Differentiation and function of group 3 innate lymphoid cells, from embryo to adult

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Received 6 July 2015, accepted 7 September 2015

Abstract

Group 3 innate lymphoid cells (ILC3) represent a heterogeneous population of cells that share the nuclear hormone receptor RORγt (retinoic acid receptor-related orphan receptor γt) as a master regulator for differentiation and function. ILC3 can be divided into two major subsets based on the cell surface expression of the natural cytotoxicity receptor (NCR), NKP46. A subset of NCRγ− ILC3 includes the previously known lymphoid-tissue inducer cells that are essential for the embryonic formation of peripheral lymph nodes and Peyer’s patches. After birth, the NCR− and NCRγ+ ILC3 contribute to the maintenance of health but also to inflammation in mucosal tissues. This review will describe the differentiation pathways of ILC3, their involvement in the development of the adaptive immune system and their role in the establishment and maintenance of gut immunity.

Keywords: gut immunity, lymph node development, Ncr, NKp46, RORγt

Introduction

Innate lymphoid cells (ILC) are the most recently discovered subset of immune cells. Many recent studies have already highlighted their role in the development of immunity and maintenance of health, leading to a wealth of reviews on these novel players in immunity (1–9). The first innate immune cells of lymphoid origin that were described were the natural killer (NK) cells (10–12) and the lymphoid-tissue inducer (LTi) cells (13). However, in the last few years it has become clear that other lymphoid cells also exist that lack lineage markers (Lin−) and lack somatic rearrangement of the T-cell and B-cell antigen receptors (TCR and BCR) (3). All ILC express the interleukin 7 (IL-7) receptor α subunit (IL7Rα; CD127) and can be classified into cytotoxic ILC, such as NK cells, and helper-like ILC, such as the ILC1, ILC2 and ILC3 groups (Fig. 1).

ILC1 and NK cells are subsets of ILC that express the transcription factor T-bet and produce interferon-γ (IFN-γ). NK cells also depend upon the transcription factor Eomes and can be seen as an innate counterpart of CD8+ T cells, whereas ILC1 are more like T1 CD4+ T cells. In humans and mice, ILC1 are best defined in the liver as CD127+NKP46+TRAIL−CD49a−CD49b− non-T, non-B lymphocytes (1, 2, 14). In other organs, they remain ill-defined and their link to NK cells is a matter of debate (15).

ILC2 maturation depends on the transcription factor GATA-3, and these cells mostly produce IL-4, IL-5 and IL-13, for example in response to helminthic infection (16, 17). They can be seen as the innate counterparts of T2 CD4+ T cells.

ILC3 are a heterogeneous population of cells. They comprise natural cytotoxicity receptor (NCR)− cells, including LTi before birth and LTi-like cells after birth, and NCR+ ILC3. The expression of the nuclear hormone retinoic acid receptor-related orphan receptor γt (RORγt) is necessary for the generation and function of all ILC3, which resemble T17/T22 cells by their secretion of IL-17 and IL-22. The LTi appear during embryonic development of the adaptive immune system, and are essential for the formation of lymph nodes. The lymph nodes are pivotal organs for the adaptive immune system, as this is where naive immune cells are instructed and immune responses toward antigens are mounted. As such, ILC3 are at the foundation of the adaptive immune system. In humans and mice, the ILC3 subset that is critical for lymph node development is IL7Rα+cKit+RORγt+NCR−TRANCE+IL-22+IL-17+ and was previously known as the LTi cell (13, 18, 19). The LTi cells that are located within the area where the lymph node is formed in the embryo (lymph node anlagen) are likely derived from the fetal liver (20).

After birth, other ILC3 subsets, which can be NCR− or NCR+, are involved in many aspects of mucosal immunity, such as in gut and skin. One of the key effector molecules of these ILC3 is IL-22, of which the receptors are mainly found in epithelial and stromal cells (21). Via IL-22, ILC3 can affect the expression profile of the epithelial and stromal cells and aid the anti-microbial defense in mucosal tissues.
In this review, we will describe the ontogeny of the ILC3 subset during embryogenesis, and the activity of ILC3 master regulator RORγt during differentiation. Subsequently, we will discuss the role of the ILC3 subset in embryogenesis during lymph node formation. Finally, we will describe the role of the heterogeneous ILC3 subset in gut immunity in the adult.

**ILC3 in the embryo**

Hematopoietic stem cells (HSC) appear at the blood vessel endothelium around mouse embryo gestation day 9.5 (E9.5) until E14. In the area between the aorta, gonads and mesonephros (AGM), CD31+Sca1+ cKit+ hemogenic endothelial cells appear along the aortic vessel wall and bud off as HSCs that will express the pan-hematopoietic marker CD45 (22–24) (Fig. 2). These cells migrate toward the fetal liver, the location within the embryo where hematopoietic progenitor cells reside and undergo a first differentiation step to become the common lymphoid progenitor (CLP) for all lymphocytes, including ILC.

CLP require Id2, Nfil3 and Tox to commit toward the ILC lineage. Although these transcription factors were initially described to be necessary for differentiation of NK and LTi cells (25–28), it became clear that they are at the root of the differentiation of CLP into the ILC/NK precursor (27–31). The common progenitors for all helper-like ILC (CHILP) also express the transcription factor Plzf (30, 31).

Id2 is a helix-loop-helix protein that is able to bind to E proteins (transcription factors, e.g. E2A), which are involved in the formation of B cells (32). The binding of Id2 to E proteins prevents the binding of the E proteins to their E box motif within the DNA and so prevents differentiation to the B-cell lineage. However, the role of Id2 for differentiation toward T cells is not well-defined, but it appears to inhibit T-cell potential in precursors (33). By using mice with the genes for Id2 and/or E2A knocked out, it was shown that the lack of Id2 leads to loss of LTi, and subsequently the loss of lymph nodes and Peyer’s patches (PP). However, when both E2A and Id2 are deleted, LTi were present (34), indicating that Id2 is required to counteract the function of E2A and necessary to induce ILC differentiation. The balance between Id2 and E2A levels determines the amount of ILC3 formed (34).

Within the differentiation pathway of ILC3, most α4β7+RORγt+ (CHILP) cells in the fetal liver expressed Id2, showing that these cells were already committed to the ILC phenotype (35). Subsequently, RORγt expression is induced and commits the progenitor cells to the ILC3 cell fate. It was described that this occurs within the fetal liver, being modulated by Notch signaling (35, 36). However, induction of RORγt expression also occurs in a progenitor population located within lymph node anlagen in the periphery. The population in the periphery in which RORγt is induced might be a separate progenitor ILC3 population that responds to retinoic acid, whereas the progenitor population in the fetal liver does not respond to retinoic acid (37).
The expression, binding and functions of RORγt

Within the ILC family, RORγt is distinctive for the ILC3 branch and its expression required for ILC3 differentiation and function (38). However, an ILC2 population has also been reported that expresses RORγt and differentiates into an IL-17-producing cell (39). Depending on promoter usage, there are two proteins transcribed from the RORc locus, either RORγt or RORγt (40). Initially, it was shown that the isoform RORγt was involved in the survival of thymocytes and it was noted that RORγt was also important for the survival of the then recently discovered ILC3 cells (41). Later, it was shown that RORγt is necessary for ILC3 differentiation (42).

RORγt binds as a monomer to the so-called RORγ-responsive elements (RORE) and via either co-repressors or co-activators regulates gene-expression (40, 43). Target genes of RORγt are also associated with T17 cell differentiation (3, 43, 44). These genes include Il17a, Il17f, Il23r, Cc120, Il1r1, Ltb4r1, Cxcr5, Ccr6, and Cxcl10 (37, 43) and are associated with functioning and activity of ILC3. The aryl hydrogen receptor (Ahr), important for the differentiation of NCR+RORγt+ ILC3 cells, was described to act cooperatively with the RORγt to induce expression of Il22 (45). Moreover, it was shown that isoflavone, a dietary ligand for Ahr, could stabilize RORγt interactions with co-activators (46). This indicates that Ahr is not involved in RORγt-mediated induction of gene expression, but rather stabilizes the expression repertoire that RORγt has induced to maintain the phenotype.

The ligand for RORγt has long been elusive. However, recent studies have shown that the RORγt ligand domain can bind to several intermediates of the cholesterol pathway (47, 48). Interestingly, RORγt participates in regulation of genes involved in lipid metabolism (49), pointing to a close relationship of this nuclear receptor with cholesterol and lipid synthesis. Deletion of Cyp51, an enzyme involved in the metabolic conversion of lanosterol to zymosterol, resulted in a significant reduction of ILC3 numbers within the embryo and resulted in smaller peripheral lymph node anlagen (47). Since RORγt is also required for the differentiation of T17 cells, the number of T17 cells were also significantly reduced in the absence of Cyp51, paving a path to new targeting possibilities in the treatment of inflammatory diseases associated with ILC3 or T17 cells.

The natural occurring Digitalis purpurea product digoxin was shown to inhibit RORγt activity (50). The use of this antagonist in both human and mouse models greatly reduced the amount of T17 cells (50) and blocked ILC3 differentiation (37). While digoxin is not toxic to mice, it is very poisonous to humans. A non-poisonous variant was chemically synthesized to be able to use this antagonist in human cells as a potential drug for T17-associated auto-immune diseases (50). Chemically synthesized ligands were also produced that bound to the ligand-binding domain of both RORα and RORγt and inhibited function (51, 52).

Although several studies have reported the regulation of RORγt expression within T17 cells (53–55), it is not clear...
whether this regulation is similar in ILC3. Indeed, retinoic acid was shown to negatively affect RORγt expression in T_{17} cells (53, 56), whereas during embryonic lymph development retinoic acid was essential for the induction of RORγt expression in ILC3 (37). The precise mechanisms by which RORγt is regulated in ILC3 remain therefore unclear.

Formation of secondary lymphoid organs

In one of the earliest described events of embryonic lymph node formation, Cxcl13 expression is induced in mesenchymal organizer cells by retinoic acid which attracts the first NCR^−/− ILC3 toward the niche (57, 58).

As well as inducing Cxcl13 in mesenchymal cells, retinoic acid induces RORγt expression in NCR^−/− ILC3 precursors (IL7Rα^−/− RORγt^−/− TRANCE-NCR^−/− ILC3 precursors also known as LTi cells) in the periphery, but not in the fetal liver (37). The expression of RORγt is required for their differentiation into NCR^−/− ILC3 (IL7Rα^−/− cKit^−/− RORγt^−/− TRANCE-NCR^−/− ILC3, also known as LTi), in mice so that TRANCE (also known as RANK-L or Tnfsf11) will interact with TRANCE-R (also known as RANK or Tnfrsf11a) on adjacent ILC3 (Figs 1 and 2). Together with IL-7, TRANCE induces the expression of the lymphochoxin isoform LTα1β2 on NCR^−/− ILC3 (59, 60), which can then interact with LTβR (which binds LTα1β2) on the mesenchymal organizer cells (61). LTβR signaling within the mesenchymal cells results in increased IL-7 and TRANCE production by the mesenchymal cells, leading to survival and differentiation of more NCR^−/− ILC3. Consequently, this process leads to more expression of chemokines, cytokines and adhesion molecules from the mesenchymal cells to attract, maintain and retain more ILC3 (58, 59, 61, 62). In the mouse, this aggregate is colonized by T and B cells leading to the development of the different cell compartments within the lymph node in the first week after birth (63, 64).

In humans, there are differences as compared with mouse development. The most notable difference is that the mature cKit^−/− IL7Rα^−/− NCR^−/− RORγt^−/− TRANCE^−/− LTi cell in humans does not express CD4 (19). The role for CD4 in the function of mouse NCR^−/− ILC3 is unknown and it is not essential for LTi functioning, but the expression within mouse NCR^−/− ILC3 provides an efficient tool to specifically isolate and analyze this subset. The second difference is that human lymph node formation occurs completely within the fetus, with the colonization by B cells and T cells and formation of concomitant clusters. Therefore, humans have fully functional lymph nodes at birth (65).

The size of the initial cluster determines the size of the lymph node in the adult at steady state. Essentially, the amount of ILC3 within this niche determines how large the lymph node will become, since the amount of ILC3 within the niche determines how much interaction with the mesenchymal organizer cells occurs. IL-7 is essential for the amount of ILC3, not only as a survival factor, but also in promoting ILC3 proliferation (58, 66) and it was shown that over-expression of IL-7 leads to larger and ectopic lymph node formation (67, 68).

Changing the proliferation rate of the ILC3 (67, 68), and differentiation via RORγt (37) or Id2 (34), will also affect the size of the lymph node. Changing the amount of vitamin A, from which retinoic acid is synthesized, in the maternal diet results in changing the ILC3 differentiation rate during embryogenesis. Consequently, the lymph node size is also affected, which has lifelong consequences for the offspring. The smaller size of the lymph nodes as a result of a vitamin A-deficient maternal diet during embryogenesis cannot be rescued by adding extra vitamin A in the diet of the offspring (37).

NCR^−/− ILC3 are also important for the embryonic formation of the PP in the gut, although the mechanism by which these organs are formed is slightly different. Initially, around mouse embryonic gestation day E12.5, so-called lymphoid tissue initiator (LTi) cells that are CD45^−/− IL7Rα^−/− CD43^−/− CD11c^−/− NK1.1^− colonize the small intestines, guided by the neuronal guidance factor RET (69). Also, an extrahepatic arginase-1^− Id2^− fetal ILC precursor population has been described in the fetal gut that could differentiate into ILC1, 2 and 3 and which was involved in PP development (70).

The lack of RET resulted in the absence of the LTin cells, which subsequently prevented the formation of PP (64). Because of their high lymphochoxin β subunit expression, these cells will interact with mesenchymal cells at evenly spaced locations within the intestines that will subsequently express chemokines and adhesion molecules (58, 71). This mechanism will attract and retain more NCR^−/− ILC3 that will also interact via the lymphochoxin pathway to stimulate synthesis of more adhesion and attracting molecules by the mesenchymal cells leading to the formation of PP before birth. Similar to the definitive formation of the B-cell and T-cell compartments within the lymph nodes, this also occurs after birth for the PP in the mouse.

NCR^−/− ILC3 are thus essential for lymphoid organ development in mice, and lack of NCR^−/− ILC3, such as in RORγt^−/− mice, resulted in lack of PP and of peripheral and mesenteric lymph nodes (41, 42, 61). Although the fetal spleen contains NCR^−/− ILC3 (36, 42), the lack of these cells did not affect spleen development. Therefore, other hematopoietic cells are likely involved in the formation of the fetal spleen that can counteract the lack of NCR^−/− ILC3. However, NCR^−/− ILC3 cells were required for regeneration the spleen after a viral infection (72).

ILC3 in the adult

NCR^−/− ILC3 and NCR^−/− ILC3 were observed in the gut and tonsils of mice and humans (19, 42, 73–77). Initially described for NK cells, NCRs are surface receptors that activate cells (78). The NCR family is composed of three molecules: Nkp30 (NCR3, CD337) and Nkp44 (NCR2, CD336) in humans, and Nkp46 (NCR1, CD335), the only NCR conserved in all mammals. Although NCR^−/− ILC3 and NK cells share the expression of some molecules, such as cell surface receptors including NCRs, transcriptional analyses of these cell subsets show that NCR^−/− ILC3 are more closely related to NCR^−/− ILC3 cells than they are to NK cells, and are not derived from NK cells, nor do they give rise to NK cells (15, 79). In particular, NCR^−/− ILC3 do not express IFN-γ or the typical perforin and other cytotoxic proteins that are characteristic of NK cells.

Cryptopatches are lymphoid organs that reside within the gut, mainly the small intestines. At steady state, cryptopatches
are small aggregates of NCRγ and NCRδ ILC3 (45, 74, 80, 81). In inflammatory conditions, the cells proliferate and attract B cells and dendritic cells to form an isolated lymphoid follicle (82). During the formation of cryptopatches after birth in mice and during the second trimester in humans, NCRγ ILC3 differentiate into NCRδ ILC3 (45). It was shown that the NCRγ ILC3 participate in the formation of cryptopatches, but not in the formation of lymph nodes or PP (81, 83), which require the NCRγ-CD4δ ILC3.

The origin of the NCRγ ILC3 is under debate at the moment. In elegant RORγt lineage-tracing experiments, it was observed that fetal NCRγ-CD4δ ILC3 (LTi) cells could not differentiate in vitro or in vivo toward the NCRγ ILC3 cells, but a small population of α4β7-RORγt progenitor cells obtained from the fetal liver and gut could differentiate into all RORγt+ ILC3 subsets (35, 37, 38). These RORγt+ precursor cells could be Id2-PLZFhigh CHILP (30, 31). It is possible that CHILP are present in the fetal liver and periphery where they could locally differentiate into NCRγ-CD4δ ILC3 and NCRδ ILC3. Indeed, NCRγ-RORγt-CD4δ ILC3 precursors were observed within the lymph node anlagen (37) and gut (37, 38) that locally differentiated into NCRγ-CD4δ (LTiγ) ILC3 (37) or NCRδ ILC3 (19, 36, 77, 84, 85) (Fig. 2).

The NCRγ ILC3 population is heterogeneous and can be divided into a CCR6+ and a CCR6- population (38, 75). Although it has been proposed that CCR6 serves as a marker of fetus-derived ILC3, this possibility remains to be formally proven. Irrespective of this issue, NCRγ ILC3 can differentiate into NCRδ ILC3, and this process involves T-bet and Notch (30, 38, 75, 86) (Fig. 3). It was also suggested that NCRγ ILC3 could further lose RORγt expression (the so-called ‘ex-ILC3’) and harbor an NK/ILC1-like phenotype (84) (Fig. 3). The general consensus is that the NCRγ ILC3 are formed within the gut after birth in the mouse, and that the microbiota impact on their differentiation. ILC3 have been mainly found associated with gut and skin. Studies of human skin revealed that the proportion of NCRγ ILC3 in psoriatic lesions was greater than that in healthy controls (87), prompting the dissection of their role in this disease. In the gut, ILC3 are present in cryptopatches and isolated lymphoid follicles, but also as scattered cells in the lamina propria. NCRγ ILC3 have been shown to promote colitis in a model of pathological adaptive immune cell responses to commensal bacteria (90, 91). It has also been proposed that T cells function as antigen-specific sensors for the activation of ILC3 to amplify and instruct local immune responses (92). Moreover, ILC3 were directly involved with oral tolerance by regulation of Treg numbers via secretion of granulocyte macrophage colony-stimulating factor which was induced in ILC3s by microbiota-activated macrophages (93). Although intestinal T cells and ILC3 use different pathogen-recognition strategies, they appear to exert redundant function to some extent for the maintenance of gut homeostasis. It is thus tempting to speculate that ILC3 and T17/T22 cells have co-evolved, leading to the selection of robust fail-safe mechanisms ensuring the adequate control of the commensal gut microbiota and protection against intestinal infections. The selective pressure for redundancy might have led to the striking similarities between ILC and T cells in terms of localization and effector function.

**Conclusion and perspectives**

Many challenges remain on the road to gaining a full understanding of the biology of ILC3. Their role in natura outside of gut and skin, such as in the respiratory tract and in the urogenital tract or during gestation and in the formation of lymphoid structures associated with auto-immune disorders, represent many interesting questions to be addressed. Their
clinical potential also stands among the most pressing questions concerning ILC3. Indeed, ILC3 are involved in many homeostatic processes and disease conditions, but other than for NK cells, no ILC-based therapies have yet been proposed. Harnessing the activity of ILC3 may lead us to decipher novel immune pathways to improve immunotherapy and vaccines, considering, in particular, the unique tropism of subsets of ILC3 toward the mucosa.

**Funding**

An A*MIDEX chaire d’excellence grant (ANR-11-IDEX-0001-002) and FRM jeunes équipes (AUE20150633331) to S. A. vdP; the European Research Council (THINK Advanced) Grant and the Ligue Nationale contre le Cancer (Equipe Labellisée) to E. V.; INSERM, CNRS and Aix-Marseille University (institutional grant to CIML).

**Acknowledgements**

The authors thank Cyril Seillet (WEHI, Melbourne, Australia) for (institutional grant to CIML).

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