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Layered origins of lymphoid tissue inducer cells

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Summary

The Innate Lymphoid Cell (ILC) family is a relatively recently described immune cell family involved in innate immune responses and tissue homeostasis. Lymphoid Tissue Inducer (LTi) cells are part of the type 3 (ILC3) family. The ILC3 family is the main ILC population within the embryo, in which the LTi cells are critically associated with embryonic lymph node formation. Recent studies have shown more insights in ILC origin and residency from local embryonic and tissue resident precursors. Embryonic LTi cells originating from a different hemogenic endothelial source were shown to be replaced by HSC derived progenitors in adult. This review will discuss the layered origin of the ILC3 family with an emphasis on the LTi cell lineage.

KEYWORDS

aorta-gonad-mesonephros, fate-mapping, hematopoiesis, hemogenic endothelium, ILC, innate lymphoid cells, yolk-sac

1 | INTRODUCTION

Cell populations from the Innate Lymphoid Cell (ILC) family are involved in many early response defense mechanisms and tissue homeostasis.¹ They resemble T-lymphocytes in cytokine responses, but lack T-cell receptor and recombinant activating gene (RAG) re-arrangement and thus respond directly to stimuli locally by producing large amounts of cytokines. Based on their expression of lineage-defining transcription factors, the ILC are divided into three large groups: the type 1 ILCs expressing T-bet (ILC1 and NK cells) implicated in defense against tumors and intracellular pathogens like viruses, the type 2 ILCs (ILC2) expressing GATA3 which are important in the defense against extracellular parasites, allergens and adipose tissue homeostasis, and the type 3 ILCs (ILC3) expressing ROR γ t implicated in gut homeostasis as well as immune responses against extracellular microbes such as bacteria and fungi.¹ In addition, among the ILC3 the Lymphoid Tissue Inducer (LTi) cells play a critical role in the embryonic lymph node (LN) formation² (Figure 1).

Beside this classification, ILC are highly heterogenous within and across tissues. Numerous studies have shown that ILC are highly plastic and transdifferentiate in response to environmental cues depending on the tissue and the immune context. Also, in addition to tissue resident ILC deriving from the bone marrow (BM),³ lineage restricted ILC can differentiate locally from progenitor cells in the fetal liver⁴ and gut,⁵ or from circulating precursors.⁶

Most studies on the ILC ontogeny have been conducted in the adult mouse or human, mainly by in vitro differentiation or using transgenic and humanized mouse models (reviewed eg here^{1,7}) but increasingly more information becomes available on the presence of the ILCs within embryos (reviewed eg here⁸⁻¹⁰). Summarized, all ILCs derive from the Flt-3⁺ common lymphoid progenitor (CLP) which resides either in the fetal liver (FL) or the bone-marrow. The expression of the transcription factors Tox and Nfil3 direct their differentiation toward an Il7R⁺ $\alpha_4\beta_7$ integrin⁺ α -Lymphocyte Progenitor (α LP), and a subsequent α LP2 stage where *Flt3* expression is downregulated, although the protein can still be observed.

This article introduces a series of reviews covering Ontogenic Waves of Immune Cell Development appearing in Volume 315 of *Immunological Reviews*.

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The next Early Intermediate Lymphocyte Progenitor (EILP) stage requires the expression Tcf1 and transiently loses IL7R α expression.^{11,12} At this stage, the NK cell precursor (NKP) cells diverge from the general ILC differentiation pathway. The EILP then gives rise to the Common Helper ILC Progenitor (CHILP) marked by the high levels of *Id2* expression, which differentiate into either the LTi precursor (LTiP) or the ILC precursor (ILCP). The LTiP gives rise to the LTi₀ which will mature into LTi₄ cells, while all three ILC lineages except the NK cells emerge from the differentiation of the ILCP (Figure 1).

Most studies on ILC ontogeny have used hematopoietic stem cells (HSC) obtained from the adult mouse bone-marrow,^{13,14} human bone-marrow¹⁵ or progenitors from human blood^{6,16} as model. However, since some ILCs are already present within the embryo⁸ and well before the establishment of the definitive hematopoiesis, this suggests that embryonic ILCs may differentiate from other progenitors than those occupying the bone-marrow. It could also imply that embryonic ILCs fulfill different functions, as was shown before for macrophages^{17,18} or mast cells.¹⁹ In this review, we will focus on the ontogeny of ILC3s with a special attention to the LTi cell, and describe the layered generation of these ILCs observed within the embryo and adult.

2 | EMBRYONIC HEMATOPOIESIS

The earliest hematopoietic progenitors arise in either the yolk sac or embryonic tissue from endothelial cells, as was described in mouse^{20,21} and human.^{22,23} Specific endothelial cells named hemogenic endothelial cells transform into hematopoietic cells in a process called Endothelial-to-Hematopoietic transition (EHT)^{24,25} in which RUNX1 plays a critical role,²⁶ although it remains unknown what initiates this transition. A consequence of hematopoietic progenitor release from different locations (yolk sac vs. embryonic arteries) and at different time points during embryogenesis (between E7.5 and E11.5) is that these waves of progenitor release generate specific hematopoietic lineages. This forms the basis of the layered immune system.²⁷

2.1 | The first hematopoietic progenitors for ILCs appear from the hemogenic endothelium

An embryo consists of the embryo-proper, from which the fetus will be formed, and the extra-embryonic tissues, such as the placenta and yolk-sac, which support embryo growth. Within the yolk-sac,

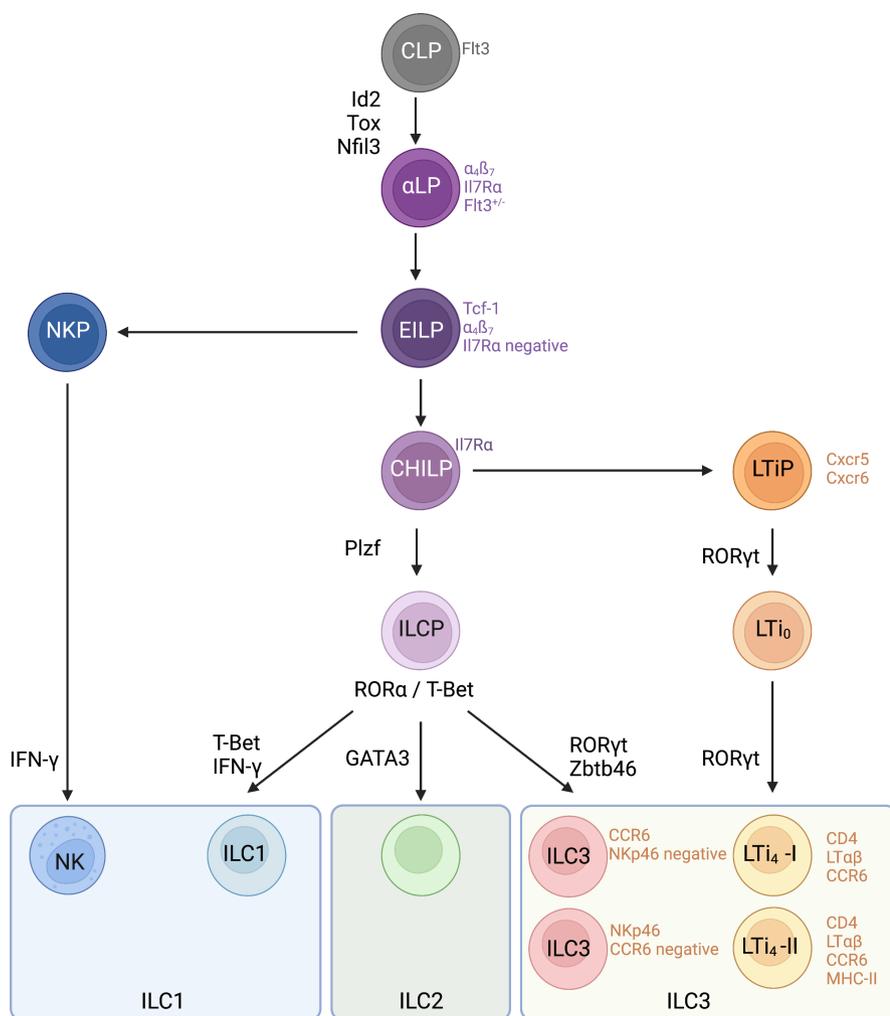


FIGURE 1 A schematic overview of the ILC lineage, which appears to be conserved between embryo and adult. Ontogeny starts with the Common Lymphoid Progenitor (CLP) from which the first ILC dedicated progenitor arises, the αLP. From the Early Intermediate Lymphoid Progenitor (EILP), the NK precursor (NKP) arises, while from the Common Lymphoid progenitor (CHILP), the Lymphoid Tissue Inducer (LTi) lineage segregates. The Innate Lymphoid Cell Progenitor (ILCP) gives rise to the ILC1, 2 and 3 s, mediated by transcription factors ROR α and T-Bet. Transcription factors and cytokine involved in the differentiation of a specific cell type are shown next to the arrows. Characteristic markers are shown at the right, with an emphasis for markers of the ILC3 lineage. There are three main types of ILC3s, the Ncr(NKp46)⁻CCR6⁻, the Ncr(NKp46)⁻CCR6⁺ and the LTi cells which are CD4⁺CCR6⁺. The LTi cells within the embryo can be subdivided into the MHCII negative (LTi₄-I) and positive (LTi₄-II) population

erythroid-myeloid progenitors (EMP) sprout from so-called blood islands and yolk-sac hemogenic endothelium between embryonic day 7 (E7) until around E8.5 and migrate toward the embryo-proper. This is considered the first wave of hematopoiesis. Within the embryo-proper, there are multiple areas of hemogenic endothelial cells. Most of the hematopoietic progenitors arise from the aortic endothelial cell wall within the aorta-gonad-mesonephros (AGM), a region highly conserved in mouse^{21,28} and zebrafish.^{29,30} In the mouse embryo, from this area hematopoietic progenitors start emerging from around E8.5 until E11.5. Lymphoid cells and definitive immune cells will emerge from the AGM during the so-called second and a third wave of hematopoiesis. Notably, HSC progenitors, which will colonize the bone marrow, are derived from the third wave. To date, studies on the appearance of the first ILC progenitors from the hemogenic endothelium are limited to NK or NK-related cells, and LTi cells. For the NK cells, it was described that the mouse and human yolk-sac hemogenic endothelium could differentiate into cells with a cytotoxic potential and expressing the NK cell marker NK1.1.³¹ LTi progenitors arise from the mouse embryonic hemogenic endothelium around E8.³² We recently showed that these progenitors were not derived from the yolk-sac and are most likely not appearing from the AGM, since progenitors from this region start to emerge after E8.5. Besides the AGM, hematopoietic progenitors were shown to emerge from other arteries such as the vitelline artery which runs from the yolk-sac to the embryo.³³ Therefore, a candidate region for the LTi progenitor emergence could be the vitelline artery endothelial cell wall. If this is true and whether possibly progenitors for other immune cells appear from vitelline hemogenic endothelial cells remains to be further established. Unfortunately, to specify progenitor emergence from the vitelline artery specifically, differentiating from the yolk-sac or AGM, would require cell fate-mapping models not yet available.

2.2 | Fetal liver hematopoiesis

After the progenitors emerge from the hemogenic endothelium they migrate through the bloodstream toward the FL.³⁴ The FL was initially considered as a site for expansion of the 500 to 700 progenitors seeding the FL.^{35,36} However, a recent elegant study using better lineage tracing models concluded instead that HSC progenitors modestly expand and rather differentiate within the FL.³⁷ Thus, the FL plays an important role in hematopoiesis for the first differentiation toward more committed precursor cells for the different hematopoietic lineages. In the FL, progenitors undergo the first steps toward a CLP stage, characterized by *Flt3* expression, able to differentiate into all lymphocytes, including ILC.^{32,38,39} Commitment toward the ILC lineage starts with the expression of the Inhibitor of DNA binding 2 (*Id2*), which primary function is to inhibit Single-stranded DNA-binding protein (E2A) to prevent the commitment toward the B-cell lineage.^{40,41} ILCs share transcription factors for their differentiation with T-cells, such as *Id2*. However, they follow a distinctive different ontological pathway, especially during embryogenesis when ILCs are the major lymphocyte population present. In mice⁴² and humans⁴³

the separation between T-cells and ILCs rely on ILC-specific chromatin remodeling and transcriptomic changes associated with the expression of the $\alpha_4\beta_7$ Integrin and the progression to the α LP stage. In the α LP population *Flt3* is downregulated but the protein was still detected on an $IL7R\alpha^- \alpha$ LP2 subset,³⁹ closely resembling the EILP.^{32,38} Notch has been involved in the differentiation the α LP progenitors and specifically of α LP2 progenitor toward a ILC2 lineage.³⁹ From the subsequent CHILP stage, the expression of the zinc-finger transcription factor *Plzf* (encoded by *Zbtb-16*) is required for the generation of ILC-Precursor (ILCP) which will eventually generate ILC1, 2, and 3 cells.^{9,38,44} In the mouse, the LTiP does not require *Plzf* for its differentiation indicating that LTi cells are not derived from ILCP but rather directly from CHILP³⁸ (Figure 1). The α LP and ILCP were observed within the FL and shown in vitro to have the potential to become ILC1, 2, and 3s.³⁸

By using single cell- and bulk- RNA sequencing, we observed that the α LP and LTiP in the FL compared to those observed within the region of the LN anlagen had similar expression profiles. Therefore, those in the LN anlagen did not descend from the population within the FL. We proposed that α LP and LTiP, still lacking *ROR γ t*, migrate from the FL toward the periphery.³² Within the periphery and within their niche, *ROR γ t* expression is induced by retinoic acid.⁴⁵ This transcription factor sets in motion the LTi cell related cell programming and maturation toward the final steps in their ontogeny. Even though some mature LTi cells were observed within the fetal liver,^{38,46,47} they probably represent mature LTi cells migrated back from the periphery.

2.3 | Mouse models to specify different LTi cell waves of origin

To study the origin and lineage of the hematopoietic progenitors, mouse models are used in which specific waves are labeled and their descendants traced. A well-known model is the inducible VE-Cadherin (encoded by *Cdh5*) *Cre-Ert2* model.^{48–50} A single tamoxifen pulse provided to the pregnant mother will label all embryonic endothelial cells with a high efficiency (more than 90%) within 24- or 48-h time frame, depending on the Tamoxifen form used.^{48,51} Even though this model is one of the most efficient to label the cells, the downside is that it targets all endothelial cells, regardless of their location within the yolk-sac or embryo-proper. Since there is an overlap in emergence of the progenitors from both yolk-sac and embryo around E8–E9, this model would thus label descendants from both areas. The *Csf1r^{MeriCreMer}* is another lineage-tracer model with enhanced tamoxifen activation of the Cre-recombinase, and has been used to label all *Csf1r* expressing hematopoietic cells such as myeloid cells from the yolk-sac¹⁸ and later HSC derived cells.⁵² The *Mx4a3^{CreErt2}* model labeled embryonic derived myeloid progenitors.⁵³ Another recent described model to label only embryo-proper derived hematopoietic progenitors makes use of the lack of *Cxcr4* expression within the yolk-sac.⁵⁴ Therefore, the inducible *Cxcr4^{CreErt2}* model resulted in labeling of non-yolk sac derived progenitors for monocytes at a high labeling efficiency more than 80%, but not macrophages derived from

the EMP wave.^{55,56} We previously used this model to exclude the LTi cell origin from the yolk-sac and observed it was derived from a very early embryonic or vitelline arterial source.³² Using these models will therefore allow a more precise determination of the progenitors to a specific location for many immune cells. However, it would be of great interest to develop models which could even better segregate the different waves, such as those derived from the vitelline artery compared to the AGM in order to identify regional differences of hematopoietic progenitor emergence within the embryo-proper.

3 | EMBRYONIC ILC3

The ILC3s are the main ILC population observed in mouse embryos³² and human fetuses.⁵⁷ ILC3 progenitors from the FL colonize different tissues within the embryo, such as thymus,⁵² gut^{58,59} or LN anlagen.³² These tissues are seeded with specific progenitors for their respective lineage. Progenitors seeding the fetal thymus around E13.5 in mouse embryos share characteristics with the CLP from the fetal liver. These cells have the potential to differentiate into all ILCs, but preferentially give rise to thymic LTi cells.⁵² Within the gut, Arg1⁺ expressing ILC precursors (ILCP) were observed.⁵⁸ It is most likely that these cells are derived from a FL progenitor such as the α LP but a direct correlation has not been shown yet. The gut associated Arg1⁺ ILCP were shown to differentiate into ILC1, 2 and 3 populations by fate-mapping experiments.⁵⁸ There is a dramatic expansion of the LTi(like)/ILC3 subset within the fetal gut after E16.5 and in neonates, while the CD4⁺ ILC3/LTi cell population decreased.^{46,59,60} This expansion was mediated by T-Bet (encoded by *Tbx21*) and ROR α .^{59,60} T-Bet and ROR γ t expression regulate each other, as was shown by deleting *Tbx21* (encoding T-Bet) in *Rorc* (encoding ROR γ t) deficient mice which resulted in the rescue of LN formation⁵⁹ (Figure 1).

RORC^{Cre} fate-mapping experiments showed that ILC1, 2 and 3 s observed within the gut were associated with a precursor stage expressing ROR γ t.^{58,59} ROR α and T-Bet were subsequently required to downregulate ROR γ t in the precursor and mature ILC2 and ILC1 lineages.^{39,58,59} Another transcription factor recently found during differentiation of mouse and human CCR6⁺ ILCs, and present within the embryo, is *Zbtb46*, which is under control of ROR γ t. It was shown that these ILC3 cell expressed *Zbtb46* in ILC progenitor stages, although not required for LTi functioning⁶¹ (Figure 1).

An important ILC3 member during embryogenesis is the LTi cell, which play a critical role in embryonic LN and Peyer's patch formation in mouse embryos⁶²⁻⁶⁴ and human fetuses.⁶⁵ As mentioned before, we have described the hemogenic endothelial origin of the LTi cell involved in embryonic LN formation to be the embryonic hemogenic endothelium around embryonic stage E8.0-8.5.³² Another study described that the LTi cells observed within the fetal thymus were derived from HSC cells originating from hemogenic endothelial around E10.5.⁵² These lineage tracing studies reveal that there are potentially multiple waves of (embryonic) LTi cells.

It was shown that FL LTiP express chemokine receptors Cxcr5 and Cxcr6,^{9,38,66} which would aid migration of the LTiP toward LN

anlagen within the periphery. This location is important for the maturation of the LTiP. Indeed, the ligand for Cxcr5, chemokine Cxcl13, is expressed specifically within the LN anlagen and was shown to be essential for the formation of most LN.⁶⁷⁻⁶⁹ When the LTiP enter the area where the LN are formed, they encounter retinoic acid within this niche, possibly initially produced by nearby neurons.⁶⁷ Retinoic acid subsequently induces the expression of ROR γ t to establish LTi cell fate.⁴⁵ At this stage, the cells are named LTi₀ cells, still lack the necessary ligands and receptors to interact with the mesenchymal Lymphoid Tissue Organizer (LTo) cells. The designation LTi zero stems from the notion that these cells do not express the mature LTi marker CD4. CD4 is only expressed on mature mouse LTi cells but not human mature LTi cells⁶⁵ and has an unknown function. TNF-related activation-induced cytokine (Trance, or Receptor activator of nuclear factor kappa-B ligand, Rank-L) and the receptor Trance -R (Rank) are expressed on LTi cells during these stages.^{70,71} The activation of this pathway initiates the last step in the LTi cell maturation. The mature, CD4 expressing, LTi₄ cells express the Lymphotoxin ligands which are required for interacting with the Lymphotoxin-receptor on the LTo cells.⁷²⁻⁷⁴ This interaction results in increased expression of cytokines, chemokines and integrin receptors to attract, retain and maintain more LTi (precursor) cells (reviewed in²). Two different LTi₄ populations have been reported to reside within the embryo, defined by MHCII⁺ expression and the second population lacking MHC but increased *Ii22* and *Arg1* expression.^{10,32,62,63} Interestingly, LTi cells have no known role in antigen presentation. Moreover, antigen presentation within an embryo is contradicting with the notion of a fetal tolerogenic immune system.⁷⁵ Therefore, the role for MHCII on the LTi₄ cell subpopulation remains elusive. It also remains to be established if one of the two LTi₄ populations is the populations responsible for LN formation. It has to be noted that the deletion of MHC-II on ROR γ t expressing LTi cells, using the *Rorc*^{Cre}; *H2-Ab1*^{flox/flox} model, did not result in lack of LNs.⁷⁶ Therefore, it seems unlikely that MHC-II on the LTi₄ subpopulation plays an essential role in LN formation.

It appears that there are at least two trajectories for the LTi cells observed within the embryo. One seeding the LN anlagen and another observed within the fetal thymus, originating from two different hemogenic endothelial sites. Besides the specific LTi cell lineage, there are other LTi (like) and ILC3 cell lineages within the embryo and neonate, mainly present within the gut to become the ILC3 cells. These cells require *Zbtb46*, T-Bet, and ROR α . All populations can be observed within the embryo, with the LTi cells abundantly present during early embryogenesis, and the ILC3s depending on ROR α and T-Bet to expand after E16.5.

4 | REPLACEMENT OF ILC3-LTI CELLS IN ADULT

In the adult, ILC3s have been described as tissue resident and slowly replaced as parabiosis or migration experiments showed a limited migration.^{3,77} However, studies have indicated replacement by Id2⁺

precursors,¹³ from gut residing progenitors⁵ or from bone-marrow residing HSC.¹⁴ Their ontogeny is mostly similar as described within the fetal liver, requiring a CLP, α LP, CHILP and ILC3P.⁷⁸ One difference was that Notch was shown to be involved in the adult ILC3 ontogeny, while not in the fetal liver.^{66,79} Three ILC3 population can be observed in the adult; CD4⁺CCR6⁺ LTi cells, CD4⁺NKp46⁺CCR6⁺, and NKp46⁺CCR6⁻ ILC3s which all share ROR γ t requirement for their maintenance. A subpopulation of the embryonic LTi cells and CCR6⁺ ILC3 populations within the gut were shown to express MHCII. These MHCII⁺CCR6⁺ ILC3 are important in antigen transfer during gut infection^{80–82} or in lungs⁷⁶ while the role for MHCII on LTi cells within the embryo is completely unknown.^{32,62,63} Precise fate-mapping experiments, in which a MHCII gene drives a reporter in all daughter cells, are required to pinpoint a relation between the embryonic MHC-II⁺ LTi cells and ILC3s and their possible descendants within the adult.

The expression of ROR γ t was assumed to be specific for the ILC3 family. However, using a ROR γ t (*Rorc*) fate mapping mouse model showed that the ROR γ t fate mapped cells could become ILC1, 2 and 3 s in embryos, losing ROR γ t expression in precursors and mature ILC1 and 2 s due to upregulation of ROR α and T-Bet.^{58,59} Accordingly, our single cell RNA sequencing data of FL and LN enriched areas showed that all ILC clusters expressed *Rorc*, except for the CLP stage (Figure S4 and S5 in reference³²). Similarly, a CD34⁺ progenitor in humans was described which expressed ROR γ t.^{16,83} This would imply that fate-mapping ROR γ t mouse models should be regarded in a different light and would principally label all ILCs. ROR α and T-Bet would in this case be necessary to aid the differentiation of the precursor into an ILC2 or ILC1 fate.

Since the half-life of ROR γ t⁺ ILC3s was shown to be between 22–26 days,⁴⁶ the cells need to be replaced by either a local resident or from the bone-marrow derived progenitors. Reports have described circulating progenitors replacing the ILC3, also in human.^{6,13,46} In addition, fate-mapping experiments on the origin of the LTi cells revealed that in the adult, LTi cells likely derived from bone-marrow progenitors gradually replaced embryonic derived LTi cells.³² Considering the similar timing of appearance from the hemogenic

endothelium at E10.5, LTi cells within the adult LN are from the same hemogenic endothelial source as thymic LTi cells.⁵² There is sparse information on the function of LTi cells within adult tissues, as they are not essential for tertiary lymphoid formation. Studies have implicated LTi cells in tertiary lymphoid structures in melanoma models⁸⁴ and spleen regeneration after viral infection.⁸⁵ A more recent study revealed that the highly IL-7R α expressing ILC3s, under which LTi cells, are important to regulate IL-7 availability to T-cells in order to regulate their cell numbers.⁸⁶

There are two defined waves of ILC3 and LTi cell origins. For the LTi cells, there is the first wave from hemogenic endothelium at E8.0 which gives rise to embryonic LTi cells involved in LN formation. The other embryonic trajectory occurs from the E10.5 hemogenic endothelium, most likely the being part of the definitive wave which colonizes the bone-marrow which also seed the fetal thymus. Bone-marrow derived LTi cells in the adult replace the embryonic LTi cells gradually. The hemogenic endothelial origin of the ILC3 has not been described yet. The first ILC3 progenitors described were observed within the FL³⁸ and gut.⁵⁸ The gut ILCP progenitor cells expanded and differentiated at the end of the gestation and in neonatal stages, giving rise to ILC3, but also ILC1 and ILC2.^{58,59} In a subsequent third wave, progenitors in the adult gut,⁵ blood¹³ and bone-marrow¹⁴ gradually replace the resident ILC3s.

5 | DISCUSSION

The concept of the layered immune system, with different waves of the immune cell population appearing from different hemogenic origins, has been well established for the macrophages and other immune cell types (reviewed e.g. here⁸⁷). This concept holds true for the LTi cell and ILC3^{38,46,58,59} lineages³² (Figure 2). Studies have shown that also ILC1^{4,88–90} and ILC2⁹¹ have an embryonic wave, seeding embryonic tissues such as fetal liver, LN anlagen, gut and lung and being gradually replaced during life by cells derived from bone-marrow residing progenitors. To better identify the descendants of these specific waves, precise fate-mapping models are

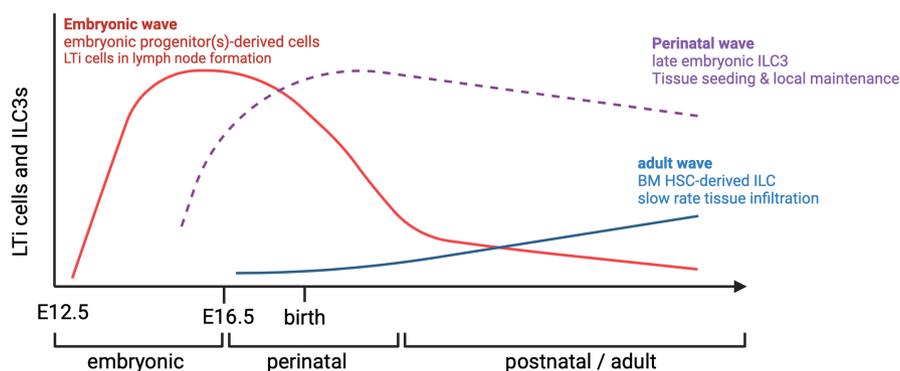


FIGURE 2 ILC3 origins can be divided into three waves. The first (LTi cell) embryonic wave originates from the E8.0–E8.5 hemogenic endothelium and gives rise to LTi cells seeding the LN anlagen in the embryo (red line). During later embryogenesis around E16.5, gut residing ILCP gives rise to ILC3 which expand during term and reside within the neonate (dotted purple line). ILC3 derived from HSCs within the bone-marrow slowly replace the tissue resident embryonic and neonatal ILC3s (blue line)

required. Until now, only the origins of LTi cells have been characterized using different inducible reporter mouse models. These models could also be applied to establish that the other ILCs are also generated in different waves.

A remaining question of ILC ontogeny is the timing of the ILC appearance. Within the embryo, most ILCs are ILC3s and notably the LTi cells are present while other ILCs appear during late embryogenesis or during neonatal stages. It is unclear how the progenitors for the ILC lineages are selected to generate specific ILC lineages or how during embryogenesis mainly LTi cells are generated. In contrast, in the adult mainly other ILCs are generated but not LTi cells. Besides Notch signaling in the α LP2 population for ILC2 lineage,³⁹ there is no information available that the progenitors or precursors like the CLP, CHILP, IELP, or α LP are different in marker expression or differentiation potential within the FL versus bone-marrow. Therefore, it could be possible that the tissue of residence provides cues for an ILCP or LTIP to differentiate toward a specific ILC lineage.

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CONFLICT OF INTEREST

The author declares that there are no competing interests.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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