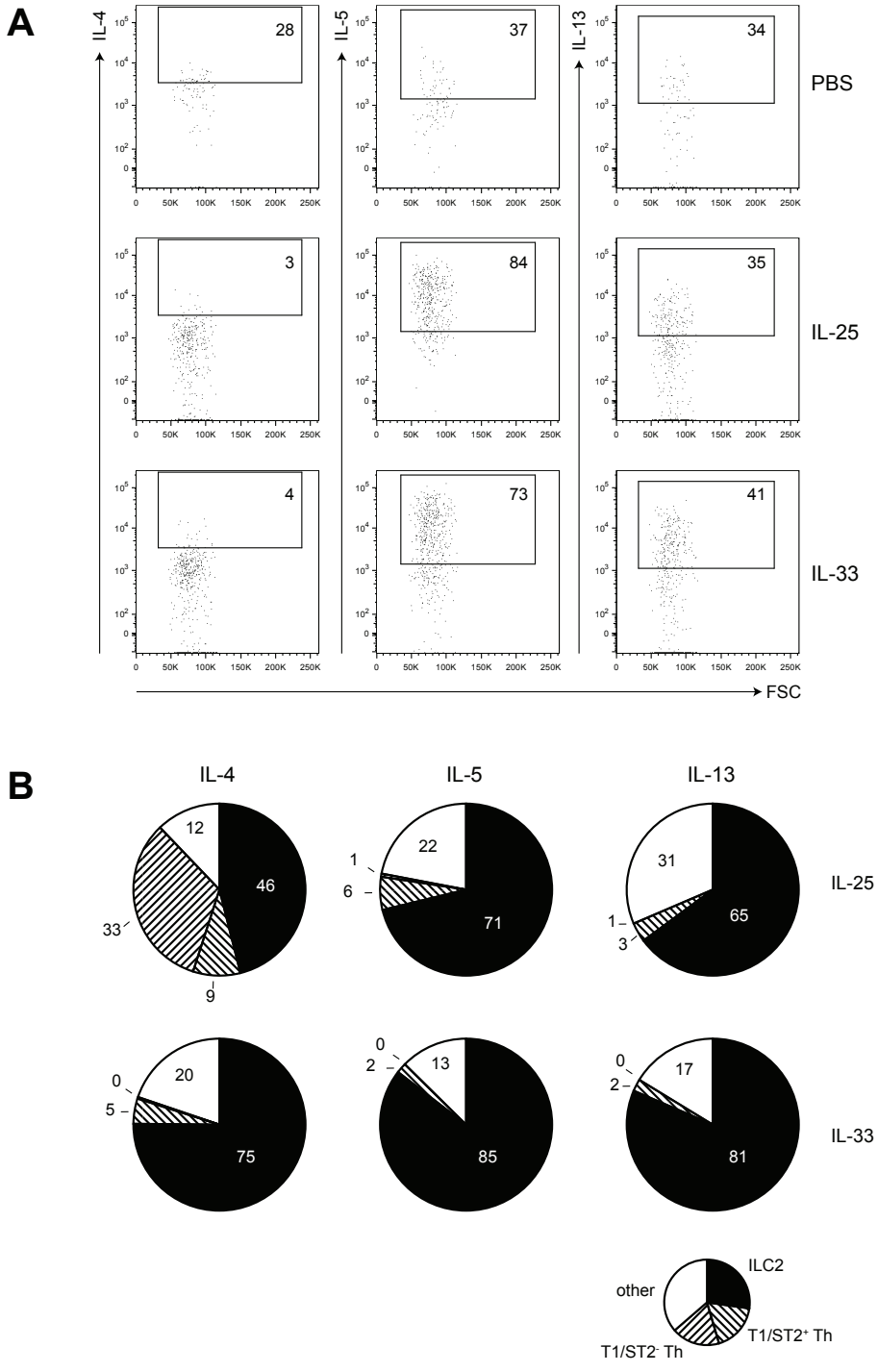
Supporting information



Supplemental Figure 1. ILC2 development does not depend on Rag, Myd88, Il22 or Rorc.
Identification of ILC2s in the lungs of mice of the indicated strains. Plots show expression of CD127 and T1/ST2 by gated Lin⁻ lymphocytes. Numbers indicate the percentage of ILC2 of Lin-negative lymphocyte population ± s.e.m.

Supplemental Figure 2 *(right page)***. ILC2 are major producers of IL-5 and IL-13 in BAL in cytokine-induced airway inflammation.**
A) Intracellular cytokine content of BAL $Lin^{-}CD25^{+}CD127^{+}T1/ST2^{+}$ ILC2s in mice treated as specified. Plots show forward scatter (FSC) versus the indicated cytokines. Single-cell suspensions of lungs were stimulated with PMA/ionomycin in the presence of monensin for 4h. Numbers indicate the proportions of cytokine-positive ILC2s. **B)** Contribution of various cell populations to production of Th2 cytokines in BAL fluid upon administration of IL-25 or IL-33. Proportions of cytokine expressing cells were determined by flow cytometry (as in panel A) in ILC2, $T1/ST2^{+}$ and $T1/ST2^{-}$ $CD3^{+}CD4^{+}$ T helper cells. Pie charts and percentages represent the contribution of various populations to the total number of cytokine-producing cells. Representative data of two independent experiments with 3-4 mice per group.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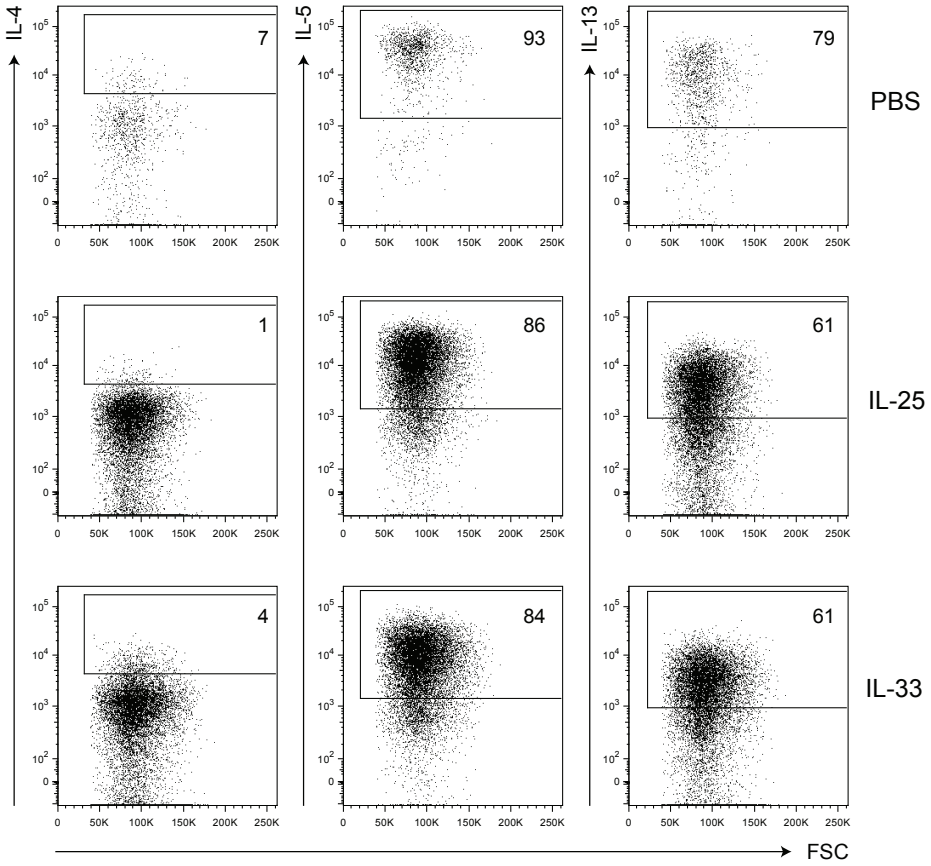


Supplemental Figure 3. The majority of ILC2s produce IL-5 and IL-13 in cytokine-induced airway inflammation. Intracellular cytokine content of $Lin^{-}CD25^{+}CD127^{+}T1/ST2^{+}$ ILC2s of *Rag2*-deficient mice treated 5 times with IL-25 or 3 times with IL-33, shown as dot plots of forward scatter (FSC) versus the indicated cytokines. Single-cell suspensions of lungs were stimulated with PMA/ionomycin in the presence of monensin for 4h. Plots show one representative sample of two experiments with $n \geq 3$ mice per group.

Supplemental Table 1. Antibodies used for flow cytometric analyses

Antibody	Conjugate	Clone	Company
B220	biotin	RA3-6B2	eBioscience
CD3	PE	145-2C11	eBioscience
CD3	PECy7	145.2C11	eBioscience
CD4	PE	GK1.5	eBioscience
CD4	PECy7	GK1.5	eBioscience
CD4	biotin	RM4-5	eBioscience
CD8a	PE	53-6.7	eBioscience
CD8a	PECy7	53-6.7	eBioscience
CD8a	biotin	53-6.7	eBioscience
CD11b	PE	M1/70	eBioscience
CD11b	PECy7	M1/70	eBioscience
CD11b	biotin	M1/70	eBioscience
CD11c	PE	N418	eBioscience
CD11c	PECy7	N418	Biolegend
CD11c	biotin	N418	BD Pharmingen
CD19	PE	1D3	BD Pharmingen
CD19	PECy7	1D3	BD Pharmingen
CD19	biotin	1D3	BD Pharmingen
CD25	PerCPCy5.5	PC61	BD Pharmingen
CD25	APCCy7	PC61	BD Pharmingen
CD45	PECy7	30-F11	eBioscience
CD45.2	PerCPCy5.5	104	eBioscience
CD117	APC	2B8	eBioscience
CD127	PE	A7R34	eBioscience
CD127	APC	A7R34	eBioscience
FceRIa	PE	MAR-1	eBioscience
FceRIa	biotin	MAR-1	eBioscience
Gr-1	PE	RB6-8C5	BD Pharmingen
Gr-1	PECy7	RB6-8C5	eBioscience
Gr-1	biotin	RB6-8C5	BD Pharmingen
IFN-g	PECy7	XMG1.2	BD Pharmingen
IL-4	PECy7	11B11	BD Pharmingen
IL-5	biotin	TRFK5	BD Pharmingen
IL-10	PerCPCy5.5	JES5-16E3	eBioscience
IL-13	Alexa Fluor 647	eBio13A	eBioscience
NK1.1	PE	PK136	eBioscience
NK1.1	PECy7	PK136	BD Pharmingen

Supplemental Table 1. Antibodies used for flow cytometric analyses

NK1.1	biotin	PK136	eBioscience
Sca-1	APC	D7	eBioscience
Sca-1	PECy7	D7	eBioscience
SiglecF	PE	E50-2440	BD Pharmingen
Streptavidin	APCCy7		BD Pharmingen
Streptavidin	Pacific Blue		Invitrogen
T1/ST2	FITC	DJ8	MD Bioproducts
T1/ST2	biotin	DJ8	MD Bioproducts
Ter-119	PE	TER119	eBioscience
Ter-119	PECy7	TER119	eBioscience
Ter-119	biotin	TER119	BD Pharmingen
TNF-a	PE	MP6-TX22	eBioscience

Chapter IV

An essential, dose-dependent role for the transcription factor GATA-3 in the development of IL-5^{+} and IL-13^{+} type 2 innate lymphoid cells

Group 2 innate lymphoid cells (ILC2, also called nuocytes, innate helper cells or natural helper cells) provide protective immunity during helminth infection and play an important role in influenza-induced and allergic airway hyper-reactivity. Whereas transcription factor GATA-3 is important for the production of IL-5 and IL-13 by ILC2 in response to IL-33 or IL-25 stimulation, it is not known whether GATA-3 is required for ILC2 development from hematopoietic stem cells (HSC). Here, we show that chimeric mice generated with *Gata3*-deficient fetal liver HSC fail to develop systemically dispersed ILC2. In these chimeric mice, in vivo administration of IL-33 or IL-25 fails to expand ILC2 numbers or to induce characteristic ILC2-dependent IL-5 or IL-13-production. Moreover, cell-intrinsic *Gata3* expression is required for ILC2 development in vitro and in vivo. Using mutant and transgenic mice in which *Gata3* gene copy number is altered, we show that ILC2 generation from common lymphoid progenitors, as well as ILC2 homeostasis and cytokine production is regulated by *Gata3* expression levels in a dose-dependent fashion. Collectively, these results identify GATA-3 as a critical early regulator of ILC2 development, thereby extending the paradigm of GATA-3-dependent control of type 2 immunity to include both innate and adaptive lymphocytes.

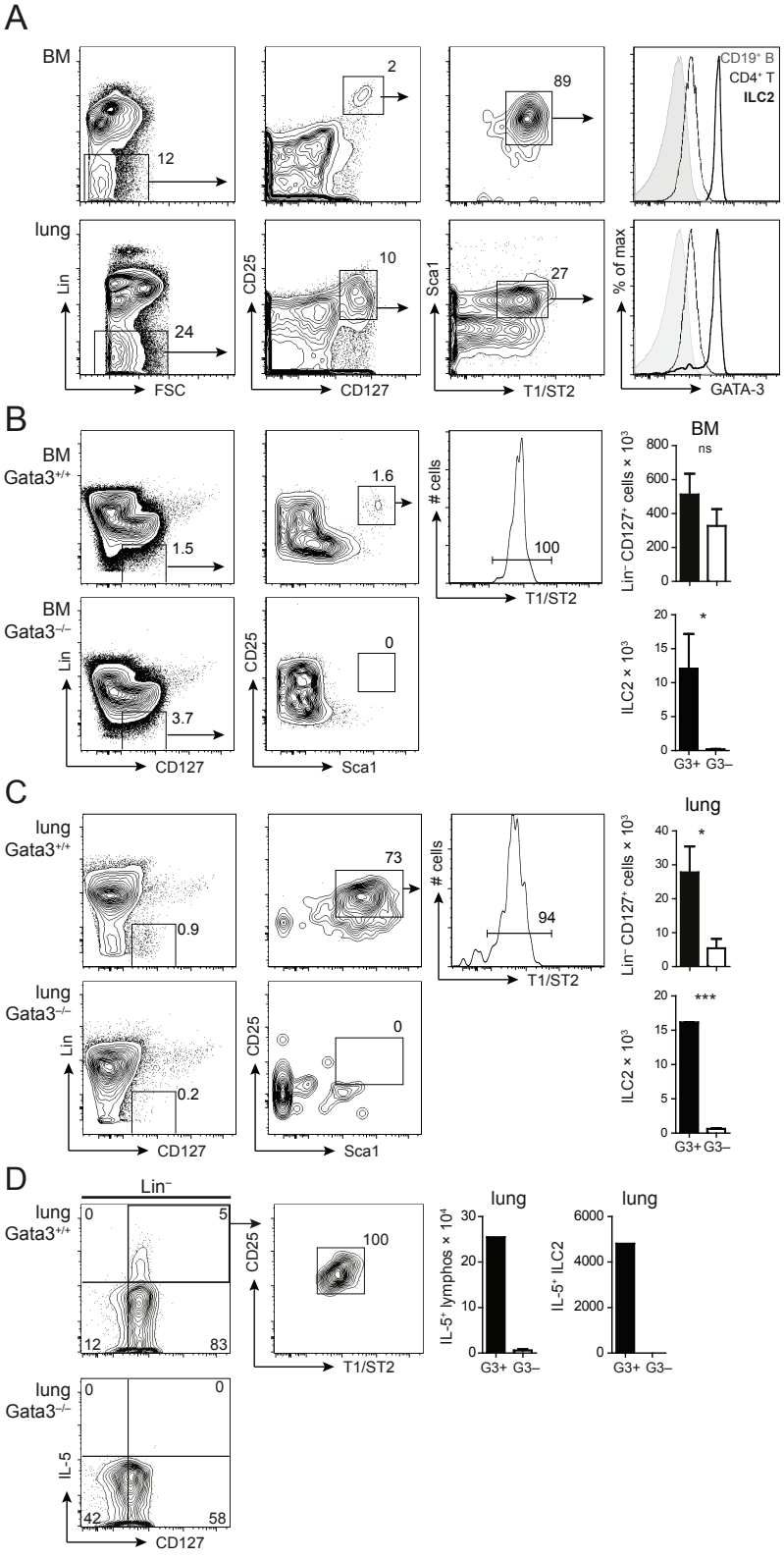
Manuscript under revision and prepared by Roel G.J. Klein Wolterink, Nicolas Serafini, Menno van Nimwegen, Christian A.J. Vosshenrich, Diogo Fonseca-Pereira, Henrique Veiga-Fernandes, Rudi W. Hendriks and James P. Di Santo** * *shared last authorship*

Introduction

Group 2 innate lymphoid cells (ILC2, also known as natural helper cells, nuocytes or innate helper cells) represent a population of non-T/non-B lymphocytes that promptly produce large amounts of the T helper-2 (Th2) cytokines IL-5 and IL-13. These IL-25- and IL-33-responsive innate cells have a broad distribution (1,2) and were shown to be essential for the innate response to the helminth parasite Nippostrongylus brasiliensis (2–4). ILC2 can be distinguished by co-expression of CD25, CD127, T1/ST2 (IL-33R), IL-17RB (IL-25R) and Sca1. ILC2 cells in the respiratory tract play a crucial role in virus-induced airway hyper-reactivity and in maintenance of epithelial barrier integrity during influenza infection (5,6). Damaged epithelial cells or infected myeloid cells release inflammatory cytokines including IL-25 and IL-33 that can potently expand and activate ILC2 in mucosal tissues (7–10). Human ILC2 have been found in the lung and are associated with chronic rhinosinusitis (5,11). In addition, we and others showed that ILC2 are major IL-5 and IL-13 producers in various murine allergic asthma models (7–10,12).

ILC2 belong to an emerging family of innate lymphoid cells that includes ILC1 (NK cells and other innate IFN-γ producing cells) as well as a distinct group 3 innate lymphoid cell (ILC3) that includes subsets expressing the orphan transcription factor RORγt and producing IL-17 or IL-22 or having lymphoid tissue inducer (LTi) function (reviewed by (13–15)). The transcriptional regulator Id2 has been identified as a common factor required for all ILC subsets (13,14,16), however the developmental stages from common lymphoid progenitors (CLP) to fully mature ILC subsets have not been elucidated. Transient Notch signals appear critical for generation of LTi or ILC2 from CLP in vivo (17–19). This process can be recapitulated in vitro using stromal cell based systems and have been useful to identify precursors for distinct ILC3 subsets (20). Nevertheless, the regulatory factors that orchestrate this process are poorly understood, although recently the transcription factor RORα was identified as an essential factor for efficient ILC2 development and in particular for IL-25-mediated expansion of the ILC2 compartment (10,19).

Like Th2 cells, ILC2 promptly produce IL-5, IL-13 and to a lesser extent, IL-4 (1–4). Regulation of *Il4*, *Il5*, and *Il13* expression is complex and in Th2 cells requires the zinc-finger transcription factor GATA-3, which is selectively up-regulated during Th2 differentiation ((21,22) and reviewed in (23)). Conditional deletion of the *Gata3* gene in established Th2 cells demonstrated that *Gata3* is critical for IL-5 and IL-13 but not IL-4 production by Th2 cells (24). Likewise, deletion of the *Gata3* gene in ILC2 abolished their IL-5 and IL-13 production (25). In addition to its role in regulation of Th2 cytokine expression in Th2 cells and ILC2, GATA-3 is required at multiple stages of T cell development and has an essential role in the specification of the earliest T cell progenitors in the thymus (26–28). In this report, hematopoietic chimeras, as well as mutant and transgenic mice in which *Gata3* gene copy number is altered, are used to address the essential, dose-dependent role for GATA-3 in ILC2 differentiation from lympho-hematopoietic precursors.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Results and Discussion

ILC2 express high levels of GATA-3 protein and require Gata3 expression for development in vivo

We first analyzed GATA-3 protein expression in ILC2 present in different tissue sites **(Figure 1A)**. ILC2 were identified as lineage (CD4, CD8α, CD11b, CD11c, CD19, B220, NK1.1, FcεRIα, Gr-1 and Ter-119)-negative cells that co-express CD25, CD127, IL-33R (T1/ST2) and Sca1 as previously described (3,4). We found that bone marrow (BM) and lung ILC2 expressed GATA-3 levels that exceeded by about 10-fold the levels expressed by mature $CD4^+$ T cells **(Figure 1A)**. It remains unclear whether ILC2 in the BM represent immature ILC2 precursors or a 'reservoir' of peripheral ILC2 (2), although BM ILC2 clearly produced cytokines (see below) and had similar GATA-3 levels as lung ILC2 **(Figure 1A)**. GATA-3^+ ILC2 were also identified in spleen, LN and intestine (see below).

In order to assess the requirement for GATA-3 in the development of ILC2 *in vivo*, we analyzed chimeric mice generated using *Gata3*-deficient hematopoietic precursors cells as previously described (29,30). To control for the resultant T cell deficiency in these chimeras (31), we generated T cell-deficient *Gata3*-proficient chimeras using $Cd3e^{-/-}$ hematopoietic precursors (32). Both $Gata3^{+/+}$ and $Gata3^{-/-}$ hematopoietic precursors engrafted to a similar extent leading to robust B, NK cell and myeloid cell reconstitution **(Supplemental Figure 1A** and (29)); no significant differences in the overall hematopoietic reconstitution were found when comparing the two sets of chimeric mice.

As $Rag2^{-/-}Il2rg^{-/-}$ hosts lack ILC2 (3), all ILC2 in the chimeric mice were donor-derived, as expected **(Supplemental Figure 1B)**. Both BM and lung ILC2 developed in $Gata3^{+/+}$ chimeras **(Figure 1B** and **Figure 1C)** and these ILC2 normally expressed CD127, CD25, Sca1 and T1/ST2 as expected (5,33). In $Gata3^{-/-}$ chimeras, we found a similar number of BM Lin-$CD127^+$ cells, although these cells failed to express characteristic surface markers of ILC2 **(Figure 1B)**. In the lung of $Gata3^{-/-}$ chimeras, Lin^-CD127^+ cells were severely reduced and cells expressing CD25, Sca1 and T1/ST2 were essentially absent **(Figure 1C)**.

Analysis of IL-5 production by Lin^- cells in the lung and BM of $Gata3^{+/+}$ chimeras showed that all cells that produced IL-5 were ILC2 **(Figure 1D)**. In contrast, IL-5 production was undetectable in lung and BM cell preparations from $Gata3^{-/-}$ chimeras **(Figure 1D)**, consistent with the absence of ILC2 in these tissues **(Figure 1B** and **Figure 1C)**.

Figure 1 (*left page*)**. GATA-3 is expressed by ILC2 and is required for ILC2 development in vivo.**
A) Gating strategy for analysis of BM and lung ILC2. Histograms show intra-cellular expression of GATA-3 of ILC2 (bold line) compared to $CD3^+$ $CD4^+$ T cells (black line) and $CD19^+$ B lymphocytes (gray area). Numbers indicate relative frequencies of gated populations. Representative results from 3 independent experiments ($n \geq 6$ total mice analyzed). **B-C)** Analysis of lymphocytes from BM **(B)** and lung **(C)** of $Rag2^{-/-}Il2rg^{-/-}$ hosts transplanted with $Gata3^{+/+}$ and $Gata3^{-/-}$ precursors for expression of the indicated surface markers. Plots show gating strategy for ILC2. Bar graphs show quantification of the indicated donor-derived populations in chimeras. **D)** Analysis of pulmonary Lin-lymphocytes for CD127 and intra-cellular IL-5 expression. Plots show gating strategy for IL-5^+ lymphocytes and ILC2. Bar graphs show quantification of the indicated populations in $Gata3^{+/+}$ and $Gata3^{-/-}$ chimeras. **B-D)** Representative results from 2 independent experiments with n=3 ($Gata3^{+/+}$) or n=5 ($Gata3^{-/-}$) total mice analyzed per genotype.

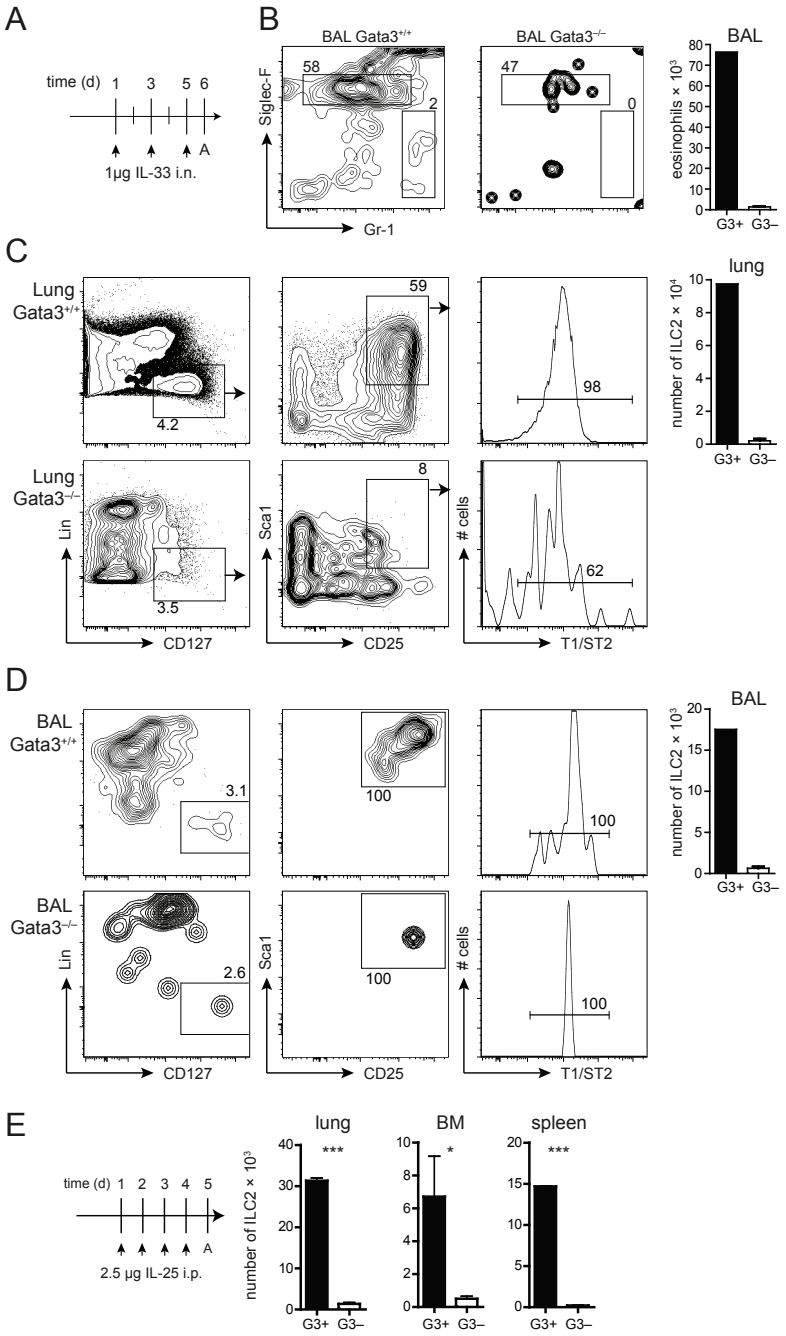

Figure 2. GATA-3 is required for in vivo responses mediated by intra-nasal IL-33 and systemic IL-25 challenge
A) Protocol of intra-nasal administration of recombinant IL-33 on the three days indicated with arrows. Mice were analyzed (A) on day 6. **B)** Flow cytometric analysis and quantification of BAL fluid granulocytes for expression of indicated markers in IL-33-challenged *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ hosts transplanted with *Gata3*$^{+/+}$ and *Gata3*$^{-/-}$ precursors. **C-D)** Flow cytometric identification (contour plots) and quantification (bar graphs) of ILC2 in donor-derived lymphocytes in lung **(C)** and BAL fluid **(D)** of challenged chimeras. **E)** *Gata3*$^{+/+}$ and *Gata3*$^{-/-}$ chimeric mice were challenged with recombinant IL-25 protein as outlined in the protocol. Bar graphs show quantification of donor-derived ILC2 of the indicated tissues one day after the last challenge. Figure shows representative results from 2 independent experiments with n≥6 total mice analyzed per genotype.

GATA-3 is required for characteristic innate responses to exogenously IL-33 or IL-25

Intranasal (i.n.) administration of IL-33 *in vivo* results in a dramatic increase in the absolute numbers of ILC2 in lung and broncho-alveolar lavage (BAL) fluid (2,7–10,34). Intranasal IL-33 **(Figure 2A)** triggered recruitment of Siglec-F$^+$ eosinophils and Gr-1$^+$ neutrophils cells in the BAL fluid in *Gata3*$^{+/+}$ but not in *Gata3*$^{-/-}$ chimeras **(Figure 2B)**. This inflammatory reaction was accompanied by a dramatic increase in ILC2 numbers within the lung tissue and BAL fluid of *Gata3*$^{+/+}$ but not *Gata3*$^{-/-}$ chimeras **(Figure 2C** and **Figure 2D)**. *Gata3*$^{+/+}$ ILC2 in the lung promptly produced IL-5 and IL-13 following stimulation, whereas cytokine production was not detected in Lin$^-$ cells from *Gata3*$^{-/-}$ chimeras **(Supplemental Figure 2)**. We conclude that lung-resident IL-33-responsive ILC2 require *Gata3* for their generation and/or function.

As the expression of the IL-33R component T1/ST2 is regulated by GATA-3 (35), the failure of *Gata3*$^{-/-}$ chimeras to respond to IL-33 *in vivo* may in part be explained by the lack of T1/ST2 expression by *Gata3*$^{-/-}$ ILC2 **(Figure 1** and **Figure 2)**. Systemically administered IL-25 can trigger an inflammatory response from ILC2 that provokes multi-system myeloid cell recruitment and mucus production (36). IL-25 injection resulted in the expansion of ILC2 in the BM and lung of *Gata3*$^{+/+}$ but not *Gata3*$^{-/-}$ chimeras **(Figure 2E**, **Supplemental Figure 3A)**. While we found that ILC2 were the major source of IL-5 produced by lung Lin$^-$ lymphocytes, IL-5 was not detected in these cells in the absence of *Gata3* **(Supplemental Figure 3B)**. Collectively, these results demonstrate that characteristic inflammatory responses following triggering of ILC2 by IL-25 and IL-33 are not elicited in chimeric mice made with *Gata3*$^{-/-}$ hematopoietic precursors.

Failure of ILC2 differentiation from Gata3$^{-/-}$ hematopoietic precursors in vitro

Notch-triggered differentiation of ILC2 from common lymphoid progenitors (CLP) can be achieved in vitro using OP9 stromal cells expressing Delta-like ligand-4 (OP9Δ4; (37)) in the presence of IL-7, Flt3L and IL-33 (19). We found that culture of *Gata3*$^{+/+}$ fetal liver hematopoietic precursors generated abundant ILC2 using this approach **(Supplemental Figure 4A)** whereas cells expressing characteristic surface markers of ILC2 were markedly reduced in the absence of Gata3 **(Supplemental Figure 4A)**. RT-PCR analysis of these FL cultures showed that in the absence of *Gata3*, *Id2* and *Rora* expression were not induced **(Supplemental Figure 5)**; these transcription factors are highly expressed in ILC2 and critical for their development (3,19,38). Moreover, we could not detect IL-5 or IL-13 production in these ILC2 *Gata3*$^{-/-}$ cultures, whereas ILC2 cultures of *Gata3*$^{+/+}$ fetal liver hematopoietic precursors gave rise to cells with characteristic IL-5 and IL-13 production **(Supplemental Figure 4B)**.

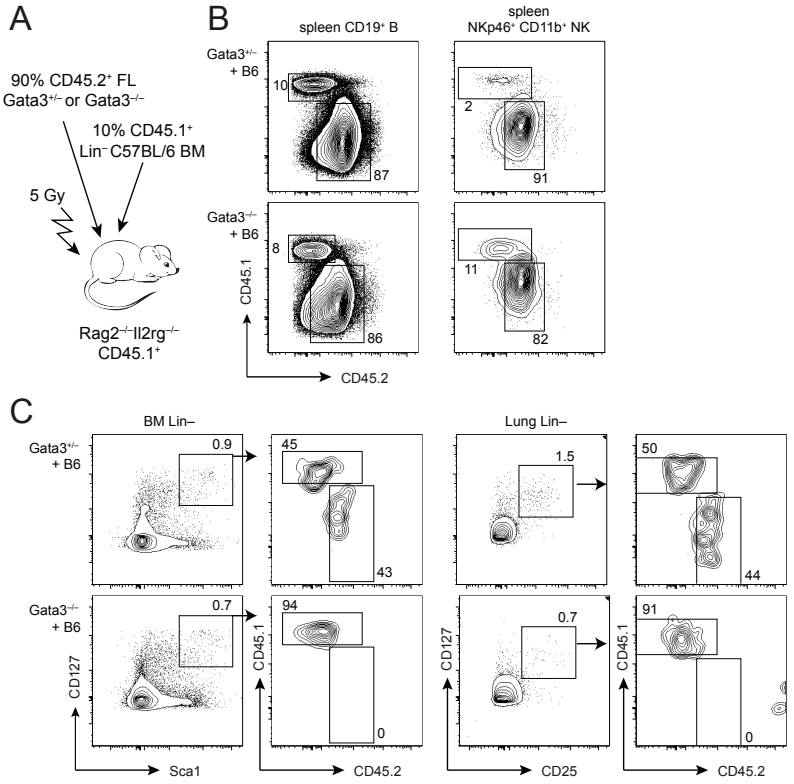

Figure 3. Cell-intrinsic GATA-3 is required for ILC2 development
A) Mixed chimeras were generated by injection of 90% *Gata3*$^{-/-}$ (knockout) or *Gata3*$^{+/-}$ (control) CD45.2^{+} fetal liver precursor supplemented with lineage-depleted CD45.1^{+} C57BL/6 BM precursor into irradiated CD45.1^{+} *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ hosts. Analysis was performed 5 weeks post-transplantation. **B)** Chimerism was analyzed in spleen of chimeric mice. Plots show expression of the allogenic markers by CD19^{+} B and NKp46^{+} CD11b^{+} NK cells for control and knockout mixed chimeras. **C)** Flow cytometric analysis of lineage-negative lymphocytes in BM and lung for ILC2 markers. ILC2 were subsequently analyzed for expression of CD45 allotypes. Numbers in contour plots indicate relative frequencies of gated populations.

Cell-intrinsic Gata3 is required for ILC2 development

To exclude the possibility that cell-extrinsic effects caused by *Gata3*-deficiency underlie the observed defects in ILC2 development, we generated and analyzed BM chimeras generated using mixtures (90%/10%) of CD45.2^{+} *Gata3*$^{-/-}$ and CD45.1^{+} wild type C57BL/6 hematopoietic precursors, respectively. Mice injected with (90%/10%) mixtures of CD45.2^{+} *Gata3*$^{+/-}$ and CD45.1^{+} *Gata3*$^{+/+}$ precursors served as controls **(Figure 3A)**. Both sets of mixed chimeras reconstituted to a similar extent **(Supplemental Figure 6A)**. We confirmed that B and NK cell development occurred normally from both *Gata3*$^{-/-}$ and *Gata3*$^{+/+}$ precursors **(Figure 3B** and **Supplemental Figure 6B)** and phenotypically mature ILC2 were generated in BM, lung, spleen and small intestine **(Figure 3C** and **Supplemental Figure 6C)**. Further analysis revealed that all ILC2 in *Gata3*$^{-/-}$ mixed BM chimeras expressed CD45.1^{+} (origi-

nating from *Gata3*$^{+/+}$ precursors), while ILC2 in Gata3$^{+/-}$ mixed chimeras originated from both CD45.2^{+} Gata3$^{+/-}$ as well as CD45.1^{+} *Gata3*$^{+/+}$ precursors **(Figure 3C** and **Supplemental Figure 6D)**. This result demonstrates that Gata3 is required in a cell-intrinsic fashion for ILC2 development. Interestingly, while analysis of the origin of B cells and NK cells recapitulated the ratio in which precursors were injected (i.e. 86-87% of the B cells and NK cells were CD45.2^{+} independent of *Gata3* genotype), ILC2 derived from *Gata3*$^{+/-}$ precursors were under-represented relative to those derived from their *Gata3*$^{+/+}$ counterparts in control chimeras **(Figure 3C)**. This result suggests that the *Gata3* gene dosage may influence overall ILC2 development.

Gata3 gene copy positively correlates with ILC2 development, homeostasis and function

We next compared the effects of modifying *Gata3* gene copy number on ILC2 development and function. We characterized ILC2 homeostasis and function in three mouse strains: C57BL/6 mice with two functional *Gata3* alleles, *Gata3*$^{lacZ/+}$ mice with one functional *Gata3* allele (the second allele is disrupted by insertion of a LacZ reporter; (39)) and transgenic C57BL/6 mice that harbor a *Gata3* transgene under the control of the human CD2 promoter (40). We found that Gata3 protein levels in BM and lung ILC2 were reduced in Gata3$^{lacZ/+}$ mice compared to C57BL/6 mice, indicating that both Gata3 alleles are apparently active in ILC2 **(Supplemental Figure 7A)**. In contrast, ILC2 from *hCD2-Gata3* Tg mice expressed similar levels of GATA-3 protein as WT C57BL/6 mice **(Supplemental Figure 7A)**.

We next assessed the impact of alterations in *Gata3* gene copy number on overall ILC2 homeostasis. Absolute numbers of mature ILC2 in the lung and BM were clearly reduced in *Gata3*$^{lacZ/+}$ mice, while *hCD2-Gata3* Tg mice had a roughly 3-fold increase in BM and lung ILC2 compared to *Gata3*$^{+/+}$ C57BL/6 mice **(Figure 4A)**. A dose-dependent function of *Gata3* in mouse ILC2 would parallel previous findings that GATA-3 haplo-insufficiency in human is associated with markedly decreased Th2 frequencies (41). ILC2 function also positively correlated with *Gata3* gene copy number: ILC2 from *hCD2-Gata3* Tg mice produced higher levels of IL-5 and IL-13 following stimulation compared to ILC2 from B6 WT mice, whereas cytokine production was reduced in ILC2 from *Gata3*$^{lacZ/+}$ mice having only one functional *Gata3* allele **(Figure 4A** and **Supplemental Figure 7B)**. Moreover, *CD2-Gata3* Tg ILC2 showed higher surface expression of T1ST2 and IL-17BR **(Supplemental Figure 7C)**.

These results suggest that *Gata3* expression levels can modify ILC2 generation from hematopoietic precursors and thereby influence ILC2 homeostasis. While absolute numbers of CLP were normal in *CD2-Gata3* Tg mice, we found that *Gata3* levels in CLP were modestly but significantly increased in CLP in *hCD2-Gata3* Tg mice compared to non-transgenic controls **(Figure 4B)**. This would be consistent with hCD2 Tg control elements being active in CLP, a hypothesis we confirmed using *hCD2*-GFP mice (42) **(Figure 4C)**. To assess the influence of increased *Gata3* levels in determining ILC2 potential from CLP, we cultured

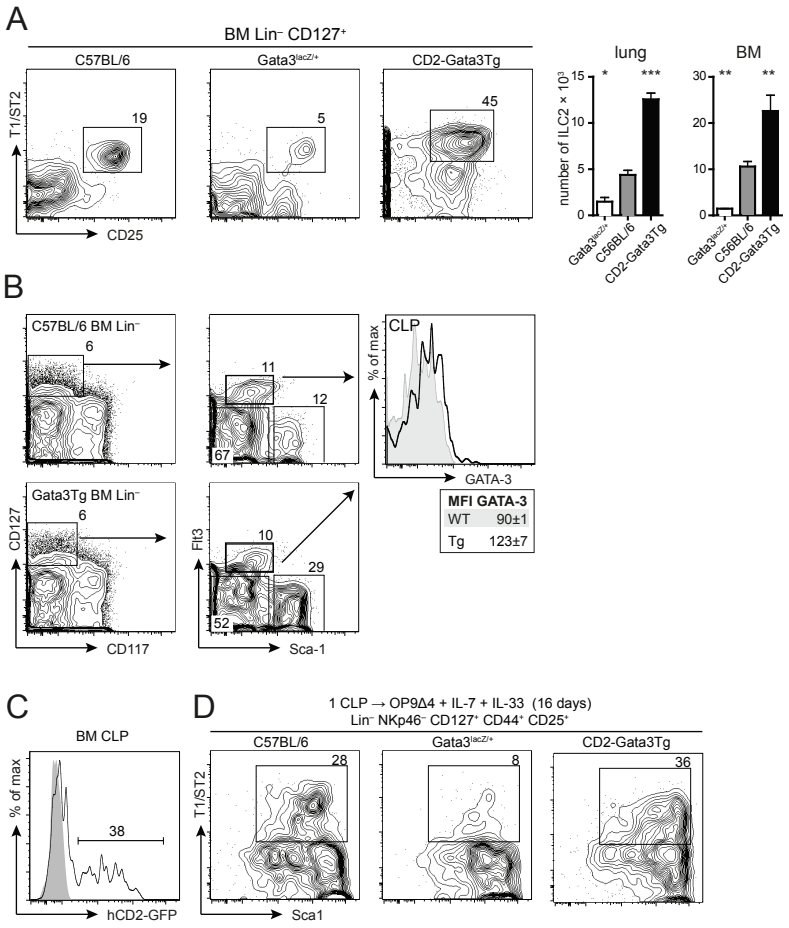

Figure 4. ILC2 development and function correlates with Gata3 gene copy number
A) Identification of ILC2 in BM of mice with different *Gata3* gene copy number, as indicated (top panels). Bottom panels show quantification of the total numbers of ILC2, IL-5$^+$ and IL-13$^+$ ILC2 in lung and BM of the indicated mouse strains. Significance levels indicate comparison of the indicated strain with C57BL/6. Representative results from 4 independent experiments with n≥8 total mice analyzed per genotype. **B)** Identification of common lymphoid precursors (CLP) in BM of wild type and *hCD2-Gata3*-Tg mice. Expression of GATA-3 in both mouse strains is shown as a histogram and mean fluorescence intensity (MFI) is shown as mean intensity ± SEM. Representative sample from 3 independent experiments is shown. **C)** GFP expression in BM CLP of *hCD2*-GFP Tg mice. Analysis of a non-transgenic age-matched control mouse is shown and used to determine background GFP levels. Representative results from 2 independent experiments are shown. **D)** Single CLP (Lin$^-$ CD127$^+$ CD117^{low} Sca1^{low} CD135$^+$) from mice with various *Gata3* copy number were sorted and co-cultured with OP9Δ4 stroma in the presence of IL-7 and IL-33 for 14-18 days and analyzed for ILC2 development. Plots show identification of ILC2 based on the indicated markers among Lin$^-$ CD127$^+$ CD44$^+$ CD25$^+$ lymphocytes. One representative sample from 3 independent experiments with n≥144 individual cultures per genotype tested in total.

single CLP from either *Gata3*$^{lacZ/+}$ mice, *Gata3*$^{+/+}$ mice or *hCD2-Gata3* Tg mice on OP9Δ4 stromal cells in the presence of IL-7 and IL-33. ILC2 developed from *Gata3*$^{+/+}$ CLP at the expected frequency (f = 1/5) (19), while *hCD2-Gata3* Tg CLP were almost twice as efficient in generating ILC2 (f = 1/2.6). In contrast, *Gata3*$^{lacZ/+}$ CLP showed a 2.6-fold reduction in

ILC2 potential (*f* = 1/13) compared to their wild type counterparts **(Table 1** and **Figure 4D)**. Collectively, these results strongly support a model in which *Gata3* levels in CLP control initial commitment to the ILC2 lineage and confirm the important role for sustained *Gata3* expression in maintenance of type 2 cytokine secretion from mature ILC2 (25).

Concluding remarks

Little is known about the mechanisms by which GATA-3 promotes T or ILC2 specification in lymphocyte precursors. In contrast, GATA-3 acts to direct cytokine production in differentiating Th2 cells (and by analogy in ILC2) by binding directly to *Il4*, *Il5* and *Il13* promoters and the Th2 locus control region (43,44), and through regulation of T1/ST2 expression (40). Concurrently, GATA-3 acts in a repressive complex to downregulate *Ifng* transcription through inhibition of *Runx3* (45), an important regulator of *Ifng* expression, and through addition of H3K27me3 suppressive marks (46). In a similar way, GATA-3 may promote early stages of T and ILC2 development through simultaneous gene activation and gene repression. Previous studies on GATA-1 clearly demonstrated the capacity of this transcription factor to participate in activating and repressive transcriptional complexes (reviewed in (47)). Similarly, GATA-3-mediated gene regulation works in a context-dependent fashion that is strongly influenced by transcription factor co-expression. Specific targets of GATA-3 in developing lymphocyte precursors are poorly defined. A recent analysis of Gata3-binding sites in early thymocyte progenitors (28) identified RORα as a GATA-3 target potentially involved in T cell specification. As RORα is critical for ILC2 homeostasis (10, 19), it is possible that GATA-3-mediated activation of RORα provides a mechanism to promote early ILC2 development. Indeed, analysis of FL cultures showed that in the absence of *Gata3*, *Rora* expression was not induced **(Supplemental Figure 5)**.

Amongst transcriptional activators, transient Notch signaling appears required for LTi and ILC2 development from lymphoid progenitors in vitro (18, 19). The role for Notch in this process is not understood, but could involve transient up-regulation of RORγt

Table 1.

ILC2 containing cultures 1 CLP → OP9Δ4 + IL-7 + IL-33 (14–18 days)			
Genotype	**Frequency (1/x)**	**95% CI**	**χ2 (p value)** versus WT
Gata3$^{lacZ/+}$	12.99	7.92 – 21.31	13.8 (<0.001)
C57BL/6	4.95	3.82 – 6.43	n/a
CD2-Gata3 Tg	2.63	2.05 – 3.38	12.4 (<0.001)

χ2 test for differences between any of the groups

$\chi^2 = 42.4$ ($p = 6.09 \times 10^{-10}$)

or GATA-3, respectively that are essential for LTi and ILC2 generation. Sustained Notch signaling, however, seems detrimental to LTi and ILC2 development, as neither of these ILC subsets is generated to any appreciable degree within the thymus. The role for Notch signaling in vivo for ILC2 development is not known, while normal homeostasis of ILC3 subsets requires Notch signals (48). One possible model is that common or dedicated ILC precursors are generated in adult mice in the bone marrow following transient Notch triggering of CLP; this may be important for *Gata3* up-regulation in CLP that generates precursors with ILC2 potential. In h*CD2-Gata3* transgenic mice, these precursors might accumulate as the initiating event in ILC2 specification (*Gata3* up-regulation) that is enforced in the bone marrow. In contrast, in vivo overexpression of *Gata3* in the presence of Notch signaling, in fetal thymocytes at the DN1 or DN2 stage, induced their re-direction into the mast cell lineage in vivo (49). Although evidence for direct action of Notch at the *Gata3* locus in lymphoid precursors is lacking, this mechanism has been amply demonstrated in Th2 cell differentiation (50,51).

While preparing this manuscript, two reports on the role of GATA-3 in mouse and human ILC2 appeared (52,53). Mjösberg et al. (52) show that GATA-3 is crucial for function of human ILC2 and that thymic stromal cell-derived lymphopoietin (TSLP) via activation of STAT5 can induce GATA-3 expression leading to cytokine production. Hoyler et al. (53) used an inducible *Gata3* ablation strategy in mice to show that intestinal ILC2 development and homeostasis requires constitutive *Gata3* expression. Our results provide independent evidence that Gata3 is essential for development of intestinal as well as pulmonary ILC2 (5–9). Moreover, since our analysis involved generation of hematopoietic chimeras in hosts devoid of endogenous ILC2 (2,3), we can conclude that GATA-3 is strictly required for ILC2 development as we exclude the possibility that absence of *Gata3*-deficient ILC2 results from growth disadvantage compared with *Gata3*-proficient cells. Importantly, we demonstrate that GATA-3 acts in a cell-intrinsic and dose-dependent fashion in vivo and in vitro to induce ILC2 development from isolated CLP; *Gata3* gene copy number directly correlated with subsequent ILC2 numbers and cytokine production capacity. This finding suggests that *Gata3* expression levels in CLP are decisive for induction of ILC2 fate.

Together, our results extend the understanding of the roles of GATA-3 in ILC2 development and function. We demonstrate a critical cell-intrinsic and dose-dependent role for GATA-3 in early ILC2 development that mirrors the role played by this transcription factor in early T cell development in the thymus (26, 27). These observations provide another example of the remarkable similarities between the molecular mechanisms that regulate the differentiation of innate and adaptive lymphocytes (13).

Material and Methods

Mice and generation of chimeric mice

Rag2-Il2rg-double deficient on the C57BL/6 background, *Cd3e*-deficient, *Gata3*^{lacZ}, *hCD2-Gata3* and -GFP Tg mice (32, 39, 40, 42) were maintained at the animal facilities of the Institut Pasteur, Erasmus MC and the Faculdade de Medicina de Lisboa. C57BL/6 mice and BALB/c *Rag2-Il2rg* mice were obtained from Harlan and Charles River, respectively. Mice were analyzed at 8-16 weeks of age. 5 Gy irradiated *Rag2-Il2rg*-double deficient animals were used as hosts for HSC grafts and were analyzed 5-10 weeks post-transplantation. All animals were kept under specific pathogen free conditions and provided with food and water ad libitum. All experiments were approved and performed in accordance with local regulations.

IL-25 and IL-33 administration

For IL-25-induced inflammation, mice received 2.5µg recombinant mouse IL-25 (BioLegend) in PBS for four subsequent days intra-peritoneally (36). Three doses of 1µg recombinant mouse IL-33 (BioLegend) in PBS were administered intra-nasally to anesthetized mice to induce eosinophilic airway inflammation (9).

ILC2 phenotypic and functional analysis

Lamina propria lymphocytes from small intestine were isolated as described previously (54). Single cell suspensions from lungs, BAL, spleen, lymph nodes and bone marrow were prepared and analyzed by FACS as described previously (9). Antibodies used for analysis of cell surface phenotype, intra-cellular cytokines and transcription factors are described in Supplemental Information.

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Supporting information

Supplemental Material & Methods

Generation of chimeric mice

Sub-lethally irradiated (5 Gy), 6-12 week-old *Rag2-Il2rg*-double deficient mice were used as hosts to generate *Gata3*-deficient and *Cd3e*-deficient chimeras. *Gata3*-deficient chimeras were generated by injection of *Gata3*-deficient fetal liver cells (E15.5) obtained from noradrenalin treated pregnant $Gata3^{+/-}$ dams mated to $Gata3^{+/-}$ males as described previously (1). The genotype of the embryos was confirmed by PCR. For *Cd3e*-deficient chimeras, bone marrow cells from 4-6 week old mice were depleted of lineage-positive cells (CD4, CD8α, CD11b, CD11c, CD19, B220, Ter-119, Gr-1, NK1.1, FcεRIα, TCRβ, TCRγδ) using magnetic beads (Miltenyi) and 1-2 × 10^6 cells were injected into the immunodeficient hosts. For some experiments, $Ly5.1^+$ *Rag2-Il2rg* mice were used as hosts and CD45.1/CD45.2 were used as congenic markers. In other experiments, *Rag2-Il2rg* deficient hosts were on the BALB/c background ($H2Dd^+$) and transplanted with cells on the C57BL/6 background ($H2Db^+$). Mixed chimeras were generated by injection of a mix of $Gata3^{+/-}$ or $Gata3^{-/-}$ fetal liver precursors supplemented with C57BL/6 lineage-depleted bone marrow precursors. Levels of chimerism were verified by screening B lymphocytes, NK and myeloid cells in spleen. Chimeric mice were analyzed 5-10 weeks after transplantation.

Antibodies for extra-cellular flow cytometric analysis

Antibodies used for flow cytometry were against CD4 (GK1.5), CD8α (53-6.7), CD11b (M1/70), CD11c (N418), CD19 (1D3), CD25 (PC61.5), CD44 (IM7), CD45 (I3/2.3), CD45.1 (A20), CD45.2 (104), CD117 (2B8), CD127 (A7R34), B220 (RA3-6B2), NK1.1 (PK136), FcεRIα (MAR-1), Gr-1 (RB6-8C5), Ter-119 (TER-119) Sca-1 (D7), T1/ST2 (DJ8; MD Bioproducts), H2Db (28-14-8), H2Dd (34-1-2S), Siglec-F (E50-2440), ICOS (C398.4A). Different conjugated were purchased from eBioscience, BD Biosciences or BioLegend, unless indicated otherwise. Biotin-conjugated antibodies were detected using Pacific Blue (Invitrogen), APC-eFluor 780 or PerCPCy5.5 (both eBioscience) conjugated streptavidin.

Intracellular flow cytometric analysis

For measurement of intracellular cytokines, cells were stimulated for 4 hours at 37°C using PMA and ionomycin (Sigma-Aldrich) in the presence of GolgiStop (BD Biosciences). After extracellular staining, cells were fixed using 2% paraformaldehyde (Electron Microscopy Sciences) and stained for intra-cellular cytokines in PBS buffer containing 0.5% saponin (Sigma-Aldrich). Antibodies against IL-4 (11B11), IL-5 (TRFK5) and IL-13 (eBio13A) were purchased from BD Biosciences and eBioscience. For GATA-3 protein detection, cells were fixed and permeabilized using the Foxp3 staining kit (eBioscience) according to the manufacturer's protocol. PE-, AlexaFluor647- or eFluor660-conjugated antibodies against GATA-3 (TWAJ or L50-823) were purchased from BD Biosciences or eBioscience.

Flow cytometric analysis and data analysis

After antibody staining, cells were analyzed using an LSRII (3 lasers) or LSRFortessa (4 or 5 lasers) flow cytometer (both BD Biosciences) equipped with FACSDiva software. Sorting was performed using FACSAria or FACSAriaII (both 3 lasers; BD Biosciences), equipped with FACSDiva software. Data analysis was performed using FlowJo 8.8.7 (Treestar Inc.).

In vitro development of ILC2 from hematopoietic precursors

OP9 stroma expressing Notch ligand Delta-like 4 (OP9Δ4) were grown in OptiMEM medium (Invitrogen) supplemented with 10% FCS (Thermo Scientific), 2-mercapto-ethanol (Sigma or Invitrogen) and penicillin/streptomycin (Invitrogen) and seeded onto multi-well plates (BD Biosciences or TPP).

Hematopoietic cell precursors were cultured on OP9Δ4 cells in IL-7 (5ng/mL; in-house hybridoma production) alone or in combination with IL-33 (20ng/mL; Biolegend). For CLP cultures, single Lin^- $CD127^+CD117^{low}Sca1^{low}CD135^+$ cells were sorted directly into 96 well plates containing OP9Δ4 cells and IL-7/IL-33. Medium and cytokines were refreshed every 3 or 4 days and cultures were split when necessary. Cultures were screened for growth using an inverted phase microscope and positive cultures were analyzed by flow cytometry on the indicated days.

Gene expression analysis

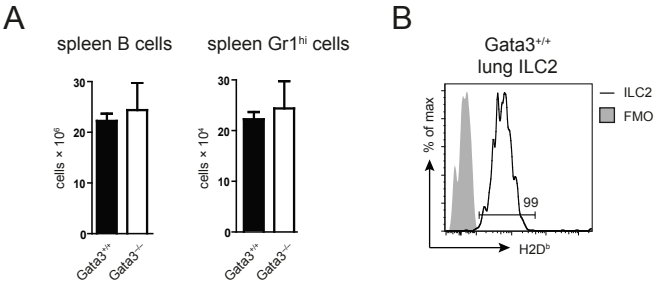
RNA was purified (RNeasy Micro Kit, Qiagen, Venlo, the Netherlands) from OP9Δ4 stromal cell cultures at various time points and cDNA was synthesized using RevertAid H Minus Reverse Transcriptase and random hexamer primers in the presence of Ribolock RNase inhibitor (all Thermo Scientific, MA, USA). qRT-PCR was performed using an ABI Prism 7300 Sequence Detection System (Applied Biosystems, Life Technologies) using gene-specific primers (Invitrogen) combined with a Universal Probe Library amplicon-specific probe (Roche Diagnostics, Lelystad, the Netherlands). Primers sequences are available upon request.

Statistical analysis

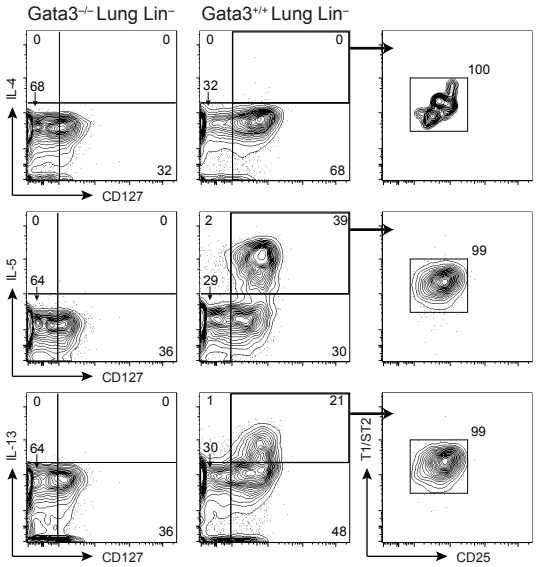
Unpaired t tests were used for comparisons between two normally distributed groups. For groups that were not normally distributed, significance of difference between groups was determined using Mann-Whitney U tests. For comparisons between multiple groups, one-way ANOVA was used, followed by a Dunnett post-test. Differences were considered significant at $p<0.05$. All statistical analyses were performed using Prism 4 and 5 (GraphPad). Bars indicate mean ± SEM. Significance levels of statistical tests are shown as ns = not significant, * $p<0.05$, ** $p<0.01$ or *** $p<0.001$.

References for Supplemental Materials & Methods

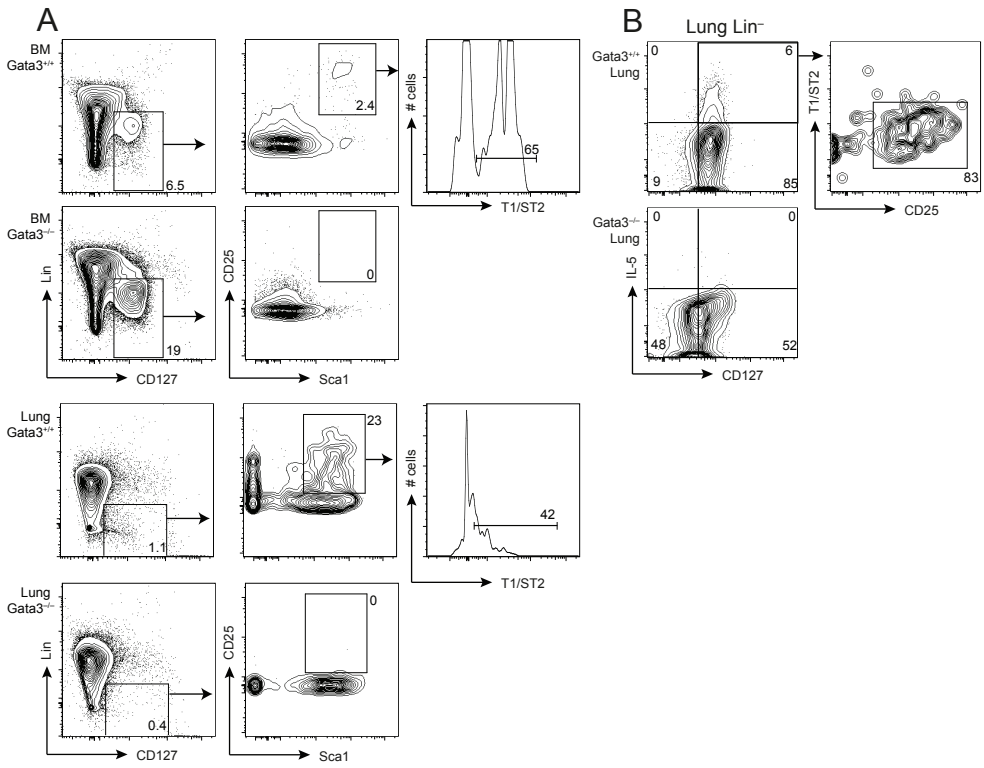
1. Kaufman CK et al. (2003) GATA-3: an unexpected regulator of cell lineage determination in skin. Genes Dev 17:2108–2122.



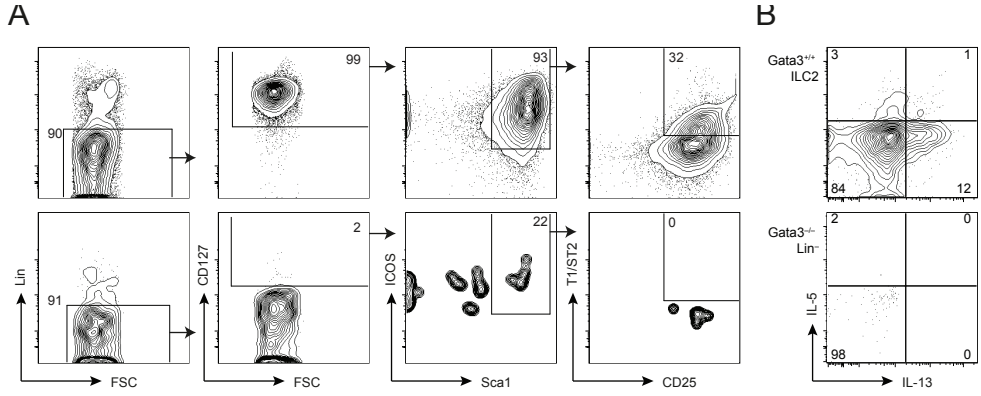
Supplemental Figure 1. Engraftment of Gata3$^{+/+}$ and Gata3$^{-/-}$ hematopoietic precursors.
A) Analysis of donor-derived (H2Db) CD19^{+} B cells and Gr-1^{+} myeloid cells in spleen from hosts transplanted with *Gata3*$^{+/+}$ or *Gata3*$^{-/-}$ hematopoietic precursors. **A)** Analysis of H2Db expression by Lin^{-} CD127^{+} CD25^{+} Sca-1^{+} T1/ST2^{+} lung cells (*Gata3*$^{+/+}$ chimeric mouse). Results from 2 independent experiments, each with n=3 mice (*Gata3*$^{-/-}$) or n=2 (*Gata3*$^{+/+}$) mice per group.



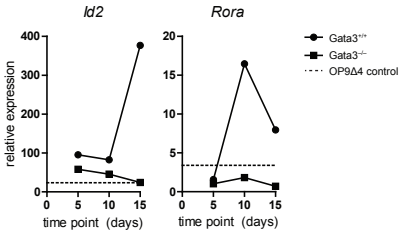
Supplemental Figure 2. Pulmonary IL-5 and IL-13 production by Lin^{-} lymphocytes upon IL-33 challenge is ILC2 derived.
Lin-negative lymphocytes from lungs of IL-33 challenged chimeras were analyzed for the expression of CD127 and the indicated intra-cellular cytokines. Expression of the indicated ILC2 markers is shown for the boxed *Gata3*$^{+/+}$ population and relative frequencies are indicated. Figure shows representative results from 2 independent experiments with n≥6 total mice analyzed per genotype.



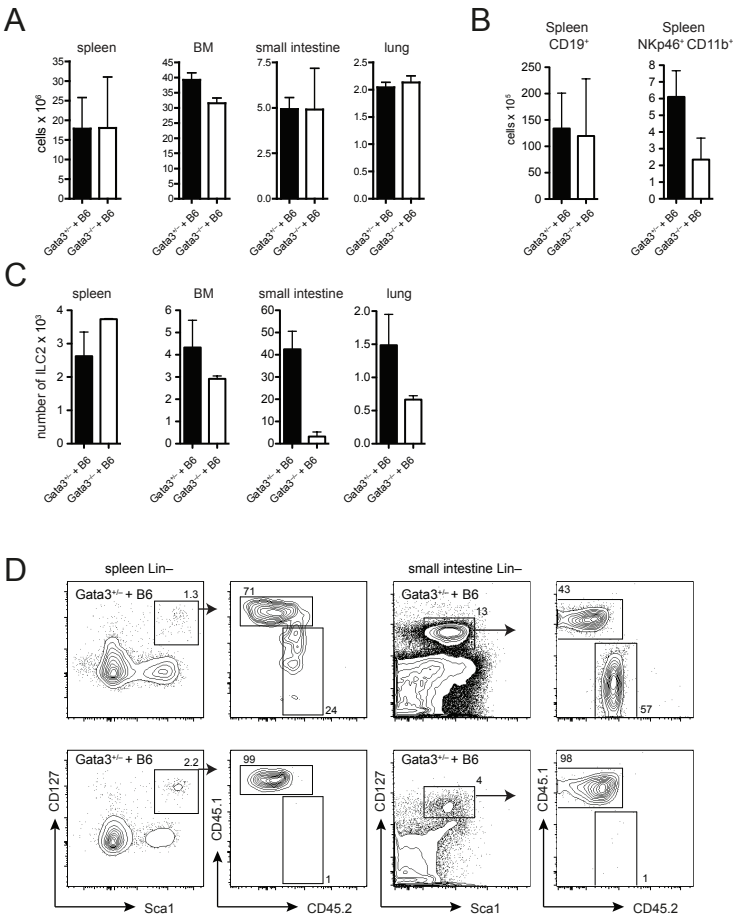
Supplemental Figure 3. Cytokine production from ILC2 in IL-25 challenged chimeras.
A) Gating strategy for identification of ILC2 in BM and lung of IL-25 challenged chimeras. **B)** B) Lin-negative lymphocytes from lungs were analyzed for IL-5 production. Cytokine-positive cells (boxed) were analyzed for expression of ILC2 markers. Figure shows representative results from 2 independent experiments with n=4-6 total mice analyzed per genotype.



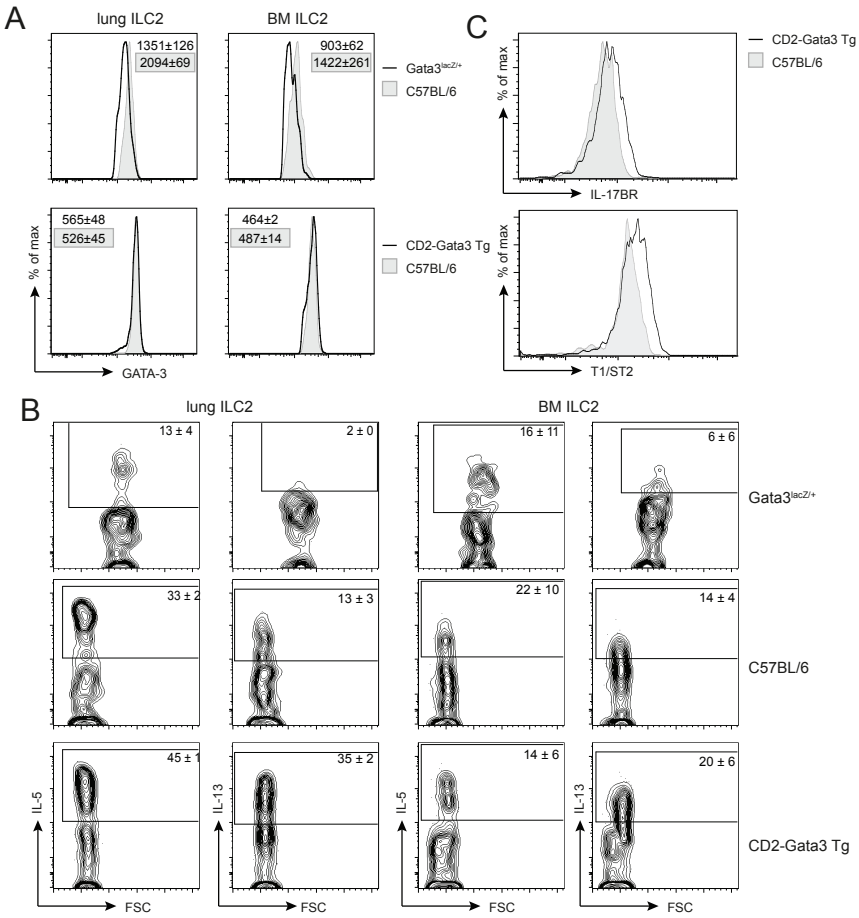
Supplemental Figure 4. ILC2 differentiation in vitro requires GATA-3.
A) *Gata3*-deficient or -competent fetal liver precursors were co-cultured with OP9Δ4 stromal cells in the presence of IL-7 and IL-33 and Lin-negative lymphocytes were analyzed by flow cytometry for the indicated markers after 17 days. **B)** B) Analysis of intra-cellular cytokine expression by ILC2 (*Gata3*-competent culture) or all lineage-negative lymphocytes (*Gata3*-deficient culture) after cultures as in A). Relative frequencies of the gated populations are indicated. Representative plots of 3 independent experiments with n≥2 samples per group.



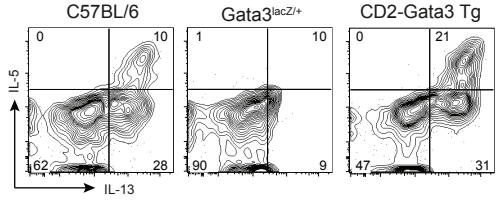
Supplemental Figure 5. No induction of Id2 and Rora expression in FL cultures in the absence of Gata3.
Gata3-deficient or -competent fetal liver precursors were co-cultured with OP9Δ4 stromal cells in the presence of IL-7 and IL-33. Fractions of the cultures were analyzed at the indicated time points using qRT-PCR for expression of the indicated genes. Expression levels were normalized to *Gapdh* expression levels. Gene expression levels in OP9Δ4 stroma cells are shown as negative control.



Supplemental Figure 6. Cell-intrinsic GATA-3 is required for ILC2 development.
A) Cellularity of the various organs of mixed chimeric mice (90% *Gata3*$^{+/-}$ or *Gata3*$^{-/-}$ FL precursors supplemented with 10% B6 BM). **B)** B) Levels of chimerism in reconstituted mice are shown as absolute number of CD4$^-$ CD8$^-$ CD19$^+$ B and CD4$^-$ CD8$^-$ CD19$^-$ NKp46$^+$ CD11b$^+$ NK cells in spleen. **C)** Quantification of the total number of Lin$^-$ CD127$^+$ CD25$^+$ Sca1$^+$T1/ST2$^+$ ILC2 in the indicated organs of mixed chimeric mice. **D)** Flow cytometric identification of ILC2 in the lineage-negative populations of spleen and small intestine lamina propria cells. ILC2 (boxed) are then probed for expression of allogenic markers.

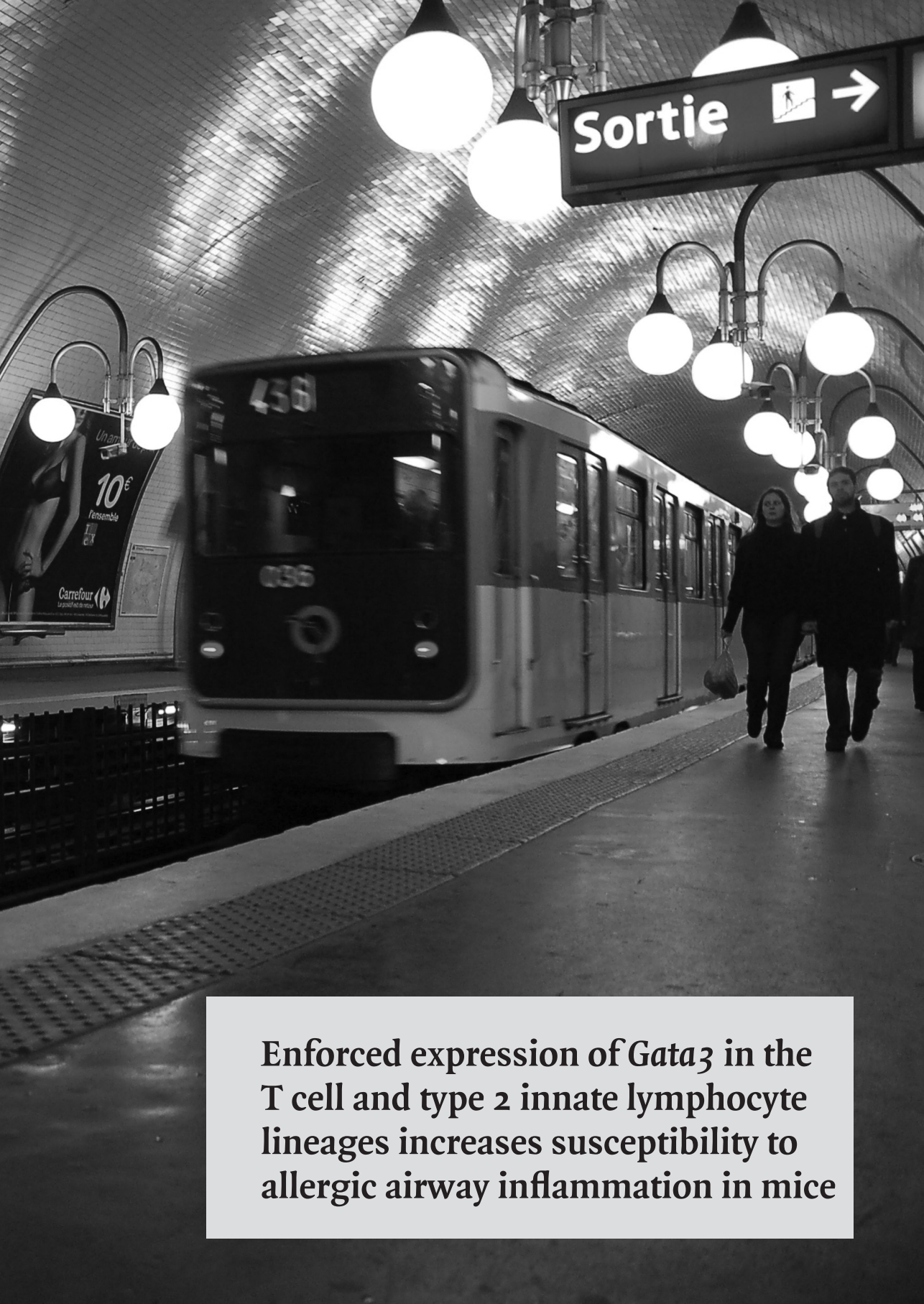


Supplemental Figure 7. ILC2 cytokine production correlates with Gata3 gene copy number.
A) Intra-cellular expression levels of GATA-3 ware analyzed after fixation and permeabilization of ILC2 from lung and BM from the indicated mouse strains. GATA-3 levels were determined using PE- or AlexaFluor660-connjugated antibodies as indicated. MFI for GATA-3 expression by ILC2 are indicated as mean ± SEM. One representative plot is shown from 4 independent experiments with n≥2 mice per genotype. **B)** Analysis of intra-cellular cytokine expression by lung and BM ILC2s gated as Lin$^-$ CD127$^+$ CD25$^+$ T1/ST2$^+$ cells. Numbers in plots indicate percentages of cytokine+ ILC2 ± SEM. **C)** C) Expression of IL-17BR and T1/ST2 by BM ILC2 gated as in B) by wild type and *CD2-Gata3* Tg mice. B and C show representative plots of four independent experiments with n=2-4 mice per group.



Supplemental Figure 8. Cytokine expression by in vitro CLP-derived ILC2.
Single Lin- CD127$^+$ Sca1^{low} CD117^{low} CD135$^+$ CLP were sorted from BM of mice with various *Gata3* copy numbers and co-cultured for 14-18 days with OP9Δ4 stroma cells in the presence of IL-7 and IL-33. Intra-cellular cytokine expression was analyzed for Lin$^-$ CD127$^+$ CD25$^+$ Sca1$^+$ T1/ST2$^+$ ILC2. One representative sample is shown with numbers indicating relative frequencies of gated ILC2. Data from 3 independent experiments with n≥144 cultures in total.

Chapter V



Enforced expression of *Gata3* in the T cell and type 2 innate lymphocyte lineages increases susceptibility to allergic airway inflammation in mice

RATIONALE The inflammation in allergic asthma is likely to be a threshold response of the innate immune system followed by the adoptive immune system, whereby T helper 2 (Th2) cells are thought to play a central role as producers of the pro-inflammatory Th2 cytokines IL-4, IL-5 and IL-13. Recently, type 2 innate lymphocyte cells (ILC2) were identified as a new cellular members of the innate immune system that contribute to the production of IL-5 and IL-13 in allergic inflammation. As transcription factor GATA-3 is essential for differentiation of both Th2 and ILC2, we hypothesized that increased *Gata3* expression in T cells and ILC2 is sufficient to increase susceptibility to allergic airway inflammation.

METHODS We used *CD2-Gata3* transgenic mice with enforced *Gata3* expression driven by the CD2 promoter, which is active in the T cell lineage and during ILC2 development. *CD2-Gata3* Tg mice and WT littermates were analyzed in mild models of allergic airway inflammation, involving intratracheal ovalbumin (OVA) or house dust mite (HDM) sensitizations without adjuvant.

RESULTS OVA allergen exposure did not induce inflammation in WT controls, while OVA-challenged *CD2-Gata3* transgenic mice showed clear eosinophilic airway inflammation and enhanced levels of IL-5 and IL-13 in bronchoalveolar lavage (BAL). Similarly, in a HDM-driven asthma model, *CD2-Gata3* Tg mice were significantly more susceptible to allergic airway inflammation than WT littermates. In HDM-driven asthma models both ILC2 and Th2 cells are important cellular sources of IL-5 and IL-13 in BAL and lung tissue. Compared to WT littermates, *CD2-Gata3* transgenic mice contained higher numbers of ILC2. These ILC2 contributed significantly to early production of IL-4, IL-5 and IL-13, as well as IFNγ, which is normally not produced by ILC2.

CONCLUSION Enforced *Gata3* expression is sufficient to enhance Th2 and ILC2 activity and leads to increased susceptibility to eosinophlic inflammation after mild exposure to inhaled harmless antigens that otherwise induce antigen tolerance.

Manuscript in preparation by Alex Kleinjan, Roel G.J. Klein Wolterink*, Y. Levani, Marjolein J.W. de Bruijn, Henk C. Hoogsteden, Menno van Nimwegen and R.W. Hendriks *equal contribution*

Introduction

Asthma is characterized by reversible airway obstruction, pulmonary inflammation and bronchial hyperreactivity (BHR) (1). Patients manifest symptoms of asthma in response to inhalation of airborne allergens, such as house dust mite (HDM), molds or animal dander. Many cell types participate in allergic inflammation, including eosinophils, dendritic cells (DCs), B and T lymphocytes, mast cells, macrophages, epithelial cells, smooth muscle cells and fibroblasts. Nevertheless, $CD4^+$ T helper 2 (Th2) cells, which produce a unique profile of IL-4, IL-5, IL-9 and IL-13 pro-inflammatory cytokines, are thought to be central in the orchestration and amplification of allergic inflammatory events. Th2 cytokines explain many hallmarks of allergic asthma: immunoglobulin E (IgE) synthesis (IL-4), airway eosinophilia (IL-5), mast cell accumulation (IL-9), goblet cell hyperplasia (IL-4, IL-13) and BHR acting on bronchial smooth muscle cells (IL-13) (1).

Although adaptive Th2 cells have been identified as important sources of IL-4, IL-5 and IL-13, many studies emphasize the importance of innate cells such as mast cells, eosinophils and basophils, in cytokine production. Most importantly, a population of non-B/non-T innate lymphoid cells (ILC) that have the capacity to produce large amounts of IL-5 and IL-13 upon stimulation by IL-33 or IL-25 was recently identified in the intestine and the lung (see for reviews: Ref. 2-3). Because of their Th2 cytokine profile, they have been named ILC2. These cells can be identified in the lineage-negative fraction as cells that co-express the receptors for IL-2, IL-7 and IL-33/T1ST2 (4). The involvement of ILC2 in asthma was recently demonstrated in various mouse models, including house dust mite (HDM)-driven allergic airway inflammation (5-7,8). ILC2 have also been identified in human lung and are associated with chronic rhinosinusitis (9-11).

Differentiation of the Th2 subset is induced by the key nuclear factor GATA-3, which acts as a transcription regulator of the Th2 cytokines IL-4, IL-5 and IL-13 and concomitantly suppresses Th1 development (12-14). A suggested mechanism for this activity is that GATA-3 causes chromatin remodeling in the Th2 cytokine locus, in conjunction with various other transcriptional regulators including C-maf, Irf4, SatB1 and Ctcf (14-16). Apart from its function in T helper cells, GATA-3 is also essential for early stages of T cell development in the thymus (14, 17).

Conditional deletion of the *Gata3* gene in established Th2 cells demonstrated that GATA-3 is critical for IL-5 and IL-13, but not IL-4 production (18). Deletion of the *Gata3* gene in murine ILC2 also abolished their IL-13 production (19). Moreover, studies of *Gata3*-deficient hematopoietic chimeras (this thesis, **Chapter IV**) and conditional targeting experiments showed that the Gata3 gene is required for ILC2 development in mice (20).

To date, more than 100 genes have been associated with asthma genome-wide, including the Th2 locus, thymic stromal lymphopoietin (TSLP), IL-33 and IL-33R/T1ST2, but typically the impact of each of these genes is mild (21-23). Interestingly, haplotype analysis

of the *GATA3* locus showed associations with asthma and atopy-related phenotypes (24). Nakamura et al. (25) showed that GATA3 expression is increased in lung mucosa and BAL cells of asthmatic patients, compared with healthy controls. Moreover, GATA-3 expression was increased after local allergen provocation, in the absence of inflammatory cell recruitment (26).

Mouse studies indicated that blocking *Gata3* expression by knock down strategies can reduce allergic inflammation in mouse model (27), suggesting *Gata3* an important target for therapeutic strategies (28). Conversely, we have previously shown that enforced expression of *Gata3* in T cells under the control of the CD2 promoter in transgenic (Tg) mice resulted in enhanced formation of a IL-33R/T1ST2 expressing Th2-committed T cell compartment in vivo (29), consistent with direct regulation of ST2 gene expression by Gata3 (30). These *CD2-Gata3* Tg mice lacked Th1–mediated hypersensitivity responses (29), and showed reduced differentiation or activity of the Th17 cell subset and protection from autoimmune encephalomyelitis and rheumatoid arthritis (31-32). Overexpression of *Gata3* in T cells has been associated with enhanced Th2-responses and eosinophilic airway inflammation in vivo (33-36). However, effects of Gata3 overexpression on the ILC2 population in allergic airway inflammation remain unknown.

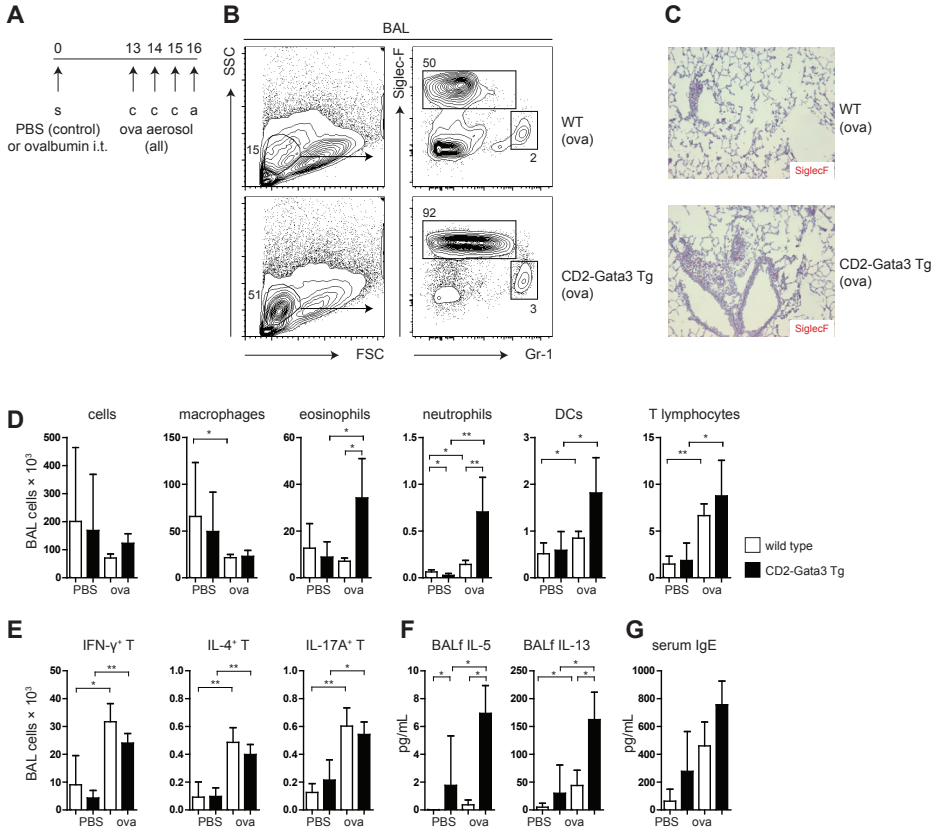
In the current study we investigated the effects of *Gata3* overexpression on Th2 cytokine production by T cells and ILC2, and the effects on the susceptibility of mice to develop asthma. We took advantage of *CD2-Gata3* Tg mice, since we recently found that these mice contain increased numbers of ILC2 (this thesis, **Chapter IV**). This might be explained by increased *Gata3* levels in ILC2 precursors in these mice, due to the activity of the hCD2 Tg control elements in common lymphoid progenitors (this thesis, **Chapter IV**).

In particular, we found that enforced *Gata3* expression enhanced eosinophilia in acute models of allergic airway inflammation, and increased both Th2 and ILC2 involvement. Both Th2 and ILC2 cells contributed to the earliest onset of allergic inflammation by the production of IL-4, IL-5 and IL-13.

Results

Enforced Gata3 expression does not enhance symptoms in a severe asthma model

First, we compared asthma susceptibility of *CD2-Gata3* Tg mice and WT littermates in an ovalbumin (OVA)-driven murine allergic airway inflammation model. Sensitization to inhaled OVA was induced by intratrachial (i.t.) injection of bone marrow (BM)-derived DCs, using PBS-pulsed DCs as a control (37). Upon three OVA aerosol challenges at day 10-12 after sensitization, mice were analyzed at day 13 **(Supplemental Figure 1A)**. Whereas only a few inflammatory cells were observed in the broncho-alveolar lavage (BAL) fluid of mice that received unpulsed PBS-DC, significant eosinophilic airway inflammation developed



Enforced Gata3 expression induces susceptibility to allergic airway inflammation upon i.t. OVA sensitization
A) *CD2-Gata3* Tg mice and WT littermates were intratracheally (i.t.) sensitized (s) with ovalbumine (OVA) or PBS as control sensitization followed by OVA aerosol challenges (c). One day after the last challenge mice were sacrificed and analyzed (a). **B)** Flow cytometric analysis of bronchoalveolar lavage (BAL) showing forward and side scatter profile (FSC/SSC) and cells with eosinophil FSC/SSC characteristics were gated and analysed for Siglec-F and Gr-1. Proportions of cells in the indicated quadrants are given (in %). **C)** Bronchial mucosal sections were stained for murine Siglec F (red), a specific marker for eosinophils. Murine eosinophils have a ring-shaped or a bilobular nucleus (blue). WT OVA/OVA mice lack eosinophilic infiltrates (upper panel) and *CD2-Gata3* Tg OVA/OVA mice show some peribronchial and perivascular eosinophilic infiltrates (lower panel). **D)** Quantification of flow cytometric analysis of the indicated populations of leukocytes in BAL of WT (white bars) and *CD2-Gata3* Tg mice (black bars). The results shown are expressed as means ± SEM. *p<0.05, **p<0.01. **E)** Quantification of flow cytometric analysis of $CD4$ T-cells positive for the indicated cytokines upon 4hrs of PMA/ionomycin stimulation. The results shown are expressed as means ± SEM. *p<0.05, **p<0.01. **F)** IL-5 and IL-13 concentrations in BAL fluid measured by ELISA. **G)** Total IgE in serum by ELISA . The results shown represent one out of two independent experiments with 3-6 animals per group. *p<0.05, **p<0.01.

upon OVA aerosol challenge of mice that had received OVA-pulsed DCs **(Supplemental Figure 1B)**. In this OVA-driven asthma model, we observed no significant differences between *CD2-Gata3* Tg mice and WT littermates in the numbers of the individual immune cell populations present in the BAL, as determined by flow cytometry **(Supplemental Figure 1B)**. Likewise, using intracellular FACS staining no differences were found in the numbers of Th1, Th2 or Th17 cells, typified by IFNγ, IL-4 or IL-17 production, respectively **(Supple-**

mental Figure 1C). Therefore, we conclude that enforced *Gata3* expression in *CD2-Gata3* Tg mice did not enhance asthma symptoms in a severe allergic airway inflammation model based on Th2 sensitization by OVA-pulsed DCs.

Enforced Gata3 expression induces susceptibility to allergic airway inflammation upon i.t. OVA sensitization

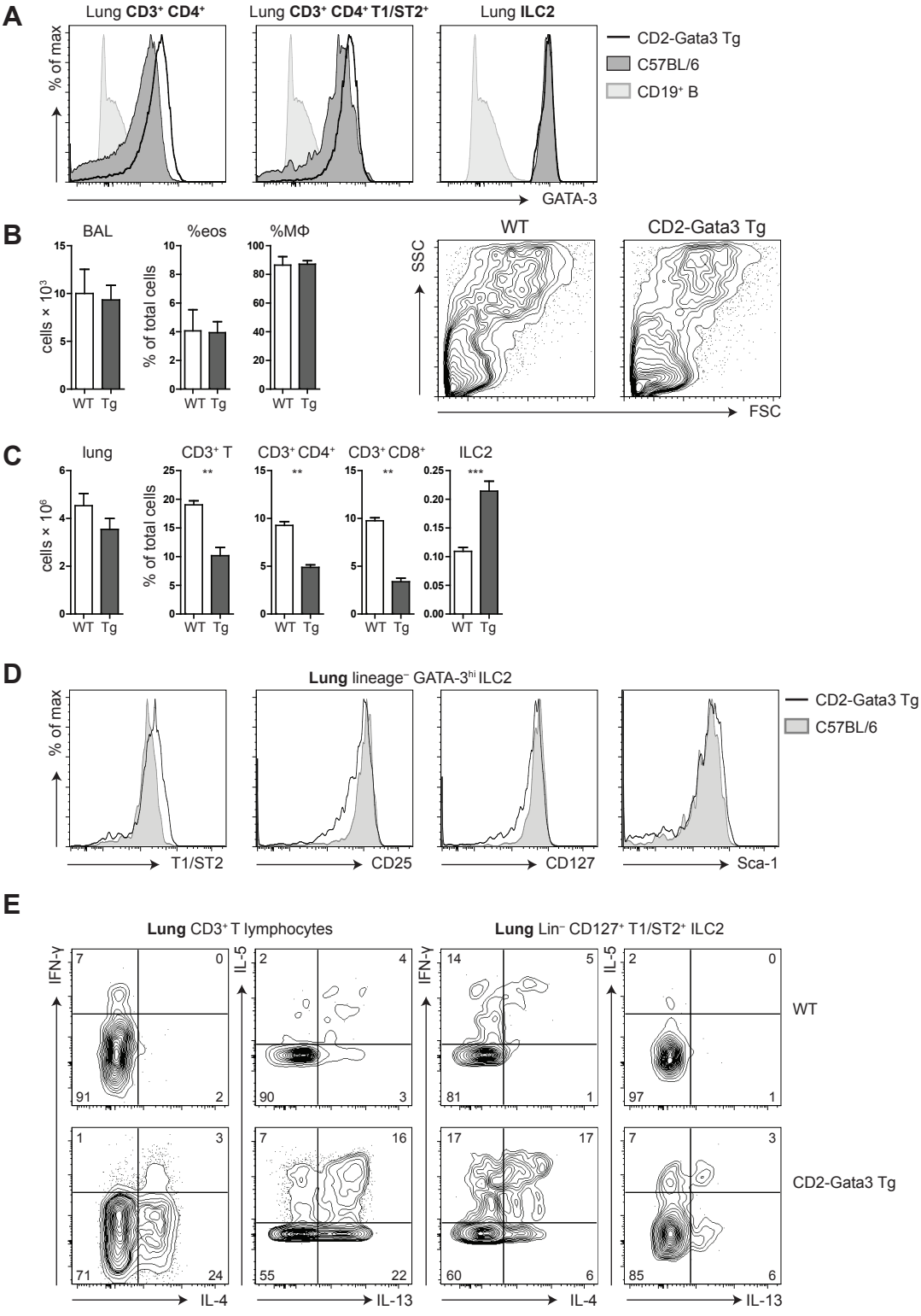
Next, we determined whether *CD2-Gata3* Tg mice were susceptible to eosinophilic airway inflammation in a very mild asthma model, based on i.t. sensitization with OVA followed by OVA aerosol challenges **(Figure 1A)**. When we compared OVA-sensitized and PBS-sensitized WT mice one day after the last OVA aerosol challenge, we did not find evidence for eosinophilic inflammation by quantification of BAL cells by flow cytometry, or by immunohistochemical staining of the lungs for Siglec-F, which is specifically expressed by eosinophils **(Figure 1B-1D)**. In contrast, we identified substantial peri-bronchial and peri-vascular eosinophilic infiltrates in sections of lungs from OVA-sensitized/OVA-challenged *CD2-Gata3* Tg mice **(Figure 1C)**. Numbers of eosinophils in BAL from OVA-sensitized *CD2-Gata3* Tg mice were significantly higher than in BAL samples from OVA-sensitized WT littermates (p=0.009) **(Figure 1B, 1D**). Moreover, quantification of BAL cell populations showed that – in contrast to our findings in WT littermates – *CD2-Gata3* Tg mice manifested significantly higher numbers of eosinophils (p=0.03), neutrophils, DCs and T lymphocytes after OVA-sensitization than after PBS-sensitization.

We therefore conclude that enforced *Gata3* expression induces susceptibility to eosinophilic airway inflammation upon i.t. sensitization in an adjuvant-free OVA model that is associated with immune tolerance in WT control mice.

Enforced Gata3 expression results in increased IL-5 and IL-13 levels in BAL fluid during allergic airway inflammation upon i.t. OVA sensitization

Although we did not find evidence for eosinophilic inflammation one day after the last OVA aerosol challenge, we did observe an increase in the number of T cells in BAL when we compared OVA-sensitized and PBS-sensitized WT mice **(Figure 1D)**. Subsequently, we

Figure 2 (*right page*). **Effects of enforced Gata3 expression on naive mice**
A) GATA-3 protein expression by intracellular flow cytometry in $CD3^+CD4^+$ T cells and in $CD3^+CD4^+T1ST2^+$ T-cells and ILC2 in lungs of *CD2-Gata3* Tg mice (black line) and WT (C57bl/6) mice (dark gray). Histogram of $CD19^+$ B cells (light gray) is shown as a control. **B)** Quantification of flow cytometric analysis of the indicated populations of leukocytes in bronchoalveolar lavage (BAL) of WT (white bars) and *CD2-Gata3* Tg mice (gray bars). . The results shown are expressed as means ± SEM. *p<0.05, **p<0.01. Flow cytometric analysis of bronchoalveolar lavage (BAL) showing forward and side scatter profile (FSC/SSC). **C)** Quantification of flow cytometric analysis of the indicated populations of leukocytes in collagenase-digested lungs of WT (white bars) and *CD2-Gata3* Tg mice (gray bars). The results shown are expressed as means ± SEM. *p<0.05, **p<0.01. ***p<0.001. **D)** Flow cytometyric analysis of surface expression of IL-33R/T1ST2, IL-2R/CD25, IL-7R/CD127 and Sca-1 on lung ILC2 from *CD2-Gata3* Tg mice (black line) and WT (C57bl/6) mice (gray) mice, shown as histogram overlays. **E)** Cytokine expression profiles of lung T-cells and ILC2 of naïve wild-type (WT) or *CD2-Gata3* Tg (Tg) mice upon 4hrs of PMA/ionomycin stimulation. Data are shown as contour plots; proportions of cells in the indicated quadrants are given (in %). The results shown represent one out of two independent experiments with 3-6 animals per group.


A
Lung CD3+ CD4+
Lung CD3+ CD4+ T1/ST2+
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BAL
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Lung Lin− CD127+ T1/ST2+ ILC2
IFN-γ
IL-5
IL-4
IL-13
WT
CD2-Gata3 Tg

determined cytokine expression profiles by intracellular flow cytometry upon four hours of stimulation by PMA/ionomycin. These analyses showed that the observed T cell increase was accompanied by an increase in the numbers of IL-4, IFN-γ as well as IL-17A positive T cells **(Figure 1E)**. In these analyses, no differences were observed between *CD2-Gata3* Tg mice and WT littermates **(Figure 1E)**. However, quantification of IL-5 and IL-13 levels in BAL fluid showed significantly elevated levels of IL-5 and IL-13 in *CD2-Gata3* Tg OVA-sensitized mice, compared with WT OVA-sensitized mice or PBS-sensitized mice **(Figure 1F)**. Finally, serum levels of total IgE tended to be higher in *CD2-Gata3* Tg mice than in WT littermates **(Figure 1G)** upon OVA-challenge in OVA-sensitized mice, but in these comparisons no statistical significance was reached.

Taken together, these data in an adjuvant-free OVA-sensitization model demonstrate that the high susceptibility of *CD2-Gata3* Tg mice to eosinophilic airway inflammation is associated with increased IL-5 and IL-13 levels in BAL fluid.

Characterization of lung T cells and ILC2 in naïve CD2-GATA3 transgenic mice

The increased levels of IL-5 and IL-13 in BAL fluid of *CD2-Gata3* Tg mice, compared with WT littermates, in the i.t. OVA sensitization model may point to cytokine production by T cells. This would be in agreement with our previously reported findings that the presence of the *CD2-Gata3* transgene enhances Th2 cytokine production, both in *ex vivo* analyzed T cells and in *in vitro* activated T cells (29, 31-32). However, as we recently found that the *CD2-Gata3* transgene is also expressed during ILC2 development (this thesis, **Chapter IV**), we also investigated ILC2. We quantified Gata3 protein expression by flow cytometry in $CD3^+CD4^+$ T cells, in $T1ST2^+CD3^+CD4^+$ Th2 memory cells as well as in ILC2. The two T cell populations showed higher *Gata3* levels in *CD2-Gata3* Tg mice, when compared with WT littermates or C57BL/6 controls **(Figure 2A)**. ILC2 expressed very high levels of GATA-3, whereby no differences between CD2-Gata3 Tg mice and WT control littermates were detectable **(Figure 2A)**.

Analysis of the immune cells in the BAL fluid revealed no differences (absolute numbers, or proportions of T and B cells, neutrophils, eosinophils or macrophages) between naïve *CD2-Gata3* Tg mice and WT control littermates **(Figure 2B** and data not shown). The two groups of mice did not differ in their numbers of total lung cells upon collagenase digestion. However in *CD2-Gata3* Tg mice, both $CD4^+$ and $CD8^+$ T-cell numbers were significantly reduced **(Figure 2C)** and the $CD4^+$ T cell population contained increased proportions of IL-33R/T1ST2-expressing cells **(Supplemental Figure 2)**, in agreement with similar findings in spleen and lymph nodes that we reported previously (29). Quantification of ILC2 showed a ~2-fold increase in the proportions of ILC2 in *CD2-Gata3* Tg mice, compared with WT control littermates. Also, their surface expression of IL-33R/T1/ST2 was slightly but reproducibly enhanced, whereas expression of CD25, CD127 or Sca-1 was similar in *CD2-Gata3* Tg and WT ILC2 **(Figure 2D)**.

Consistent with previously analyses in lymph node and spleen cells (29,31-32), lung T cells in naïve *CD2-Gata3* Tg mice showed increased proportions of IL-4, IL-5 and IL-13 expressing cells, compared with WT littermates. Despite our inability to detect increased expression of GATA-3 protein in *CD2-Gata3* Tg ILC2 by intracellular flow cytometry **(Figure 2A)**, these cells contained increased proportions of IL-5 and IL-13 expressing cells, when compared with WT ILC2 **(Figure 2E)**. Remarkably, naïve *CD2-Gata3* Tg ILC2 also contained significant numbers of cells that were positive for IL-4 and IFN-γ, cytokines that are generally not expressed by ILC2 **(Figure 2E)**.

In summary, these findings show that *CD2-Gata3* Tg mice manifest no spontaneous eosinophilic airway inflammation, but the T cell and ILC2 populations in the lungs of these mice contain higher proportions of Th2 cytokine positive cells than WT mice do.

Enforced Gata3 expression enhances allergic airway inflammation in a mild HDM model

Next, we investigated the effects of *Gata3* overexpression in a HDM-driven allergic inflammation model, whereby toll-like receptor-4 (TLR4) on epithelial cells helps drive the development of of allergic reactions to a common allergen (42). As is shown in **Figure 3A**, we performed PBS or HDM sensitizations, followed by three HDM challenges at day 13-15. To focus on the early phase of inflammation, we analyzed *CD2-Gata3* Tg mice and WT littermates one day after the last challenge. Histological analyses of lung tissue showed more peri-bronchial and peri-vascular eosinophilic infiltrates in *CD2-Gata3* Tg HDM-sensitized and challenged (HDM/HDM) mice, than in the WT HDM/HDM mice or in *CD2-Gata3* Tg PBS/HDM mice.

Quantification of BAL fluid cells showed that HDM sensitization and challenge did not induce any signs of eosinophilic airway inflammation in WT mice **(Figure 3B)**. In contrast, *CD2-Gata3* Tg mice in the HDM/HDM group showed a ~5-fold increase in eosinophils in BAL fluid, when compared with the WT HDM/HDM group **(Figure 3B)**. In the HDM/HDM *CD2-Gata3* Tg mice this eosinophlia was accompanied by a more generalized inflammation of innate cells, evidenced by elevated numbers of macrophages, neutrophils and DCs **(Figure 3B)**. We observed a similar trend in the lungs: the highest numbers of eosinophils were present in the HDM/HDM *CD2-Gata3* Tg mice **(Figure 3C)**.

Both in *CD2-Gata3* Tg mice and WT littermates, we observed a significant increase of the total numbers of T lymphocytes in the HDM/HDM group, compared with the PBS/HDM group **(Figure 3D)**. In BAL fluid of *CD2-Gata3* Tg mice, we observed a substantial influx of ILC2 after HDM exposure, irrespective of PBS or HDM sensitization **(Figure 3D)**. In the lungs the numbers of ILC2 upon HDM challenge was ~2-fold higher in *CD2-Gata3* Tg mice than in WT mice **(Figure 3D)**.

Collectively, these findings show that enforced CD2-driven *Gata3* expression enhances HDM-driven allergic airway inflammation. In this model, the BAL fluid of *CD2-Gata3* Tg

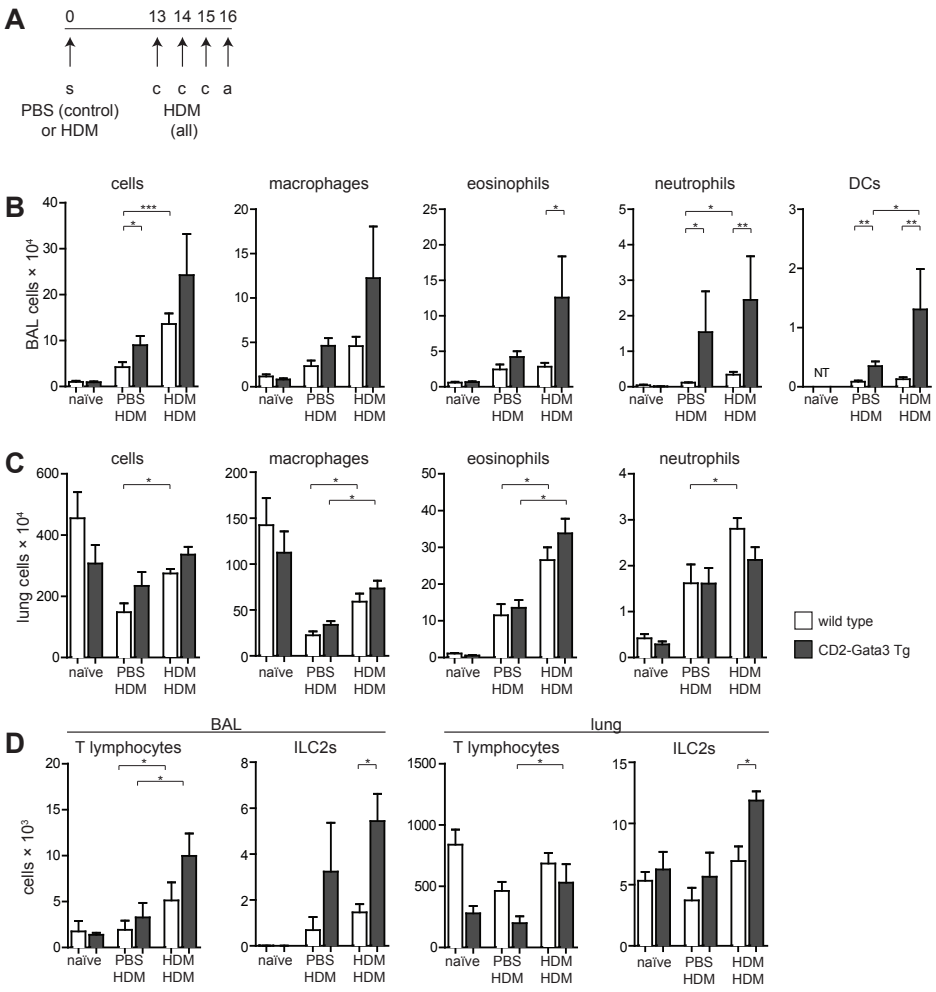

Figure 3. Enforced Gata3 expression enhances allergic airway inflammation in a mild HDM model
A) Mild experimental HDM asthma design showing intratracheal sensitisation (s) of 10 µg HDM or PBS as control and challenge (c) with 10 µg HDM intra-nasally. **B)** Quantification of flow cytometric analysis of the indicated populations of leukocytes in bronchoalveolar lavage (BAL) of WT (white bars) and *CD2-Gata3* Tg mice (gray bars). The results shown are expressed as means ± SEM. **C)** Quantification of flow cytometric analysis of the indicated populations of leukocytes in lungs of WT (white bars) and *CD2-Gata3* Tg mice (gray bars). The results shown are expressed as means ± SEM. **D)** Numbers of T-cells and ILC2 in BAL fluid (left) and collagen-digested lungs (right). Data are shown as median and SEM. The results shown represent pooled data of two independent experiments with 3-6 animals per group. Statistical evaluations were performed only between different treatments of groups of the same genotype, or between the two genotypes within one treatment-group. *p<0.05, **p<0.01, ***p<0.001.

mice contains increased numbers of leukocytes, in particular eosinophils and ILC2.

Enforced Gata3 expression enhances cytokine production by Th2 cells and ILC2

Next, we used intracellular FACS analyses to investigate cytokine profiles of Th2 and ILC2 in BAL fluid and lungs in HDM-driven allergic airway inflammation. **Figure 4A** shows an

example of the cytokine analysis of gated $CD3^+$ T cells and ILC2 from the lungs WT and *CD2-Gata3* Tg mice.

When we quantified cytokine-expressing cells in BAL fluid, we observed that enforced expression of Gata3 increased the numbers of T cells expressing the Th2 cytokines IL-4, IL-5 and IL-13, whereby differences between *CD2-Gata3* Tg and WT littermates reached significance in the HDM-sensitized group **(Figure 4B)**. In contrast, the presence of the *CD2-Gata3* transgene did not affect the numbers of T cells positive for the key Th1 cytokine IFNγ. Remarkably, enforced expression of *Gata3* did not only increase the numbers of ILC2 expressing IL-5 and IL-13, both of which are typically produced by ILC2, but also the numbers of $IL\text{-}4^+$ and $IFN\gamma^+$ ILC2 **(Figure 4B)**. Again, differences between *CD2-Gata3* Tg and WT littermates reached significance only in the HDM-sensitized group **(Figure 4B)**.

Whereas *CD2-Gata3* Tg and WT littermates had similar numbers IFNγ T cells in the lung, *CD2-Gata3* Tg mice showed an increase in the numbers of IL-4, IL-5 or IL-13 expressing T cells. Remarkably, the average number of Th2 expressing T cells was not different between the groups of naïve, PBS/HDM-treated and HDM/HDM-treated *CD2-Gata3* Tg mice **(Figure 4C)**. Parallel to our findings in the BAL fluid, we observed significantly increased numbers of IFNγ, IL-4, IL-5 and IL-13 expressing ILC2 in HDM-sensitized/HDM-challenged *CD2-Gata3* Tg mice, compared with WT littermates.

Finally, we measured cytokine levels in BAL fluid by ELISA. We found a moderate increase for IFNγ, IL-4 and IL-5 in *CD2-Gata3* Tg mice compared with WT littermates, whereby observed differences were significant for IFNγ and IL-5.

Taken together, these findings in our HDM-driven allergic airway inflammation show that the presence of the *CD2-Gata3* transgene is associated with enhanced Th2 cytokine production by both T cells and ILC2 in BAL and lung. Enforced expression of Gata3 in vivo does not affect the numbers of IFNγ-expressing T cells, but remarkably results in an accumulation of IFNγ-producing ILC2 in BAL and lung.

Discussion

The transcription factor GATA-3 is highly expressed in Th2 cells and ILC2, is required for the differentiation of these cells and acts as a key regulator of Th2 cytokine expression. In this study we used transgenic mice expressing *Gata3* under the control of the human CD2 promoter region to show that enforced *Gata3* expression increases susceptibility to allergic airway inflammation. We found that in mild models (i.t. sensitizations with OVA or HDM without adjuvant) that fail to induce airway inflammation in WT mice, eosiniphilic airway inflammation was induced in *CD2-Gata3* Tg mice. This was accompanied by an increase of IL-4, IL-5 and IL-13 producing Th2 cells and ILC2 in BAL and lung.

Remarkably, the presence of the in *CD2-Gata3* transgene resulted in a significant increase of the expression of IL-4, IL-5 and IL-13 (but not IFNγ) in pulmonary T cells al-

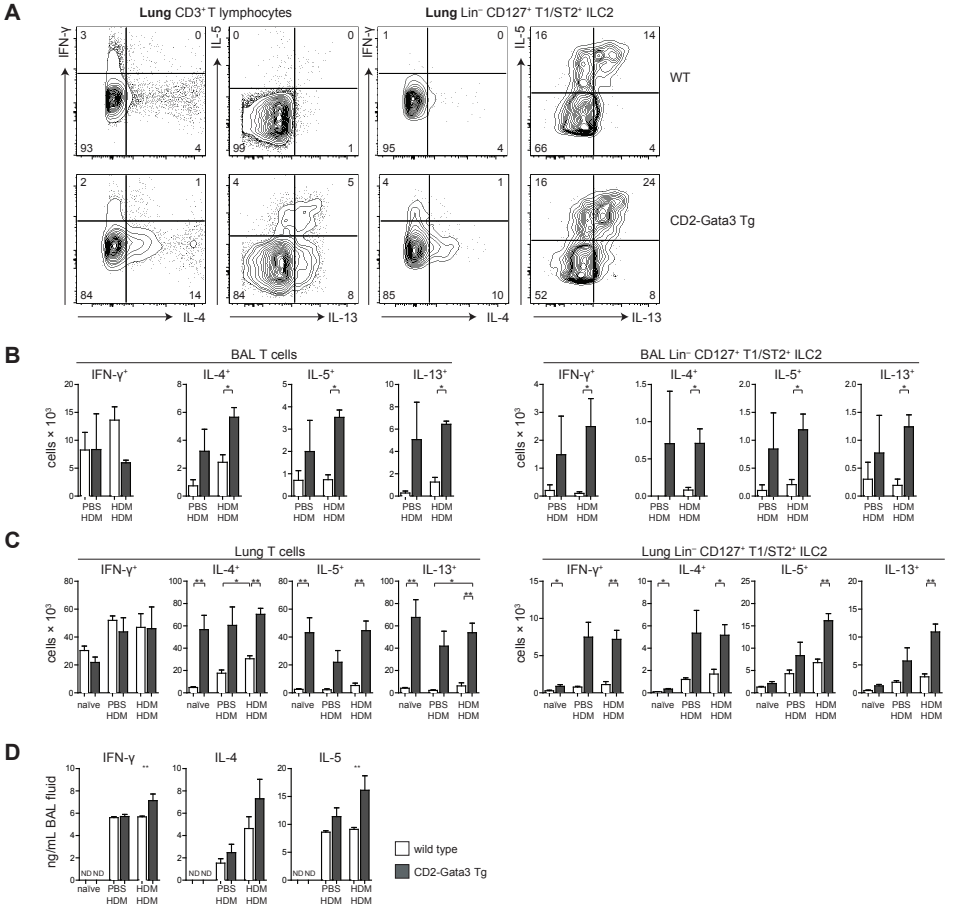

Figure 4. Enforced Gata3 expression enhances cytokine production by airway Th2 cells and ILC2
A) Cytokine expression profiles of gated lung T cells (left) and ILC2 (right) of naïve wild-type (WT) or *CD2-Gata3* (Tg) mice upon 4hrs of PMA/ionomycin stimulation. Data are shown as contour plots; proportions of cells in the indicated quadrants are given (in %). **B)** Quantification of flow cytometric analysis of T cells (left) and ILC2 (right) positive for the indicated cytokines upon 4hrs of PMA/ionomycin stimulation from bronchoalveolar lavage (BAL) WT (white bars) and *CD2-Gata3* Tg mice (gray bars). The results shown are expressed as means ± SEM. *p<0.05. **C)** Quantification of flow cytometric analysis of T cells (left) and ILC2 (right) positive for the indicated cytokines upon 4hrs of PMA/ionomycin stimulation from collagenase-treated lung WT (white bars) and *CD2-Gata3* Tg mice (gray bars). The results shown are expressed as means ± SEM. *p<0.05. **D)** Quantification of levels of IFNγ, IL-4 and IL-5 in BAL fluid from WT (white bars) and *CD2-Gata3* Tg mice (gray bars) as measured by ELISA. Data are shown as average values ± SEM. **p<0.01. The results shown represent one out of two independent experiments with 3-6 animals per group.

ready in naïve mice. Furthermore, the elevated numbers of Th2 cytokine expressing T cells did not change upon HDM-challenge in PBS-sensitized or HDM-sensitized mice. Together with the findings that the T cell population in *CD2-Gata3* Tg mice contains increased proportions of T cells with a memory phenotype and increased expression of IL-33R/T1ST2 (29), our results indicate that *Gata3* supports the formation of a Th2-commited memory T cell compartment in vivo in the lung.

In contrast, ILC2 cells in the lungs of naïve *CD2-Gata3* transgenic mice do not manifest increased cytokine expression, compared to WT mice. However, upon HDM-challenge their cytokine expression is induced to levels that are significantly higher than those of WT ILC2. Consistent with the innate characteristics of ILC2, i.e. the absence of an antigen receptor, their cytokine expression profile is not different between mice from the PBS- or HDM-challenged group.

The increased cytokine production by ILC2 may be explained by a direct effect of *Gata3* overexpression on the Th2 cytokine locus in mature ILC2. However, we were unable to detect higher levels of GATA-3 protein in pulmonary or intestinal ILC2 cells by our intracellular flow cytometric assay **(Figure 2A** and R.K.W., unpublished results). Alternatively, increased *Gata3* levels in developing ILC2 cells might affect epigenetic modifications at the Th2 locus (14), which may enhance cytokine production in ILC2 upon activation during HDM challenge. Nevertheless, we found that *CD2-Gata3* Tg ILC2 expressed higher levels of the IL-33R/T1ST2, thereby increasing their sensitivity to respond to IL-33, which is a potent ILC2 activator induced during HDM challenge (1-3). It is not likely that the presence of the CD2-Gata3 transgene affected IL-33 induction, because we did not detect differences in IL-33 expression between *CD2-Gata3* Tg and WT littermates in lung homogenates and observed a similar increase upon HDM challenge in both mouse groups (data not shown). An important role of GATA-3 in enhancing IL-33R-mediated ILC2 activation would be consistent with the finding that in our asthma model based on i.t. instillation of OVA-primed DCs (thus bypassing the role of IL-33-expressing epithelial cells or activated macrophages) we did not detect differences between *CD2-Gata3* Tg and WT littermates **(Supplemental Figure 1)**. The differences between the DC-installation and OVA/HDM sensitization models would point to an important role of IL-33 as a non-specific trigger in allergic airway inflammation in susceptible individuals. In this context, it was shown that IL-33 is a steroid resistant cytokine and because levels are elevated in steroid resistant asthma patients (39), the IL-33-IL33R/T1ST2 axis could be a potential therapeutic target to inhibit Th2 cytokine induction in susceptible individuals (39,40). Finally, we found that already in the absence of sensitization, HDM-challenged *CD2-Gata3* Tg mice manifested significant recruitment of DCs, next to some eosinophilic inflammation **(Figure 3B)**. It is therefore conceivable that ILC2 may act in parallel with epithelial cells that are induced by HDM to produce CCL20 and GM-CSF to support recruitment and activation of DCs (41). Further experiments should identify chemokines and cytokines that are produced by activated ILC2. These factors, like IL-4, IL-5, IL-13, could be regulated by GATA-3.

Several other groups have generated transgenic mice with overexpression of *Gata3* and have consistently reported enhanced eosinophilic airway (33-36). In chronic models that involve repeated allergen exposure, enforced *Gata3* expression was associated with increased subepithelial firbrosis and airway smooth muscle hyperplasia (35). Although in most of these published models *Gata3* expression was also driven by the human CD2 promoter,

the effects of *Gata3* overexpression on cytokine profiles appeared variable: both limited effects on IL-4 but increased IL-5/IL-13 expression (33), increased IL-4 but similar IL-5 levels (35) and increased production of IL-4, IL-5 and IL-13 (36) have all been described. Because we observed the largest effects of GATA-3 in naïve or non-sensitized mice, it is conceivable that acute and more chronic models for allergic inflammation may show diverging effects of GATA-3. Furthermore, *Gata3* overexpression may differentially affect Th2 cells and ILC2. In this context, a paradoxical finding was that enforced *Gata3* expression, which does not affect IFNγ production by circulating (29,31-32) or pulmonary T cells **(Figure 4A** and **Figure 4C)**, resulted in increased expression of IFNγ by pulmonary ILC2 **(Figure 4A** and **Figure 4C)**. Since it has been reported that Gata3-deficient NK cells produce less IFNγ (45), our observation may point to similarities between ILC2 and other innate lymphocytes and suggests that ILC2 cells show plasticity in cytokine expression. This plasticity is also reflected by the apparently variable capacity of ILC2 to produce IL-4 (2,3,11) **(Figure 4B** and **Figure 4C)**.

In summary, we have shown that enforced expression of *Gata3* is sufficient to enhance Th2 and ILC2 activity. As a result, due to joint efforts between Th2 cells and ILC2, production of the pro-inflammatory cytokines IL-4, IL-5 and IL-13 is enhanced and mice become remarkably susceptible to eosinophilic inflammation, even after mild exposure to inhaled harmless antigens that usually leads to inhalational tolerance.

Material and methods

Mice and genotyping

CD2-Gata3 Tg mice (29) were backcrossed on the C57BL/6 background for >10 generations and genotyped by PCR (5'- CAG CTC TGG ACT CTT CCC AC-3' and 5'- GTT CAC ACA CTC CCT GCC TT-3'). Animals were kept under specific pathogen free conditions, provided with water and food ad libitum and were used at the age of 6-11 weeks. All experiments were approved by the Erasmus MC Animal Ethics Committee.

OVA and House Dust Mite (HDM) allergic airway inflammation model

To induce airway inflammation, mice were anaesthetized with isofluorane and treated intratracheally (i.t) with 80µl ovalbumin (OVA, Worthington) solution (10 mg/mL in PBS, Life Technologies) or PBS as a control on day 0. Ten days later, all mice were exposed to OVA aerosols 10 mg/ml during 30 min for 3 consecutive days. In some experiments, house dust mite (HDM; Greer Laboratories) extract was used as an allergen: on day 0, isoflurane-anesthetized mice were sensitized i.t. using 10µg HDM extract in 80µL PBS or using PBS only. Ten days later, anesthetized mice were challenged with 10 µg HDM in 50µL PBS intranasally (i.n.) for 3 consecutive days. 24 hours after the last challenge, mice were sacrificed and BAL was performed by flushing the lungs 3 times with 1 ml PBS containing EDTA (Sigma-Aldrich). Lungs were collected and digested using collagenase I or IV (Life Technologies) containing DNase-I (Life Technologies) for FACS analysis and partly snap-frozen or inflated with PBS/OCT (TissueTek®, Sakura Finetek Europe, Zoeterwoude, the Netherlands), snap-frozen in

liquid nitrogen and stored at -80°C until further processing for histological analysis.

In some experiments, DCs were transferred i.t. to induce asthma. In brief, myeloid DCs were grown for 8 days from C57BL/6 BM in the presence of recombinant GM-CSF 20ng/ml (PeproTech) and subsequently pulsed with OVA or PBS. 10^6 DCs were injected i.t. and subsequently challenged 3 times using OVA aerosols as described above.

Flow cytometric analysis

Brochoalveolar lavage (BAL), lung and mediastinal lymph node cells were collected for cellular differentiation by flow cytometry as previously described (46).

In some experiments, a fraction of the isolated cells were stimulated with ionomycin (Sigma), phorbol-12-myristate-13-acetate (PMA, Sigma) and GolgiPlug (BD Biosciences) at 37°C for 4 hours. Next, cells were stained for CD3, CD4 and intracellularly for IL-4, IL-5, IL-10, IL-13, IL-17 and IFNγ, after fixation with 2% PFA and permeabilisation using a saponin-containing buffer. Fixable Aqua Dead for 405 nm (Invitrogen, Molecular Probes) was used to distinguish live and dead cells. Cells were analyzed using a LSRII Flowcytometer (BD Biosciences) and analyzed with FlowJo software (Tree Star Inc, USA).

Immunohistochemistry

Immunohistochemical stainings were performed in a half-automatic stainer (Sequenza) as previously described (47). Acetone-fixed slides were washed with PBS and incubated and blocked in diluted normal goat serum (CLB, Amsterdam, the Netherlands). Sections were stained with PE-conjugated-anti-SiglecF (BD Pharmingen, E50-2440). The primary antibody was detected using an appropriate alkaline phosphatase-conjugated secondary antibody. After rinsing, slides were incubated with New Fuchsin substrate. Finally, the sections were counterstained with Gills triple strength haematoxylin and mounted in Vecta Mount (Vector).

Cytokine level determination

Cytokine levels in lung homogenates and BAL fluid were determined using commercial ELISA kit for IFNγ (BD), IL-4 and IL-5 (both eBiosciences), IL-13, IL-17 and IL-33 (all R&D) according to manufactures' protocol.

Statistical analysis

Reported values are shown as mean ± SEM. Statistical analyses were performed with SPSS (SPSS Inc., Chicago, IL) using a Mann-Whitney U-test. Resulting p values less than 0.05 (*), 0.01 (**) and 0.001 (***) are indicated and considered significant. Tests that did not reach significance (p>0.05) are not indicated.

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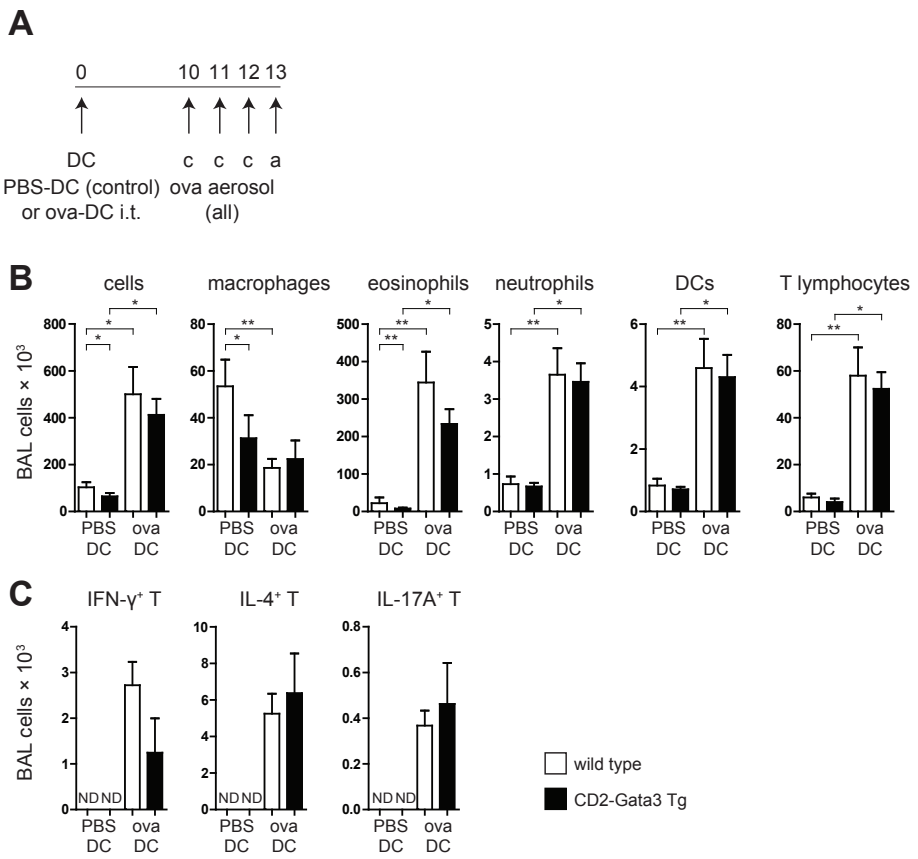
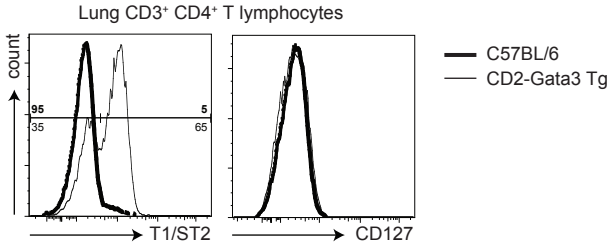
Supplemental information





Supplementary figure 2. Increased expression of IL-33R/T1ST2 on CD2-Gata3 Tg CD4+ T cells.
Flow cytometyric analysis of surface expression of IL-33R/T1ST2 and IL-7R/CD127 on collagenase-digested lung $CD3^+CD4^+$ T cells from *CD2-Gata3* Tg mice (thin black line) and WT (C57BL/6) mice (bold line), shown as histogram overlays.

Supplementary Figure 1 (*left page*)**. Enforced Gata3 expression does not enhance symptoms in a severe asthma model**
A) Mice were intratracheally (i.t.) sensitized with PBS or OVA-pulsed dendritic cells (ova-DC). One day after the last OVA aerosol challenge (c), allergic airway inflammation was investigated in wild-type (WT) (white bars) and *CD2-Gata3* Tg (black bars) mice. **B)** Quantification of flow cytometric analysis of the indicated populations of leukocytes in bronchoalveolar lavage (BAL) of WT (white bars) and *CD2-Gata3* Tg mice (gray bars). The results shown are expressed as means ± SEM. *p<0.05, **p<0.01. **C)** Quantification of flow cytometric analysis of T-cells positive for the indicated cytokines in bronchoalveolar (BAL) fluid upon 4hrs of PMA/ionomycin stimulation. The results shown are expressed as means ± SEM. The results shown represent one out of two independent experiments with 3-6 animals per group.

Chapter VI

Discussion

In this thesis, we addressed the role of transcription factor GATA-3 in the development and function of T cells and the recently identified group 2 innate lymphoid cells (ILC2). We characterized the role of GATA-3 in T cell commitment and demonstrated that GATA-3 acts in concert with Notch signaling to suppress the B cell potential in developing thymocytes (**Chapter II**). Using in vivo and in vitro approaches, we found that GATA-3 is a crucial factor for development of ILC2 (**Chapter III**) that, next to Th2 cells, play an important role in mouse models of asthma (**Chapter IV**). We demonstrated that in Th2 cells and in ILC2, GATA-3 levels regulate cytokine production and thereby influence airway inflammation (**Chapter V**).

In the first part of this chapter, we will (i) summarize the various murine models of allergic airway disease in which ILC2 were studied, (ii) review findings of human ILC2 in airway disease and (iii) discuss the implications of the discovery of ILC2 for our understanding of the pathogenesis of asthma and asthma exacerbations.

In the second part of this chapter, we will focus on the transcription factor GATA-3 and discuss how GATA-3 fulfills stage-specific functions in T cell and ILC development.

Lastly, we will consider how the findings described in this thesis may contribute to the development of novel therapeutic strategies for asthma and allergic disorders.

ILC2 in murine allergic airway inflammation models

Our understanding of the pathogenesis of allergic asthma has greatly advanced thanks to the development of various animal models for asthma. The initially studied models included mice that were sensitized to protein antigens, such as ovalbumin (OVA). Later, models based on more physiological antigens such as house dust mite (HDM) have been developed. While our studies described in **Chapter III** were ongoing, other groups also found that ILC2 are important players in various models of allergic asthma. The various models in which ILC2s have been studied are summarized in **Table 1**.

Cytokine-based murine lung inflammation models

ILC2 are commonly identified as lineage-negative cells that, among other markers, express the receptors for the cytokines IL-7 (IL-7Rα and the common γ chain), IL-25 (IL-17RA and IL-17RB) and IL-33 (T1/ST2 and IL1RAcP) (reviewed by Spits et al.; (1,2)). While the absence of ILC2 from *Il7ra*$^{-/-}$ and the strong reduction of ILC2 in *Il7*$^{-/-}$ mice indicates that IL-7 probably functions in development and homeostasis of ILC2 (3–5), IL-25 and IL-33 were shown to induce proliferation and production of the effector cytokines IL-5 and IL13 (5–10).

We and other groups have found that local (intra-nasal) or systemic administration of IL-25 or IL-33 to mice induced the expansion of ILC2 in lungs, BAL fluid and mediastinal lymph nodes (5–10). While both IL-25 and IL-33 can induce proliferation of and cytokine expression by ILC2, IL-33 induces 5-fold more ILC2 than a similar dose of IL-25 (10). This response is independent of B and T lymphocytes, as similar levels of airway inflammation, eosinophil recruitment and cytokine production were observed in the absence of B and T cells (*Rag*$^{-/-}$ mice) as compared with wild type mice (5,6,8,9). In addition, experiments in which wild type ILC2 were transferred to *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ double deficient animals show that ILC2 are sufficient to induce airway inflammation in response to IL-33 (11).

Importantly, we found that IL-25 and IL-33 increase the fraction of cytokine-producing ILC2 to up to 82% and 94% IL-5+ ILC2 respectively, while in PBS control animals only 28% of ILC2 produced IL-5 and IL-13 (**Chapter III**). Transfer experiments illustrate that ILC2-derived IL-13 is required for AHR induction in cytokine-induced airway inflammation models, as transfer of wild type but not *Il13*$^{-/-}$ ILC2 to *Il13*-deficient mice could restore IL-25-induced AHR (9,10).

Ovalbumin-induced asthma

Ovalbumin-induced airway inflammation is a long-used model for studying allergic inflammation and is thought to mimic important aspects of human allergic asthma. Barlow et al. (10) observed that the number of ILC2 increases to 2000 or 4000 cells in BAL fluid in a 12- or 25-day ovalbumin treatment regimen respectively. The lung ILC2 population increased 2- to 4-fold in the two models respectively. Ovalbumin asthma models in IL-4- and

Table 1. ILC2 in pulmonary inflammation models

Mouse		
Allergen-based models		
Allergen (type)	**Mechanism**	**Ref.**
Papain (protease)	ILC2-dependent eosinophilia and AHR. IL-2-dependent IL-9 production drives IL-5 and IL-13 production.	(6,7)
Alternaria alternata (fungus)	ILC2 required in initial, innate phase of inflammation.	(5)
House dust mite	ILC2 required for inflammation in acute response. During challenges, similar numbers of IL-5$^+$/IL-13$^+$ ILC2 and Th2	(6,8)
Sphingomonas spp. (Gram-negative bact.)	NKT cells activated by Cd1d-ligands from Sphingomonas spp. directly induce AHR via IL-13 and ILC2/Mϕactivation via IL-33.	(9)
Ovalbumin	Similar contribution of ILC2 and Th2 to IL-5 and IL-13 production. Limited IL-4 production by ILC.	(8,10)
Influenza virus infection models		
Influenza strain	**Mechanism**	**Ref.**
Influenza PR/8 (H1N1)	ILC2 crucial for tissue repair by production of EGF-family member amphiregulin	(32)
Influenza H3N1	IL-13 from ILC2 induces AHR, irrespective of adaptive immunity	(4)
Other pulmonary inflammation models		
Model	**Mechanism**	**Ref.**
Cytokine-based IL-25/33	Strong expansion of ILC2 and induction of cytokine production, mainly IL-5 and IL-13, but also IL-4.	(5–10)
Nippostrongylus brasiliensis (helminth)	After N. brasiliensis infection, pulmonary ILC2 (and CD4+ T cells) produce IL-13 in a GATA-3-dependent fashion	(106)
Strongyloides venezuelensis (helminth)	Pulmonary S. venezuelensis stimulates alveolar epithelial cells to produce IL-33. ILC2 accumulate and produce IL-5 and IL-13 in response to IL-33, and can expel the worms.	(153)
HUMAN		
Model	**Mechanism**	**Ref.**
n/a	Mechanism unknown; ILC2 are found in both fetal and adult lung and intestine and in peripheral blood. ILC2 are increased locally in chronic rhinitis patients.	(31)
n/a	Mechanism unknown; ILC2 are found in lung and BAL fluid of lung transplantation patients.	(32)

IL-13-reporter mice showed that a substantial proportion (44% or 75% after 12 or 25 days, respectively) of IL-13-producing cells were ILC2, while most IL-4-producing cells were lineage-positive cells.

In **Chapter III**, we describe similar findings in an ovalbumin-based airway inflammation model. Analysis of the IL-5$^+$ and IL-13$^+$ populations in the lung showed a similar number-wise contribution of ILC2 and Th2. In BAL fluid, ILC2 were found to be the most abundant IL-5 producers. Overall, ILC2 produced very little IL-4 in this model.

House dust mite (HDM) asthma

Although house dust mite (HDM)-based asthma models classically involve a sensitization and a challenge phase, HDM also induces an acute inflammatory reaction. Halim et al. (6) administrated 3 daily doses of 100µg HDM to WT, *Rag1*$^{-/-}$ and *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice. Analysis the next day showed increased mucus production and a mild increase in BAL eosinophils (5000 cells) only when ILC2 were present (WT and *Rag1*$^{-/-}$ mice). This effect could be abolished by depletion of ILC2 using an anti-CD25 antibody in *Rag1*$^{-/-}$ mice. This data suggests that ILC2 may play a role in the early, innate response to HDM, providing an early source of Th2 cytokines, although it remains unclear what cytokines were produced in this model.

In **Chapter III**, we compared the contribution of ILC2 and Th2 cells in a classic HDM-model in which mice were sensitized and then challenged 1, 3 or 10 times with HDM. We found that the number of ILC2 increased with increasing numbers of challenges in both lung and BAL. ILC2 importantly contributed to the production of IL-5 and IL-13 in lung and BAL, but did not significantly contribute to the production of IL-4. Together with our finding that IL-5 transcription levels per cell are very high in ILC2, compared to Th2 cells, we conclude that ILC2, next to Th2 cells, are important cytokine producers in HDM- and ovalbumin-induced asthma.

Fungal allergens: Alternaria alternata

Fungal allergens, especially Alternaria alternata, have been reported both clinically and epidemiologically to be associated with asthma (12,13). In a mouse model in which mice were exposed to A. alternata, BALB/c and *Rag1*$^{-/-}$ mice responded with classical allergic lung inflammation to a similar level in the early phase (5). In BALB/c mice only, airway eosinophilia further increased at day 5. This indicates that that the adaptive immune system is involved at later time points to further amplify the initial innate response. Interestingly, using adoptive transfer experiments in which ILC2 were transferred to *Il7r*$^{-/-}$ mice that lack ILC2, it was shown that A. alternata-mediated airway inflammation is mediated mainly by ILC2, although a contribution of other Il7r-dependent populations cannot be excluded (5).

Glycolipid-mediated airway inflammation

Glycolipid antigens that can stimulate NKT cells have been found in pollen (14), house

dust (15) and bacteria (16,17). Administration of the NKT ligand α-GalCer to wild type mice rapidly induces AHR and airway inflammation. This response relied on IL-33 produced by alveolar macrophages, DCs and type II pneumocytes and signals via the IL-33-ST2 pathway, as shown by using ST2-blocking antibodies and *St2*$^{-/-}$ mice (18,19). Interestingly, in vitro experiments show that α-GalCer-activated NKT cells, but not Th2 cells, stimulate macrophages to produce IL-33 that induce activation and expansion of ILC2 (9).

This study contributed to our understanding of the pathogenesis of asthma caused by atypical allergens, such as Gram-negative *Sphingomonas* bacteria that are widely distributed in nature. These bacteria are increasingly exploited in the food industry because of their unique ability to degrade toxins, secrete useful polysaccharides and serve as antagonists to phytopathogenic fungi (20). Some Sphingomonas species, however, can cause infections in humans. Furthermore, these bacteria are often found in poorly controlled asthma and have been associated with AHR (21). Glycolipid antigens from Sphingomonas are known to activate NKT cells that produce both IL-13 and IL-33. Kim et al. (9) showed that most of the IL-13 produced in Sphingomonas-challenged mice was derived from ILC2. These ILC2 relied on NKT-derived IL-33 for their activation and expansion. This effect was seen even in the absence of the adaptive immune system. Innate-like NKT cells thus serve a double role in the pathogenesis of asthma. NKT cells directly contribute to AHR by producing IL-13, but via IL-33 also activate other cells, such as ILC2 **(Figure 1)**. It remains to be shown whether NKT-cell derived IL-33 is also important for the function and expansion of other cells, such as IL-33R^{+} Th2 cells.

Papain-induced airway inflammation

The protease papain is a known cause of occupational asthma (22) and was reported to cause asthma symptoms in *Rag*-deficient mice (18). Halim et al. (6) demonstrate that papain-induced airway inflammation, characterized by eosinophilia and increased levels, IL-5 and IL-13 in BAL fluid and mucus production can be mediated by ILC2. Depletion of ILC2 from *Rag1*$^{-/-}$ mice using an anti-CD25 antibody abolishes the papain-induced response, and transfer of ILC2 to *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice restores the inflammatory response to papain administration.

Wilhelm et al. (7) employed an IL-9-reporter mouse (*Il9*^{Cre} x *Rosa26*$^{flox\text{-}eYFP}$) in a papain-induced model of airway inflammation to show that IL-9 is strongly induced upon administration of the protease. Interestingly, most IL-9 derives from lineage-negative cells that express Thy1 and T1/ST2 and varying levels of Sca-1, MHCII and CD25, but no IL-7Rα. In these experiments, all IL-9^{+} Lin^{-} cells co-expressed IL-13 and most expressed IL-5, but not IL-4. Kinetic experiments show that IL-9 production by ILC2 was rapidly lost (within 24 hours), while the cells continue to express IL-5 and IL-13. The authors went on to show that the observed IL-9 production depends on IL-2 derived from adaptive immune cells. Whether IL-9 production is a common trait of ILC2, or whether expansion of this popula-

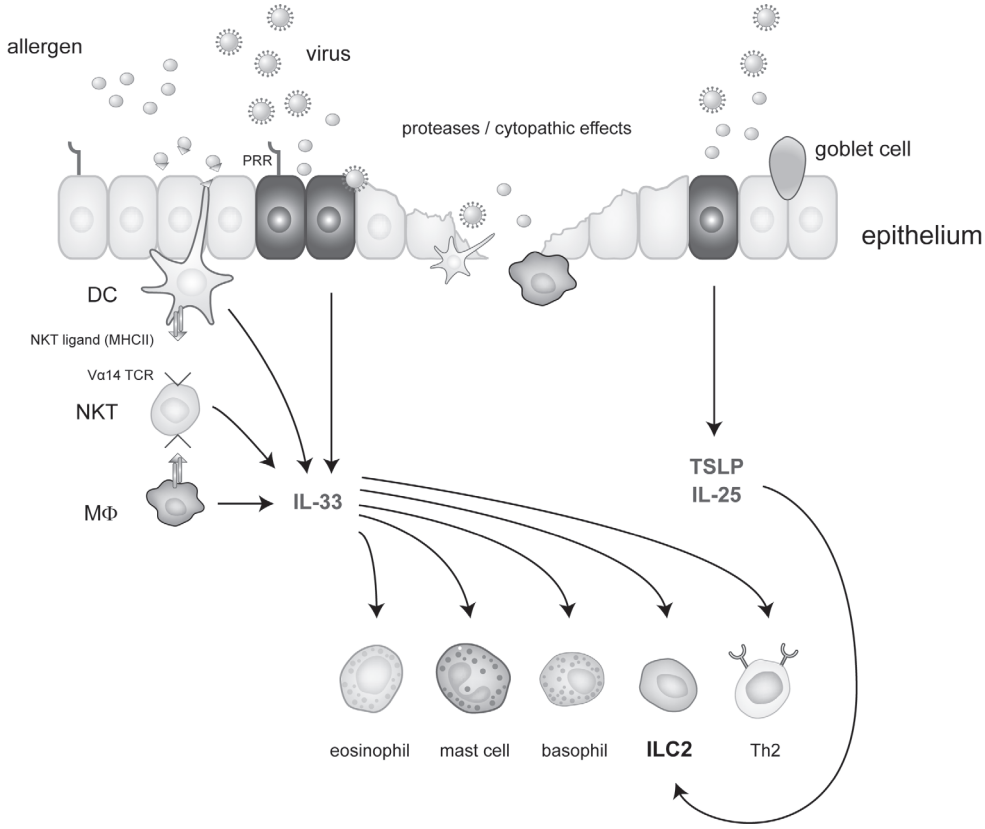


Figure 1. Allergen- and virus-induced expression of IL-25, IL-33 and TSLP. *(full color version: page 236)*
When allergens or influenza virus particles enter the airway, they activate airway epithelial cells via pattern recognition receptors (PRR), including Toll-like Receptors (TLR). Activated epithelial cells (in red) produce TSLP, IL-25 and IL-33 that activate a wide variety of immune cells. In addition, dendritic cells (DC) constantly sample the airway lumen and may directly produce IL-33. Furthermore, many allergens contain NKT ligands that are also sampled by DCs and then presented in the context of a MHC class II molecule to NKT cells, which will also produce IL-33. ILC2 can thus be activated via multiple pathways in response to allergen or influenza virus exposure.

tion is confined to papain-based models of allergic airway inflammation remains to be shown.

Next to its function as a growth factor for mast cells, IL-9 was also shown to induce the production of IL-22 (23). This IL-10 family member is normally produced in lung tissue of healthy individuals, but is found at lower levels in patients with sarcoidosis and acute respiratory distress syndrome (ARDS) (24). IL-22 mainly acts on epithelial cells and leads to expression of host defense genes, such as the anti-microbial peptides Reg3β and Reg3γ in the intestine. Recent studies indicated that RORγt$^+$ ILC3 are the main producers of IL-22 in this organ system [(25–29) and discussed further on]. In an ovalbumin asthma model, Taube and colleagues (30) found that IL-22 increased upon allergen exposure and was mainly produced by Lin$^-$ RORγt$^+$ CD25$^+$ CD44$^+$ ILC, thus resembling intestinal IL-22$^+$

RORγt$^+$ ILC3. Expression of the IL-22-specific IL-22R1 chain was only found on the immortalized murine clara cell line C22 and not on any hematopoietic cell. Administration of recombinant IL-22 to ovalbumin-exposed mice reduces AHR, eosinophilia and IL-13 levels, while *Il22*$^{-/-}$ mice show an aggravated asthma phenotype, including higher levels of IL-33. ILC3 may thus dampen airway inflammation via the effects of IL-22 on epithelium, which reduces IL-33 secretion and subsequent activation of Th2 cells and ILC2 **(Figure 2)**. Indeed, IL-22 reduced the secretion of TNF-α and IL-13 by a murine Clara-cell line. The mechanisms by which ILC3 are activated in allergic airway inflammation remain to be elucidated. Furthermore, it is not known whether these cells play a role in human disease.

ILC2 in the human respiratory system

Studies of human lung tissue or BAL fluid from lung transplantation patients identified a population of lineage-negative lymphocytes that expresses CD25, CD127 and ST2, thus sharing important cytokine receptors with murine ILC2 (31,32). A report by Mjosberg et al. (31) shows that human ILC2 express CD161 (a NK cell marker) and CRTH2 (a chemoattractant, also expressed by Th2 cells; (33)). These cells were also detected in fetal tissue, including lung and intestine. Interestingly, human ILC2 can also be found at low frequencies in peripheral blood, suggesting that these cells recirculate. Similarly, Monticelli et al. identified Lin$^-$ populations in healthy lung parenchyma and BAL fluid from patients that underwent lung transplantation (32). Like murine ILC2, these cells co-express CD127, CD25 and ST2.

Mjösberg et al. also reported that ILC2 were increased locally in nasal polyps of chronic rhinitis patients compared to healthy controls. Transcript analysis revealed that IL5 and IL13 levels are increased in patients compared to healthy controls, suggesting that ILC2 are an important source of type 2 cytokines and therefore constitute a potential new therapeutic target in chronic rhinitis. Although chronic rhinitis belongs to the so-called "Th2 diseases", it remains to be shown whether ILC2 play a role in human asthma and whether these cells will be a target for novel therapeutic strategies.

The finding that ILC2 numbers are increased in chronic rhinitis patients may indicate that ILC2 are increased in other Th2-type diseases as well. Analysis of bronchial mucosal biopsies from patients with atopic asthma can be analyzed histologically (e.g. for CRTH2$^+$ ICOS$^+$ ST2$^+$ CD3$^-$ cells) or using gene expression profiling (e.g. GATA3, RORA, ST2 and PTGDR2 encoding CRTH2) for the presence of ILC2. Even though broncho-alveolar lavages are not regularly performed in asthmatic patients, flow cytometric analysis of BAL fluid may yield important information about the abundance and activation status of Th2 cells and ILC2 in patients. Meta-analyses of genome-wide association studies (GWAS; (34,35)) indicated several asthma susceptibility loci that are also linked to ILC2: *TSLP* and *IL33* (both activators of ILC2), *RORA* (essential transcription factor for ILC2 development in mouse; (36,37)), *IL2RB* (part of the IL-2R, expressed by ILC2), *IL13* (cytokine produced by

ILC2).

Next to its effect on goblet cells, IL-13 also induces epithelial hyperplasia. This effect of IL-13 is seen in response to lung infections, but has also been observed in pulmonary fibrosis (38–40). Additional patient studies and analyses of experimental animal models will reveal whether ILC2 also play a role in chronic obstructive pulmonary disorder (COPD), sarcoidosis, acute lung injury and bronchitis.

ILC2 in virus-induced asthma exacerbations

In most patients in the western world, adequate control of asthmatic disease can be achieved using glucocorticoid or other long-term control therapy complemented with quick-relief medication to treat acute symptoms of wheezing and shortness of breath. However, exposure to respiratory pathogens can trigger an asthma exacerbation, which is an important cause of morbidity and often requires hospitalization (41). Rhinoviruses, especially respiratory syncytial virus (RSV), are the most common pathogens found in asthma exacerbations, but influenza virus and the atypical bacterial pathogen *M. pneumoniae* are often found in asthma exacerbations (42–44).

In healthy individuals, infections with these pathogens are usually self-limiting, but in patients at risk for asthma and in patients with established disease, viral respiratory tract infections can have a profound effect on the expression of disease (41). Most hospitalisations of asthmatic patients correspond with seasonal increases in the prevalence of rhinoviruses (45). Along the same lines, rhinovirus-induced episodes of wheezing early in life are an important risk factor for development of asthma in later life (41).

Interestingly, infection in mice with Sendai virus, a murine parainfluenza-virus similar to RSV, was shown to induce chronic inflammation in the lung while the infection was cleared to trace levels of non-infectious virus (46). This type of disease arose independently of an adaptive response, but rather was driven by IL-13 produced by macrophages that were stimulated by NKT cells. Interestingly, Umetsu and colleagues showed that NKT cells can drive activation of ILC2 that, together with NKT cells, induce IL-13-driven AHR (9). Therefore, NKT cells may, in addition to the mechanisms described in their study (46), have an additional role to active ILC2 by secreting IL-33 **(Figure 1)**.

ILC2 are potent cytokine producers and were found to increase in mouse models of asthma and in patients with allergic rhinitis. Therefore, it is interesting to determine the function of ILC2 in pulmonary virus infection. Next to rhinoviruses, influenza viruses are another common cause of virus-induced asthma exacerbations. Several groups have already shown that influenza virus infection induces IL-33 production in the lung (32,47). Two groups have specifically asked what role ILC2 play in influenza virus infections in mice. Chang et al. (4) used influenza A virus subtype H3N1 to show that H3N1 influenza induces IL-13 mediated AHR and airway inflammation, irrespective of the presence of Th2 cells or other cells of the adaptive immune system. Instead, the ILC2 population expands

5-fold in response to IL-33 production by alveolar macrophages and was responsible for most of the IL-13 produced. While *Il13*$^{-/-}$ animals or ILC2-depleted animals are protected from AHR, adoptive transfer of Il13-competent ILC2 to *Il13*$^{-/-}$ hosts restores the AHR phenotype, indicating that ILC2 are responsible for the production of IL-13 that induces AHR in response to H3N1 influenza virus infection.

Importantly, in influenza virus infection with the H1N1 PR/8 strain (32), ILC2 also increase. In this study, however, ILC2 were shown to fulfill a protective role, as depletion of ILC2 impaired lung function and tissue repair, leading to decreased body temperature, decreased oxygen saturation levels in blood and decreased integrity of lung epithelium. This protective function is independent of IL-13 and IL-22, but instead relies on the production of amphiregulin. ILC2-derived production of this epidermal growth factor family member, that has been linked to tissue remodeling and repair in various diseases of acute epithelial damage and asthma (39,40,48,49), was further enhanced by IL-33 stimulation. The seeming contradiction in the detrimental role for ILC2 observed in the first report of influenza virus infection (4) and the beneficial role described in the second study (32), may be caused by the properties of the virus proper. Even though Monticelli et al. (32) do not report whether H1N1 virus infection induces AHR, H1N1 virus infection has not been reported to cause AHR (50), while this was demonstrated for H3N1 infection (4).

Important parts of the abovementioned studies were carried out in *Rag*-deficient animals, thus eliminating B cells. Class-switched memory B lymphocytes that can quickly produce large amounts of pathogen-specific immunoglobulins (Ig) are crucial for the generation of immunologic memory. Upon secondary infection, these memory B cells quickly expand and differentiate to help control the infection, even before clinical symptoms become apparent. As this process takes several days, these so-called B2 lymphocytes are not important in the first days of influenza infection. In contrast, large quantities of B1 lymphocytes are found in human and mouse near mucosal surfaces, especially in the peritoneal and pleural cavity (reviewed by Baumgarth; (51)). B1 cells produce natural antibodies of the IgM isotype that respond to common pathogen-associated patterns. Experiments in mice show that B1 lymphocytes are the most important producers of IgM in influenza virus infection and are crucial for control of infection (52). IL-5 is an essential growth factor for B1 cells (53) and IL-6 functions as a general B cell growth factor (54,55). While most ILC2 reports focus on the production of IL-5, ILC2 also produce IL-6 (3). ILC2 may thus serve another, indirect protective role in influenza virus infection by the stimulation of B1 cells via production of IL-5 and IL-6 **(Figure 2)**.

The present reports on the role of ILC2 in influenza infection focused on early time points. We observed that the accumulation of ILC2 upon H3N2 (X31) influenza infection persisted even after clearance of the virus (18 days post infection; Klein Wolterink, Hendriks et al., unpublished results). Furthermore, gene expression profiling of total lung at 6 hours, 1, 2, 4 and 8 days after influenza infection showed induction of Il13, T1st2

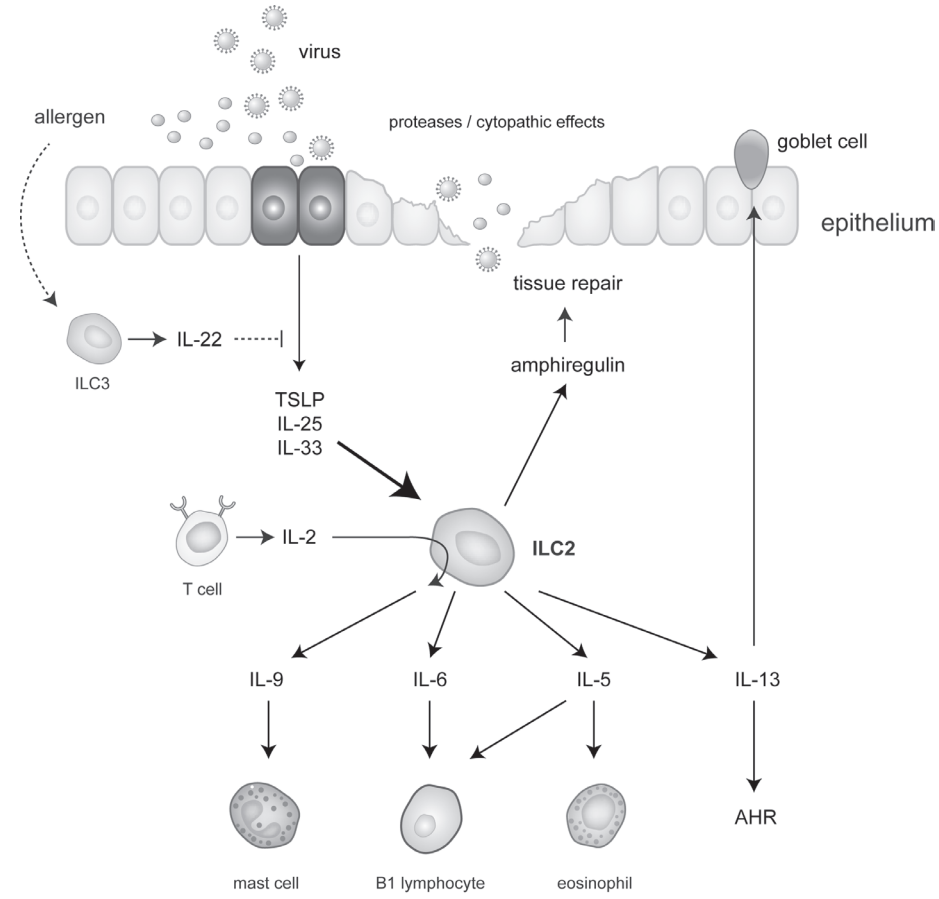


Figure 2. The multifaceted role of ILC2 in allergic airway inflammation. *(full color version: page 237)*
Upon allergen exposure or influenza virus infection, the epithelium and immune cells that guard the epithelial wall rapidly produce important amounts of IL-25, IL-33 and TSLP. These cytokines activate ILC2 that will expand and produce various cytokines. ILC2 contribute to an asthma phenotype by production of IL-5 and IL-13 that induce eosinophil recruitment and AHR, respectively. If ILC2 are co-stimulated with IL-2 derived from T cells, ILC can also transiently produce IL-9 that will activate mast cells. ILC2 may also serve protective roles by stimulating tissue repair via amphiregulin and through stimulation of B1 lymphocytes that produce virus-neutralizing antibodies. In ovalbumin-induced airway inflammation, lung ILC3 are stimulated to produce IL-22 which limits the production of IL-33.

(IL-33R) and amphiregulin in the lung (Geurts van Kessel, Hendriks et al.; unpublished data). While amphiregulin may have beneficial roles in tissue repair (39,40,48,49), it is also reported to upregulate mucin expression (56) and is found at increased levels in sputum of asthmatic children (57). Therefore, experiments in mice in which influenza virus infection is combined with house-dust mite or ovalbumin models of asthma could provide new insights in the role of ILC2 in asthma exacerbations. An interesting hypothesis to test is whether asthma induction and challenge followed by influenza infection leads to increased morbidity, measured as increased airway resistance, influx of eosinophils and impaired clearance of the virus.

Outstanding questions

- **Kinetics**: When are ILC2 important in asthma sensitization, challenge and exacerbation? How quickly do they respond and are there differences in their response (e.g. cytokine expression) at different time points? How does the number of ILC2 change over time?
- **Localization and trafficking**: What is the spatial distribution of ILC2 and what drives their migration? What chemokines attract ILC2 to the lung and BAL? Are there differences in chemokine receptor expression by ILC2 present at different locations (respiratory tract, intestine, bone marrow)?
- **Exacerbations**: Are ILC2 implicated in virus-induced and other asthma exacerbations? How long are ILC2 increased after pulmonary virus infection and what is the role of these cells?
- **Human disease**: What is the role of ILC2 in human allergic airway disease? Are ILC2 present (in increased numbers) in asthmatic patients and other type 2 conditions?

GATA-3 in development and function of T cells and ILCs

In **Chapter II** we showed that GATA-3 is an essential transcription factor in early T cell development. Using in vitro culture systems, we identified a previously unknown role for GATA-3 in suppression of a latent B cell potential that is revealed in the absence of Notch. In **Chapter III** we used a lacZ-knock-in mouse strain (58) to discover that GATA-3 is an essential transcription factor for development of ILC2. We failed to detect cells in vivo and in vitro that fit the phenotype of ILC2 cells or ILC2-like cells that lack markers under the control of GATA-3 (e.g. T1/ST2). Using chimeric mice bearing both *Gata3*-deficient and Gata3-competent wild type cells, we show that that ILC2 require GATA-3 for development in a cell-intrinsic manner. Interestingly, using mutant mice in which *Gata3* copy numbers were altered, we show that ILC2 development from CLPs correlated with GATA-3 levels.

As described in the Introduction (**Chapter I**), GATA-3 is a widely expressed transcription factor. It is involved at multiple stages of development and function of T cells and ILC2 **(Chapters II–V)**. Furthermore, GATA-3 is also involved in the development of thymic NK cells, a specific, IL-7-dependent subset of NK cells (**Chapter I**). Therefore, it is interesting to consider how GATA-3 is able to perform this variety of functions at different stages of development in specific cell types. Recent studies describing genome-wide analysis of GATA-3 binding sites importantly contribute to our understanding of the mechanisms of action of GATA-3 (59,60). In this part of the Discussion, we will (i) introduce RORγt+ ILC3

as the last members of the innate lymphoid cell family, (ii) consider the developmental requirements of the different ILC subsets and (iii) consider in more detail the stage-specific functions of GATA-3 in T cells and ILC2.

Group 3 RORγt+ innate lymphoid cells (ILC3) mediate generation of lymphoid tissue and mucosal homeostasis

ILC2 belong to the family of innate lymphocytes. For a long time, NK and LTi cells were thought to be the only members of this family, but recent research has shown that there are more lymphocytes that have properties of innate immune cells and that play an important role mainly in mucosal homeostasis. Next to ILC2 that mediate type-2 immunity, several populations of RORγt$^+$ ILC have been identified. This family of innate lymphoid cells include lymphoid tissue inducer (LTi) cells, that have been identified almost two decades ago (61,62), and more recently identified cells that produce important amounts of IL-17A and IL-22 **(Table 2)**. In this Discussion, we will comply with the recent suggestion to refer to this subset of innate lymphoid cells as group 3 ILC (ILC3) (63).

Lymphoid tissue inducer (LTi) cells

LTi cells are essential in human and mouse fetal life for the formation of secondary lymph nodes (64–66). LTi cells are phenotypically characterized by expression of the TF RORγt and IL-7Rα (CD127). In mice, about half of the LTi cells express CD4. The two populations (CD4$^-$ and CD4$^+$) are thought to be developmentally related, with the CD4$^+$ population representing the more mature cells (67). Next to their role in generation of lymph nodes in the fetus, LTi cells in adults are involved in the formation of isolated lymphoid follicles (ILF), T-cell independent IgA expression and tissue remodeling (64,68–70). Unexpectedly, adult LTi cells were found to produce IL-17A and IL-22 and play a role in mucosal immunity (29,64,71). In contrast to other ILCs that produce IL-17A and IL-22, LTi cells lack the expression of the NK cell receptor NKp46 (mouse) or NKp44 (human).

IL-22 producing ILCs (ILC22)

ILCs that are RORγt+ and produce IL-22 are phenotypically different from LTi cells, because they also express NK cell-associated receptors (NCR): NKp44 (human) or NKp46 (mouse) **(Table 2)**. These cells are found in relatively high numbers in the small intestine and to a lesser degree in the colon. Since several groups have identified these cells separately, a wide variety of names for these cells has been introduced in literature: NK22 (28), NCR22 (72), NKR-LTi (26), LTi-like cells (64) and ILC22 (1). Here, we will refer to these cells as IL-22$^+$ ILC3 (63).

The importance of IL-22$^+$ ILC3 is exemplified in a mouse model of *C. rodentium* infection. In the absence of IL-22$^+$ ILC3, this intestinal infection induces fatal colits and wasting

disease, while in the presence of IL-22+ ILC3 colitis is contained and mice recover normally, even in the absence of adaptive immunity (25,28,73). In this model, IL-22 acts mainly on epithelial cells in which it induces the secretion of anti-microbial peptides, such as Reg3β and Reg3γ. The production of IL-22 by ILC3 was shown to be mediated by IL-23 and IL-1β (68,74).

Next to IL-22, these cells have also been found to secrete IL-2 and CXCL8 (68,75). Strikingly, the production of the type-2 immunity-related factors IL-13, GM-CSF and BAFF has also been reported (68,75).

IL-17 producing ILCs and ILCs co-producing IL-17A and IL-22

ILCs that produce IL-17A, but not IL-22 have been described in the fetal mouse intestine (71) and during intestinal inflammation in mice (76) and humans (77). In contrast to ILC22, these ILCs lack the expression of NKp46 (76).

In addition to IL-17A-producing ILCs, a subset of RORγt-expressing ILC that produces IL-17A and IL-22 simultaneously has been described in the mouse spleen (29,78) and inflamed human tonsils (68). It remains to be shown if IL-17$^+$ and IL-22$^+$ ILC3 are developmentally related and/or if these cells are plastic and change their cytokine profile based on environmental clues.

Table 2. Phenotype and function of ILC3

Subset (other names)	Phenotype	Tissue distribution	Function	Ref.
LTi cells	CD127$^+$ CD117$^+$ NCR$^-$ LTα/LTβ+ *Mouse*: Thy1$^+$ 50% CD4$^+$	Fetal: LN anlagen (fetal) Adult: intestine, spleen, tonsil (human)	*Fetal*: lymphoid organ development *Adult*: ILF formation, mucosal immunity	(62, 64, 68–70, 154)
IL–22$^+$ ILC3 (NK22, NCR22, NKR–LTi, ILC22)	CD127$^+$ CD117$^+$ NCR$^+$ CD4$^-$	Intestine, Peyer's patches, tonsil (human)	Epithelial homeostatsis and immunity (IL–22–induced anti–microbial peptides), exacerbation of ileitis	(25–27, 72, 75, 77, 155–157)
IL–17A$^+$ (IFN–γ$^+$) ILC3 (ILC17)	CD127$^+$ *Mouse*: CD117$^-$ NCR$^-$ Thy1$^+$ CD4$^-$ NK1.1$^+$	Intestine, spleen (mouse), tonsil (human)	Yeast immunity, intestinal pathology (IBD), induction of β–defensins and anti–microbial peptides	(29, 76, 77)

Overview of the three types of ILC3 in mouse and human. Ref = references, LTi = lymphoid tissue inducer, NCR = Natural Cytotoxicitiy Receptor (i.e. NKp44 in human, NKp46 in mouse), LN = lymph node, ILF = isolated lymphoid follicle, IBD = inflammatory bowel disease.

Shared features of ILC development: dependency on Id2, γc and Notch signals

In this thesis, we describe that GATA-3 is an essential factor for development of ILC2 (**Chapter IV**). In order to place this finding in a broader context of transcriptional regulators required for ILC development, we will first summarize our current knowledge of prerequisites for ILC development that are shared between the different ILC subsets (ILC1, ILC2 and ILC3) **(Table 3)**.

Id2

Id proteins have been introduced in **Chapter I** as proteins that sequester E proteins, thus inhibiting B and T cell development and promoting the development of NK cells. *Id2*-deficient mice lack NK cells (79), but are also devoid of ILC2 (3) and ILC3 (72). Interestingly, mice deficient for both Id2 and E47 have normal development of NK and LTi cells (80), but the impact on other ILCs remains unknown. Overexpression of Id2 in lymphoid precursors favors development of NK and LTi cells and inhibits the development of pDCs, T and B cells (81–83). Together, these results suggest that ILCs, including NK cells, may share an *Id2*-dependent precursor, although it cannot be excluded that Id2 is required at differential stages in various ILC populations.

Using an *Id2*-reporter mouse strain, Carotta and colleagues recently identified two BM populations of Lin$^-$ CD127$^+$ CD135$^-$ Id2^{hi} that are efficient NK cell precursors in in vitro experiments using IL-7 and IL-15 (84). The two populations, called 'pre-pro-NK', differentially expressed CD117 (negative or intermediate) and Sca1 (high or low, respectively), but lacked markers of mature NK cells. Interestingly, the population of Lin$^-$ CD127$^+$ CD117^{interm} Sca1^{low} largely resembles CLP, except for the lack of expression of CD135 and the high expression levels of Id2 (CLP are negative for Id2). This population may thus constitute a NK-biased precursor derived from CLP, although a developmental relationship remains to be shown. The other precursor population that was identified (Lin$^-$ CD135$^-$ CD117$^-$ CD127$^+$ Sca1^{hi} Id2^{high}) resembles ILC2. In this report, the cells were not tested for expression of receptors of IL-25 or IL-33 or for cytokine production. The authors observed very efficient NK cell generation from both precursors (frequency: 1/2.4), although the latter precursor was less well studied. Furthermore, these precursors have not been tested in vivo for their developmental capacities and the possible ILC potency of these cells was not addressed.

Notch signaling and integrin α4β7 expression

T and NK cells

In Introduction of this thesis (**Chapter I**) and in **Chapter II**, we described that Notch signals are required for initiation of the T cell program in ETPs. In in vitro culture systems, T cell development can be induced by co-culture of CLPs or other hematopoietic stem cell

populations bearing T cell potential with OP9 stroma cells expressing Notch ligands. As discussed in **Chapter I**, both splenic and thymic NK cells develop normally in the absence of Notch (85,86).

ILC2

The OP9 culture system can also be used to induce development of ILC2 and RORγt+ ILCs. Wong et al. (36) first described that ILC2 can be cultured in vitro from CLP in the presence of IL-7 and IL-33 using OP9 cells that express Notch ligands (OP9Δ1). In line with these results, we also found that ILC2 development from CLPs was dependent on Notch signals (**Chapter III**). Interestingly, cells that were grown on OP9Δ1 stroma could be transferred to OP9 stroma after 3-6 days and still developed efficiently into cytokine-secreting ILC2, indicating that only the first stages of ILC2 development are Notch-dependent. Yet, the phenotype of an intermediate ILC2 precursor remains to be demonstrated.

ILC3

Several in vitro assays using OP9 stroma have been developed to study the development of ILC3 in vitro. A RORγt-GFP-reporter mouse strain (87) importantly enhanced this field of research and allowed the identification of a subpopulation of Lin^- $CD117^+$ $CD127^+$ CLPs that expresses RORγt. If analysis of RORγt is combined with expression analysis of integrin α4β7, several populations with differential developmental capabilities can be identified. Already in 2001, it was shown that $\alpha4\beta7^+$ fetal CLPs have lost B cell potential, but can still give rise to T, NK and LTi cells (88). Cells that are $ROR\gamma t^+$ $\alpha4\beta7^-$ constitute the precursor population for $IL\text{-}22^+$ ILC3 (89). Interestingly, this precursor population is not af-

Table 3. Effects of transcription factor deficiencies on ILC subsets

Deficiency	NK cells	ILC2	ILC3	References
Id2	Absent	Absent	Absent	(3,32,72,79)
Notch	No effect	Absent (only tested in vitro)	Reduced (no effect in fetus)	(36,85,90,91)
Gata3	Thymic NK absent cNK functionally impaired	Absent	No effect	(108,158,159), this thesis
Rora	NT	Reduced	No effect	(36,37)
Rorc	No effect	No effect	Absent	(3,25,27,105,154,157)
Tox	Strongly reduced	NT	Strongly reduced	(121)
Ahr	No effect	NT	Reduced (adult)	(92–94)

NT = not tested

fected by the absence of *Id2* (89), even though further development of IL-22$^+$ ILC3 is strictly *Id2*-dependent (72) **(Table 2)**.

The group of Eberl also characterized the developmental potential of fetal α4β7$^+$ RORγt- cells and found that these cells can give rise to bona fide NK cells (NK1.1$^+$ NKp46$^+$ RORγt$^-$) and α4β7$^+$ RORγt$^+$ cells in vitro (90). These α4β7$^+$ RORγt$^+$ cells resembled LTi cells, because they produced IL-17A and IL-22, but did not express NKp46. Only very few NKp46$^+$ RORγt$^+$ cells that produced IL-17A or IL-22 (ILC17/ILC22) were generated in these cultures.

Detailed analysis of the α4β7$^+$ RORγt$^-$ population showed termination of the B cell program (*Pax5*, *Ebf1*, *E2a*), while ILC-related genes were upregulated (*Id2*, *Tox*). Low levels of Rorc were also detected, but were 10- to 100-fold lower than in α4β7$^-$RORγt$^+$ cells. Interestingly, the authors also found an induction expression of *Il17rb*, which is related to ILC2 development. To further investigate the requirement for Notch for RORγt$^+$ ILC development, CLPs were first cultured on OP9Δ4 stroma (Notch$^+$) to induce α4β7$^+$ cells. In these cultures, almost all cells were RORγt$^-$. Subsequent culture of purified α4β7$^+$ RORγt$^+$ cells on OP9, thus removing Notch-signaling, efficiently induced differentiation into α4β7$^+$ RORγt$^+$ LTi cells. It remains to be determined if Notch signaling controls expression of *Id2* and *Rorc*.

The role of Notch in development ILC3 has also been addressed by Possot et al. (91). Their findings seemingly in contrast with the findings of Cherrier et al. (90), as in this study, development of RORγt$^+$ ILC from fetal CLPs was independent of Notch signals. In contrast, ILC development from BM CLPs strictly required Notch2 but not Notch1 signals. A possible explanation for these contradicting findings is that fetal CLPs were already primed by Notch signals when they were isolated and put in culture. Indeed, the authors could find *Hes1* and *Notch2* transcripts in fetal CLPs. Furthermore, Cherrier et al. also observed LTi cell development in the absence of Notch, but this process was very inefficient and generated few cells.

Other groups have investigated the interaction between Ahr and Notch signaling (92–94). AhR is a bHLH transcription factor that senses environmental ligands (e.g. the toxin dioxin), dietary phytochemicals (e.g. natural flavonoids) and several endogenous ligands (e.g. metabolites of tryptophan) (95–97). Several groups have demonstrated that *Ahr*-deficiency in mice results in significantly reduced numbers of ILC3 (both LTi cells and IL-22$^+$ ILC3) (92–94). Strikingly, this quantitative reduction was not seen during fetal development and early after birth, but only in adult mice (93). Importantly, only after weaning were the numbers of RORγt$^+$ ILCs reduced in AhR-deficient mice compared to wild type mice (92). Furthermore, cryptopatches and isolated lymphoid follicles (ILFs) were also absent in *Ahr*-deficient mice (92), probably due to the strong reduction in LTi cell numbers. In addition to the developmental defects, *Ahr*-deficient ILC3 failed to produce IL-22 (92–94). This is similar to the observation that *Ahr*-deficient Th17 cannot produce IL-22 (98,99).

Micro-array analysis comparing Ahr-deficient and wild type mice indicated that Notch1 is a possible target of AhR. Indeed, inducation of AhR-signaling using the toxin TCCD (2,3,7,8-tetrachlorodibenzo-p-dioxin) upregulated Notch1 and Notch2 levels in intestinal ILC3 (92). In a conditional knockout for *Rbpjk*, a critical mediator of Notch signaling, numbers of intestinal RORγt^{+} were reduced in a similar fashion as in *Ahr*-deficient animals. The number of LTi cells was also reduced, although less pronounced. However, the remaining LTi cells were sufficient to generate and conserve ILFs and cryptopatches. It is possible that the partial phenotype is caused by inefficient Notch-deletion mediated by *Vav*-induced Cre.

Taken together, there is emerging evidence that both ILC2 and ILC3 require Notch signals for their development. This feature is shared with T cell development, but not with NK cell development (ILC1), in which Notch signals neither promote, nor inhibit development.

ILC2-specific developmental requirements

Next to the abovementioned shared developmental conditions, ILC type-specific transcription factors have been identified. For ILC2, RORα and GATA-3 have been implicated as essential factors for development. Furthermore, signaling via IL-25 and IL-33 is also important for ILC2 development, although not absolutely required.

Transcription factor RORα

RORα is a transcription factor that is structurally related to RORγt, but has limited function in the immune system. Its main function lies in the development of Purkinje cells of the cerebellum (100) and cones of the retina (101). RORα-deficient 'staggerer' mice suffer from severe neurological defects and succumb soon after weaning. These mice are small, have smaller spleens and thymi and reduced numbers of lymphocytes (102). In the immune system, Th17 cells express high levels of *Rora* and *Rorc* (103,104). Adoptive transfer of *Rora*-deficiency only has a mild effect on Th17 cell differentiation, while the absence of RORγt has a much greater impact. However, Th17 cell differentiation was only completely blocked when both RORα and RORγt were absent (104).

Multiple groups have shown that *Rorc*-deficient mice have normal numbers of ILC2 (3,105). Two groups have investigated the role of RORα in nuocyte development and function and found that ILC2 express high levels of RORα, but not RORγt (36,37).

In the first report, wild type and *Rora*-deficient mice were challenged intra-peritoneally with IL-25. In wild type mice, this induced a rapid and siginifcant increase in the number of ILC2, while in the absence of RORα, the population of ILC2 failed to expand (36). Using BM chimeras, the investigators showed that RORα was required in a cell-instrinsic manner for IL-25-induced expansion of ILC2 and mediation of type-2 immunity (i.e. eosinophilia) (36). In in vitro cultures, ILC2 development from *Rora*$^{-/-}$ CLP was very inefficient, although

not completely abolished.

A similar observation was made somewhat later by the group of Takei (37). ILC2 were shown to express high levels of Rora but not Rorc. In Rora-deficient mice, the authors report a more than 10-fold reduction of ILC2s in lung and intestine, while RORγt$^+$ ILCs were not affected. Conversely, in *Rorc*$^{-/-}$ animals, ILC2 numbers are similar or increased compared to wild type, while RORγt$^+$ ILCs are absent. In vitro treatment of lung explants with IL-25 failed to induce efficient IL-5 production in the absence of RORα. After adoptive transfer of BM into *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice, most lymphocytes, including Th2 cells, developed normally, but ILC2 numbers were significantly reduced. Challenge of *Rora*-deficient mice with the allergen papain failed to induce eosinophilia and provoked less mucus production.

These two studies clearly demonstrate an important role for RORα in ILC2 development and function. Interestingly, both groups find that ILC2 are not completely absent in Rora-deficient mice and report that expansion of ILC2 in response to stimuli is particularly affected. One possible explanation is that the function of RORα can be compensated by the low-level expression of RORγt. This compensatory effect is probably less efficient than bone fide RORα signaling, and could explain a failure in expansion when high-efficiency signaling is required. Analysis of *Rora*-*Rorc*-double deficient animals could provide more insight in the role of RORα and possible compensatory mechanisms during development.

Transcription factor GATA-3

Induced deletion of *Gata3* shows a role for GATA-3 in ILC2 homeostasis and confirms GATA-3 requirement for ILC2 development

Next to our report describing GATA-3 as an essential factor for ILC2 development (**Chapter III**), three other groups have reported on the function of GATA-3 in ILC2. One report shows that GATA-3 is required for the expression of IL-13 following infection with the helminth N. brasiliensis (106). The second report investigated the function of GATA-3 in human ILC2 and found that GATA-3 controls expression of the cytokine IL-13 (but not IL-5) and the receptors for IL-33 (ST2) and TSLP ((107); discussed further on). In a third report, Diefenbach and colleagues (108) use an inducible knockout for *Gata3* to show that ILC2 development requires GATA-3, consistent with our findings.

In this report, Gata3 deletion was induced by the administration of tamoxifen that induces nuclear translocation of *Id2*-controlled Cre-ERT (108). These *Id2*$^{CreER/+}$ *Gata3*$^{fl/fl}$ mice were further crossed to a reporter mouse strain carrying Cre-inducible YFP under the *Rosa26* promotor (these mice were called Gata3$^{i\Delta ILC,T}$). This elegant approach allowed the researchers to compare cells that expressed Cre-ERT and thus deleted *Gata3* (YFP$^+$) with cells that had not deleted Gata3 (YFP$^-$). Consistent with previous reports (25,91), this study shows that RORγt$^+$ ILC also express *Gata3*, albeit at lower levels. In both RORγt$^+$ and RORγt$^-$ (ILC2) populations YFP$^+$ GATA-3 protein expression was abolished, while YFP-

cells continued to express GATA-3.

In order to study the effects of Gata3 deletion on homeostasis of ILCs, $Gata3^{i\Delta ILC,T}$ mice were treated for 3 weeks with tamoxifen. In the YFP^+ subset that deleted *Gata3*, almost no ILC2 could be observed, while $ROR\gamma t^+$ ILC were not affected. In the YFP^- subset, ILC2 were maintained at normal numbers. In the intestine, the entire pool of ILC2 was lost within a week, indicating that ILC2 homeostasis is severely affected in the absence of *Gata3*. This suggests that GATA-3 is required for maintenance of ILC2 and that development of ILC2 depends on GATA-3. The developmental requirement for GATA-3 was also directly tested in vitro. Culture of BM $Id2^{hi}$ $Sca1^+$ $CD25^+$ ILC2 precursors in which *Gata3* deletion was induced ($Gata3^{fl/fl}$ x $Id2^{CreER/+}$) showed hardly any development of mature ILC2 and no production of IL-5 or IL-13 was observed, while *Gata3*-competent cell gave rise to cytokine-producing ILC2 normally. Together, this study shows that GATA-3 is required for the maintenance of ILC2, but not of other ILC. In addition, it shows that GATA-3 expression is required for development of ILC2 from $Id2^+$ BM precursors.

GATA-3 in human ILC2

A recent report by the group of Spits showed that GATA-3 is highly expressed by human ILC2 (107). Strikingly, transcriptional analysis showed that GATA3 expression was not confined to ILC2, but was also expressed by conventional NK cells and IL-22 producing ILC3, although GATA3 levels were two-fold lower than in ILC2. Analysis of protein expression revealed that only a small subset of ILC2 expressed GATA3 (16% upon IL-2 stimulation), but most ILC2 upregulated GATA3 protein expression after TSLP stimulation (around 60%). This induction of GATA3 expression was not seen in NK cells, which lack the expression of TSLPR.

Overexpression of GATA-3 in Lin^- $CD127^+$ $CD117^+$ $NKp44^-$ $CRTH2^-$ precursors induced the expression of ST2 and TSLPR and these cells rapidly produced high amounts of IL-4, IL-5, IL-13 and GM-CSF upon TSLP or IL-33 stimulation, suggesting that these cells had differentiated into ILC2. Likewise, a knockdown using shRNA targeting of *GATA3* reduced the expression of IL-13 (while no significant decrease of IL-4 or IL-5 was observed), but did not directly impact on the survival of ILC2. It remains to be determined whether GATA-3 has a role in long-term survival of human ILC2.

Cytokines IL-25 and IL-33

The cytokines IL-25 and IL-33 are important for the activation and expansion of ILC2s. Local or systemic administration of either cytokine potently and rapidly induces expression of the effector cytokines IL-5 and IL-13 and promotes expansion of the ILC population, with IL-33 being a more potent stimulus than IL-25 [(5–7,9,10) and **Chapter IV**]. In vitro experiments show that ILC2 development can be induced in CLPs by stimulation with IL-7 and IL-33 (36,109). We and others found that in vitro culture with IL-7 alone does not

induce perceptible ILC2 differentiation [(36) and **Chapter III**]. This might be the result of stochastic events in differentiation (i.e. B and T lymphocytes develop from CLPs at a much higher frequency than ILC2) or could be due to limitations of in vitro culture systems (e.g. lack of appropriate signals other than IL-25/IL-33 or overgrowth by other cells). The latter could indicate that ILC2 development requires signals via the IL-25/IL-33 pathway, but this is unlikely, since in the initial description of the nuocyte (ILC2), ILC2 are present (but reduced) in *Il1r1*$^{-/-}$ *Il17rb*$^{-/-}$ mice (110). Thus, IL-25 and IL-33 are critical cytokines for ILC2 expansion, but are not required for ILC2 development.

Developmental requirements for ILC3

Transcription factor RORγt

RORγt is the third member of the family or RAR (retinoid acid receptor)-related orphan receptors. Two isoforms of the Rorc have been identified: RORγ, which is highest expressed in muscle tissue (111) and the shorter isoform RORγt, first cloned from thymus and T cell lines (112). Several functions of RORγt in various lineages have been reported since. RORγt is essential in DP cells in the thymus for upregulation of the anti-apoptotic protein Bcl-xL (113). The observation that Rorc-deficient animals lack lymph nodes and Peyer's patches (113,114) led to the discovery of RORγt as a critical TF for LTi cell development (reviewed in (115)). Furthermore, as discussed in the Introduction of this thesis (**Chapter I**), RORγt is the key TF for development of Th17 cells (103).

The development of LTi cells and IL-17- and IL-22-producing ILC3 is critically dependent on RORγt. In Th17 cells, the expression of RORγt is induced by TGF-β, IL-6 and IL-21 (**Chapter I** and (116)). However, IL-1, IL-6 and IL-23 are not required for expression of RORγt in ILCs (26). In T cells, RORγt expression is induced by the TF Runx1 (117,118). Similarly, complexes of Runx1 and Cbfβ2 were shown to be essential for regulation of RORγt in fetal LTi cells (119). Furthermore, Notch signaling has been implicated in mediation of RORγt expression (91), but the exact mechanisms that regulate RORγt expression remain to be elucidated.

Transcription factor Tox

TOX, thymocyte selection-associated high mobility group (HMG)-box, is a transcription factor that, in the immune system, is involved in multiple stages of T cell development. One of the functions of TOX is the regulation of the transition of $CD4^+$ $CD8^+$ DP thymocytes to $CD4^+$ T cells. Therefore, $CD4^+$ T cells are absent in *Tox*$^{-/-}$ mice (120).

TOX expression has also been reported in NK and LTi cells and both cell types are significantly reduced in *Tox*-deficient animals (121). Because of the lack of LTi cells, *Tox*$^{-/-}$ mice have virtually no lymph nodes and reduced numbers of (smaller) Peyer's patches (121). TOX may thus be expressed by a common precursor for NK and LTi cells. Other intestinal

RORγt$^+$ ILCs were reported to be normal, but this was not functionally addressed (121).

Transcriptional analysis revealed that *Id2* expression in mature BM NK cells was lower in the absence of *Tox*. However, forced expression of an *Id2* transgene in precursors did not rescue development of NK and LTi cells. This could indicate that TOX does not directly regulate *Id2*, but other, more complex regulation mechanisms cannot be excluded. Furthermore, it remains to be shown whether TOX is involved for ILC2 development.

Identification of a common ILC precursor?

As alluded to earlier, several TF and signals appear essential for development of the particular ILC subsets. The requirement for some factors (Id2, Notch and γc cytokines) are shared between the different subsets, while others (e.g. RORγt, GATA-3 and Tox) are specific for one or another group. While Id2 has been proposed as a determinant factor for ILC development (distinguishes developing ILC from T and B lymphocyte **(Figure 3)**, it remains unclear how and when Id2 expression is induced and how further differentiation of CLP or other lymphoid progenitors into ILC1, ILC2 or ILC3 is achieved.

α4β7 and CXCR6 as markers for a common ILC precursor?

Possot and colleagues identified integrin α4β7 and CXCR6 as markers that define subsets of CLPs (91). Traditionally, CLPs are described as Lin$^-$ CD117^{int} Sca1^{int} CD127$^+$ cells that reside in the BM (adult) or fetal liver (122). The authors show that B cell potential is only present in the α4β7$^-$ subset and is extinguished in any α4β7-expressing population (91). Cells that have upregulated α4β7 can be further divided using CXCR6. T cell potential is present in α4β7$^+$ CXCR6$^+$ cells, but is extinguished in the CXCR6$^+$ subset that only maintains NK cell and RORγt$^+$ ILC potential. Both the CXCR6$^+$ and CXCR6$^-$ subset can give rise to RORγt$^+$ ILCs.

Interestingly, the subsequent acquisition of α4β7 and CXCR6 is accompanied by upregulation of *Gata3* and *Id2* levels, showing that even precursors for RORγt+ express *Gata3*. In this study, *Gata3* levels in mature ILC3 were not tested. Furthermore, the authors report that α4β7$^+$ CXCR6$^+$ very efficiently developed into RORγt$^+$ ILCs, but the ILC2 potential of these cells was not addressed. We obtained preliminary results using the CXCR6 reporter mouse strain and found that about 30% of BM ILC2 express CXCR6, whereas most mature ILC2 in lung express CXCR6 **(Figure 4**; Klein Wolterink, Satoh-Takayama and Di Santo, unpublished results). Future experiments should clarify whether differential expression of CXCR6 reflects different stages of development and whether these populations are developmentally related. Another possible explanation is that CXCR6 expression purely reflects migratory capacity and does not relate to development of this subset.

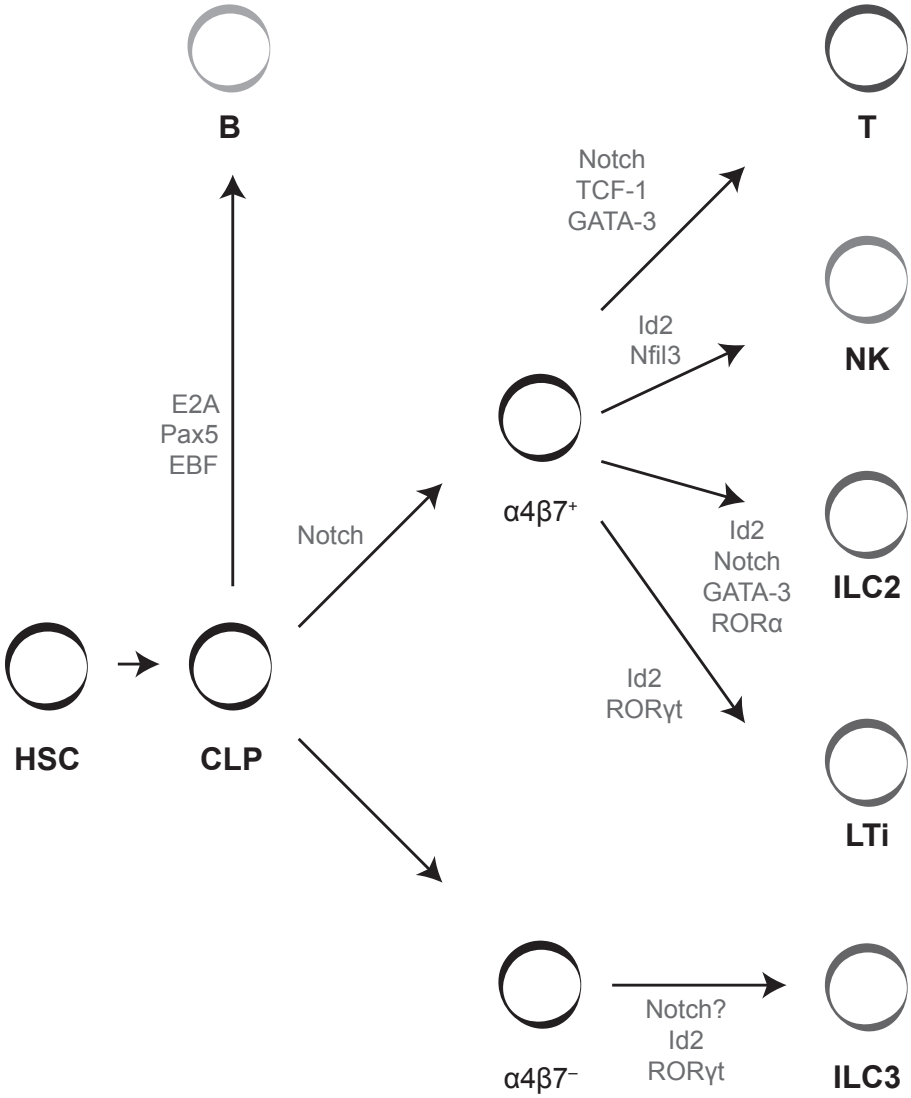


Figure 3. Schematic overview of ILC development from hematopoietic precursors. *(full color version: page 238)* Common lymphoid precursors (CLP) develop from hematopoietic stem cells (HSC) and can give rise to all lymphoid lineages. The B cell lineage depends on expression of EBF and Pax5 that drive the B lineage program and inhibit development of T, NK and LTi cells. CLP that receive Notch signals develop into $\alpha4\beta7^+$ progenitors that have potential to develop into T cells, NK cells, ILC2 and LTi. These lineages have both specific (e.g. TCF-1, RORγt) as well as shared (GATA-3, Id2) requirements for transcription factors. ILC3 develop from $\alpha4\beta7^-$ precursors that, like LTi cells, depend on Id2 and RORγt expression.

How does GATA-3 perform stage-specific functions?

In this thesis, we have described multiple roles for the transcription factor GATA-3 in lymphoid cell development and function. GATA-3 has long been known for its multiple roles in T cell development. We demonstrated that GATA-3 is an essential factor in early T

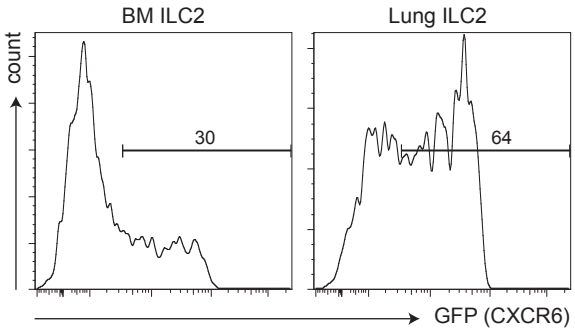


Figure 4. CXCR6 expression by BM and lung ILC2
Lin$^-$ CD127^{hi} Sca1$^+$ CD25$^+$ T1/ST2$^+$ ILC2 from BM (left panel) and lung (right panel) of CXCR6$^{GFP/+}$ mice were analyzed for GFP (CXCR6) expression. Numbers show GFP-positive fractions.

cell development (**Chapter II**) and summarized the role of GATA-3 in later stages of T cell development: β-selection, CD4 specification and Th2 cell differentiation. Furthermore, we found that GATA-3 is an essential factor for development of ILC2 (**Chapter III**). Using Id2-induced deletion of *Gata3*, similar results were obtained (108), suggesting that GATA-3 may be required at multiple stages of ILC2 development. Lastly, we described the importance of GATA-3 levels for the expression of type 2 cytokines by Th2 cells and ILC2 in a mouse model of allergic asthma (**Chapter V**). One of the major outstanding questions is how GATA-3 performs these functions in different cell types at different stages of development.

GATA-3 targets: insights from genome-wide analyses of binding sites

Despite the numerous reports on the functions of GATA-3, a limited number of targets has been identified. Most studies investigated GATA-3 binding in Th2 cells (116,123–127), but two recent genome-wide analyses of GATA-3 binding in distinct T cell types (59) and early thymocytes (60) provide unique insight in the operational mechanisms of GATA-3.

The group of Zhao investigated GATA-3 binding sites in distinct T cell subsets, and the various Th subsets in particular (59). Using stringent criteria for GATA-3 binding, 7187 binding sites were identified in Th2 cells, while Th1 and Th17 cells contained 987 and 266 GATA-3 binding sites respectively. Together with results from other T cell subsets (immature thymocytes, CD4 and CD8 T cells, Tregs, NKT cells), this markedly cell-specific number of binding sites correlated with the overall expression level of GATA-3. Combined analysis of GATA-3 binding sites, general analysis of expression levels and detailed analysis of activating and repressing histone modifications shows that GATA-3 can act as a repressor (e.g. for *Rorc*, *Foxp3* and E2A-encoding *Tcf3*) and an activator (e.g. for *Il5*, *Il13* and T1/ST2-encoding *Il1rl1*). In the Th2 locus, significantly less activating H3K4me2 marks were observed in the absence of GATA-3, while most H3K27me3 repressing marks

were lost in the *Tbx21* (T-bet) and *Ifng* loci. Deletion of GATA-3 from Th2 cells changed the expression of 2039 genes, of which 45% correlated with changes in H3K4me2 (activating; 11%) and H3K27me3 (repressive; 34%) modifications. Interestingly, only 16% of all genes that were bound by GATA-3 and showed changes in histone marks after GATA-3 deletion significantly changed expression levels. Together, these results show that GATA-3 binds in a highly cell-specific manner and controls many genes at the same time. GATA-3 binding correlates with both repression and activation, but does not change gene expression in a large number of bound genes. GATA-3 thus acts in a more subtle way than simply as an on/off-switch.

The Rothenberg laboratory performed a genome-wide analysis of GATA-3 binding sites, focusing on T cell commitment, thereby analyzing various DN subsets in detail (60). In accordance with previous results from our laboratory (58), this group reports that GATA-3 levels change importantly during development (128), although not as dramatically as other factors, such as Bcl11b and Tcf7. These results are confirmed in their present study using RNA sequencing (60). In developing thymocytes, about 1500 loci showed GATA-3 binding, but the distribution of GATA-3 occupancy was considerably different between the earliest precursors (DN1), committed precursors (DN2b) and DP thymocytes **(Table 4)**. There is a strong correlation between GATA-3 binding in DN1 and DN2b cells (r=0.61), but this correlation is poor between DN2b and DP cells (r=0.22) and completely absent between DN1 and DP (r=-0.0064). The changes in GATA-3 binding throughout development show a high correlation with changes in gene expression and activating and repressing histone modifications at those sites, e.g. *Tcf7*, *Zbtb7b* and *Rag1*/*Rag2*. Interestingly, GATA-3 binding is observed at sites that are not yet transcribed, such as *Cd3d* and *Rag*, suggesting that GATA-3 may serve as a pioneer to prepare the locus. Sites that are silenced with H3K27me3 marks and show no mRNA expression, e.g. *Zbtb7b*, also remained bound by GATA-3.

GATA-3 targets and GATA-3 regulation in DN thymocytes

The role of GATA-3 in T cell development is complex, because it does not always regulate the same targets (59,60). GATA-3 levels seem to correlate with the number of binding sites, suggesting that GATA-3 concentration is a crucial determinant of GATA-3 function (59). For instance, Th2 cells express high levels of GATA-3 and 7,187 GATA-3 peaks were identified, while GATA-3 levels were low in Th17 cells in which only 266 peaks were identified (59). Still, there are other factors that influence GATA-3 binding, as subsets with similar GATA-3 levels show distinct binding patterns [(60) and **Table 4**]. In DN1 cells, GATA-3 binding is mostly restricted to stemness genes, while in DP cells GATA-3 binding is mostly found in T cell identity genes **(Table 4)**.

It has been challenging to untangle the mechanisms that regulate expression of GATA-3 in T cell development. Two GATA-3 promotors have been identified: a proximal one that

Table 4. GATA-3 binding sites in thymocytes
Selection of loci that encode genes expressed by developing T cells and ILC that were identified as GATA-3 binding sites in a recent genome-wide analysis of binding sites (60) (left table). DN1 and DN2b cells are derived from FL cells expanded on OP9Δ4 stroma cells. DP cells were directly sorted from freshly isolated thymocytes. Values represent reads per million (RPM) and are sorted from highest RPM to lowest for DN2b cells. Binding sites are considered positive >2 RPM. Strike-through values do not pass this threshold. Right table shows loci for which no GATA-3 binding was detected.

GATA-3 binding sites				
Gene	**DN1**	**DN2b**	**DP**	**Other name**
Zfpm1	30.17	19.73	26.23	FOG-1
Tcfe2a	14.26	18.87	25.63	E2A
Rora	8.3	14.50	38.15	
Il9r	8.09	14.04	11.24	
Runx3	8.99	8.14	1.28	
Il2rb	1.48	7.22	3.15	
Cd3d	4.24	6.75	3.35	
Rorc	2.17	5.30	4.34	ROR-gt
Itgb7	6.44	4.97	0.99	integrin b7
Zbtb7b	3.20	4.57	4.83	ThPOK
Itga4	1.79	4.44	1.77	integrin a4
Id2	8.89	3.44	0.79	
Icos	3.89	3.38	4.34	
Rag1	0.52	3.38	2.96	
Runx1	6.41	3.31	0.20	
Tox	1.89	3.18	1.38	
Tcf12	0.69	2.98	9.66	HEB
Bcl11b	0.21	2.25	8.68	
Notch2	1.00	2.05	0.39	
Il17rb	0.76	1.72	4.73	
Notch1	0.79	1.32	5.52	
Tcf7	0.72	1.19	2.46	TCF-1
Cd3e	0.34	0.79	2.86	
Tbx21	2.45	0.73	0.30	T-bet

No GATA-3 binding detected	
Gene	Other name
Ahr	
Areg	Amphiregulin
Bcl11a	
Cxcr6	
Flt3	Flk2, CD135
Foxp3	
Gata1	
Gata2	
Il15ra	
Il17ra	
Il1rl1	T1/ST2, IL-33R
Il7ra	CD127
Ly6a	Sca-1
Ly6e	Sca-1
Nfil3	E4bp4
Rag2	

is used by most T cells and precursors and a distal one that is specifically used by Th2 cells (129,130). Only in the distal promotor Notch binding was demonstrated (129,130). In line with the idea that Notch only regulates GATA-3 levels in Th2 cells, the Notch-binding site is covered with repressive H3K27me3 marks in all T cells, except Th2 cells (60,131,132).

Importantly, Engels and colleagues have recently identified an enhancer for GATA-3 that is specific for the T cell lineage (133). This enhancer is located 280kb downstream (3') of the *Gata3* gene and was shown to be necessary and sufficient for expression of *Gata3* transgene in cells of the T cell lineage. This region now must be explored for the mecha-

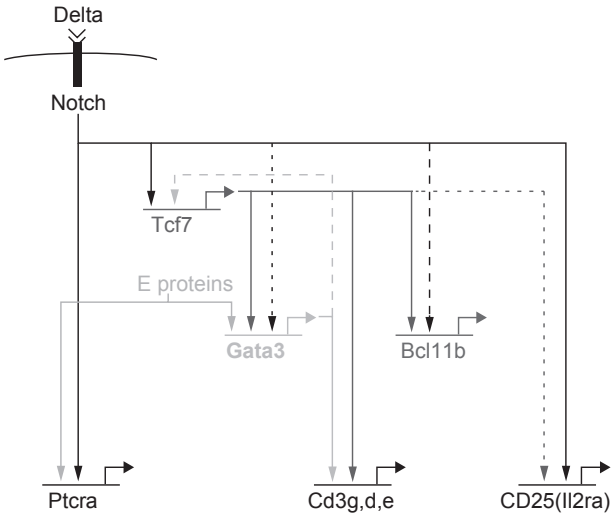


Figure 5. Gene network of transcription factors in initiation of T cell development *(full color version: page 239)* Schematic overview of the relationship between Notch, TCF-1 (encoded by *Tcf7*), GATA-3, Bcl11b and their target genes. Notch signals are required for initiation of the T cell program, as they induce TCF-1, GATA-3 and (at later stages) Bcl11b expression. The program then uses a feed-forward mechanism in which GATA-3 and TCF-1 enhance each other's expression levels, ultimately inducing expression of Bcl11b which is required for further T cell development and for maintenance of T cell identity. Solid arrows indicate evidence from perturbation and binding studies. Dashed arrows indicate evidence from perturbation studies only. Adapted from Rothenberg (160).

nism by which Notch and other signaling molecules are involved in regulation of *Gata3* expression.

Two groups recently showed that TCF-1 is one of earliest initiators of the T cell program (134,135). Interestingly, *Tcf7* (the gene encoding TCF-1) is directly activated by Notch via its enhancer and is required for T cell development. Further evidence for TCF-1 acting downstream of Notch is provided by Weber et al. (135): if Notch is absent, forced expression of *Tcf7* still induces most of the T cell identity program, including GATA-3 and Bcl11b, but fails to activate the Notch target *Ptcra*. Further analysis showed that overexpression of TCF-1 strongly enhances GATA-3 levels and that TCF-1 directly binds to *Gata3* and *Bcl11b* (135). Remarkably, GATA-3 can also bind to the *Tcf7* promotor [(60) and **Table 4**], suggesting a feed-forward mechanism, involving Notch, GATA-3 and TCF-1. These initiators of the T cell program then jointly regulate the T cell program (i.e. CD25 upregulation, Bcl11b expression, initiation of β-rearrangement). The role of TCF-1 in ILC development and function has not been investigated, but it would be interesting to test whether TCF-1 plays a role in the regulation of GATA-3 and/or Notch1 in this cell type as well. Transcriptional analysis revealed that ILC2 express high levels of *Tcf7* (36).

These emerging data and hypotheses have been elegantly integrated into a schematic overview of initiation of T cell development by Rothenberg **(Figure 5)**. Indeed, our results

in *Gata3*-deficient DN2 cells (Chapter 1) are in line with this scheme. We observed slightly lower levels of *Cd3e* and *Tcf7* in *Gata3*-deficient DN2 cells, probably due to absence of GATA-3. Importantly, *Bcl11b* levels were strongly reduced in *Gata3*-deficient cells. This indicates that GATA-3 levels are crucial at this stage, possibly for upregulation of TCF-1, which is important for *Bcl11b* expression. However, it is likely that TCF-1 fulfills other functions at this stage as well, since ectopic *Bcl11b* expression (combined with Bcl-x_L or not) did not rescue T cell development (data not shown). Together, these results importantly contribute to our understanding of initiation of T cell development. They reiterate once more that delicate regulation of expression levels of transcription factors is required for a successful launch of the T cell program.

GATA-3 targets in ILC2

The recently published genome-wide analyses of GATA-3 binding sites show that GATA-3 binding is both cell-specific and stage-specific (59,60). Still, some of these results may be applied to ILC development, and ILC2 development in particular, as DN1 and DN2 cells were shown to still bear a potential to develop into ILC2 (36). Several genes that are required for ILC development and differentiation contain important binding sites of GATA-3 in DN1 and DN2b cells **(Table 4)**. Interestingly, the locus of the ILC2-related transcription factor RORα is one of the key targets of GATA-3 in DN1 and DN2b cells. Furthermore, GATA-3 binds to the *Id2*, *Tcfe2a* (E2A), *Rorc* and *Il1rl1* (T1/ST2) regions. Together, these results indicate that GATA-3 may serve as a pioneer at these loci, either preparing for transcription or by silencing expression. One interesting hypothesis is that GATA-3 controls expression of *Id2* and *Heb*/*Tcf7* and thereby serves as a lineage-determining factor, deciding between ILC/NK and T cell development respectively. Furthermore, GATA-3 may control mutually exclusive expression of RORα and RORγt, thereby promoting development of ILC2 or RORγ^t+ ILC.

Although GATA-3 may be involved in regulation of RORα in developing ILC2, data that emerged from analysis of human ILC2 suggests that GATA-3 does not directly control RORα in mature ILC2 (107). Similar to mouse ILC2, human ILC2 express high levels of *RORA*. Surprisingly, substantial *RORA* levels were also observed in human NK cells and ILC22. Overexpression of *GATA3* in human ILC2 enhanced the expression of TSLPR and ST2. This results are in accordance with mouse studies that already demonstrated that *Il1rl1* (T1/ST2) is a direct target of GATA-3 in Th2 cells (136). Curiously, ectopic expression did not enhance the production of IL-4, IL-5 and IL-13 by ILC2 stimulated with IL-2. Instead, cytokine production was only observed after stimulation with TSLP and IL-33, indicating that GATA-3 alone is not sufficient to drive cytokine production in human ILC2. Furthermore, RORα expression was not upregulated in cells that overexpress GATA3, suggesting that the regulation of RORα in mature human ILC2 is probably more complex.

Genome-wide analysis of GATA-3 binding sites, combined with analysis of histone

modifications and gene expression data will prove to be invaluable in unraveling the role of GATA-3 in ILC development. Results from the group of Zhao indicate that GATA-3 levels are crucial in regulation of GATA-3 function in developing T cells (59). Interestingly, we also observed dose-dependent effects of GATA-3 on ILC2 development (**Chapter III**). Detailed analysis of *Gata3* levels in the various ILC subsets and intermediate precursors may provide valuable new insights in the role of GATA-3 in development. Furthermore, as RORα was shown to be an essential TF for ILC2 development and expansion (36,37), complementation studies in *Gata3*-deficient precursors could provide valuable information about the role of GATA-3 in early ILC2 development. These results, however, suggest that the role of GATA-3 may be much more complex than the regulation of one or two single genes.

How is GATA-3 expression regulated in ILC2?

Little is known about the mechanisms that regulate GATA-3 expression in ILC2. Some indirect evidence comes from a study investigating the role of GATA-3 in ILC2 during worm infections (106). In this study, GATA-3 was shown to be essential for the production of IL-13 by ILC2. All ILC2 expressed high levels of GATA-3 and GATA-3 levels did not change upon N. brasiliensis infection.

In humans, only a fraction of ILC2 derived from human cell lines expresses GATA-3 protein at steady state (16%), but the fraction of GATA3^{+} ILC2 increased markedly after stimulation with TSLP (over 60%) but not IL-33. TSLP can phosphorylate STAT3 and STAT5 (137,138). In human ILC2, TSLP stimulation induced STAT5 only (107). ChIP assays on Th2 cells showed that STAT5 directly binds the *Gata3* gene (137), suggesting that STAT5 directly regulates *Gata3* expression. It remains to be tested whether STAT5 also regulates *GATA3* in ILC2.

As described before, regulation of GATA-3 in T cells has been studied extensively. Since Th2 cells and ILC2 show important similarities, it is attractive to consider that the knowledge of GATA-3 regulation in Th2 cells can be applied to ILC2 as well. Traditionally, Th2 cells are characterized by the production of large amounts of IL-4. ILC2, however, produce very little IL-4 and are mainly characterized by the production of IL-5 and IL-13. GATA-3 is recognized as the most important regulator of the Th2 locus, but regulation of the Il4 locus in particular requires co-factors, including STAT6 (**Chapter I**). This signal transducer is also the most important inducer of GATA-3 expression in differentiating Th2 cells. A study by Doherty et al. suggests that GATA-3 is regulated differently in ILC2, as IL-5 production and GATA-3 levels in ILC2 were not reduced in Stat6-deficient animals exposed to Alternaria (139). Nonetheless, in the absence of *Stat6*, ILC2 numbers and proliferation were reduced, showing that STAT6 might be important for cell cycle regulation in ILC2, possibly independent of GATA-3. Analysis of epigenetic modifications and binding of

Th2-associated TF (e.g. GATA-3, c-Maf, STAT5, STAT6) at the Th2 locus may help to better understand why Th2 cells and ILC2 produce different sets of cytokines. Furthermore, ILC2 have been shown to produce other Th2-associated cytokines, such as IL-6, IL-9 and GM-CSF, but very little is known about how production of these cytokines in ILC2 are regulated.

One of the most striking differences between Th2 cells and ILC2 is that GATA-3 levels in ILC2 are more than 10-fold higher than those found in Th2 cells **(Chapters IV and V)**. Even though ILC2 express higher levels of GATA-3 than T cells, flow cytometric analysis of IL-5 and IL-13 protein production per cell after PMA/ionomycin stimulation showed no increase in cytokine production compared to Th2 cells (**Chapter V**). It remains to be tested whether there are differences in IL-5 and IL-13 production without stimulation (spontaneous) or after stimulation with other cytokines (e.g. IL-7, IL-25, IL-33, TSLP). Interestingly, Paul et al. have previously shown that in T cells, GATA-3 expression can be induced by the IL-33/STAT5-inducers IL-2, IL-7 and TSLP (136), and it remains to be shown whether GATA-3 levels in ILC2 change in response to these cytokines.

Interestingly, early thymocytes (DN2 cells) and ILC2 show significant phenotypic similarities. Both cell types lack the expression of lineage markers, express high levels of CD25, CD44 and CD127. Furthermore, both populations derive from CLP, express GATA-3 and depend on Notch signaling during development. McKenzie and colleagues have shown that DN2 cells still possess a potential to develop into ILC2 in vitro (105). Therefore, it is interesting to consider the possibility that early thymocytes and ILC2 are developmentally related and share a precursor downstream of the CLP. Nevertheless, most thymic precursors will give rise to T cells in vivo, while only very few give rise to ILC2. What factors influence the choice between the T cell and ILC lineage?

Thymopoiesis relies on the thymus as a dedicated organ for T cell development (**Chapter I**). During development, thymocytes migrate through the thymus and are exposed to different signals, such as interaction with stroma, Notch ligands and cytokines (e.g. IL-7 and SCF) (reviewed by (140)). Not only does the thymus provide thymocytes with optimal conditions for development, it also efficiently suppresses development of other hematopoietic cells. While ILC2 and DN2 cells appear very similar, the thymus produces many T cells every day, but there have been no reports of thymus-derived ILC2. Furthermore, ILC2 numbers are normal in nude mice (3). Thus, early thymocytes must be instructed to develop into T cells at the expense of ILC development.

One of the unique features of the thymus is the high availability of Notch ligands and production of IL-7. These factors may divert precursors from ILC development and promote T cell development, but Notch signals are also required for the first stages of ILC2 and ILC3 development (36,90) and IL-7 may represent an important survival factor for ILC. However, for ILC3 development, it has been shown that sustained Notch signals are

detrimental (90). The effects of continuous Notch signaling on ILC2 development remains to be tested, but McKenzie and colleagues have shown that Notch signals are only required during the first days of ILC2 development in vitro (36).

In early thymocytes, GATA-3 levels need to be tightly regulated: lack of GATA-3 blocks T cell development [**Chapter II** and (58,141)], while overexpression of GATA-3 diverts thymocytes to the mast cell lineage (142). As ILC2 express high levels of GATA-3 (as well as other ILC subsets that also express GATA-3, albeit at lower levels), expression levels of this TF may be important in fate determination. High GATA-3 levels may induce the ILC2 development program, while intermediate GATA-3 levels promote T cell development. In that case, it is interesting to consider whether Notch may play a role in regulation of GATA-3.

The interaction between Notch and GATA-3 has been extensively studied and is still subject of debate: Notch1 was shown to directly regulate expression of GATA-3 in developing Th2 cells (129), but was found not to be a direct target for Notch1 in human thymocytes (143,144). It remains to be elucidated whether Notch has a role in the regulation of GATA-3 in ILC. One possibility to consider is that prolonged Notch signals downregulate GATA-3 levels, thereby shutting down the ILC2 development program that depends on high GATA-3 levels.

Another important question is how high GATA-3 levels are achieved and maintained in ILC2. It is attractive to speculate that in ILC2, GATA-3 expression is important for induction of IL-33R expression, as it is the case in Th2 cells (136). Signaling via this cytokine receptor may then further upregulate GATA-3 levels, thus generating a feed-forward loop. The initial description of the nuocyte, however, already described that ILC2 development is less effective, but not abolished in the absence of IL-33 and/or IL-25 or their receptors (105). Thus, while this pathway may be important for homeostasis and proliferation of mature ILC2, it is unlikely that GATA-3 levels are controlled via IL-25/IL-33-signaling in developing ILC2. Consequently, other mechanisms that ensure high GATA-3 levels must be considered. Careful analysis of the role of the STAT family members that are essential in GATA-3 regulation in T cell biology may provide valuable analysis. STAT5 has already been implicated in the regulation of GATA-3 in human ILC2 (107), while STAT6 seems to play a limited role in murine ILC2 (139). Other cytokines, such as IL-2, IL-7 and TSLP are known to signal via the STAT5 pathway and may prove to be instructors of ILC development, homeostasis and function. Furthermore, the role of RORα in ILC2 development and homeostasis needs to be investigated further, as absence of RORα strongly reduced ILC2 numbers, but does not completely abolish ILC2 development (36). Genome-wide analysis of GATA-3 binding shows that RORα is a potential target in thymocytes and therefore it must be tested whether GATA-3 plays a role in RORα regulation in ILC2 as well.

Outstanding questions

- **Common precursor**: Do the different ILC subsets share a common precursor and what is the phenotype of that precursor? How are common ILC transcription factors (e.g. Id2) regulated and what is their role in further ILC specification?
- **Notch signaling**: What are the targets of Notch in development of ILC2 and ILC3? Is Notch involved in regulation of GATA-3?
- **GATA-3 in ILCs**: At what stages of development is GATA-3 required for ILC development? How is *Gata3* regulated in developing and activated ILC2? What is the developmental relationship between Notch, GATA-3 and Id2? What are the targets of GATA-3 in developing ILC2; e.g.. is RORα directly regulated by GATA-3? Does GATA-3 have a role in ILC3 development and/or function?
- **GATA-3 in T cell development**: How do Notch, GATA-3 and TCF-1 regulate early T cell development? Is GATA-3 directly regulated by Notch via its recently identified T/NK-cell specific enhancer?

Developmental of novel therapeutic strategies

ILC2 modulation as a novel therapy for asthma?

Many studies have illustrated that the Th2 pathway is crucial in allergic asthma. This prompted several groups to develop biological targeting of the key type 2 cytokines in asthma: IL-4, IL-5, IL-9 and IL-13. The overall efficacy of humanized antibodies directed against these cytokines in patient trials is low [reviewed by Holgate; (145)]. In most asthmatic patients, the disease is very heterogeneous, possibly explaining the lack of improvement of patient-related outcome measures. In most studies, a small, but generally well-defined group of responders can be defined, but even in this group generally no significant differences in clinical outcome are reported (145).

In these studies, monoclonal antibodies directed against a single cytokine were used. Given the recent descriptions of ILC2 and its significant involvement in the production of both IL-5 and IL-13 in various models of allergic airway responses and influenza **(Table 1)**, these cells could prove to be an interesting target for the development of new therapeutics that prevent exacerbations and limit symptoms of acute disease.

Targeting the inducer cytokines IL-25 and IL-33

Several groups have addressed the importance of the IL-25 and IL-33 pathways by using antibodies directed against the cytokines or their receptors. Blocking of the IL-33 pathway using a neutralizing antibody in ovalbumin asthma models resulted in a significant reduction in BAL cellularity, IL-4, IL-5 and IL-13 and ovalbumin IgE in serum (19). Antibodies against IL-33 and ST2 (IL-33R) were also effective in an Aspergillus fumigatus model of airway inflammation (146). Similarly, anti-IL-25 antibodies were effective when used during the sensitization and challenge phases in an ovalbumin-alum asthma model: reduction of allergic inflammation, ovalbumin-IgE and AHR (147). Interestingly, IL-25 seems to have divergent functions during the sensitization and challenge phase in this model, as IL-25 neutralization during challenges, as opposed to anti-IL-25 therapy during the sensitization phase, had no effect on lung cellularity or Ig production (but did results in a reduction in AHR). Anti-IL-25 treatment also reduced the production of Th2 cytokines in lung at transcription level in a model of RSV-associated airway inflammation (148). In summary, targeting the IL-25 and IL-33 pathways that are important regulators of ILC2 activation and expansion is beneficial in mice. It remains to be tested whether this is applicable to the human situation as well.

Targeting key transcription factors: RORα and GATA-3

The recent identification of RORα and GATA-3 as key transcription factor for ILC2

[(36,37,107,108) and **Chapter III**] could open a road for development of small molecule inhibitors of these transcription factors. Interestingly, genome-wide association studies have linked asthma with mutations in *RORA* (34). Previously, *Rora*-deficient mice were shown to have impaired type 2 immune responses (149).

Two recent publications describe RORγt inhibitors that potently antagonized Th17 differentiation (150,151). In the first publication, administration of digoxin or two synthetic, non-toxic derivatives inhibited differentiation of Th17 cells in mouse and human via direct inhibition of RORγt transcriptional activity (150). Consequently, in a Th17-driven model of EAE, disease onset was delayed and disease severity was reduced. In the second study, the synthetic RORγt ligand SR1001 also prevented Th17 differentiation and impaired EAE development (151). Interestingly, the compound SR1001 also inhibited RORα function, while administration of digoxin or its derivatives did not impact on RORα function. Importantly, although RORγt and RORα are widely expressed, the authors observed no obvious toxicity is animals treated with SR1001 (151). Therefore, SR1001 could be an interesting target for the development of strategies targeting ILC2 (and RORγt^{+} ILC).

GATA-3 performs two critical functions in ILC2: it is required during development of ILC2 [(108) and **Chapter III**] and it is essential for the production of type 2 cytokines (106,107), probably using similar mechanisms as Th2 cells, although this remains to be demonstrated. A study by the group of Barnes et al. shows that the glucocorticoid fluticasone inhibits the translocation of GATA-3 from the cytosol to the nucleus where it performs its functions as a transcription factor (152). Fluticasone inhibits nuclear translocation of GATA-3 via two distinct mechanisms. First, it competitively binds to importin-α, a protein that is bound by GATA-3 and is required for nuclear import. Secondly, fluticasone induces expression of MAP kinase phosphatase-1, an inhibitor of the p38 MAP kinase that is required for phosphorylation of GATA-3. Asthmatic patients are regularly treated with corticosteroids. Interestingly, peripheral blood lymphocytes of patients that were treated with inhaled fluticasone also showed inhibited GATA-3 nuclear translocation.

ILC2 express high levels of GATA-3. Preliminary results obtained using immunohistochemical analysis of IL-33 treated mice shows that GATA-3 in ILC2 primarily localized in the cytosol. Additional human studies are required to determine GATA-3 localization in ILC2. Fluticasone treatment could also impact on GATA-3 nuclear translocation in ILC2. This could be addressed in asthma patients that are treated with fluticasone or not, but could also be tested using in vitro assays. Flow cytometric analysis combined with microscopy can be a valuable approach to study whether fluticasone treatment inhibits GATA-3 nuclear translocation in activated ILC2.

In this thesis we have characterized the various roles of GATA-3 in development and function of T cells and ILC2. These two cell types were studied in the context of mouse models of allergic asthma. In both T cells and ILC2, GATA-3 is an important determinant in

development and function and GATA-3 levels directly correlate with production of type-2 cytokines. Therefore, inhibition of GATA-3 may prove to be an important novel therapeutic strategy, because inhibition of GATA-3 will target both T cells and ILC2. Most cellular players in asthma are localized in the lung and draining lymph nodes. Therefore, inhibition of GATA-3 by means of local administration of small molecule inhibitors will directly impact on antigen-specific T cells and ILC2 as important players in asthma. Local administration also reduces the risk of unwanted side-effects, such as general impairment of T cell development.

Outstanding questions

- **Innate cytokines**: Can IL-25/IL-33 inhibition in humans serve as a novel therapy for (certain types) of asthma or other type-2 conditions ?
- **ROR inhibition**: What is the potential of RORα/RORγt-specific inhibitors for development of novel therapies for allergic airway disease (Th2-mediated) or Th-17 mediated diseases (rheumatic disorders, psoriasis)?
- **GATA-3 inhibition:** Where is GATA-3 localized in ILC2 and Th2 cells? Do inhibitors of GATA-3 translocation improve allergic airway disorders in mice and humans?

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Chapter VII

Summary

English summary

Transcription factors (TFs) control the complex process of differentiation of hematopoietic stem cells into functional and mature blood cells. During a stepwise program, multipotent hematopoietic stem cells that reside in the bone marrow gradually lose differentiation potential and commit to different blood cell lineages, including T lymphocytes. T lymphocytes are white blood cells and constitute an important arm of the mammalian immune system that defends the body against pathogens and tumors. T lymphocytes recognize antigens via the T cell receptor (TCR) and respond by clonal expansion and further differentiation, thereby providing lifelong protection against reinfection by the same pathogen.

In contrast to other blood cells, T lymphocyte development primarily takes place in the thymus. Interactions with the environment of developing T cells trigger developmental programs that are tightly controlled by TFs. One essential TF for T cell development is GATA-3. This factor is critically involved at various stages of T cell development and was shown to play a role during commitment to the T cell lineage, during the process of TCRβ-chain selection, in $CD4$ T cell development and during Th2 differentiation (**Chapter I**).

Although the role of GATA-3 in T cell development has been extensively studied, the exact role of GATA-3 during T cell commitment remains unknown. In **Chapter II**, we used hematopoietic chimeras and in vitro culture systems to show that GATA-3 orchestrates a transcriptional repertoire that is required for thymocyte maturation up to and beyond the pro-T-cell stage. Furthermore, we demonstrated that GATA-3 is essential to suppress a latent B cell potential that is revealed in the absence of Notch. As such, GATA-3 is essential to seal Notch-induced T-cell fate in early thymocyte precursors by promoting T cell identity through repression of alternative developmental options.

Recently, previously unknown populations of innate lymphoid cells (ILCs) have been identified. These non-B/non-T lymphocytes reside primarily at mucosal sites where they rapidly produce cytokines upon triggering via cytokine receptors. Strikingly, the cytokine signature of the newly identified subsets of ILCs show important parallels with T helper cell subsets. Group 2 ILCs (ILC2) found in the intestine and lung produce important amounts of the T helper-2 (Th2) cytokines IL-5 and IL-13 upon triggering with IL-25 and IL-33.

Allergic asthma is characterized by chronic airway inflammation and hyperreactivity. Th2 cells have been recognized as key regulators of this response. The type-2 cytokines IL-4, IL-5 and IL-13 are critical in the pathogenesis of asthma. In **Chapter III**, we identified a population of ILC2 in the pulmonary tract that is increased in various murine models of allergic asthma. Using these models, we compared the contribution of Th2 and ILC2 in the production of type-2 cytokines and found that in house dust mite and ovalbumin-induced asthma, the contribution of ILC2s to the total population of intracellular IL-5^+ and IL-13^+

cells in the lung was in the same range as found for Th2 cells. Therefore, we conclude that both ILC2s and Th2 cells produce large amounts of IL-5 and IL-13 that contribute to allergic airway inflammation.

GATA-3 is the characteristic TF for the Th2 lineage, as it is required both for Th2 differentiation and for production of IL-5 and IL-13. Similarly, the production of IL-13 by ILC2 is controlled by GATA-3. However, it is not known whether GATA-3 is required for ILC2 development from hematopoietic stem cells (HSC). In **Chapter IV**, we use in vitro approaches and different models of chimeric mice generated with Gata3-deficient stem cells to show that GATA-3 is required for ILC2 development in a cell-intrinsic manner. Using mutant and transgenic mice in which *Gata3* gene copy number is altered, we show that ILC2 generation from common lymphoid progenitors, as well as ILC2 homeostasis and cytokine production is regulated by *Gata3* expression levels in a dose-dependent fashion. Collectively, these results identify GATA-3 as a critical early regulator of ILC2 development, thereby extending the paradigm of *Gata3*-dependent control of type 2 immunity to include both innate and adaptive lymphocytes.

As GATA-3 controls both function and development of Th2 cells and ILC2, we investigated the effects of altered *Gata3* gene copy numbers on the acute phase of murine asthma models. In **Chapter V**, we use ovalbumin and house dust mite-induced asthma models in mice transgenic for *Gata3*. Increased *Gata3* levels correlated with increased numbers of ILC2 and Th2 cells, increased production of type-2 cytokines and increased eosinophilia. Taken together, these results indicate enforced expression of *Gata3* in the T and ILC2 lineage induces increased susceptibility to allergic airway inflammation.

The results described in this thesis provide new insights in the role of TF GATA-3 at various stages of development and function of T cells and ILC2. The diverse roles of GATA-3 in development and function of various lymphocyte lineages indicate that GATA-3 acts in a cell-specific and stage-dependent manner. These findings are in accordance with two recent genome-wide analyses of GATA-3 binding sites (discussed in **Chapter VI**) that show that GATA-3 binding sites are highly variable at different stages of T cell development. Further characterization of the role of GATA-3 in lymphocytes using classic immunological and cell biological approaches can now be combined with new techniques, such as genome-wide analysis of gene expression and binding sites. These analyses will provide valuable information that may pave the way for new therapeutic strategies for a wide variety of diseases including allergic asthma.

Nederlandse samenvatting

Transcriptiefactoren zijn eiwitten die de complexe ontwikkeling van hematopoietische stamcellen tot functionele, rijpe bloedcellen sturen. Deze ontwikkeling van multipotente stamcellen verloopt in verschillende stappen en begint in het beenmerg. Tijdens de verschillende stappen van het ontwikkelingsproces wordt de uiteindelijke identiteit van de cel steeds meer vastgelegd en verliest de cel de mogelijkheid om zich te ontwikkelen tot andere typen bloedcellen. T lymfocyten zijn een type witte bloedcellen die een belangrijke rol spelen in het immuunsysteem van zoogdieren. Deze cellen beschermen het lichaam tegen infecties met pathogenen en tegen onstaan van maligniteiten. Zodra een T cel een specifiek antigen herkent via de T cell receptor (TCR), raakt de cel geactiveerd en zal zich vermenigvuldigen en verder differentiëren. Op deze manier zorgen T cellen voor een levenslange bescherming tegen herinfectie door hetzelfde pathogeen.

T cellen zijn uniek, omdat de ontwikkeling van deze cellen voor het grootste deel van de thymus plaatsvindt, terwijl andere cellen zich ontwikkelen in het beenmerg. De interactie tussen T cellen met de gespecialiseerde omgeving van de thymus beïnvloeden het ontwikkelingsproces dat gestuurd wordt door verschillende transcriptiefactoren. GATA-3 is een van de transcriptiefactoren die essentieel zijn voor T-celontwikkeling. Dit eiwit speelt een rol tijdens verschillende stappen van T-celontwikkeling: op het moment dat een stamcel vastlegt T cel te worden, tijdens β-selectie, bij ontwikkeling van CD4 T cellen en bij Th2 differentiatie **(Hoofdstuk I)**.

Hoewel de functie van GATA-3 tijdens T-celontwikkeling uitgebreid is bestudeerd, is het onbekend welke rol GATA-3 precies speelt tijdens het vastleggen van het T-celontwikkelingsprogramma. In **Hoofdstuk II** tonen we met behulp van hematopoëtisch chimere muizen en in vitro kweeksystemen aan dat GATA-3 een cascade van TF aanstuurt die nodig zijn voor T celontwikkeling tijdens en na het pro-T-celstadium. Bovendien laten we zien dat GATA-3 noodzakelijk is om het B-celpotentieel, dat tot uiting komt als Notch afwezig is, te onderdrukken. GATA-3 versleutelt dus de Notch-geïnduceerde T-celontwikkeling door het voorlopercellen onmogelijk te maken zich tot een ander type bloedcel te ontwikkelen.

Tot voor kort wisten we niet van het bestaan van zogenaamde ‘innate lymphoid cells’ (ILC) af. Deze niet-B/niet-T lymfocyten maken deel uit van het immuunsysteem en worden met name gevonden in mucosale organen waar ze snel cytokines produceren als ze geactiveerd worden via cytokinereceptoren. Er zijn verschillende populaties van ILC beschreven die elk een uniek cytokineprofiel hebben. Deze cytokineprofielen komen grotendeels overeen met de profielen van de verschillende T-helpercel families. Zo produceren groep-2 ILCs die als eerste werden beschreven in de darm, net als Th2 cellen, grote hoeveelheden IL-5 en IL-13 als ze gestimuleerd worden met IL-25 of IL-33.

Allergisch astma is een ziekte die gekenmerkt wordt door chronische luchtwegontsteking en –hyperreactiviteit. Onderzoek heeft aangetoond dat Th2 cellen bij deze aandoe-

ning een sleutelrol spelen en dat de type-2 cytokines IL-4, IL-5 en IL-13 essentieel zijn bij de pathogenese van asthma. In **Hoofdstuk III** beschreven we een populatie van ILC2 in de longen die toeneemt in grootte in verschillende muismodellen voor allergisch astma. Met deze modellen hebben we de relatieve bijdrage van Th2 cellen en ILC2 aan productie van type-2 cytokines vergeleken. We concluderen dat in astmamodellen met huisstofmijt en ovalbumine het aantal ILC2 dat IL-5 of IL-13 maakt in dezelfde orde van grootte is als het aantal Th2 cellen dat deze cytokines produceert. We concluderen daarom dat zowel Th2 cellen als ILC2 grote hoeveelheden IL-5 en IL-13 maken en zo bijdragen aan allergische luchtwegontsteking.

GATA-3 is de kenmerkende TF van Th2 cellen, omdat GATA-3 vereist is voor zowel Th2-cel differentiatie als voor productie van IL-5 en IL-13. Ook in ILC2 is GATA-3 noodzakelijk voor productie van IL-13. Het is echter niet bekend of GATA-3 ook noodzakelijk is voor ontwikkeling van ILC2 vanuit hematopoietische stamcellen. In **Hoofdstuk IV** laten we met behulp van in vitro systemen en chimere muismodellen gemaakt met *Gata3*-deficiënte stamcellen zien dat cel-intrinsiek GATA-3 noodzakelijk is voor ontwikkeling van ILC2. Daarnaast tonen we met behulp van mutante en trangene muizenstammen waarin het aantal *Gata3* genkopieën is veranderd aan dat GATA-3 op een dosis-afhankelijk manier de ontwikkeling van ILC2 uit de lymfoïde stamcel, ILC2 homeostase en cytokineproductie reguleert. Concluderend laten we in deze studie zien dat GATA-3 een essentiële vroege regulator is van ILC2 ontwikkeling. Dit toont aan dat het paradigma van *Gata3*-afhankelijke sturing van type-2 immuniteit niet alleen opgaat voor lymfocyten van het verworven immuunsysteem, maar ook voor die van het aangeboren immuunsysteem.

Gegeven dat GATA-3 zowel ontwikkeling als functie van Th2 cellen en ILC2 stuurt, hebben met behulp van muismodellen voor astma onderzocht wat de effecten zijn van een veranderd aantal Gata3-genen. In **Hoofdstuk V** hebben we transgene muizen met een hoger aantal *Gata3*-genkopieën onderworpen aan astmamodellen met ovalbumine en huisstofmijt. We tonen aan dat meer *Gata3* leidt tot grotere aantallen ILC2 en Th2 cellen, toegenomen productie van type-2 cytokines en toegenomene eosinofilie. Deze resultaten tonen aan dat geforceerde overexpressie van *Gata3* in T cellen en ILC2s de gevoeligheid voor het ontwikkelen van allergische luchtwegontsteking vergroten.

De resultaten beschreven in dit proefschrift geven nieuwe inzichten in hoe GATA-3 functioneert tijdens verschillende ontwikkelingsstadia van T cellen en ILC2. We tonen aan dat de functie van GATA-3 niet alleen afhangt van het ontwikkelingsstadium van een cel, maar ook van het celtype waarin GATA-3 tot expressie komt. Deze bevindingen sluiten aan bij twee recente genoom-brede analyses van bindingsplaatsen van GATA-3 die beschreven worden in **Hoofdstuk VI**. Deze studies laten zien dat de bindingsplaatsen van GATA-3 sterk verschillen tussen verschillende stadia van T-celontwikkeling. De combinatie van klassieke immunologische en celbiologische technieken met nieuwe technieken, zoals genoom-brede analyse, zal in de toekomst leiden tot meer inzicht in de functie van GATA-3. Deze kennis zal leiden tot belangrijke nieuwe inzichten die de opmaat kunnen vormen voor ontwikkeling van nieuwe therapieën voor aandoeningen zoals astma.

Résumé français

Les facteurs de transcription (FT) régulent le développement et la différentiation des cellules souches hématopoïétiques en cellules matures du système sanguin. Au cours d'un processus graduel, les cellules souches dans la moelle osseuse perdent progressivement leur multipotence et se différencient vers des lignages distincts. Les lymphocytes T sont des globules blancs qui constituent une partie importante du système immunitaire et protègent le corps contre des pathogènes ou des tumeurs. Les lymphocytes T reconnaissent les antigènes via leur récepteur (TCR) et répondent par une expansion clonale et une différenciation, apportant ainsi une protection à long terme contre la réinfection par le même pathogène.

Contrairement aux autres leucocytes, les lymphocytes T se développent principalement dans le thymus. Les interactions entre les cellules T et leur environnement thymique induisent des programmes de développement qui sont aussi régulés par les FT. L'un des FT essentiels pour la différenciation des lymphocytes T est GATA-3. Ce facteur est fondamental à plusieurs étapes du développement des lymphocytes T dont leur engagement dans le lignage, le processus de sélection de la chaîne TCRβ, le développement des T CD4, et la différenciation en cellule de type Th2 (**Chapitre I**).

Bien que le rôle de GATA-3 dans le développement des cellules T ait été largement étudié, le rôle précis de GATA-3 lors de l'engagement des cellules dans le lignage T est inconnu. Dans le **Chapitre II**, nous utilisons des chimères hématopoïétiques et des cultures in vitro pour démontrer que GATA-3 régule un ensemble de FT qui sont nécessaires pour la maturation des thymocytes jusqu'au, et au delà, du stade pro-T. De plus, en absence des signaux Notch, nous montrons que GATA-3 est nécessaire pour inhiber la différenciation des lymphocytes B. De cette manière, GATA-3 est essentiel pour verrouiller le développement des lymphocytes T induit par les signaux Notch dans les précurseurs des thymocytes, en favorisant l'identité T par la répression des autres voies de différenciation.

Récemment, des cellules du système immunitaire inné (ILC) ont été découvertes. Ces lymphocytes non-T, non-B, résident principalement dans les tissus muqueux, où ils sécrètent rapidement des cytokines à la suite de leur activation via leurs récepteurs de cytokines. Étonnamment, les signatures cytokiniques des différentes classes d'ILC ont des similitudes importantes avec les différentes sous-populations de lymphocytes T. Les ILC du group 2 (ILC2) ont été identifiés en premier dans l'intestin et produisent des cytokines de type Th2 IL-5 et IL-13 après leur activation par l'IL-25 et l'IL-33.

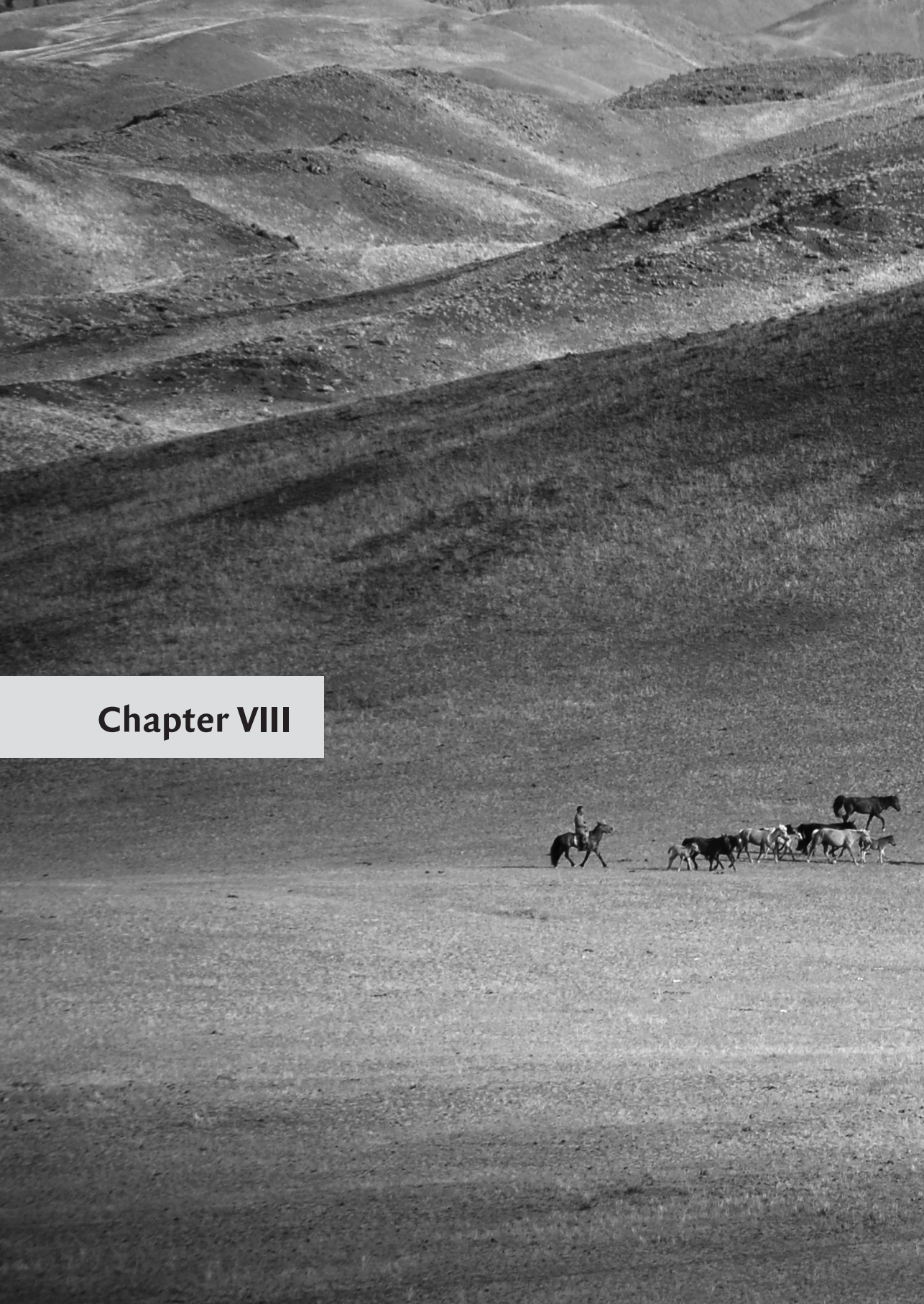
L'asthme allergique est caractérisé par une inflammation chronique et une hyperréactivité des voies respiratoires. Les Th2 sont des régulatrices essentielles de cette réponse. Les cytokines de type Th2 IL-4, IL-5 et IL-13 sont primordiales dans la pathogenèse de l'asthme. Dans le **Chapitre III**, nous avons mis en évidence une population de cellules ILC2 qui augmente dans l'appareil respiratoire dans différents modèles murins d'asthme allergique. En utilisant ces modèles, nous avons comparé la contribution des Th2 et des ILC2 dans leur production de cytokines de type Th2: dans l'asthme induit par les mites et l'ovalbumine, la contribution des ILC2 à la présence de cellules IL-5+ et IL-13+ était similaire à celle des cellules Th2 dans le poumon. Donc, nous concluons que les ILC2 et les Th2 produisent de grandes quantités d'IL-5 et IL-13, ce qui contribue à l'inflammation des voies respiratoires au cours de l'asthme allergique.

GATA-3 est un FT caractéristique du lignage Th2, puisqu'il est nécessaire à la différenciation des Th2 et à la production d'IL-5 et d'IL-13. De même, la production d'IL-13 par les ILC2 est contrôlée par GATA-3. Or, nous ne savons pas si GATA-3 est nécessaire pour le développement des ILC2 à partir des cellules souches hématopoïétiques. Dans le **Chapitre IV**, nous avons utilisé des expériences in vitro, et des modèles de souris chimériques reconstituées avec des cellules souches *Gata3* déficientes, pour montrer que GATA-3 est nécessaire pour le développement des ILC2 de manière intrinsèque. Grâce à des souris transgéniques ou mutantes chez lesquelles le nombre de copies de *Gata3* est modifié, nous montrons que le développement des ILC2 à partir des cellules progénitrices lymphoïdes, ainsi que l'homéostasie des ILC2 et leur production de cytokines, est régulé par le niveau d'expression de *Gata3*. Pris ensemble, ces résultats mettent en évidence le rôle essentiel de GATA-3 dans les étapes précoces du développement des ILC2, et élargissent le paradigme dans lequel GATA-3 control l'immunité de type 2 afin d'inclure les cellules de l'immunité innée et adaptive.

Puisque GATA-3 agit sur le développement et le fonctionnement des cellules Th2 et ILC2, nous avons étudié les effets des modifications dans le nombre de copies du gène *Gata3* lors de la phase aigue dans un modèle d'asthme murin. Dans le **Chapitre V**, nous utilisons les modèles d'asthme induits par les mites et l'ovalbumine chez des souris transgéniques pour *Gata3*. Une augmentation des niveaux de *Gata3* corrélait avec une augmentation du nombre de cellules ILC2 et Th2, de production de cytokines de type 2, et d'éosinophiles. Dans leur ensemble, ces résultats montrent qu'une sur-expression de *Gata3* dans les lignages T et ILC2 induit une plus grande susceptibilité à l'allergie des voies respiratoires.

Les résultats décrits dans cette thèse apportent de nouveaux points de vus sur le rôle du facteur de transcription GATA-3 à divers étapes du développement et des fonctions des cellules T et ILC2. Les rôles variés de GATA-3 lors du développement et des fonctions dans des lignages lymphocytaires diversifiés, suggèrent que GATA-3 agit à des étapes précises dans des cellules spécifiques selon un programme finement régulé. Ces données sont en accord avec deux analyses récentes des sites de liaison de GATA-3 sur l'ensemble du génome (discuté dans le **Chapitre VI**), qui montrent que les sites de liaison de GATA-3 sont très variables à différentes étapes du développement des lymphocytes T. Une caractérisation plus approfondie du rôle de GATA-3 dans les lymphocytes, avec des outils classiques de biologie cellulaire et d'immunologie, peut à présent être combinée avec de nouvelles technologies telles que l'analyse génomique entière de l'expression génique et des sites de liaison. Ces analyses apporteront des informations importantes, qui pourront guider les études futures, afin d'établir de nouvelles stratégies thérapeutiques contre de nombreuses pathologies dont l'asthme allergique.

Chapter VIII



Portfolio

List of publications

- Klein Wolterink RGJ, García-Ojeda ME, Vosshenrich CA, Hendriks RW, Di Santo JP. The intrathymic crossroads of T and NK cell differentiation. *Immunol Rev* 2010.

- Klein Wolterink RGJ, Kleinjan A, van Nimwegen M, Bergen I, de Bruijn M, Levani Y, Hendriks RW. Pulmonary innate lymphoid cells are major producers of IL-5 and IL-13 in murine models of allergic asthma. *Eur J Immunol* 2012

- García-Ojeda ME*, Klein Wolterink RGJ*, Lemaître F*, Richard-Le Goff O, Hasan M, Hendriks RW, Cumano A, Di Santo JP. GATA-3 promotes T cell specification by repressing B cell potential in pro-T cells. *Blood* 2013. *equal contribution

- Klein Wolterink RGJ, Hendriks RW. Group two innate lymphocytes in allergic airway inflammation. *Curr Allergy Asthma Rep* 2013.

- Klein Wolterink RGJ , Serafini N, van Nimwegen M, Vosshenrich CAJ, Fonseca-Pereira D, Veiga-Fernandes H, Hendriks RW*, Di Santo JP*. Transcription factor GATA-3 is essential for the development of IL-5 and IL-13-producing type 2 innate lymphoid cells. *Pending revision*. *shared last authorship

- KleinJan A*, Klein Wolterink RGJ*, Levani Y, de Bruijn M, Henk C Hoogsteden, van Nimwegen M, Hendriks RW. Enforced expression of Gata3 in the T cell and type 2 innate lymphocyte lineages increases susceptibility to allergic airway inflammation in mice. *In preparation*. *equal contribution

- Klein Wolterink RGJ*, Hassan M*, Di Santo JP. Gfi1 has multiple roles in NK cell development. *Manuscript in preparation*. *equal contribution

About the author

"It's a pity to do only one thing at a time."

"Somehow it has always been possible to schedule my journeys just around exams and compulsory classes."

"Cycling in Paris is a fabulous way to explore this city."

"Although bureaucracy is annoying, being able to deal with it in French can be quite satisfying."

"In fact I have no idea what the job of my dreams is."

Roel is our eldest son. He was born in The Hague on 4 May 1985, two years before his brother. In 1988 we moved to Dordrecht, where in 1993 and 1995 his two sisters were born.

Roel attended high school at the Johan de Witt Gymnasium in Dordrecht from 1997 to 2003. In 2003 he started his medical studies at the Erasmus University in Rotterdam, and combined this with an MSc programme in Molecular Medicine from his second year on. He became an active member of the international student organization IFMSA, and served as their national president for a year in 2006, while successfully completing the first year of a BA in French language & literature at the University of Amsterdam. From June 2007 till January 2008 he lived and worked in Mundri, a very remote and small village in Southern Sudan. In 2009 he finished the theoretical part of this medical studies and left for Paris to do a research internship at the Institut Pasteur as part of his MSc degree. That project eventually led to his combined PhD research in Paris and Rotterdam under supervision of Prof. James Di Santo and Prof. Rudi Hendriks, resulting in the thesis in front of you. In January 2013 he left his Paris apartment to start his clinical rotations in Rotterdam, aiming to pursue his medical degree in December 2014.

Looking back at the ten years that have gone by since Roel began his studies at Erasmus University, we can fairly say that he has made the most of those years. His studies and research, his travels all over the world, living and working in Rotterdam, South Sudan and Paris, have provided him with opportunities to learn, to teach, to organize, to take on responsibilities. It has enabled him to meet with people from many different backgrounds and to broaden his horizon time and again. For Roel it has been an exciting challenge to work near and even shift the boundaries of human knowledge. He has grasped the opportunities that he found along the way. He has worked hard and enjoyed doing so.

We are very proud of his achievements and his receiving a doctor's degree and we are very happy and thankful that Roel enjoys his life and is able to do the things he wants to do.

Liesbeth Richters
Jan Klein Wolterink
Roel's parents

PhD Portfolio

Roel Klein Wolterink
Joint PhD program Erasmus Universiteit Rotterdam (NL) and Université Paris-Diderot (FR)

Project title:	Stage-dependent functions of GATA-3 in lymphocyte lineage determination and type-2 immunity
Thesis directors:	Prof. Dr. Rudi W. Hendriks *Dept. of Pulmonary Medicine, Erasmus MC Rotterdam, The Netherlands* Prof. Dr. James P. Di Santo *Innate Immunity Unit, Institut Pasteur, Paris, France*
Research schools:	Molecular Medicine Postgraduate School *Erasmus MC Rotterdam, The Netherlands* Ecole doctorale Biochimie, Biothérapies, Biologie Moléculaire et Infectiologie (B3MI), *Paris-Diderot (Paris 7), Paris, France*
PhD period:	2010 – 2013

The work performed during this PhD training was supported by a personal grant from the Ligue Nationale Contre le Cancer (LNCC), France and by the Department of Pulmonary Medicine, Erasmus MC Rotterdam, the Netherlands.

Courses

2009	Laboratory Animal Experimentation
2010	Molecular Immunology
2011	Workshop on Basic data analysis on gene expression arrays (BAGE)

Scientific presentations and meetings

October 2010	5th Int. Conference on Gene Regulation in Lymphoid Development *Chania, Crete, Greece (poster presentation)*
November 2010	Mini-symposium "From stem cells to lymphocytes" *Department of Immunology, Erasmus MC Rotterdam (oral presentation)*
December 2010	Annual meeting NVVI (Dutch society for Immunology) *Noordwijkerhout, the Netherlands (oral presentation)*
February 2011	15th Molecular Medicine Day, *Erasmus MC Rotterdam, The Netherlands (oral presentation)*
April 2011	Lung Science Conference *European Respiratory Society (ERS), Estoril, Portugal*
April 2011	National Institutes of Health, Experimental Immunology Branch *Washington DC, United States of America (seminar)*
May 2011	EUThyme/Rolduc thymus meeting *Noordwijkerhout, The Netherlands (oral presentation)*
June 2011	Journées Départementales d'Immunologie (JDI) *Institut Pasteur Paris, France (oral presentation)*
February 2012	EAACI Winter School *Are, Sweden (oral presentation)*
February 2012	16th Molecular Medicine Day *Erasmus MC Rotterdam, The Netherlands (poster presentation)*
April 2012	Netherlands Respiratory Society (NRS) Longdagen *Utrecht, the Netherlands (oral presentation)*
January 2013	IFReC-SIgN Winter School on Advanced Immunology *Singapore (oral and poster presentation)*

Awards and scholarships

2011	Best oral presentation, 15th Molecular Medicine Day
2011	Best oral presentation, Journées Départmentales d'Immunologie
2012	Best oral presentation, NRS Longdagen
2012	Travel grant EAACI Winter School
2012	Travel grant IFReC-SIgN Winter School on Advanced Immunology

Curriculumn vitae

Roel Klein Wolterink

Born 4 May 1985

Education

1997 – 2003	**Johan de Witt Gymnasium** *Dordrecht (pre-university education)*
	Medicine *Erasmus University Rotterdam, The Netherlands*
2003 – 2006	Propedeutical exam, year 2 & 3
2006 – 2007	President IFMSA-NL (Int. Fed. of Medical Students' Associations) Dutch branch of an international organisation of medical students worldwide, over 300 members on all 8 medical faculties in The Netherlands
2007 –2008	Initiator and organiser of project Mpower! *Your body, your responsibility* Training of trainers program on basic health care in Mundri, Southern Sudan
2008 – 2008	Year 4 of medical studies
2013 – 2014	Clinical rotations (year 5 & 6 of medical studies); graduation: Dec 2014
	Research master Molecular Medicine *Erasmus University Rotterdam, NL* 2yr MSc program about molecular and cellular principles of health and disease
2004 – 2006	Courses
2008 – 2009	Courses and research project (6 months) The role of B cells in the development of iBALT in the lung under supervision of prof. dr. R. Hendriks (Pulmonary Medicine)
2009	Training Laboratory animal experimentation *(Article 9 official)*
2009 – 2010	Research project (1 year) Gata3 in early T cell development, Instiut Pasteur, Paris, France under supervision of prof. dr. J. Di Santo (Innate Immunity Unit)
	PhD training
2010 – 2013	Joint program *Erasmus University Rotterdam & Université Paris-Diderot* supervised by prof. dr. R. Hendriks and prof. dr. J. Di Santo funded by the Ligue Nationale Contre le Cancer (French Cancer Foundation)
	French Language & Culture *University of Amsterdam, NL*
2006 – 2007	Propedeutical exam, 1st semester
2008	Propedeutical exam, 2st semester; propedeutical exam passed

Scholarships and awards

2009	Huygens Scholarship Program (HSP) Talents programme Scholarship to allow excellent students to study abroad
2009	Prof. Bruins award, Erasmus University Rotterdam Award for the best research master student, to be used to study abroad
2009 – 2010	Royal Neth. Academy of Arts and Sciences (KNAW) assistants program Support for talented students interested in a scientific career

Skills

Languages	Dutch: mother tongue	French: fluent (oral & written)
	English: fluent (oral & written)	German: fair
Digital media	Good computer skills, DTP for print and web publications	
Creativity	Graphic design of print and web media, Photography	

Experience

Education and language

2002 – 2003	Trainer Verbredingsproject (suppl. courses) *Johan de Witt Gymansium*
2004 – 2010	Trainer of medical students during national and international meetings
2007	Trainer project Mpower! Your body, your responsibility
2008	Running a Training of Trainers programme for medical students

International experience

2005 – 2007	9 international meetings of IFMSA en EMSA worldwide and European organizations of medical students that strive for more awareness of global health
2005 – 2006	Chair of EMSA General Assemblies *Vienna (Austria) and Pécs (Hungary)*
2006 – 2007	Delegation leader IFMSA General Assemblies *Serbia and Australia*
2007 – 2008	Worked in very basic conditions in Southern Sudan

Other activities

2008 – 2009	Art Director Global Medicine *(student magazine about global health)*
2003 – present	Graphic design of print and digital publications (freelance projects)

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This thesis is the result of a collaborative effort of many people. I would like to thank everybody that contributed to the research described here. It is impossible to list everyone individually. Nevertheless, there are some people that I would like to acknowledge in particular.

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I would like to thank the organizers of the EAACI and the IFReC-SIgN Winter Schools for supporting my participation in their excellent trainings.

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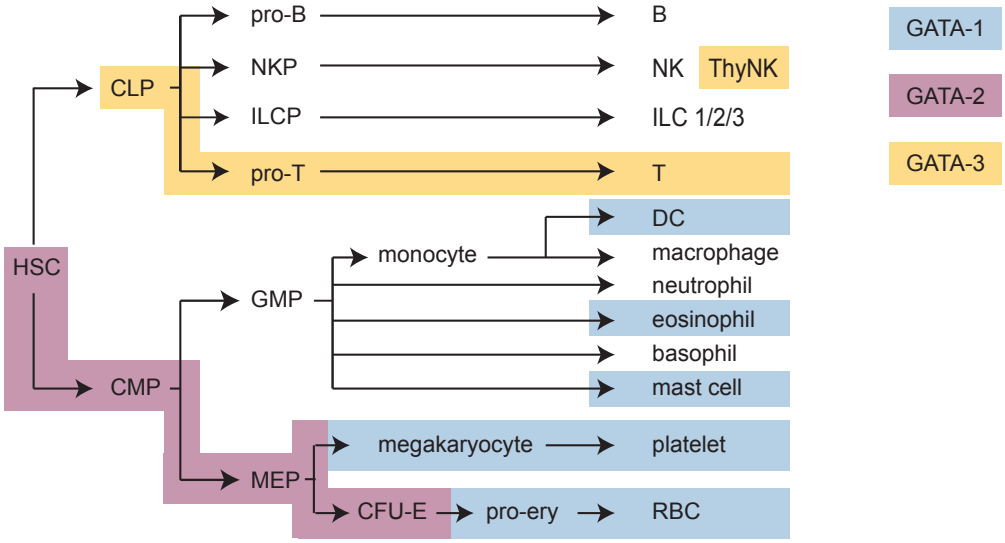
Lieve Emmaline, ik ben ontzettend blij met jou!

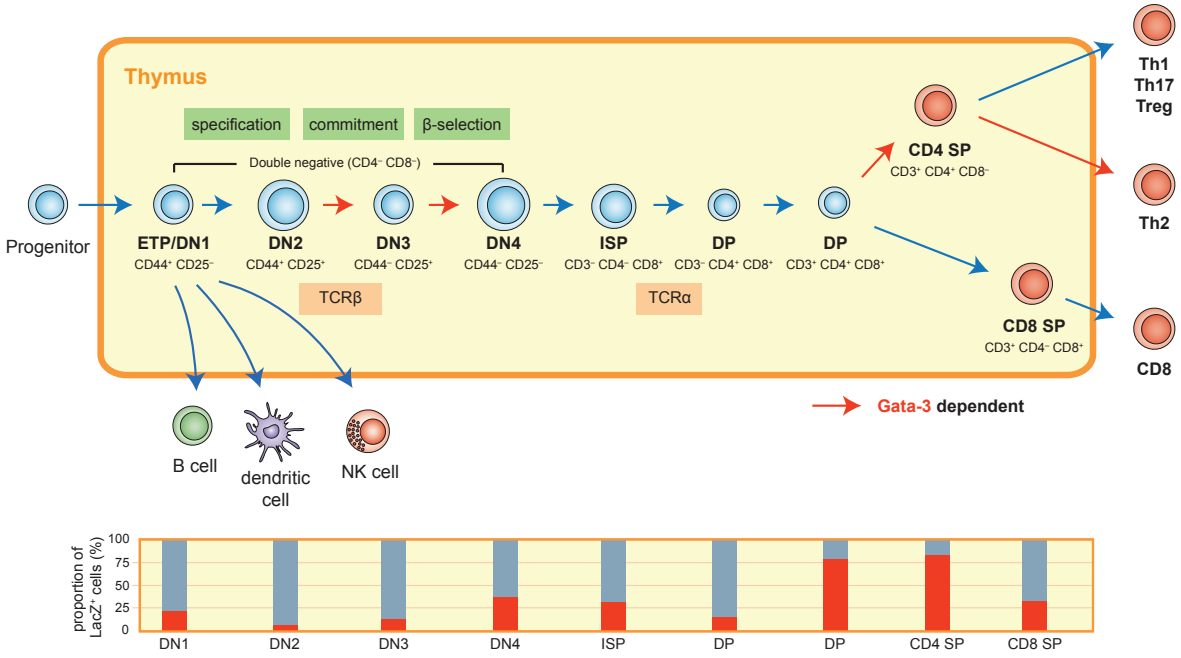
Appendix

Figures

Full color figures

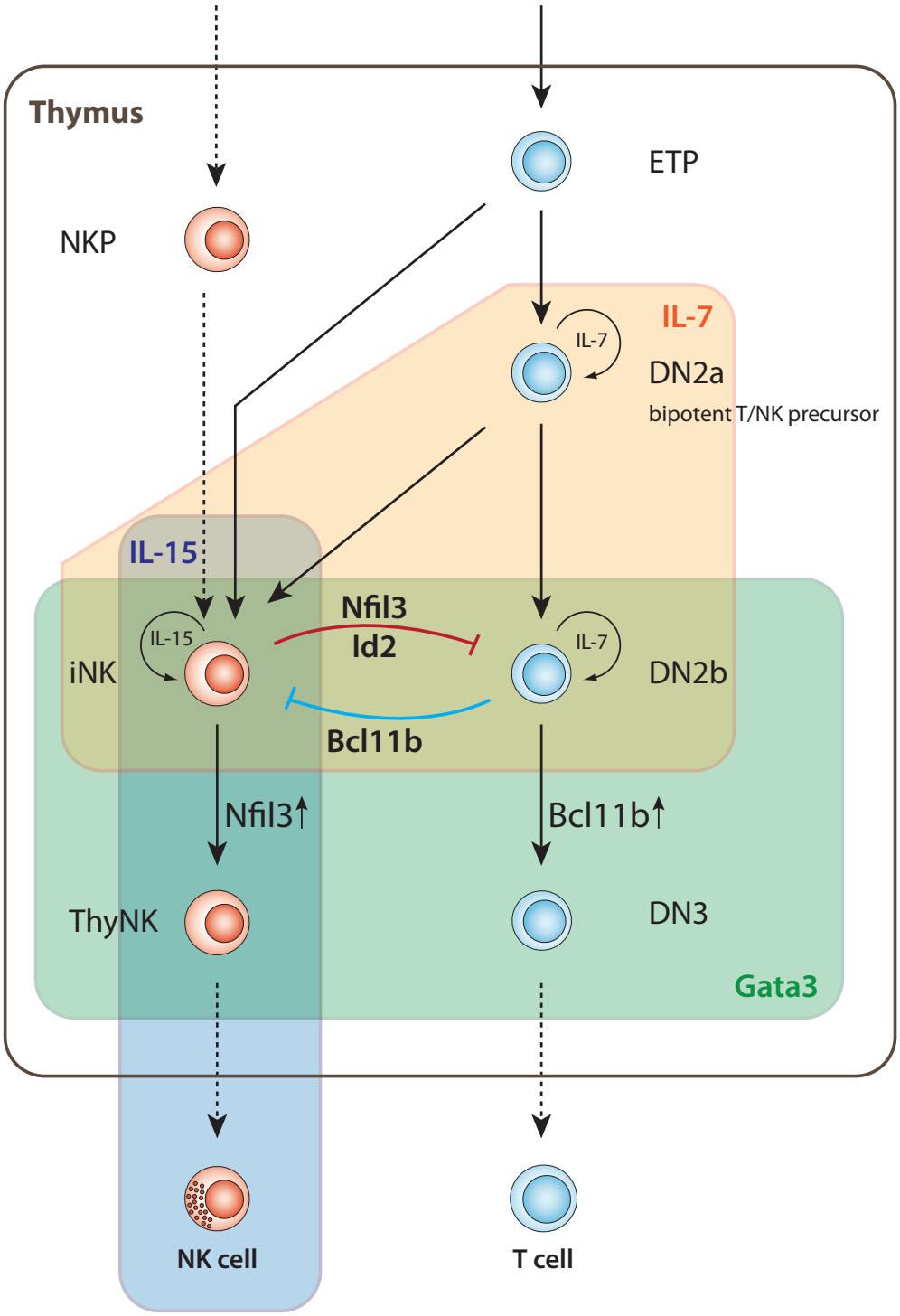


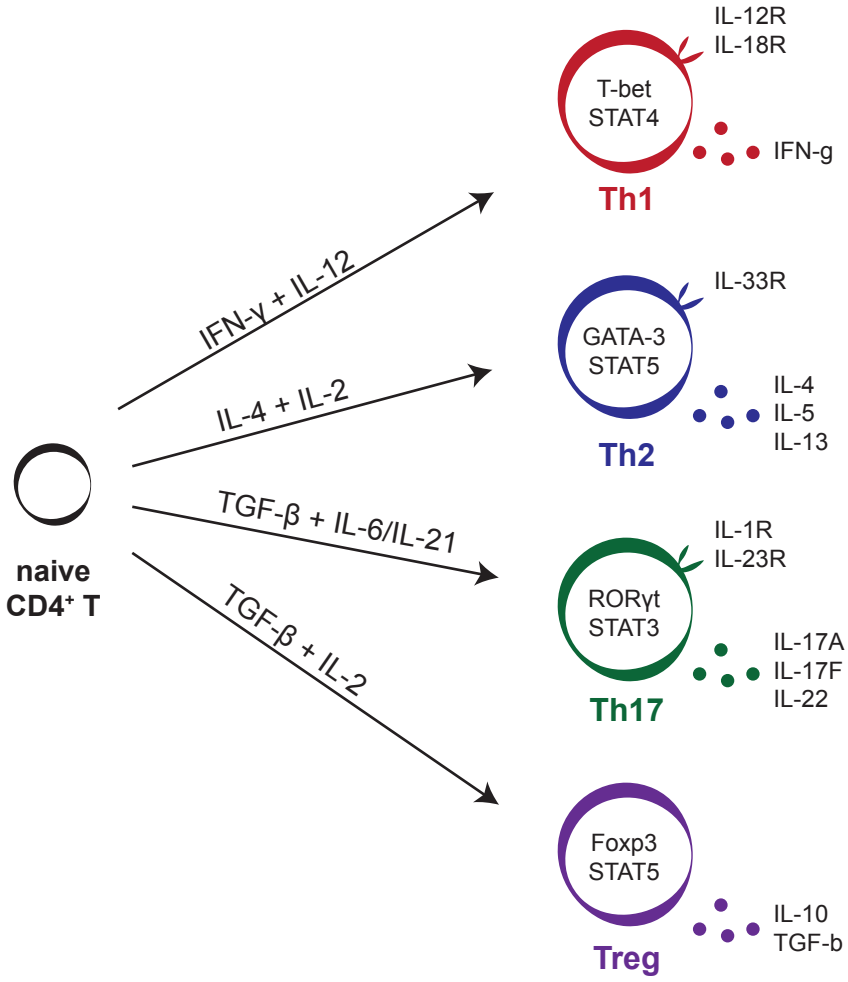
Chapter I – Figure 1. GATA transcription factors in hematopoiesis
This schematic overview shows the role of GATA transcription factors at various stages of hematopoiesis. Hematopoietic stem cells (HSC) are multipotent cells that can give rise to all lymphoid lineages via the common lymphoid precursor (CLP) and all myeloid lineages via the common myeloid precursor (CMP) (31,32,360). Other models that propose intermediate precursors between the HSC and lymphoid/myeloid-restricted precursor have also been reported (38,361). CLPs give rise to B and T lymphocytes and NK killer cells (NK), including a subset of thymic NK cells (ThyNK) that are GATA-3 dependent. Recently, CLP were also found to be precursors for innate lymphoid cells (ILC) that possibly share a common ILC precursor (ILCP). The role of GATA factors in ILC development is currently under investigation. Erythrocytes (or red blood cells; RBC) and platelets develop from a megakaryocytic-erythroid precursor (MEP) via colony forming units (CFU-E) and megakaryocytes respectively. Granulocytes, mast cells and monocytes stem from granulocyte/monocyte precursor (GMP). Monocytes can further differentiate into macrophages and dendritic cells (DC). Shaded areas show stage-dependent requirement of the indicated GATA factors.



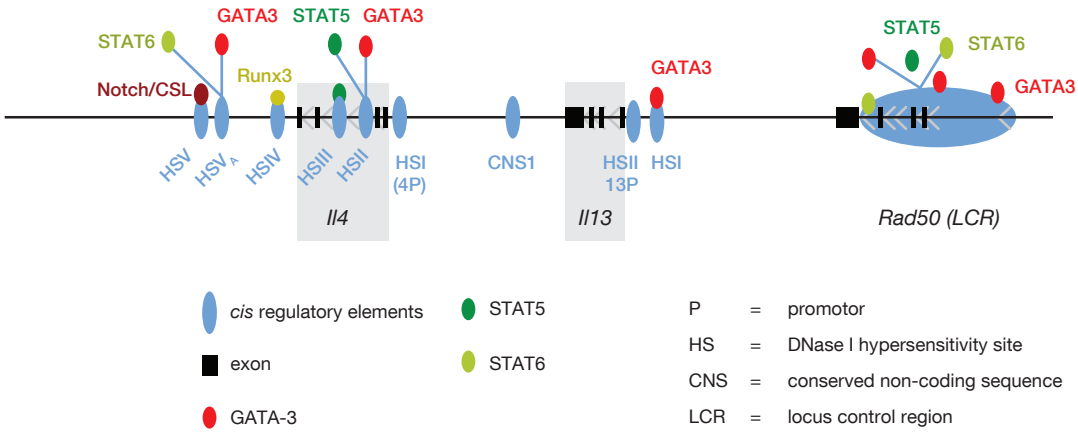
Chapter I – Figure 2. Overview of T cell development and GATA-3 expression in developing thymocytes.
T cell development starts with a multipotent progenitor that enters the thymus. During four $CD4^-$ $CD8^-$ double negative (DN) phases, early thymocytes subsequently initiate the T cell program (specification), exclude all other fates (commitment) and test their TCRβ chain for functionality after rearrangement (β-selection). DN cells then acquire CD8 to become immature single positive (ISP) cells and will start re-arrangement of the TCRβ chain. This process is completed at the double positive (DP) stage at which cells now express both CD4 and CD8. CD4- or CD8-committed cells lose the expression of the other co-receptor and become single positive (SP) cells that egress from the thymus. CD4 T cells can further differentiate into one of the T helper (Th) lineages, characterized by production of a specific set of cytokines. GATA-3 is one of the many transcription factors that steer T cell development. The various stages at which GATA-3 is crucially involved are shown with red arrows. Using a Gata3-lacZ knock-in mouse (*Gata3*$^{lacZ/+}$) and FDG substrate (146), the expression levels of GATA-3 in various T cell subsets were analyzed and are shown in the lower panel.

Chapter I – Figure 3. T and NK cell development from early thymocyte progenitors (ETPs) *(right page)*
ETPs that seed the thymus differentiate progressively towards the T-cell lineage via phenotypically defined intermediates. DN2a cells retain T and NK cell potential and expand in response to IL-7 (IL-7 responsive cells are indicated by an orange region). The DN2b subset is also IL-7 responsive, but has lost NK potential in a process including the upregulation of Gata3 (indicated by a green region). Upregulation of *Bcl11b* coincides with loss of IL-7 responsiveness and marks the progression to the DN3 stage where progenitors are fully T-cell committed. Immature NK cells (iNK) can derive from several pathways, including direct differentiation from ETP, via DN2a or from NK cell precursors (NKP) that seed the thymus. Thymic NK (ThyNK) cell differentiation depends on Nfil3 and requires IL-15 (shown by a blue region). Bcl11b and Nfil3/Id2 mutually inhibit the NK cell and T-cell pathways, respectively.

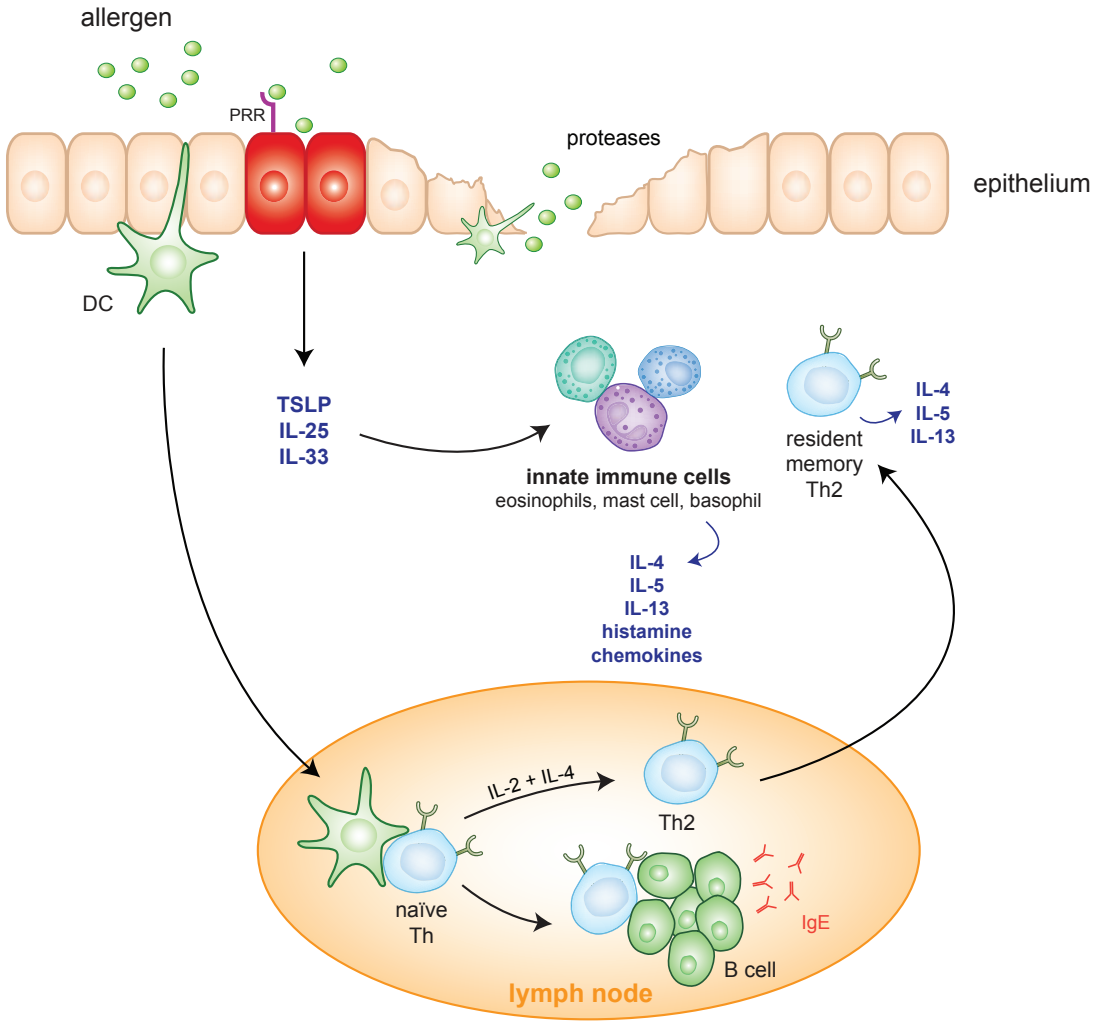

Thymus
ETP
NKP
IL-7
IL-7
DN2a
bipotent T/NK precursor
IL-15
IL-15
Nfil3
Id2
iNK
IL-7
DN2b
Bcl11b
Nfil3↑
Bcl11b↑
ThyNK
DN3
Gata3
NK cell
T cell



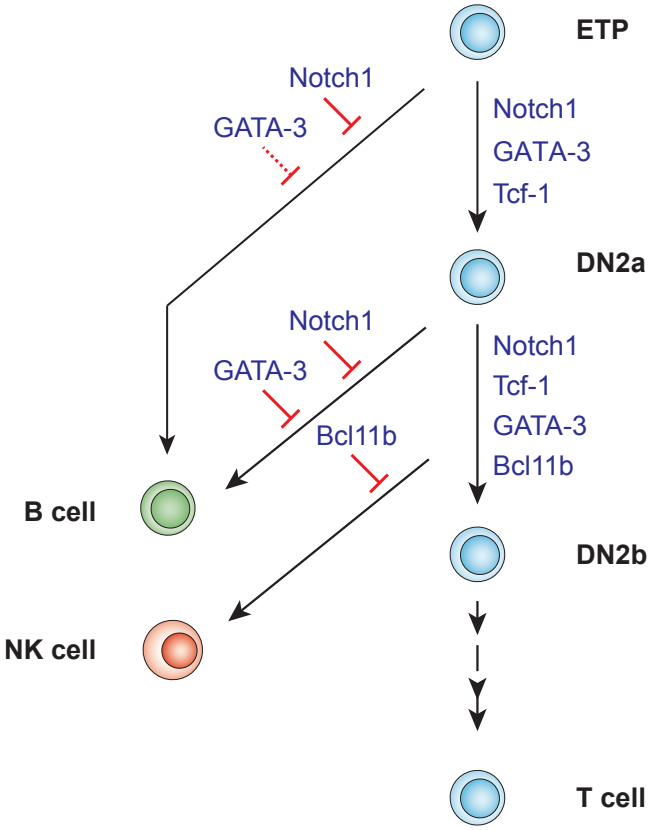
Chapter I – Figure 4. T helper cell differentiation
Schematic overview showing cytokine-induced differentiation of naïve T helper (Th) cells into various lineages. Naïve $CD4^+$ T cells are activated via TCR triggering by an antigen presenting cell (APC) such as a dendritic cell. Together with signals delivered by the APC, the cytokine milieu will steer differentiation into one of the Th lineages. Each lineage is characterized by a master transcription factor and an associated STAT protein. These master TFs are also involved in the secretion of a characteristic set of effector cytokines. All Th subsets are associated with an IL-1 family receptor that, if triggered, induced rapid secretion of cytokines independently of TCR activation.



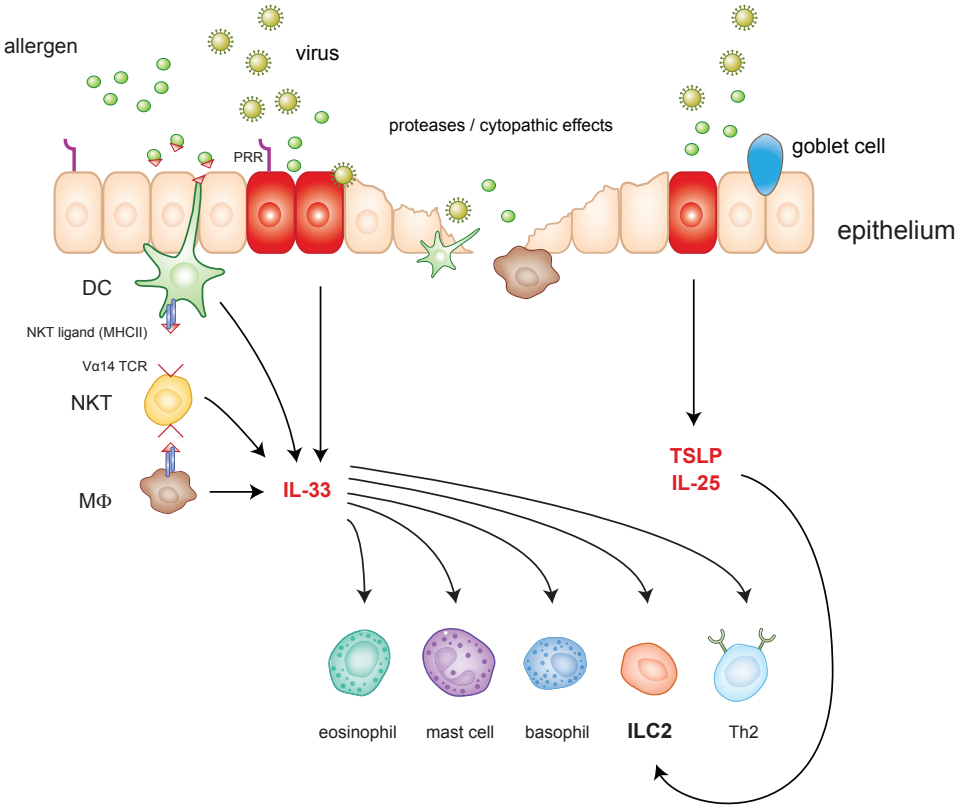
Chapter I – Figure 5. Cis-regulatory elements and binding transcription factors in the Th2 locus
The well-studied Th2 locus located on chromosome 5q23.3/5q31 (human) and 11qB1.3 (mouse) contains several regulatory elements, including the locus control region (LCR), CNS and various HS sites associated with the Il4 gene. Many transcription factors directly bind in the Th2 locus, including GATA-3, Notch/CSL, Runx3, STAT5, STAT6, c-Maf and NFAT (the latter two are not depicted). The figures represents a ~70kb region. The GATA-3 binding sites were recently confirmed in a genome-wide analysis of GATA-3 binding sites (362). Figure adapted from a review by Zhu and Paul (363).



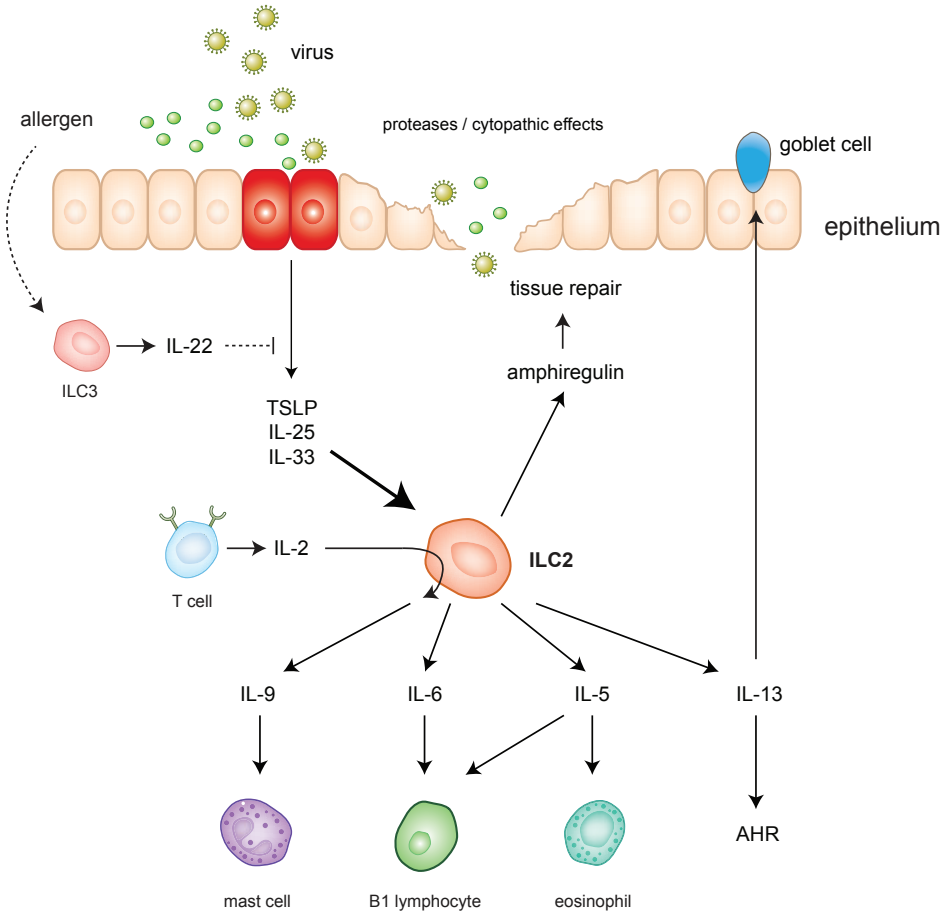
Chapter I – Figure 6. Dendritic cells link the adaptive and immune system in allergic inflammation.
Inhaled allergens, such as house dust mite, enter the airways and can activate epithelial cells and dendritic cells (DC) via pattern recognition receptors (PRR). Furthermore, DC constantly sample the airway lumen for peptides. Activated DCs migrate from the lung to the local draining lymph node where they interact with naïve T helper (Th) lymphocytes and present antigens. The combination of the cytokine milieu, namely the presence of IL-2 and IL-4, and factors expressed by DCs, such as OX40L and Notch ligand Jagged2, induces Th2 cell differentiation. Furthermore, follicular T helper cells (Tfh) promote B cell differentiation and IgE production. Differentiated Th2 cells will migrate to the lung, where they reside as memory cells and rapidly can produce important amounts of IL-4, IL-5 and IL-13. Innate immune cells are important for the initial response and produce type 2 cytokines and chemokines that attract other immune cells.



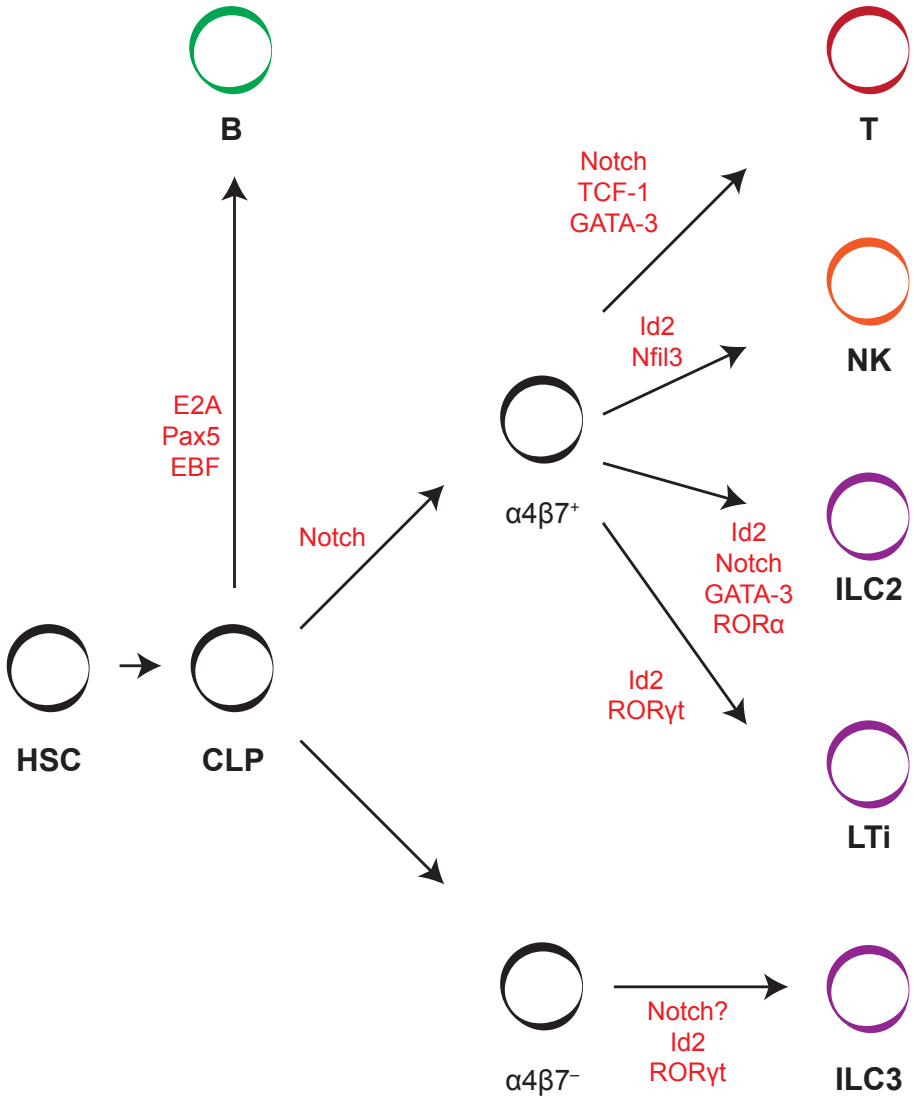
Chapter II – Supplementary Figure 9. Schematic overview of the role of GATA-3 in T cell development by suppression of a latent B cell potential in DN2a thymocytes.
Solid red lines indicate demonstrated effects and dashed lines indicate putative roles of the indicated transcription factor.



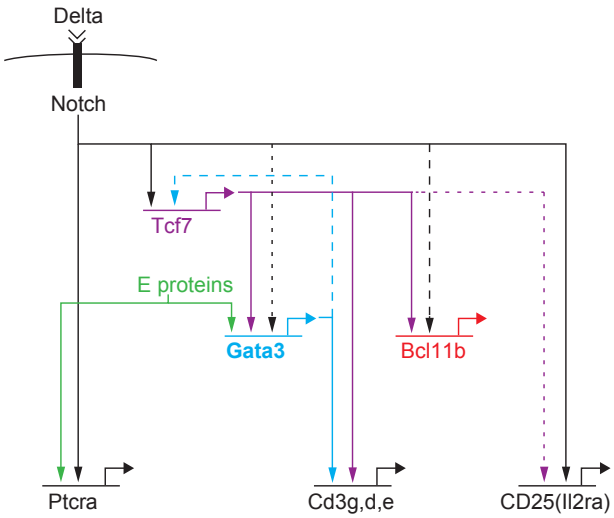
Chapter VI – Figure 1. Allergen- and virus-induced expression of IL-25, IL-33 and TSLP.
When allergens or influenza virus particles enter the airway, they activate airway epithelial cells via pattern recognition receptors (PRR), including Toll-like Receptors (TLR). Activated epithelial cells (in red) produce TSLP, IL-25 and IL-33 that activate a wide variety of immune cells. In addition, dendritic cells (DC) constantly sample the airway lumen and may directly produce IL-33. Furthermore, many allergens contain NKT ligands that are also sampled by DCs and then presented in the context of a MHC class II molecule to NKT cells, which will also produce IL-33. ILC2 can thus be activated via multiple pathways in response to allergen or influenza virus exposure.



Chapter VI – Figure 2. The multifaceted role of ILC2 in allergic airway inflammation.
Upon allergen exposure or influenza virus infection, the epithelium and immune cells that guard the epithelial wall rapidly produce important amounts of IL-25, IL-33 and TSLP. These cytokines activate ILC2 that will expand and produce various cytokines. ILC2 contribute to an asthma phenotype by production of IL-5 and IL-13 that induce eosinophil recruitment and AHR, respectively. If ILC2 are co-stimulated with IL-2 derived from T cells, ILC can also transiently produce IL-9 that will activate mast cells. ILC2 may also serve protective roles by stimulating tissue repair via amphiregulin and through stimulation of B1 lymphocytes that produce virus-neutralizing antibodies. In ovalbumin-induced airway inflammation, lung ILC3 are stimulated to produce IL-22 which limits the production of IL-33.



Chapter VI – Figure 3. Schematic overview of ILC development from hematopoietic precursors.
Common lymphoid precursors (CLP) develop from hematopoietic stem cells (HSC) and can give rise to all lymphoid lineages. The B cell lineage depends on expression of EBF and Pax5 that drive the B lineage program and inhibit development of T, NK and LTi cells. CLP that receive Notch signals develop into $\alpha4\beta7^{+}$ progenitors that have potential to develop into T cells, NK cells, ILC2 and LTi. These lineages have both specific (e.g. TCF-1, RORγt) as well as shared (GATA-3, Id2) requirements for transcription factors. ILC3 develop from $\alpha4\beta7^{-}$ precursors that, like LTi cells, depend on Id2 and RORγt expression.



Chapter VI – Figure 5. Gene network of transcription factors in initiation of T cell development.
Schematic overview of the relationship between Notch, TCF-1 (encoded by *Tcf7*), GATA-3, Bcl11b and their target genes. Notch signals are required for initiation of the T cell program, as they induce TCF-1, GATA-3 and (at later stages) Bcl11b expression. The program then uses a feed-forward mechanism in which GATA-3 and TCF-1 enhance each other's expression levels, ultimately inducing expression of Bcl11b which is required for further T cell development and for maintenance of T cell identity. Solid arrows indicate evidence from perturbation and binding studies. Dashed arrows indicate evidence from perturbation studies only. Adapted from Rothenberg (160).

Pictures in this thesis

Chapter I

Chapter II

Chapter III

Chapter IV

Chapter V

Chapter VI

Chapter VII

Chapter VIII

Appendix

Chapter I – A 'ger' that is still used today by the nomadic majority in Mongolia. Ger simply means 'house' in Mongolian. **Chapter II** – The 5033m high Mount Kazbeg is one of the highest Caucasus mountains in northern Georgia. **Chapter III** – The White House in Washington DC, United States of America. **Chapter IV** – One of the clocks in the Musée d'Orsay in Paris, France. The Basilique du Sacre Coeur can be seen in the background. **Chapter V** – Cité is a metro station on line 4 of the Parisian metro system and is located underneath the Ile de la Cité. **Chapter VI** – Mountain landscape near the Erdene Zuu Monastry, near the site of the old Mongolian capital Kharkhorin. **Chapter VII** – View from the Novosibirsk train station in the southwestern part of Siberia, Russia. **Chapter VIII** – Horses in the mountains near Yolyn Am in southern Mongolia. **Appendix** – Montains in Svaneti, the highest inhabited area in the Caucasus, located in northern Georgia.