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Review

Development and regulation of RORγt$^+$ innate lymphoid cells

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ABSTRACT

RORγt$^+$ innate lymphoid cells (ILCs), or ILC3, play a fundamental role in the development of lymphoid tissues, as well as in homeostasis and defence of mucosal tissues. These cells produce IL-22, IL-17A and LTα1β2, key cytokines for the activation of epithelial defences and the recruitment of polymorphonuclear phagocytes. In the absence of ILC3, the early defence to infection and resistance to injury are compromised. Given the importance of ILC3 in mucosal immunity, significant efforts are made to discover their multiple functions and decipher their mode of action and regulation.

1. Introduction

ILCs have only recently been recognized as a major population of immune effector cells through the discovery of new subsets and the demonstrations that early responses to infections are largely dependent on cytokines they produce [1–3]. ILCs are defined by their developmental origin from the common lymphoid progenitor that also gives rise to B and T cells. However, ILCs do not express an antigen receptor, and therefore, do not undergo clonal selection and expansion as lymphocytes do. Rather, ILCs react to cytokines and chemokines produced in tissues following infection and injury, and respond by expansion and the production of effector cytokines.

The first type of ILCs described more than 30 years ago is natural killer (NK) cells, innate cells that, similar to CD8$^+$ cytolytic T cells, lyse target cells and produce IFNγ [4]. More recently, IFNγ-producing yet non-cytolytic ILCs, similar to T helper (Th) 1 cells, were described to reside in the mucosal epithelium [5]. In the 1990s, another lymphoid cell type that did not express an antigen receptor was found colonizing nascent lymph nodes and Peyer's patches, or lymphoid tissue anlagen [6,7]. These cells were termed lymphoid tissue inducer (LTi) cells, shown to be required for the activation of local stromal cells and the subsequent recruitment of lymphocytes to the developing lymphoid tissues [8]. After birth, LTi cells cluster into cryptopatches in the intestinal lamina propria and induce the formation of isolated lymphoid follicles (ILFs) [9–11]. In 2008, it was found that LTi cells are members of a larger family of LTi-like ILCs that express pro-inflammatory cytokines, such as IL-17A and IL-22, playing a role in mucosal homeostasis and defence similar to Th17 cells [12–16]. Finally, a third subset of ILCs was discovered to duplicate the activity of Th2 cells in the production of IL-4, IL-5, IL-6 and IL-13, and to be involved in early defence against worms and in allergic responses [17–19]. Altogether, ILCs are grouped into three categories according to their cytokine expression pattern mirroring the activity of Th cells: type 1, 2 and 3 ILCs, also termed ILC1, ILC2 and ILC3, reflecting Th1, Th2 and Th17 cells [1].

With the exception of LTi cells, ILCs were first described in the context of infection and inflammation, during which they expand from initially small populations, or at steady state in the intestine where they establish a dynamic crosstalk with the resident microbiota. Even though the generation of LTi cells is programmed [20,21], the differentiation, expansion and activity of LTi cells, and of ILCs in general, is closely linked to the microbial environment and the local perturbation induced by infection and injury. The identification of microbes and molecules involved in this crosstalk are subject of intense investigation as they may hold keys to the control of early immunity to infection and injury, and thus to the regulation of the developing adaptive immune response.

In this review, we will focus on the development, subset analysis and regulation of ILC3, which are dependent on expression of the nuclear hormone receptor RORγt [22].

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2. The development of RORγt$^+$ ILCs

All ILCs are derived from common lymphoid progenitors, which arise from fetal liver or bone marrow hematopoietic stem cells [1,2]. Sequential expression of transcription factors and cytokine receptors orchestrates the commitment of multi-potent progenitors towards the ILC3 cell fate [3]. This developmental program is framed by interactions with environmental cues such as Notch ligands and components of the intestinal microbiota that could also account for some degree of plasticity among ILC3 subsets (see Fig. 1).

A common requirement for the development of lymphoid cells is activation of the IL-7 receptor α (IL-7Rα). The common lymphoid progenitor is dependent on IL-7Rα, and mice deficient in IL-7Rα or in the common γ_c chain associated with its signalling are characteristically lymphopenic [23]. The first step in the differentiation of the common lymphoid progenitor to ILCs is the expression of the inhibitor of DNA binding Id2 [1,22,24]. In the absence of Id2, ILCs fail to develop as the common lymphoid progenitor is induced by E-box transcription factors to generate lymphocytes. Id2 blocks these E-box proteins, and an absence of both Id2 and the E-box protein E2A restores ILC development [25]. However, Id2 is not a marker of ILCs, as it is also expressed in mature T cells and is involved in the generation of memory cells [26]. Another transcription factor, Gata3, initially shown to be specifically required for the development of ILC2 [27–29], also appears to play a more general role in the early differentiation of ILC3 [30].

Another hurdle facing the development of ILCs from the common lymphoid progenitor is the early bias towards B cell differentiation. The presence of Notch ligands, at least in some niches of the fetal liver and the bone marrow after birth, blocks this bias and allows both the development of T cells and ILCs [31]. However, the prolonged presence of Notch ligands subsequently favours the emergence of T cells over ILCs, as in the thymus. As a consequence, immature thymocytes grown *in vitro* in the absence of Notch ligands generate significantly higher frequencies of ILCs [31].

Once progenitors are solidly engaged into the ILC pathway, the decision has to be taken to generate one of the at least 3 types of ILCs. The transcription factors shown to be required for the differentiation of Th cells are also required, but not necessarily sufficient, for the differentiation of the ILCsubsets. T-bet is required to generate Th1 cells and ILC1 [5,32,33], Gata3 is required to generate Th2 cells and ILC2 [27–29], and RORγt is required to generate Th17 cells and ILC3 [22,34]. In mice deficient for RORγt, ILC3 do not develop, leading to a lack of all lymph nodes and Peyer's patches [22]. How the expression of RORγt is induced in ILC precursors remains unclear. In the differentiation of Th17 cells from naïve CD4$^+$ αβ T cells, RORγt is induced through the activation of Stat3 by cytokines such as IL-6 and IL-23, in combination with TGFβ signalling [35]. However, the involvement of these cytokines in the generation of LTi cells can be excluded as mice deficient in Stat3 in the hematopoietic lineage still develop lymph nodes and Peyer's patches. The lineage-specific transcription factors are stably expressed in their respective Th cells and ILCs, and are therefore used as specific markers. However, lineage plasticity has been observed in both T cells and ILCs. For instance, inflammatory settings induce a subset of ILC3 to downregulate RORγt, upregulate T-bet and express IFNγ [33], even though it remains to be assessed whether mature ILC3 or RORγt$^+$ immature precursors develop an ILC1 phenotype. The transcription factor Tcf-1 is required for further differentiation of ILC3 into one particular subset expressing the pan-NK marker NKp46 (see below) [36]. Finally, a recent study suggests that the development of ILC3 is not as linear as described here, as an ILC precursor expressing the transcription factor PLZF gives rise to ILC1, ILC2 and ILC3 but not to LTi cells [37].

The impact of the microbiota on the generation of ILC3 remains controversial. Whereas the generation of LTi cells in the fetus is obviously independent of microbiota [20,21], as well as of potential microbial compounds delivered by the mother through the placenta (our own unpublished data), the generation of ILC3 after birth may be induced by certain types of microbiota, as reported by some laboratories [13,15]. Clearer is the impact of nutrients

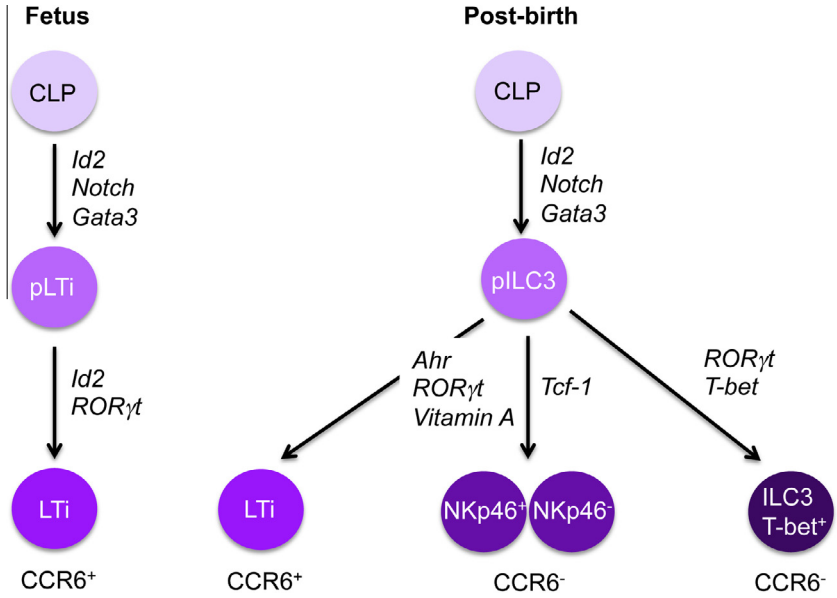

Fig. 1. The development of ILC3. In the fetus, common lymphoid progenitors (CLP) generate LTi cells, half of which express CD4. The development of all ILCs requires Id2 to block the generation of B and T cells. Notch signalling blocks the generation of B cells, but subsequently, favours precursors to generate T cells instead of ILCs, such as in the thymus. The development of ILC3 specifically requires RORγt, while Gata3 is involved in the development of both ILC3 and ILC2. After birth, ILC3 precursors generate an additional subset of ILC3 that does not express CCR6 and does not induce lymphoid tissue formation, including Tcf-1-dependent NKp46$^+$ cells. Furthermore, LTi and CCR6$^-$ ILC3 become dependent on the aryl hydrocarbon receptor (Ahr) that binds dietary metabolites, as well as on vitamin A. In the context of inflammation, CCR6$^-$NKp46$^-$ ILC3 or ILC3 precursors generate T-bet$^+$ cells that not only express IL-17 and IL-22, but also IFNγ. Recent data suggest that the development of LTi cells branches off before the common precursor to ILC1, ILC2 and CCR6$^-$ ILC3 [37].

on the generation of ILC3. Mice that are deficient for the aryl hydrocarbon receptor Ahr, required for the generation of T cells producing IL-22 [38], show a deficit in the formation of ILFs, but not in the development of lymph nodes and Peyer's patches [39–41]. Ahr appears to be required for the maintenance of ILC3 subsets after, but not before birth, as ligands for Ahr are provided through food by cruciferous vegetables, such as broccoli and Brussel's sprouts. Another nutrient required for the generation of ILC3 is vitamin A. In its absence, precursors develop preferentially into ILC2, thus linking vitamin A and nutrition to the regulation of intestinal immunity [42].

3. Subsets and functions of RORγt$^+$ ILCs

Whereas LTi cells are programmed to develop in the fetus, another subset of LTi-like ILC3 develops mostly after birth (Fig. 2) [20]. By weaning it becomes the major subset of ILC3 in the intestinal lamina propria. These LTi-like cells do not express the chemokine receptor CCR6 [20], required for LTi cells to cluster after birth in cryptopatches to form ILFs. CCR6$^-$ ILC3 rather resemble lymphoid effector cells and produce high amounts of cytokines including IL-22 [13,21]. To further add some complexity so typical of immunology, both CCR6$^+$ and CCR6$^-$ ILC3 can be further subdivided into phenotypically and functionally distinct types of cells.

LTi cells, the first ILC3 to be characterized [6,7,22], can be subdivided into CD4$^+$ (LTi$_4$) and CD4$^-$ (LTi$_0$) cells, which reside in lymphoid tissues at a ratio of roughly 1 to 1, depending on the tissue and age of mice. It has been suggested that LTi$_0$ cells are precursors to mature LTi$_4$ cells, even though the level of effector cytokines, including IL-17A and IL-22, is similar between the two cell types [21]. The role of CD4 remains unknown, as the development of lymphoid tissues proceeds normally in CD4-deficient mice. LTi cells also express high levels of MHC class II, involved in the regulation of T cells reacting to the microbiota [43]. However, a link between the expression of CD4 and MHC class II by LTi cells has not been established. Both subsets express lymphotoxin (LT) α1β2, which activates LTβR$^+$ stromal cells to produce cytokines, such as CXCL13, and to express the adhesion molecules ICAM-1 and VCAM-1, to recruit further waves of LTi, as well as B and T cells [8,44]. A similar pathway unfolds in the formation of ILFs after birth, although this process requires the colonization of the intestine by bacteria and the induction of CCL20 production by epithelial cells, the ligand of CCR6 on LTi and B cells [9]. After birth, LTi cells are also involved in the IL-22 mediated defence against intestinal pathogens, such as the Proteobacteria *Citrobacter rodentium* [45].

Within CCR6$^-$ ILC3, the subset of NKp46$^+$ cells, and their human NKp44$^+$ counterparts, gained much attention through their identification as a population of NK-like cells expressing RORγt and IL-22 [13–16,46]. NKp46$^+$ ILCs are abundant in the intestine and play a major role in intestinal defence against infection by *C. rodentium* as well as in resistance to injury and microbial invasion induced by dextran sodium sulfate (DSS). The functional significance of NKp46 expression on ILC3 remains unclear [47], even though receptors of this family of natural cytotoxicity receptors are involved in the regulation of the activity of NK cells [48,49]. During infection and inflammation, NKp46$^+$ ILC3 expand that also express T-bet and IFNγ, therefore closely resembling ILC1 [33].

A more cryptic subset of CCR6$^-$ ILC3 is defined mostly by negative staining for CD4, NKp46, as well as low levels of IL-7Rα [20,21]. This subset was found to express transcripts coding for CD3 chains but no protein. It was also found to generate IFNγ-producing ILCs, as mentioned above [33]. Furthermore, a subset of IL-17A-, IL-22- and IFNγ-producing CCR6$^-$ NKp46$^-$ ILC3 was expanded in the inflamed colon in both mouse and human [50]. It is likely that the ill-defined population of CCR6$^-$ NKp46$^-$ ILC3 is a heterogeneous mix of progenitor and effector cells. A more detailed analysis of this population of ILC3 is warranted, as it is a subset of abundant ILC3 in the intestine of mice after weaning.

4. Regulation of RORγt$^+$ ILCs

LTi cells are the first type of lymphoid cells to be generated during fetal life [8,20]. Not only do they induce the formation of lymphoid tissues through the expression of LTα1β2 and other members of the TNF superfamily, such as TRANCE, but they also produce IL-22 and IL-17A [21]. The role of these cytokines during fetal life is unknown, as mice deficient for IL-22 or IL-17A develop a normal set of lymphoid tissues. It is possible that this expression

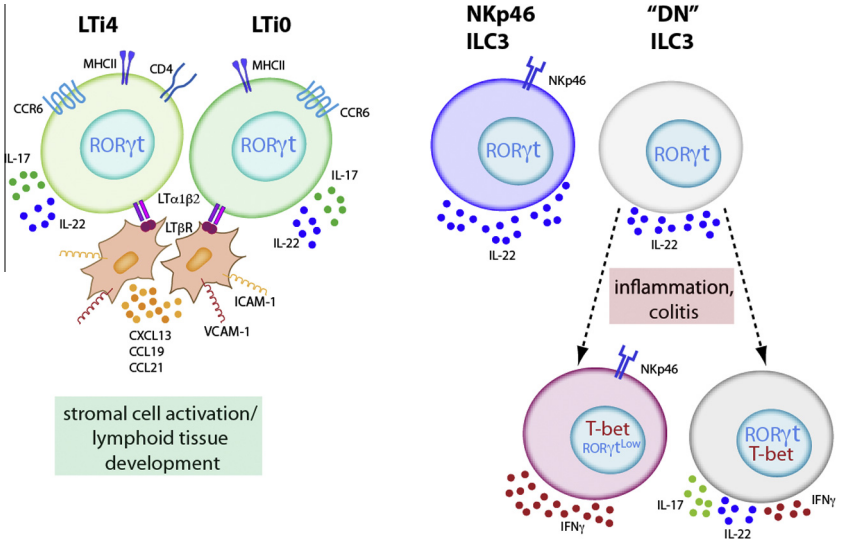

Fig. 2. Subsets and functions of ILC3. Three types of functions are provided by ILC3: the induction of lymphoid tissue development through LTα1β2, mucosal defence induced by IL-17 and IL-22, and anti-bacterial defence induced by IFNγ in the context of infection and inflammation. ILC3 can be subdivided into two main subsets that share these functions: LTi cells that express CCR6 and cluster to induce the development of lymphoid tissues, and a diverse group of CCR6$^-$ cells that do not cluster but rather resemble effector T cells. Both CCR6$^+$ and CCR6$^-$ cells express IL-22 and IL-17 before weaning and during inflammation. CCR6$^-$ ILC3 that do not express the pan-NK marker NKp46 (double negative or "DN" ILC3) include effectors that express IL-22, as well as precursors to NKp46$^+$ cells that express T-bet and IFNγ and expand during intestinal infection. A population of DN ILC3 also expands during colitis that expresses IL-17, IL-22 and IFNγ. The lineage relationship between IFNγ-producing ILC3 and DN ILC3 remains unclear, as the latter may include both mature effectors and immature precursors.

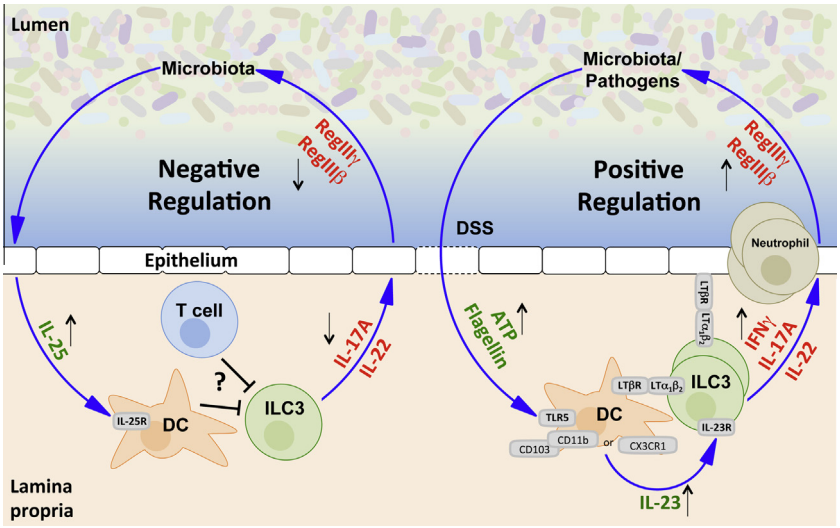

Fig. 3. Regulation of ILC3. ILC3 are programmed to express IL-17 and IL-22, independently of the microbial environment and inflammation. For instance, the highest level of ILC3 activity is found in fetal LTi cells. After birth, this activity is negatively regulated by microbiota that induces expression of IL-25 by epithelial cells. IL-25 acts on DCs to regulate the activity of ILC3. The mechanisms by which microbiota induces IL-25 expression and DCs regulate ILC3 remain unknown. T cells are also found to regulate ILC3 activity, possibly through competition for cytokines such as IL-23. Of note, particular types of microbiota have been found to rather activate than regulate ILC3. In the context of inflammation induced by epithelial damage or infection, the negative regulation of ILC3 is overridden by microbial signals, such as flagellin and possibly ATP [59], which activate CD11b$^+$ DCs and macrophages to produce IL-23. Activated ILC3 provide a positive feedback loop by activating DCs through LTα1β2, and induce the production of anti-microbial proteins by epithelial cells through IL-17, IL-22 and LTα1β2 [60], and the recruitment of neutrophils through IL-17.

reflects a general, in that case neutral, transcription profile induced by RORγt. However, it is more likely that IL-22 and IL-17A play a role in the selection of bacteria colonizing the intestine after birth by inducing anti-microbial peptides in epithelial cells [51]. Interestingly, upon bacterial colonization of the intestine after birth, the cytokine-producing activity of ILC3 is significantly reduced as compared to fetal production levels, a reduction that is alleviated in germfree mice (Fig. 3) [21]. We showed that microbiota induces the production of IL-25 by epithelial cells, which inhibits the expression of IL-17A and IL-22 by a mechanism that involves dendritic cells (DC), thereby establishing a negative feedback loop on the anti-bacterial activity of ILC3. Nevertheless, it has been observed by other laboratories that the production of IL-22 was actually increased in the presence of microbiota, indicating that different components of the microbiota have a positive or a negative effect on the activity of ILC3 [13,15]. In the context of colitis, the activity of ILC3 is unleashed to levels similar to those found during fetal life [21].

Dendritic cells (DCs) are an important element for the regulation of ILC3 activity. DCs establish a link between microbiota and ILC3 through recognition of microbe-associated molecular patterns (MAMPs). Infection by *C. rodentium* induces the production of IL-23 by CD103$^+$ CD11b$^+$ DCs [52], as well as by CX$_3$CR1$^+$ DCs [53], which then induce IL-22 production by ILC3. CD103$^+$ CD11b$^+$ DCs also produce IL-23 upon recognition of flagellin by TLR5, and induce IL-22 production by ILC3 peaking within 2–3 h upon flagellin injection [54]. As ILC3 express LTα1β2 and DCs express LTβR, a positive feedback loop is established where ILC3 further promote DCs to produce IL-23 [55]. Direct activation of ILC3 by MAMPs has been reported in the human system, where tonsil-derived ILC3 could be activated to produce diverse cytokines in response to TLR2 stimulation [56], but such a pathway has not yet been reported in mouse.

As ILC3 have functions similar to Th17 cells, it is expected that activation of these two cell types involves competition for cytokines such as IL-23. In the absence of T cells in RAG-1-deficient mice, the activity of ILC3 is increased [21,57], even though the mechanism of this lymphocyte-mediated repression remains to be determined. Conversely, ILC3 regulate CD4$^+$ T cells through their expression of MHC class II and the processing and presentation of microbiota-derived antigens [43]. As a consequence, the selective deletion of MHC class II in ILC3 leads to an increased reactivity of T cells against the microbiota and the development of intestinal inflammation [43]. Furthermore, ILC3 control the number of lamina propria T cells, and as a consequence the production of T cell-dependent IgA, through the expression of soluble LTα3 [58]. The mutual regulation of T cells and ILCs, and their respective roles in immune defence, are central questions that remain to be explored further. However, new mouse models are needed that allow for the selective depletion of ILC subsets without affecting the integrity of T cells.

5. Conclusion

ILCs play a fundamental role early in immune responses. They replicate the regulatory roles of Th cell subsets promptly after tissue injury when T cells still need to be selected and expanded. Therefore, knowing the rules that determine the development of ILC1, ILC2 and ILC3 subsets, and regulation of their activity, should allow to create immunotherapies, including vaccines, that are better targeted to specific types of injury or infection. ILC biology may thus lead to a new era of innate immunity for the control of adaptive immunity.

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