Transcription factors controlling development and function of innate lymphoid cells

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Abstract

Innate lymphoid cells (ILCs) are a heterogeneous group of lymphocytes, which play an important role in tissue homeostasis at epithelial surfaces. They are scarce in spleen and lymph nodes, but substantial numbers can be found in the intestinal mucosa even at steady state. There, they represent the first line of defence against invading pathogens and contribute to lymphorganogenesis, tissue repair and, when inappropriately activated, immune pathology. Lineage-specific development, function and maintenance of these cells depend on a restricted set of transcription factors that partially emerged as a result of diversification and selection during vertebrate evolution. The differential expression of transcription factors regulates unique developmental programs, which endow the different ILC subsets with specific effector functions. Despite this division of labour, ILCs are considered to share a common origin, as they all are progeny of the common lymphoid progenitor, rely on the common \( \gamma \)-chain (\( \gamma_c \)) used by various cytokine receptors and show a developmental requirement for the transcriptional regulator Id2 (inhibitor of DNA binding 2). Here, we review the transcriptional programs required for the development and function of ILCs and give an overview of the evolution of transcription factors and cytokines expressed by ILCs.

Keywords: evolution, GATA-3, ILC, inflammation, lymphorganogenesis, ROR\( \gamma \)-t, T-bet

Introduction

The last decade has witnessed a resurgence of research into the intestinal immune system. This was driven by the insight that the design principle of the intestinal immune system is not one built on the best possible separation of intestinal microbiota and components of the intestinal immune system to avoid inflammatory signalling. Instead, the vertebrate host continuously receives signals from the intestinal microbiota that were found to generate transcriptional circuitry supporting intestinal organ homeostasis (1–4). In addition, new technology has allowed us to appreciate the diversity of the intestinal microbiota, which drove discovery of specific bacterial species regulating tolerance (5, 6) and immune system differentiation at mucosal surfaces (7, 8). Among the important findings in this field of research was the discovery of previously unknown innate lymphocyte subsets that are located at barrier surfaces. Together with NK cells and lymphoid tissue inducer (LTi) cells, they are now collectively referred to as ‘innate lymphoid cells’ (ILCs). ILCs are a functionally diverse group of cells that are derived from the common lymphoid progenitor (CLP) but lack somatically re-arranged immune receptors, distinguishing them from cells of the adaptive immune system, such as T and B cells. The various ILC subsets may be related and may even emerge from a common ILC progenitor (CILP) because all ILC subsets require expression of the transcriptional regulator Id2 (inhibitor of DNA binding 2) for their development. Although it is unknown which receptor systems ILCs use to discriminate between ‘self’ and ‘non-self’ or ‘altered-self’, some ILC subsets express germline-encoded receptors previously identified to be expressed by NK cells (e.g. NKG2D, NKp46, NKp44). While the role of such NK receptors for development and function of ILCs is not known, ILCs can also readily respond to a vast array of cytokines such as IL-1, IL-12, IL-7, IL-23, IL-25, IL-33 and thymic stromal lymphopoietin (TSLP). It is well documented that ILCs play a relevant role in lymphorganogenesis (9–13), tissue homeostasis and integrity (14) and the immune response against invading pathogens (15–17), but they can also cause inflammatory disorders if
improperly activated (18–21). One of the most striking patterns observed was the fact that the newly identified ILC subsets express the same cell fate-determining transcription factors as the various T helper cell effector populations (i.e., T-bet, Eomes, GATA-3 and RORγt), and that these transcription factors coordinate a similar gene expression program. On the basis of these findings, a recent consensus report (22) has defined three distinct groups of ILCs: group 1 ILCs expressing T-bet and/or Eomes and producing type 1 cytokines (e.g. IFN-γ, TNF), group 2 ILCs expressing GATA-3 and producing type 2 cytokines (e.g. IL-5, IL-13) and group 3 ILCs (ILC3s) expressing RORγt and producing ‘type 17’ cytokines (e.g. IL-17A, IL-17F, IL-22).

Here, we discuss how a conserved set of transcription factors plays lineage-defining roles for the different groups of ILCs. We begin by describing early events during vertebrate evolution, which led to the emergence of the relevant transcription factors. We then detail the different ILC subsets by describing the role of transcription factors for their development and function.

**ILC fate-determining transcription factors during vertebrate evolution**

Coinciding with the appearance of vertebrates around 540 million years ago, there was a striking increase in the genomic material in early vertebrate species due to one (jawless vertebrates) or even two (jawed vertebrates) rounds of whole-genome duplication (23). From a Darwinian perspective, this allowed for a process of diversification and selection of new genes providing vertebrates with many advantages in a challenging environment. For example, T-box genes can be grouped into five subfamilies, and T-box genes within the same subfamily have arisen from duplication of a single ancestral gene (24). Interestingly, the T-brain 1 subfamily in vertebrates consists of Eomesoderm (Eomes), Tbr1 and Tbx21 (encoding T-bet), and comparative analysis has demonstrated that the major role of the single ancestral gene in deuterostomes included the specification of mesoderm and anterior neural structures, a function still maintained by Eomes and partially by Tbr1. In contrast, Tbx21 lost both ancestral roles and together with Eomes acquired novel functions in the immune system (24, 25). The fact that amphioxus, which is the single extant cephalochordate and hence the closest living invertebrate relative to vertebrates, harbours only a single member of the T-brain 1 subfamily supports the notion that T-box transcription factors with specific roles in the immune system first developed in vertebrates.

Intriguingly, the development of the T-box gene family was paralleled within the GATA family. Originating from two ancestral GATAs, which are still present in some invertebrate deuterostomes, six GATA factors have been identified in vertebrates: GATA-1, 2 and 3 comprise one subgroup, while GATA-4, 5 and 6 constitute the other (26). After the first round of whole-genome duplication, a GATA-1 and a GATA-2/3 intermediate were generated. This is also supported by transcriptional analysis of hagfish (jawless vertebrates) leukocytes, which express the GATA-2/3 intermediate but no dedicated GATA-2 or GATA-3 orthologue (27). The second round of duplication resulted in the emergence of GATA-2 and GATA-3. GATA-3 must have played a very early role in T-cell development during vertebrate evolution as even skates (Chondrichthyes), which are a representative of the earliest jawed vertebrates, express a clear ortholog to the mammalian counterpart that shows high levels of expression in the skate thymus (28).

RORγt-expressing ILCs (RORγt+ ILCs) in mammals are typically connected with the presence of lymph nodes. However, lymph nodes first appeared in birds, and the necessary genes of the TNF receptor family may not exist in the genome of lower vertebrates (29, 30). It should be noted though that lymphoid tissue formation in the gut lamina propria [cryptopatches (CPs) and isolated lymphoid follicles (ILFs)] is evolutionarily more ancient, and ILCs are a major constituent of the lymphoid compartment in the lamina propria. CPs are small lymphoid clusters containing RORγt+ ILCs and surrounded by CD11c+ mononuclear phagocytes. Perhaps, CPs facilitated communication between ancient lymphocytes and components of the myeloid system forming the ‘blueprint’ of future, more complex lymphoid structures such as Peyer’s patches, lymph nodes and ILFs that allow for communication between adaptive and innate immune system components. Another original function of RORγt+ ILCs was most likely the secretion of cytokines such as IL-22 and those of the IL-17 family. It has been demonstrated that IL-17D homologues are already present in molluscs (Crassostrea gigas), nematodes (Caenorhabditis elegans) and echinoderms (Strongylocentrotus) and stimulation of C. gigas with bacteria results in the production of IL-17 (31, 32). However, it remains to be determined whether the secretion of IL-17 homologues in lower vertebrates and invertebrates is regulated by Rorc(γt) (gene encoding RORγt) homologues, which, so far, have been cloned only from jawed vertebrates including zebra fish and trout (33, 34).

The overwhelming majority of our immune cells is situated in the intestine, and the gut-associated lymphoid tissue, together with the spleen, was the first secondary lymphoid organ to develop (29). As outlined above, lymph nodes started to develop later and are a hallmark of the mammalian immune system. The reason for this chain of events is not fully clear at this moment, but it is likely that one of the first immunological dilemmas faced by our vertebrate ancestors was to distinguish between commensal microbiota and potentially life-threatening infections caused by viruses, bacteria, yeast and worms, all trying to gain access through the intestinal lumen. Although the innate immune response is present in all metazoans (35) and can be executed by cells of haematopoietic and of non-haematopoietic origin, it lacks a specific effector response that is targeted at the invading pathogen. Therefore, it is conceivable to assume that the above-described process of diversification resulted in the selection of transcription factors that regulated development and function of new innate immune cells, cells that maintained tissue integrity and mutualism with the commensal microbiota while at the same time specifically attacking invading pathogens. Thus, the earliest vertebrates could develop sophisticated strategies, which could fill a gap in their intestinal defence mechanism. These strategies could be then tested intensively in the intestine of our vertebrate ancestors and being
denied ‘fit for purpose’ they were adopted by the adaptive immune system.

**Group 1 ILCs**

Group 1 ILCs (ILC1s) are defined by their dependency on the T-box transcription factors T-bet and/or Eomes and by the secretion of the signature cytokine IFN-γ (22). All currently recognized ILC1 subsets express NK receptors such as Nkp46, NKG2D and NK1.1 in C57BL/6 mice or CD56, NKG2D, NKP46, and CD161 in humans (36, 37). In general, ILC1s can be broadly divided into subsets that require IL-15 or IL-7 for their development and/or maintenance. IL-15-dependent ILC1 subsets represent bona fide NK cells, which are the major ILC1 populations in spleen, lymph nodes and liver (38, 39). The bone marrow is the primary site for NK cell development, where NK cells develop from the CLP (40) in a process that requires among other factors the transcriptional regulators Ets-1 (41), Pu.1 (42), MEF (43), IRF2 (44), Id2 (11), NFIL3/E4BP4 (45, 46), and Tox (47). For a detailed discussion of these factors in NK cell development, refer to excellent recent reviews (48). In contrast to IL-15-dependent NK cells, IL-7-dependent ILC1s are primarily present in the intestine and at other mucosal sites (37), but IL-7-dependent ILC1s remain a poorly explored ILC subset.

The T-box transcription factors T-bet and Eomes have important roles in NK cell development, and mice deficient in either of these T-box transcription factors show substantially impaired NK cell numbers and diminished function of splenic NK cells, which is further aggravated by the combined deficiency of the two factors (49, 50). However, T-bet and Eomes are not interchangeable in NK cell development, and the respective knockouts show relevant differences. In contrast to T-bet, the effect of Eomes on NK cell development can only be evaluated in conditional knockout mice because Eomes deficiency causes embryonic lethality. Recently, it became clear that deletion of Eomes in all haematopoietic cells (Vav-Cre x Eomes<sup>fl/fl</sup>) affects not only CD8 memory T cells but also NK cells (50). This is in line with their constitutive expression of Eomes in steady state as opposed to other haematopoietic lineages. With respect to CD8 memory T cells, conditional Eomes knockout mice lack central memory T cells (CD44<sup>+</sup> CD62L<sup>+</sup>), and infection experiments in mixed bone marrow chimeras mice could show that Eomes, by a yet unknown mechanism, determines the ability of activated CD8 T cells to compete for the memory cell niche (51). With regard to NK cell development, conditional Eomes knockout mice have dramatically reduced numbers of mature NK cells in the spleen and bone marrow and the remaining NK cells show an immature phenotype (50). Mature NK cells can be identified based on the expression of CD49b (integrin α<sub>γ</sub>, recognized by the monoclonal antibody DX5), CD11b (integrin α<sub>γ</sub>δ), CD43 and KLRG1, which are acquired in an orderly fashion during maturation (52). CD49b is of specific importance as its expression co-segregates with the acquisition of potent effector functions and is the first marker to be expressed on mature NK cells (53). Interestingly, Eomes expression in immature NK cells coincides with the appearance of CD49b<sup>+</sup> (i.e. mature) NK cells. Eomes seems to be specifically required for this maturation step in the bone marrow as conditional Eomes knockout mice have a developmental arrest at the CD49b<sup>+</sup> stage. Consequently, Eomes-deficient mice virtually lack all CD49b<sup>+</sup> NK cells in spleen, liver and lymph nodes and harbour only low numbers of immature NK cells in the periphery (50).

While Eomes is up-regulated during the transition of CD49b<sup>−</sup> immature NK cells to CD49b<sup>+</sup> mature NK cells, T-bet expression is already found in immature NK cells. In contrast to conditional Eomes knockout mice, Tbx21<sup>−/−</sup> mice show an accumulation of CD49b<sup>−</sup> NK cells in the bone marrow and lymph nodes as T-bet regulates egress of mature NK cells from the bone marrow via induction of sphingosine-1-phosphate receptor 5 (S1P<sub>5</sub>) (54). In contrast to Eomes-deficient mice, splenic NK cell numbers are only mildly reduced in Tbx21<sup>−/−</sup> mice and they express normal levels of the maturation markers CD49b and CD11b, suggesting that development and differentiation of NK cells is more dependent on Eomes than on T-bet. However, survival of Tbx21<sup>−/−</sup> NK cells was impaired, and their proliferation was increased. While Tbx21<sup>−/−</sup> NK cells produced virtually normal amounts of IFN-γ after cytokine stimulation, IFN-γ production following infection with murine cytomegalovirus was substantially reduced (49).

The liver harbours, in addition to CD49b<sup>+</sup> Eomes<sup>+</sup> conventional NK cells, a unique IL-15-dependent NK cell population that lacks CD49b expression and other maturation markers like Ly49 receptors and CD11b, but expresses TNF-related apoptosis-inducing ligand (TRAIL), also expressed by a subset of bone marrow precursors believed to be immature NK cells (55). In contrast to splenic CD49b<sup>−</sup> NK cells in spleen and liver, which uniformly express Eomes and T-bet, TRAIL<sup>−</sup> CD49b<sup>−</sup> liver NK cells do not express Eomes but are T-bet<sup>+</sup> (50). In contrast to splenic NK cells, TRAIL<sup>−</sup> CD49b<sup>−</sup> liver NK cells showed a cell-intrinsic requirement for T-bet but not for Eomes to develop (50). However, both cells are developmentally related as they both depend on IL-15 for their development and both can be derived from CD27<sup>+</sup> CD11b<sup>+</sup> bone marrow NK cells (38, 50). Deletion of Eomes in TRAIL<sup>−</sup> CD49b<sup>−</sup> T-bet<sup>+</sup> Eomes<sup>+</sup> splenic NK cells reverted them into TRAIL<sup>−</sup> CD49b<sup>−</sup> T-bet<sup>−</sup> Eomes<sup>−</sup> NK cells that migrated to the liver (50). The same study demonstrated that TRAIL<sup>−</sup> CD49b<sup>−</sup> liver NK cells could differentiate into TRAIL<sup>−</sup> CD49b<sup>+</sup> splenic NK cells, albeit at a very low frequency (50). These data imply that Eomes stabilizes the transcriptional program of mature TRAIL<sup>−</sup> CD49b<sup>−</sup> NK cells and that TRAIL<sup>−</sup> CD49b<sup>−</sup> NK cells in the bone marrow and elsewhere are the precursors to TRAIL<sup>−</sup> CD49b<sup>−</sup> T-bet<sup>+</sup> Eomes<sup>+</sup> NK cells (50). However, such a model would put T-bet upstream of Eomes. This model is not consistent with the fact that conditional Eomes knockout mice have a more severe reduction of NK cell numbers and an almost complete block in NK cell maturation (Y. Tanriver and A. Diefenbach, unpublished results). Furthermore, Tbx21<sup>−/−</sup> mice have an almost normal compartment of mature NK cells, which is not consistent with a linear differentiation of TRAIL<sup>−</sup> CD49b<sup>−</sup> T-bet<sup>−</sup> Eomes<sup>−</sup> NK cells from a T-bet-dependent TRAIL<sup>−</sup> CD49b<sup>−</sup> precursor.

As mentioned above, the IL-7-dependent subset of ILC1s is primarily found in the intestine, where it can be distinguished from bona fide NK cells based on the expression of CD127 (IL-7Rα). These cells do not express RORγt and were first found in lineage-tracing experiments for RORγt (RORγt...
fate map), a genetic method stably and heritably labelling RORγt-expressing cells even if they lose expression of this transcription factor during lineage progression (18, 56). IL-15-dependent NK cells are RORγt fate map negative, demonstrating that they do not express RORγt at any time of their lineage differentiation (18, 56). In these lineage-tracing experiments, two additional CD127− NK1.1+ NKp46+ ILC1 subsets were identified that were either RORγt fate map positive or RORγt fate map negative. We were able to show that the RORγt fate map-positive subset of ILCs are derived from bona fide ILC3s that have down-regulated RORγt expression allowing for functional and phenotypic plasticity (18), which has also been supported by data from human ILC3s (36, 57). These ‘ex-RORγt’ ILC3s will be best discussed later in the context of ILC3s. The CD127+ Lin− NK1.1+ NKp46+ RORγt fate map-negative ILCs may be bona fide ILC1s but remain to be characterized in depth.

Recent work has demonstrated that ILC1 subsets other than NK cells are also present in humans. CD3− CD56− NKp44+ CD103+ ILC1s that expressed T-bet but were negative for RORγt were found within the epithelial layer of tonsils and in the intra-epithelial lymphocyte (IEL) compartment of the intestine (37). Conspicuously, CD127 expression was absent from this ILC subset (37). This innate IEL subset failed to produce IL-22 upon stimulation with IL-23 but produced copious amount of IFN-γ after stimulation with IL-15 and IL-12. In addition, these cells were able to execute cell-mediated cytotoxicity. The authors also identified a related T-bet-expressing IEL subset in mice characterized by the expression of NK1.1, NKp46 and CD160. While NK cells are virtually absent in Il15ra−/− mice (39), this population was only marginally affected, suggesting that these cells are an ILC1 subset distinct from NK cells. Their development required expression of E4bp4 and T-bet. It remains to be established whether the described human and murine ILCs are true counterparts as the surface marker profile and effector phenotype of human CD3− CD56− NKp44+ CD103+ ILC1s would also be in agreement with epithelial NK cells. In a separate study, a CD127+ Kit− NKp44− human ILC1 subset was characterized, which primarily resides in the lamina propria of the intestine (36). These cells expressed high levels of Tbx21 and low levels of Rorc. Upon stimulation with IL-12 and IL-18, they produced IFN-γ. These cells were distinct from NK cells as they lacked expression of cytotoxic effector molecules (perforin and granzyme B) and the NK cell-related marker CD56. Intriguingly, Lin− CD127+ Kit− NKp44− human ILC1s accumulated in the lamina propria of patients with active Crohn’s disease and thus might play a role in the disease process (36). Using reconstitution of allogeneic mice with human hematopoietic stem cells, the authors found that Lin− CD127+ Kit− NKp44− human ILC1s can be derived from RORγt+ ILC3s under the influence of IL-12 signals. Thus, Lin− CD127+ Kit− NKp44− human ILC1s may be part of the ILC3 lineage, which is highly reminiscent of the previously recognized plastic ‘ex-RORγt’ ILC3s in mice (see above).

Based on surface markers, effector molecules and transcriptional regulation, it is most likely that the above-described human studies have identified two distinct ILC1 populations. Due to their different anatomic location, one might speculate that these cell subsets act at different steps during intestinal inflammation and may play distinct roles during the steady state.

Group 2 ILCs

Group 2 ILCs (ILC2s) are defined by the secretion of the signature cytokines IL-5, IL-9, IL-13 and amphiregulin, a member of the epidermal growth factor family (58). ILC2s developmentally depend on the transcription factors Id2 (59, 60), GATA-3 (60) and RORγt (61, 62) and on the cytokines IL-7 (59) and Flt3L (63). Given the ‘type 2 cytokine signature’ of ILC2s, they may play essential roles in the resistance against worm infections (59, 64), contribute to the regeneration of tissue after viral infections (14) and participate in immune pathologies such as allergic asthma (20) or airway hyper-responsiveness (21). In 2001, it was known that there was an innate immune cell type capable of promptly producing high levels of IL-5 and IL-13 (65), but only more recently three independent research groups were able to unanimously identify these cells [variably dubbed as natural helper cells (59), nuocytes (64) or innate type 2 helper cells (66)] and demonstrated their relevant role in early immunity to worm infections.

As all other ILC subsets, ILC2s are rare in secondary lymphoid organs but are substantially represented in the lamina propria of the intestine. In contrast to ILC3s, ILC2s are not well represented in the newborn gut. Indeed, ILC2s are generated in the bone marrow where they derive from the CLP (63). We were recently able to identify a lineage-specified ILC2 precursor (ILC2P) in the bone marrow, which is defined as Lin− Sca1+Id2−Gata-3+ (LSIG cells) co-expressing markers also found on mature ILC2s [e.g. IL-1RL1 (the IL-33 receptor), IL-17RB (an IL-25 receptor subunit)] or on most other ILC subsets (e.g. Thy1, CD25, CD127) (60). However, in contrast to peripheral mature ILC2s, ILC2Ps lacked expression of KLRG1 (an inhibitory immunoreceptor binding E-cadherin) and the receptor tyrosine kinase Kit and were only very poor producers of IL-5 and IL-13. However, stimulation of ILC2P with IL-33 induced KLRG1 expression, and these cells became potent producers of IL-5 and IL-13. Using an inducible conditional knockout system for Gata-3 in all ILCs, we could further demonstrate that Gata-3 had two important roles in ILC2s. First, Gata-3 was indispensable for the differentiation of ILC2Ps, which were diminished in numbers in Gata-3-deficient mice. Second, Gata-3 was vital for the peripheral maintenance of mature ILC2s because induced deletion of Gata-3 led to a rapid decline in mature ILC2s (60). Two recent studies corroborated and extended these findings (67, 68). Analysis of mice that express one or two Gata3 alleles or supernormal numbers of Gata3 alleles (i.e. Gata3 transgenic mice) established a dose-dependent effect of Gata-3 on ILC2 development, function and maintenance. Indeed, there was a direct correlation between Gata3 gene dosage and ILC2 numbers, type 2 cytokine (IL-5, IL-13) production and IL-25 and IL-33 receptor expression (67). Another recent report identified the MAPK p38 as an essential intracellular signalling mediator that activates Gata-3 via phosphorylation after stimulation of ILC2s with IL-33 or IL-2 plus IL-25 (68). Phosphorylation of Gata-3 is essential as it allows Gata-3 to translocate and bind to regulatory regions of the Il5 and Il13 genes. Inhibiting the action of p38 upon
activation of ILC2s profoundly inhibited their production and secretion of IL-5 and IL-13 (68). Collectively, these findings are highly reminiscent of the diverse roles of GATA-3 for Tc2 cell differentiation and maintenance (69, 70). GATA-3 is considered the ‘master transcription factor’ for the Tc2 cell fate by directly controlling several regulatory elements at the Tc2 cytokine gene loci (71). The relevance of GATA-3 for ILC2 function has also been demonstrated for human ILC2s. Overexpression or repression of GATA-3 in human ILCs revealed that GATA-3 is important in regulating the response of ILC2s to IL-33 and TSLP and in transactivating IL-4, IL-5, IL-13 and GM-CSF production (72). Murine studies have shown that GATA-3 regulates the expression of IL-1RL1, IL-17RB and CD25 (the IL-2Rα subunit), a cytokine receptor expression pattern that is specific to ILC2s. IL-25 and IL-33 play a partial redundant role in the activation and expansion of ILC2s during worm infection; however, no significant reduction in ILC2 numbers has been reported in mice that lack the corresponding receptor subunits (64). Transcriptional profiling of ILC2s revealed that they express high levels of RORα. Correspondingly, staggerer mice (Rora<sup>−/−</sup>), which carry a deletion within the ligand-binding homology domain of RORα (leading to a functional null mutation) (73), have an intrinsic defect in the development of ILC2s and impaired worm clearance after infection (62). Albeit reduced in numbers, the remaining ILC2s in Rora<sup>−/−</sup> mice produced similar amounts of IL-5 and IL-13 on a per cell basis compared with wild-type ILC2s when stimulated with IL-33. Finally, GATA-3 levels were unaffected in Rora<sup>−/−</sup> mice, arguing against a direct transcriptional control of Gata3 through RORα (68).

In addition to GATA-3 and RORα, T-cell factor 1 (TCF-1, encoded by the Tcf7 gene) was required for ILC2 development. TCF-1 acted downstream of Notch signalling in ILC2s, as retroviral over-expression of TCF-1 in bone marrow progenitor cells, that lacked Notch signalling, could rescue ILC2 development (74). Upon induction, TCF-1 then induced the expression of GATA-3 and IL-7Rα by directly binding to their respective regulatory regions to further propel ILC2 development (74) (Fig. 1). This chronology shows extensive parallels to early thymic T-cell development, which is further corroborated by the fact that ILC2s can be derived from DN1 and DN2 thymic progenitors and have an overlapping transcriptional profile with T cell-committed DN3 progenitors (74).

**Group 3 ILCs**

ILC3s are the most complex group of ILCs, all of which strictly depend on the transcription factor RORγt and on the cytokines IL-7 and TSLP (13, 18, 22, 75–78). ILC3s produce ‘type 17’ cytokines such as IL-17A, IL-17F and/or IL-22 (75–77, 79–81) and have been involved in immunity to intestinal infections (15) but have also been implicated in autoimmune diseases when inappropriately stimulated (18, 19).

The first subset of ILC3s identified were LTi cells (9). LTi cells play a crucial role in the formation of secondary lymphoid organs during the foetal (lymph nodes, Peyers patches) and the early neonatal (intestinal CPs, ILFs) periods. Through their surface expression of lymphotixin (LT) α<sub>4</sub>β<sub>7</sub>, LTi cells can interact with LT<sub>β</sub> receptor-expressing stromal and endothelial cells. This initiates a cascade of controlled local "inflammatory" signalling (82) that attracts different haematopoietic cell subsets, which ultimately results in the formation of secondary lymphoid organs (83). Besides their role during early development, subsets of ILC3s also participate in the structural reorganization of lymphoid structures after an assault. Originally, LTi cells were described as a non-B-/non-T-cell lymphocyte subset recruited to lymph node and Peyers patch anlagen and characterized by the expression of CD4, integrin α<sub>4</sub>β<sub>7</sub>, and CD127 (9, 84). Later, it was demonstrated that these cells continuously express RORγt (13) and that they developmentally depend on RORγt, Runx1 (85), Id2 (11) and Fox (47) (the last three are not specific to ILC3s, though). LTi cells continuously express RORγt and are therefore referred to as RORγt<sup>+</sup> ILCs (18, 86). Other ILC subsets (ILC1s, ILC2s) do not express this transcription factor at any stage during their development (59, 60). Within the foetal liver, Id2 and Notch signalling induce Lin<sup>−</sup> integrin α<sub>4</sub>β<sub>7</sub>-IL-7Rα<sup>+</sup> cells from CLPs that can further differentiate into LTi precursors (87). ILCs with a profile reminiscent of foetal LTi cells, including the expression of CD4, can also be found in adult mice, where they maintain the ability to induce secondary lymphoid organs (88). Although LTi cells are present in various organs, in adult mice the overwhelming majority is located in the lamina propria of the terminal ileum. Here, they instruct the formation of so-called CPs within the first 2 to 3 weeks of life, a process that is independent of the intestinal microbiota (89) but requires LTα<sub>4</sub>β<sub>7</sub>-induced LTβR signalling (90, 91). CPs are an accumulation of RORγt<sup>+</sup> ILCs surrounded by an ill-defined population of CD11c<sup>+</sup> mononuclear phagocytes. B cells are recruited to CPs that are then referred to as ILFs (92–95). B cell recruitment is partly orchestrated by chemokines that are produced by stroma cells once in contact with LTα<sub>4</sub>β<sub>7</sub>-expressing ILC3s (96). RORγt<sup>+</sup> ILCs and likely CPs/ILFs are required for induction of AID expression by lamina propria B cells and class switching to IgA (97).

It has recently become clear that the RORγt<sup>+</sup> ILC populations in the intestinal lamina propria are more complex than previously appreciated. In addition to CD4<sup>+</sup> LTi cells (i.e. CD4<sup>+</sup> CD127<sup>+</sup> integrin α<sub>4</sub>β<sub>7</sub>- RORγt<sup>+</sup> ILCs), other RORγt-expressing ILCs have been recognized. We refer to the entire population of RORγt<sup>+</sup> ILCs as ILC3s henceforth. Principally, ILC3s can be divided into two major subgroups based on the expression of the chemokine receptor CCR6 (16). Ligands for CCR6 are CCL20 (98, 99) and β-defensin 3 (100) that are secreted during steady state in response to the microbial flora or during acute inflammation by a variety of mucosa-associated cell types (e.g. monocytes, endothelial cells, fibroblasts, dendritic cells and intestinal epithelial cells). CCL20 and β-defensin 3 both play a non-redundant role in the recruitment of CCR6<sup>+</sup> B cells into CPs (i.e. the formation of ILFs), but CCR6 signalling seems to be dispensable for the initial generation of CPs (94, 96). Since CCR6 expression on RORγt<sup>+</sup> ILCs is not required for their postnatal clustering within the lamina propria, its physiological role on RORγt<sup>+</sup> ILCs might be primarily associated with acute inflammation (100). CCR6<sup>+</sup> cells, which include LTi cells but also a CD4<sup>+</sup> CD127<sup>−</sup> subset, can already be encountered in the foetal liver and are characterized by their production of IL-17A, IL-17F and IL-22 (86). IL-22 only acts on non-haematopoietic cells like stromal and epithelial cells, as its receptor is...
not expressed on haematopoietic cells (101). There, IL-22 induces the secretion of anti-microbial proteins (e.g. Reg3γ, S100A8, SAA3) (102) but also regulates the regeneration of epithelial structures in the thymus (103) and the intestine (104) after an inflammatory insult. IL-22 production by ILC3s has been causally linked to protection against attaching and effacing types of intestinal infections such as those with the mouse pathogen Citrobacter rodentium (15, 102). CCR6−/low ILC3s are hardly spotted in the foetal liver but display an impressive proliferation-driven expansion during the first 4 weeks of life (16, 105). In contrast to CCR6+ ILC3s, only few CCR6−/low ILC3s co-express CD4. However, CCR6−/low ILC3s can be subdivided into an NKP46+ and an NKP46+ subset. The current concept is that NKP46+ CCR6−/low ILC3s are the precursors of NKP46+ CCR6−/low ILC3s (18, 86). In contrast to CCR6+ ILC3s, the expansion of CCR6− ILC3s requires expression of the aryl hydrocarbon (AhR) receptor in ILC3s (16, 105). Ahr+/− and Ahr−/− mice (conditional deletion of Ahr in all ILC3s and T cells) have a largely normal compartment of CCR6+ ILC3s, whereas CCR6−/low ILC3s are dramatically diminished in numbers (16, 105–107). Despite the normal representation of CCR6+ ILC3s, Ahr+/− and Ahr−/− mice lack CPs and ILFs, suggesting that either CCR6−/low ILC3s contribute to lymphoid tissue formation or

AhR regulates genes required for LTI function in CCR6+ ILC3s (105, 106). The AhR is a transcription factor whose activity is controlled by a plethora of environmental ligands (108). Expansion of CCR6−/low ILC3s is driven by a group of dietary AhR ligands (i.e. glucosinolates contained in high concentrations in plants of the Brassicaceae family) linking diets to the formation of immune system components (105).

It is obvious that the transcription factors expressed by the various groups of ILCs strikingly resemble those found in the analogous T helper cell subsets. It is believed that expression of these ‘master transcription factors’ is largely mutually exclusive. Surprisingly, almost all CCR6−/low ILC3s co-expressed RORγt and T-bet (16, 109, 110). However, T-bet is not essential for the development of NKP46− CCR6−/low ILC3s but merely required for their differentiation into NKP46− CCR6−/low ILC3s (16, 109, 110). TCF-1 may also participate in this differentiation process as Tcf7−/− mice show a striking reduction of NKP46− ILC3s, while CD4+ ILC3s were not affected (111). Similar to ILC2s, TCF-1 might function downstream of Notch signalling, which may be important for the differentiation of NKP46− ILC3s as well (106, 110). A more careful analysis of Tcf7−/− and Notch-deficient mice with respect to CCR6+ and CCR6−/low ILC3s should reveal whether TCF-1 acts upstream or cooperatively with T-bet in CCR6−/low ILC3s (Fig. 1).
It is currently believed that CCR6<sup>−/low</sup> ILC3s engage an increasing T-bet gradient controlling the sequential up-regulation of NKp46 and NK1.1 while concomitantly down-regulating RORγt (16, 18). Because of the expression of T-bet, these cells are weaker producers of IL-22 compared with T-bet<sup>+</sup> CCR6<sup>−</sup> ILC3s. T-bet<sup>−</sup> CCR6<sup>−/low</sup> ILC3s do not produce IL-17A or IL-17F but gain the capacity to produce IFN-γ and TNF (16). Genetic fate mapping experiments of RORγt have revealed that NKp46<sup>+</sup> CCR6<sup>−/low</sup> ILC3s can down-regulate RORγt expression. NKp46<sup>+</sup> ex-RORγt<sup>+</sup> ILC3s now only express T-bet and do not produce any ‘type 17’ cytokines but are producers of IFN-γ and TNF (18). Thus, the increasing T-bet gradient in ILC3s is a major driver of ILC3 plasticity. NKp46<sup>+</sup> ex-RORγt<sup>+</sup> ILC3s are phenotypically hard to distinguish from bona fide ILC1s. While it remains unknown if NKp46<sup>+</sup> ex-RORγt<sup>+</sup> ILC3s can revert into NKp46<sup>+</sup> RORγt<sup>+</sup> ILC3s, the NK cell subset of ILC1s seemed to be rather stable and did not differentiate into RORγt-expressing ILC3s upon transfer into aliphoid mice (18). Similarly, ILC2s seemed to be rather stable and did not show any signs of functional or transcriptional plasticity (60). Thus, transcriptional and functional plasticity may be restricted to ILC3s.

The molecular signals that control plasticity of CCR6<sup>−/low</sup> ILC3s are only partially understood. IL-7 was found to stabilize RORγt expression in NKp46<sup>+</sup> RORγt<sup>+</sup> ILC3s (18). In contrast, IL-15 may promote RORγt down-regulation at least in <i>vitro</i> assays (18). More recent work has focussed on the factors controlling T-bet expression in CCR6<sup>−/low</sup> ILC3s. In germ-free mice and in IL-23-deficient mice, the T-bet<sup>+</sup> fraction within CCR6<sup>−/low</sup> ILC3s was dramatically reduced, indicating that IL-23 and microbiota-driven cues are, at least, partially controlling the T-bet gradient in CCR6<sup>−/low</sup> ILC3s that is required for functional plasticity of ILC3s (16). Related data for human ILC3s demonstrated that IL-7 maintains IL-22 production by ILC3s, whereas IL-2 and IL-23 were able to promote IFN-γ production by ILC3s (57).

Current data support the view that plasticity of ILC3s may support immunity to intestinal infections. Indeed, NKp46<sup>+</sup> ex-RORγt<sup>+</sup> ILC3s were one of the crucial IFN-γ sources during <i>Salmonella typhimurium</i> infection. Interestingly, IFN-γ contributed to innate immunity to <i>Salmonella</i> infection by controlling mucus release of goblet cells likely in an attempt to fortify the epithelial barrier (16, 112). On the other hand, IFN-γ production by NKp46<sup>+</sup> ex-RORγt<sup>+</sup> ILC3s also promoted intestinal inflammation in the <i>Salmonella</i> model (16) but also in the innate anti-CD40 colitis model (18). Buonocore et al. described another subset of RORγt<sup>+</sup> ILC3s that are potent producers of IL-17, IL-22 and IFN-γ and show dual expression of RORγt and T-bet based on quantitative PCR. Intriguingly, these cells are also positive for CCR6 expression, which is usually a marker for T-bet<sup>−</sup> RORγt<sup>+</sup> ILC3s during the steady state (19). Hence, it remains to be established whether these cells are a distinct subset that expands upon inflammation or whether they are derived from the two major subsets of CCR6<sup>+</sup> and CCR6<sup>−/low</sup> ILC3s.

Summary and future prospects

In summary, we argued that whole-genome duplication at the onset of vertebrate evolution has initiated a process of diversification and selection generating a unique set of core transcription factors, which play an essential role in innate and adaptive immunity. Although cytotoxic cells with functions similar to NK cells have been described in lower vertebrates, including fish, distinct ILCs have only been identified in mammals so far. We speculate that if the innate immune systems with all its cellular components pre-dated the appearance of the adaptive immune system, then distinct ILCs should be detectable in lower vertebrates, including lamprey and hagfish, but comparative studies addressing this issue are still missing. Despite the similarities between ILCs and T helper cells, there are relevant differences, which have to be considered when comparing ILCs and T cells. In addition to TCR engagement, the development of T helper cells depends on particular environmental cues that are integrated and result in the nuclear translocation of members of the protein family of signal transducers of activation and transcription (STAT) culminating in the expression of T helper cell-defining transcription factors. Interestingly, whereas the deletion of specific cytokines or STAT signalling pathways has a dominant negative effect on the differentiation of T helper cell subsets (113), it only has a marginal effect on ILCs (unpublished results from our own laboratory). Thus, the signals that ultimately regulate the transcriptional program in ILCs and T helper cells are quite different, and it will be a major future challenge to understand how these transcription factors are induced and maintained in ILCs. However, this question can probably only be addressed if an uncommitted ILC precursor can be identified, which most likely resides in the bone marrow or in the foetal liver. As all ILCs depend on Id2 and Id2 reporter strains are now available (114, 115), it might be possible to identify such a putative Id2-expressing ILC precursor. Another bewildering aspect of the transcriptional control of ILCs is the fact that at least ILC1s and ILC3s contain more that one cell type. This could imply functional redundancy or, which is more likely, a division of labour. Hence, it will be important to perform side-by-side comparisons of different ILCs within the same setting to obtain a dynamic picture of the spatiotemporal contributions of specific ILCs in various inflammatory settings. Eventually, this approach may pave the way for devising new therapeutic strategies that will harness the potency of ILCs to combat infections and to ameliorate inflammatory disorders.

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