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INVITED REVIEW

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Assembling the layers of the hematopoietic system: A window of opportunity for thymopoiesis in the embryo

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Summary

During embryonic development, several independent generations of hematopoietic cells were identified. They occur in the yolk sac and the intra-embryonic major arteries, in a narrow window of development. They arise sequentially, starting with primitive erythrocytes in the yolk sac blood islands, progressing to less differentiated erythromyeloid progenitors still in the yolk sac, and culminating with multipotent progenitors, some of which will generate the adult hematopoietic stem cell compartment. All these cells contribute to the formation of a layered hematopoietic system that reflects adaptative strategies to the fetal environment and the embryo's needs. It is mostly composed, at these stages, of erythrocytes and tissue-resident macrophages both of yolk sac origin, the latter persisting throughout life. We propose that subsets of lymphocytes of embryonic origin derive from a different intra-embryonic generation of multipotent cells occurring before the emergence of hematopoietic stem cell progenitors. These multipotent cells have a limited lifespan and generate cells that provide basic protection against pathogens before the adaptive immune system is functional, contribute to tissue development and homeostasis, and shape the establishment of a functional thymus. Understanding the properties of these cells will impact the understanding of childhood leukemia and of adult autoimmune pathology and thymic involution.

KEYWORDS
fetal hematopoiesis, hematopoietic stem cells, lymphoid tissue inducer cells, thymic seeding progenitors, thymus

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1 | FOREWORD

More than 30 years ago, Herzenberg and Herzenberg proposed that the mammalian hematopoietic system is composed of cells originated from different waves of hematopoietic stem cells (HSC) with specific differentiation potential, emerging sequentially during embryonic development.[1] This notion integrated findings suggesting the embryonic origin of some adult lymphocyte subsets that were not easily generated from adult bone marrow (BM) progenitors and was published in a paper entitled "Toward a Layered Immune System". Although the tools to analyze hematopoiesis and the knowledge on progenitor subsets evolved, the notion that the hematopoietic compartment is composed of cells that originate from developmentally restricted progenitors and that persist for life is of actuality.

2 | A HISTORIC PERSPECTIVE

2.1 | Extra-embryonic generation: the yolk sac

Colony-forming cells in the spleen (CFU-S) is an in vivo assay that measures the presence of highly proliferative hematopoietic progenitors considered, for a long time, to comprise HSC.[2]

The first multilineage colony-forming cells were identified in the yolk sac (YS) at embryonic day (E)8 as CFU-S analyzed 8 days after transplantation (CFU-S_8).[3] In vitro colony formation in semi-solid medium was also first detected at this stage, peaked in numbers at E10 and decreased thereafter.[3] These results were consistent with a YS origin of HSC. However, pioneer experiments in the avian model challenged this idea. Chicken chimeras derived from chick embryos that developed in a major histocompatibility complex (MHC) allogeneic egg showed that adult hematopoietic cells derive from the embryo proper and not from the YS.[4]

Primitive erythrocytes were initially identified as the first embryonic hematopoietic cells of YS origin.[5] Circulating primitive erythrocytes are nucleated and express specific hemoglobin chains that have increased capacity to transport oxygen compared to adult hemoglobin.[6] They can be found around E7 of mouse gestation in YS blood islands, peak by the time circulation is established (E8-8.5), and decrease after E12.5, although a few were detected after birth.[5] They circulate but are also found in the fetal liver (FL), the first main site of embryonic hematopoietic differentiation. Limited numbers of primitive macrophages and megakaryocytes have also been reported in the YS blood islands.[5]

Around E8-8.5, a second layer of hematopoietic progenitors emerges in the vasculature of the YS. It is composed of erythro-myeloid progenitors (EMP) that generate definitive erythrocytes and multiple myeloid lineages.[7,8] However, they fail to generate lymphocytes in vitro or in vivo. The YS has long been considered a provider of cells that transiently ensure embryo survival. It came therefore as a surprise that YS EMP-derived macrophages persist for life as tissue-resident macrophages.[9] The best example are microglia, the macrophages residing in the central nervous system (CNS) that, under physiologic conditions, stay throughout adult life with the same original cell composition and are not replaced by BM-derived cells (discussed below). Subsequently, YS mast cells were also found to have a long lifespan.[10] This observation indicates that mature blood cells emerging in the embryo in an HSC-independent manner persist and are functional for long periods of time, thus challenging the paradigm that all adult hematopoietic cells are derived from HSC.

2.2 | Intra-embryonic generation: major arteries

Experiments in several models including the chicken,[4,11] the mouse,[12–14] and the zebrafish[15,16] indicated an intra-embryonic origin of lymphoid progenitors that paralleled that of HSC. This generation was restricted to the major arteries (aorta, omphalo-mesenteric, and vitelline arteries)[17] and occurred in close vicinity to endothelial cells lining the vessel. The emergence of hematopoietic progenitors was directly observed in the zebrafish larvae.[15,16] These observations indicated that hematopoietic progenitors emerge from endothelial cells through a process, designated endothelial-to-hematopoietic transition (EHT), that involves progressive morphological alterations, loss of endothelial cell markers, and acquisition of hematopoietic specific transcripts. It was subsequently proposed that EMP in the YS emerge by a similar mechanism.[18] For the purpose of this review, we will use the term "embryo-derived" for all hematopoietic progenitors that are generated within the embryo proper.

The above experiments indicated that a third layer of hematopoietic progenitors emerges in the intra-embryonic dorsal aorta also called aorta-gonads-mesonephros (AGM) and in the omphalo-mesenteric and vitelline arteries that connect circulation between the embryo proper and the extra-embryonic tissues (YS and placenta).[17] These progenitors can generate erythroid, megakaryocyte, myeloid, and lymphoid cells at the clonal level. They migrate to the FL where they establish the adult HSC compartment. The emergence of the third layer is limited to the period between E9 and E11.5 and partially overlaps in time, in the mouse, with the previous EMP generation.[19]

The first emerging multipotent progenitors were assessed for their capacity to reconstitute the hematopoietic compartment of myeloablated recipient mice for long periods of time. This ability designated long-term reconstitution (LTR) activity is taken as the golden standard to evaluate HSC. These experiments revealed a poor LTR capacity of E9-E10 progenitors.[20] Although CFU-S were detected in the intra-embryonic compartment earlier than in YS,[13,21] very low numbers of cells with LTR activity were found before E12. It was subsequently shown that emerging multipotent progenitors needed to undergo maturation in an organ culture of AGM or FL in order to acquire LTR activity and that these newly generated multipotent progenitors were designated pre-HSC[22] or immature-HSC.[23] One reason for their inefficient reconstitution capacity is their low expression of MHC-class I on the surface that activates host NK cell activity,[14] but other factors are probably also involved in their fast elimination from hosts by innate immune mechanisms. These experiments dissociated CFU-S_{11} and LTR activity, indicating that the

CFU-S assay does not detect HSC.[24] They also indicated that testing the in vivo activity of the emerging multipotent progenitors should not be done in the same assays used to detect adult HSC.

2.3 | The fetal liver, the major embryonic hematopoietic organ

The FL is the main hematopoietic organ in the mouse embryo. It develops from the hepatic endodermal bud (that gives rise to the liver parenchyma), that proliferates in cords that invade the mesodermal septum transversum (giving liver fibroblasts or stellate cells, mesothelial and endothelial cells), and it provides the environmental signals that support hematopoietic differentiation and the establishment of the HSC compartment.[25,26] It was early recognized that hematopoietic cells in FL are originated elsewhere.[25,27] CFU-S_8 were detected in FL at E10.5[3] and CFU-S_{11}[21] at E11.5. Using a competitive in vivo assay, it was shown that LTR activity was first detected in FL at E12.5.[28] Later studies, using organ cultures and, again, in vivo reconstitution assays, confirmed that the FL is initially colonized at E10.5 (30 somite [S] stage), that is, shortly after being produced in the AGM, by immature HSC, which will fully develop to HSC in situ.[23] Ema and Nakauchi further showed that, in FL, the number of cells displaying LTR activity increased more than 30-fold between E12.5 and E16.5, and decreased thereafter. These observations were interpreted as reflecting a single wave of HSC expansion in FL and a progressive exit to colonize the BM or the spleen. Interestingly, E12.5 is also the time when the first B cell progenitors were detected in FL.[29,30]

Although these conclusions remain unchallenged, the extent to which HSC expand in the FL is a matter of debate (see below).

Little is known about the properties of the FL stromal cells that sustain hematopoiesis. Endothelial cells,[31,32] hepatic stellate cells,[32] hepatoblasts[33] and portal vessels associated pericytes[34] have been reported to be involved in HSC expansion and hematopoiesis in FL.[26] These apparently conflicting reports may indicate that several cell types contribute to fetal hematopoiesis and that both compartments, hepatic and hematopoietic, evolve as development progresses. It is, therefore, required to take into consideration how the properties of the different stromal cells evolve along gestation, to understand the factors governing FL hematopoiesis.

2.4 | A layered immune system

In the late 80s it was reported that a small subset of B cells (designated B1 cells to differentiate them from B2 conventional B cells), reside in the peritoneal and pleural cavities, expressed the T cell marker CD5 and were endowed with some surprising properties.[35] B1 cells could expand extensively when transferred into neonatal mice,[36] expressed B cell receptors biased toward the recognition of bacterial products,[37,38] appeared to secrete most natural antibodies[39] and originated from B1 specific B cell progenitors that reside in FL,[40] (BM cells being less efficient than FL in reconstituting the B1 compartment upon transfer[35]). These properties led to the proposal that B1 cells originated from an embryonic-specific progenitor and not from HSC.[41] However, additional experiments indicated that: 1. B1 and conventional B2 cells can share the same progenitors[42]; 2. an antigen receptor from B1 cells can confer B1 properties to conventional B2 cells[43]; 3. some properties of B1 cells can be explained by an inefficient pre-B cell receptor selection in FL, that fails to delete some autoreactive receptors[44]; 4. induction of *Rag* in the BM of *Rag*$^{-/-}$ mice results in the production of B1 cells.[45] In the absence of a consensual view, we will consider here that FL progenitors contribute more efficiently to the B1 compartment than their BM counterparts.

Around the time B1 cells were described it became also evident that subsets of mouse γδ T cells expressing invariant T cell receptors (TCR)[46] also had specific properties not shared with other T cells. They reside in tissues (skin for Vγ5Vδ1- expressing cells, also called dendritic epidermal T cells [DETC], and lung for Vγ6Vδ1-expressing cells), precede by several days conventional αβ T cells in the embryonic thymus[47] and appeared to be of strict embryonic origin.[48–50] Vγ5 and Vγ7 (residing in the intestinal epithelium) expressing T cells were later shown to be positively selected by butyrophilin-like molecules (called Skint1 for Vγ5 and *Btnl1* for Vγ7) expressed by epithelial cells in the thymus and in the intestine, respectively.[51,52] Vγ5 T cells in turn induce the up-regulation of *Skint1* in mTEC (Figure 2).[53] It should be pointed out that γδ T cells have multiple nomenclatures which can be a source of confusion. Thus, Vγ5 and Vγ6 T cells in Heilig and Tonegawa[54] now a widely used nomenclature, have been called Vγ3 and Vγ4 in Garman, Doherty, and Raulet,[55] also used by Havran and Allison. We will use here the Heilig and Tonegawa nomenclature and full correspondence between the different nomenclatures can be found in Raulet.[56]

The basis for the developmental restriction of invariant γδ T cell subsets has not been elucidated. Several possibilities to explain this developmental restriction include 1. an HSC-independent embryonic origin, possibly from YS, 2. epigenetic modifications of HSC as they transit from FL into BM that restricts rearrangement of specific segments within the TCRγ and TCRδ loci, and 3. environmental changes modulating the selection of these subsets.

Although injection of progenitors in embryonic environments is still challenging and difficult to achieve with low cell numbers, fetal thymic organ cultures (FTOC) are highly efficient. BM progenitors seeded in fetal thymic lobes generate αβ and γδ T cells but no Vγ5- or Vγ6-expressing cells indicating different cell intrinsic properties in fetal and adult thymic seeding progenitors (TSP).[49] The embryonic restriction of invariant T cells appears to be more complete than that of B1 B cells because there are no reports of a possible adult BM HSC origin for Vγ5 or Vγ6 T cells. This observation raises the possibility of different underlying mechanisms in the restricted generation of B1 or invariant γδ T cells.

Similar to B1 cells and γδ T cells, a population of lymphoid cells, called lymphoid tissue inducer cells (LTi), that induces lymph node and Peyer's patches formation, appear also to be restricted to the embryonic period. Their development depends on the expression of the orphan receptor Rorc encoded by RoRγt[57] and, although cells

with similar properties have been found in the progeny of BM HSC, only in the FL is RoRγt expressed in lymphoid progenitors before tissue residency.[58]

Thus, the immune system, like embryonic erythroid and myeloid cells, is composed of cells that were generated in a developmentally restricted manner. The origin and function of these different hematopoietic progenitors will be discussed below.

3 | WAVES OF HEMATOPOIESIS

3.1 | Yolk Sac Hematopoiesis – longer than expected

As previously mentioned, the hematopoietic system is established by multiple waves of progenitors. This includes all multipotent progenitors that may or may not undergo maturation into HSC and their progenies (Figure 1).

The YS waves generate mostly erythrocytes responsible for carrying oxygen across the developing embryo, macrophages that play important roles in tissue development and remodeling and megakaryocytes that produce platelets responsible for clotting processes. The idea that these YS populations are only produced and required during embryonic development has been recently challenged.[9,59] Two major recent contributions, using inducible lineage-tracing mouse models, directly showed that distinct tissue-resident macrophage populations, generated during embryonic development from YS progenitors, persist throughout adulthood. By pulsing Runx1^{MerCreMer}Rosa26^{EYFP} embryos with 4-hydroxytamoxifen (OH-TAM) at E7.5, that labels YS progenitors and their progeny, Hoeffel and colleagues showed that around 30% of microglia cells (resident macrophages of the brain) were labeled in adult mice.[59] Using two distinct lineage-tracing models Csf1r^{MeriCreMer}Rosa26^{YFP} pulsed with OH-TAM at E8.5 and Tie2^{MeriCreMer}Rosa26^{YFP} pulsed with OH-TAM at E7.5 or E8.5, Gomez-Perdiguero and colleagues showed that most tissue-resident macrophages (microglia cells from brain, Kupffer

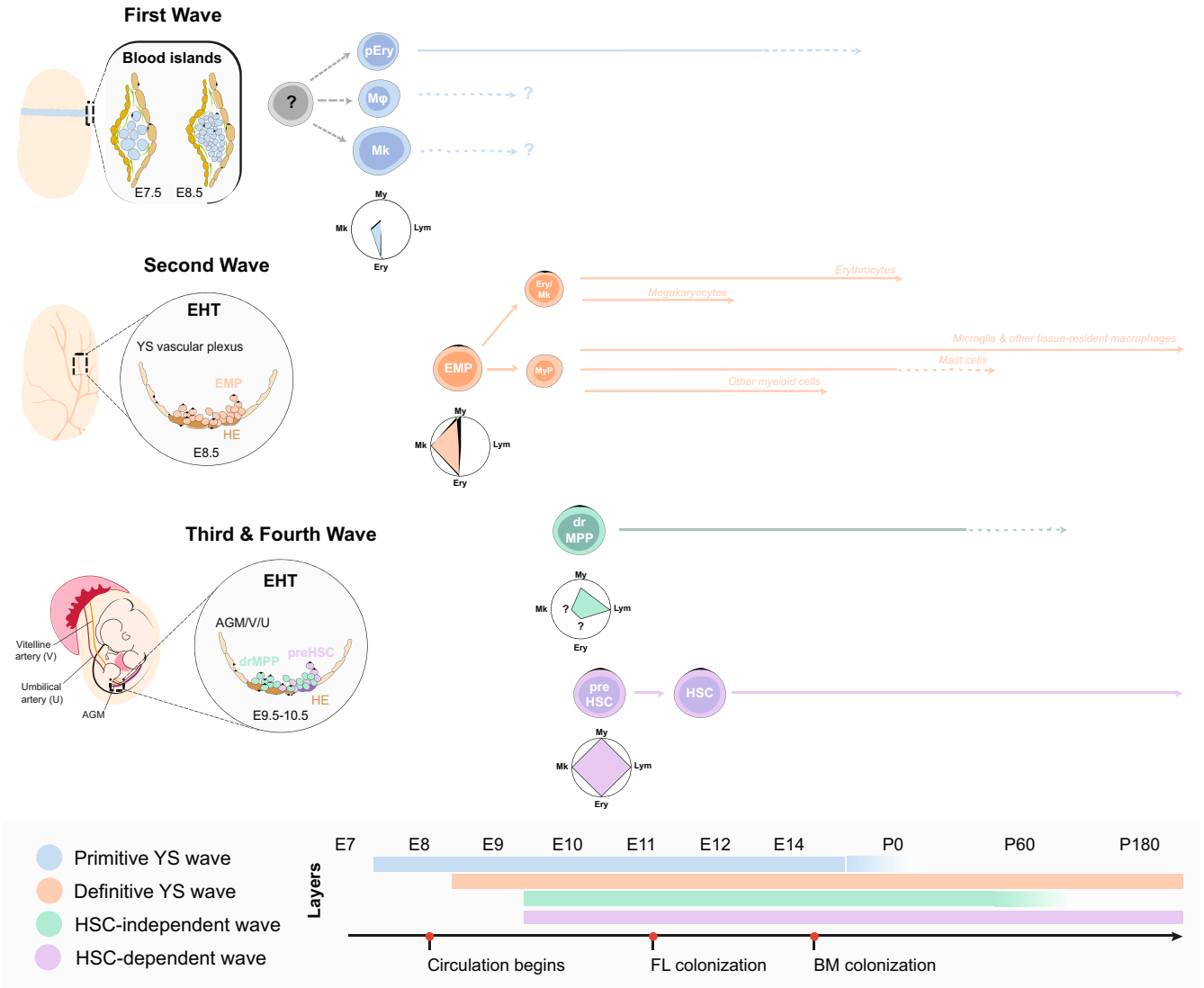

FIGURE 1 Model of hematopoietic progenitor emergence in the embryo: sequential but overlapping waves.

cells from liver, Langerhans cells from skin and alveolar macrophages from lung) originate from a Tie2 expressing EMP progenitor.[9] While about half of those cells were slowly replaced by HSC-derived progeny, microglia cells were only marginally replaced during a year period.[9,60] In fact, the contribution of BM-derived precursors and/or circulating monocytes to the microglia pool has only been observed in situations where the integrity of the blood-brain barrier (BBB) has been disrupted, e.g., using radiation, or in the context of disease.[61–63]

In the developing CNS of rodents, primates and humans, microglia cells were progressively found at key sites of neurogenesis such as the ventricular and subventricular zone,[64–66] where they regulate the size of the neural precursor pool by phagocytosis.[67,68] Dysfunctions in these cells can lead to neuropsychiatric diseases, since they have also been implicated in synaptic pruning and remodeling and have important functions in learning and memory (reviewed in 69,70). YS-derived microglia cells colonize the brain starting at E9, a time when there are still no embryo-derived macrophages, and participate in the BBB formation. This occurs at a specific window of opportunity since no more myeloid precursors colonize the CNS after this period.

YS-derived hematopoiesis has proven to also have a higher relevance than initially thought regarding other blood populations. Indeed, lineage-tracing analysis of YS versus HSC-derived hematopoiesis showed that there is a minimal contribution of HSC to various cellular subsets in the embryo. It is the case for mast cells, which are predominantly YS-derived in the embryo and at perinatal stages in most connective tissues.[10,70] Furthermore, recent evidence showed that, until birth, red blood cell production is maintained by YS EMP with minimal contribution from HSC.[71] Remarkably, YS-derived erythrocyte progenitors require 10-fold lower concentrations of erythropoietin (Epo) than their HSC-derived counterparts and have, therefore, a selective advantage in an Epo-poor environment such as the embryo. This can explain why patients with mutations altering the receptor-binding kinetics of Epo only display anemia postnatally.[72]

These studies have challenged the dogma by which HSC assume blood production from the moment they are generated and are the source of all hematopoietic lineages in the adult, thus providing evidence for the existence of a developmentally regulated "layered" hematopoietic system. The obvious selective pressure for such phenomenon lies in the differences in environments that exist in FL and BM for hematopoiesis. The FL exhibits lower cytokine concentrations and, therefore, progenitors that respond to lower concentrations of chemokines and cytokines, like YS progenitors will have a selective advantage over HSC and their progeny.[44,71,73] One can speculate that, by taking over the production of myeloid and erythroid cells throughout embryonic development, the YS-derived progenitors, or other transient progenitors, are liberating the embryo-derived progenitors, from where HSC are derived, from fulfilling the needs of the embryo, allowing them to set up the stem cell pool and to generate the lymphoid counterparts that are exclusively embryo-derived.

3.2 | Embryo-derived progenitors – multipotency versus stemness

The first embryo-derived hematopoietic progenitors appear in clusters in the dorsal aorta and other major arteries through a process of EHT performed by specialized endothelial cells named hemogenic endothelium (HE).[15,16,74] These progenitors are multipotent, giving rise to all blood lineages, and the only ones with lymphoid potential.[12,13] The EHT process starts with the up-regulation of the transcription factor Runx1, essential for HSC generation, that activates the hematopoietic transcriptional program.[75–77] This leads to a series of morphological changes that manifest as endothelial cells budding and forming intra-aortic hematopoietic cell clusters (IAHC). IAHC can be identified as c-Kit$^+$ and constitute a heterogeneous group of cells with expression of endothelial markers such as CD31 and Flk-1 at the basal level of the cluster, while CD45 can be detected in some cells of the cluster outer region. c-Kit$^+$ cells in the AGM peak at E10.5 (around 800 c-Kit$^+$ cells per aorta) and the majority localized ventrally.[78] At this stage, all c-Kit$^+$ cells are multipotent at the clonal level, but only about 20–30 are pre-HSC.[79] Studies aiming at identifying the distribution of HSC within the IAHC showed their preferential localization in the ventral side of the aorta, hinting a ventral polarity in the development of these cells.[80,81] Major efforts have been made to identify the molecular and signaling pathways that induce the specification of the HE and coordinate HSC emergence (reviewed in 82).

Whether all multipotent progenitors generated in the major arteries will give rise to HSC has recently been questioned.[83] The fact that most of the c-Kit$^+$ cells present in IAHC do not show HSC activity may have two different explanations: All c-Kit$^+$ cells may be HSC precursors at different stages of development and with different LTR activity. Alternatively, they may represent a heterogeneous population of cells generated by two developmental pathways, one that will give rise to HSC and the other fated to differentiate. Whether this heterogeneity is defined at the HE stage or in response to extrinsic cues is not yet clarified. Recently, Dignum and colleagues[84] reported that Cxcr4 expression in the HE associates with HSC activity, whereas Cxcr4$^-$ HE cells generate HSC-independent progenitors that can give rise to all lineages in vitro but lack engraftment post-transplantation. In fact, Cxcr4$^-$ HE represents a heterogeneous progenitor compartment with cells displaying distinct lineage-restricted potential (multipotent, lymphomyeloid, erythromyeloid, and myeloid-only) in culture assays. Another study points to heterogeneity in the HE in terms of HSC potential as *Mecom*, a critical regulator of HSC, is preferentially expressed in progenitors that will mature into phenotypic HSC.[83] Accumulating evidence points, therefore, to the existence of multipotent progenitors independent of HSC that will be further described below.

3.3 | The establishment of the HSC compartment

At this stage, we will discuss the attempts to evaluate the degree of expansion of the different hematopoietic compartments, as well as how the existence of multiple layers can be integrated in these views. The organization of the hematopoietic system is classically viewed as a hierarchical tree, with HSC continuously sustaining all mature blood lineages throughout life. However, this concept has only been based on a "retrospective" assessment of the two hallmarks of HSC (self-renewal and multipotency), upon isolation and transplantation into myeloablated recipients, which might not reflect hematopoiesis in intact animals. In the last years, different lineage-tracing mouse models, genetic barcoding technologies and RNA sequencing have been increasingly implemented to analyze the hematopoietic system in unperturbed settings (reviewed in 85,86). These studies were quite informative on the physiological hematopoietic flux throughout its hierarchical structure and revealed a minimal contribution of adult HSC to downstream compartments.[87–89] These findings were in line with the fact that the adult stem cell compartment is mainly quiescent at steady-state.[90,91] Whereas downstream multipotent progenitors proved robust in vivo self-renewability and sustained adult hematopoiesis,[89] their repopulating ability, when transplanted, was limited, being exhausted after a few weeks,[92] and are, therefore, not clinical targets for LTR. The mechanisms regulating the cell fate decision between self-renewal or differentiation are still largely unknown and their understanding is expected to open doors to the development of efficient HSC manipulation and expansion protocols ex vivo.

In contrast to the adult system, it has been widely accepted that HSC are highly proliferative during embryonic development, expanding significantly in the FL,[28] thus rendering this organ an ideal site to study the mechanisms driving HSC proliferation. However, this dogma was recently challenged.[93,94] To dissect how the adult HSC pool is established, from its generation in the AGM region until its settlement in the BM, reports that aimed to quantify HSC throughout development are here revisited (Table 1).

Only 1–2 HSC (cells with LTR potential) were detected in the AGM at E10.5-E11.5.[13,20] Given the extremely low repopulation activity of the emerging hematopoietic cells in this region, the term pre-HSC was introduced to identify immature precursors that acquired LTR potential after ex vivo cultures.[79] Using this system, Rybtsov and colleagues identified around 20 pre-HSC in E10.5 AGM, peaking at E11.5 (40–60 cells). They further showed that the number of pre-HSC in E11.5 AGM matched the number of mature HSC in E12.5 FL (~60 cells), suggesting that maturation, rather than proliferation, takes place during the migration of cells from the AGM to the FL. This latter value compares well with the ~50 repopulating units (RU) described by[28] in E12.5 FL.

The notion that the FL is a site of significant HSC expansion comes mainly from the work of Ema and Nakauchi who quantified LTR potential from FL cells at different developmental stages. They showed a 33-fold expansion from E12.5 (~50 cells) to E16.5 (~1500), corresponding to about 5 rounds of symmetric division. Despite variable estimates in the number of RUs in E14 FL, ranging from 440 to 2000 (Table 1), most studies converge to the conclusion that the FL contributes to the generation of 1000–2000 HSC. These numbers represent about 10% of the estimated number of HSC in the adult (Table 1). Accordingly, 3 additional rounds of symmetric division should take place after HSC leave the FL (see below).

Recent reports,[93,94] not only questioned the concept that the FL is a privileged niche for active HSC expansion without differentiation, but also concluded that the estimated cohort of HSC generated in the AGM only doubles in size during fetal stages of mouse development. Using a multi-color ROSA26 – Confetti reporter system and based on mouse-to-mouse variance in the composition of adult blood, the authors traced back the hemopoietic hierarchical tree to ~600 E7-E10.5 AGM precursors, a number 10-fold higher than the above-cited number of pre-HSC in E11.5 AGM. In *Conf-Ubiq*$^{ERT2\text{-}Cre}$ mice treated with tamoxifen (TAM) at different stages, they further refined their estimations by showing that the HSC compartment in the BM of 6-month-old mice derived from about 850 E8-E10 endothelial progenitors and 1.700 E12–14 hematopoietic progenitors, this number remaining constant up at least 9 days postnatally. Altogether, these results indicated that the size of the embryonic pool of HSC is primarily determined by the very high number of pre-HSC generated in the AGM, with not more than 1 round of division occurring at a later stage during embryonic development, eventually in the FL.[94]

Numerous studies showed that essentially all FL cells are in active division. Between E12.5 and E14.5, all FL Sca-1^{+}c-Kit^{+} cells are in cycle, as demonstrated by BrdU uptake analysis.[95] Notably, Ganuza et al. also estimated 4 rounds of division of CD45^{+}c-Kit^{+}Lin^{-} progenitors from E12.5 to E14.5[94] in line with Ema et al. This notwithstanding, an active cycling HSC population is not necessarily in contradiction with a low net expansion, as asymmetric division or differentiation might occur, not duplicating the stem cell pool at each division. This is what Ganuza et al. suggest: FL HSC are actively dividing but most daughter cells are biased for differentiation, implying that the FL is not a privileged niche for active HSC expansion without differentiation.

Although HSC migrate to the BM as early as E15.5, only minimal HSC activity is detected in this tissue during fetal stages.[96–98] This might indicate that, either the migration from the FL to the BM occurs progressively (or massively only after birth), or that the establishment in the new niche might temporarily compromise their LTR potential. Although quantification of transplantable HSC throughout the first days after birth has not been performed, the entire HSC population was reported to be in cycle during the first two weeks after birth, switching progressively to a quiescence state that is fully acquired by the end of the fourth week.[96] An increasing contribution of precursor cells to the adult BM pool could be estimated using the *Conf-Ubiq*$^{ERT2\text{-}Cre}$ model, from ~2.000 around birth to ~5.700 at 3 weeks.[94] Overall, these results are also in line with the classical notion that, after birth, the HSC compartment must expand about 10-fold (~3 rounds of symmetric division) to reach the 10.000–20.000 HSC in adult BM (Table 1).

TABLE 1 Reports quantifying HSC at different developmental stages.

	AGM						FL					BM				
Method	E8	E9	E10	E11	E12	E11	E12	E14	E16	E18	E18	2 wk	3 wk	4 wk	Adult	Reference
LTRA; LDA	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1 CRU/ 10.000 (20.000)[a]	99
LTRA; LDA	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1 CRU/ 11.200 (18.000)[a]	100
LTRA	–	–	–	–	–	–	–	1/15.700 (1300)[b]	–	–	–	–	–	–	–	101
LTRA	–	–	–	–	–	–	–	1/17.300 (1200)[b]	–	–	–	–	–	–	–	102
LTRA; LDA	–	–	–	–	–	–	–	1.100 CRU	–	–	–	–	–	–	–	103
LTRA ; LDA	–	–	–	–	–	n. d	1/39.840 (50) 40 CRU	1/40.980 (488)	1/36.900 (1.626) 1.538 CRU	1/43.487 (1.035)	–	–	–	–	–	28
LTRA	–	–	–	–	–	–	–	1/23.000 (900)[b]	–	–	–	–	–	–	1/20.000 (10.000)[a]	104
LTRA	–	–	–	n. d	<5	n. d	30	500	1.000	–	<10	–	–	–	–	105
LTRA; LDA	–	–	–	–	–	–	–	2.000 CRU 1/35.000 ($Ter119^-$)	–	1/73.000	1/95.000 or 1/6.600 (Lin^-)	–	1/6.500 (Lin^-)	1/6.300 (Lin^-)	1/5.500 (Lin^-)	96
LTRA	–	–	–	–	–	–	–	443 ESLAM[c]	–	1044 ESLAM	7 ESLAM	–	1016 ESLAM	983 ESLAM	1700 ESLAM	106
LTRA; LDA	–	–	–	–	–	<1	35 30 CRU	600 SLAM[c]	600 (E15.5)	–	–	–	–	–	–	107
LTRA (cultured explants)	–	–	–	55	10	n. d	60	–	–	–	–	–	–	–	–	79
MtMV of Confetti—reporter labeling	600 Pre. (E7.5)	850 Pre.	–	–	–	1700 Pre.	–	–	–	3.000 Pre.	5.700 Pre.	–	1/18.320 (11.000)[a]	93,94		

Note: Unless otherwise stated, cell numbers or frequencies are indicated as RUs relative to the respective organ's total cell number and some values were rounded for simplification.

Abbreviations: CRU, competitive repopulating unit; LDA, limiting dilution assay; LTRA, long-term repopulation assay (transplantation assays) – repopulation potential was considered for at least 16 weeks after transplantation; n.d, not detected; Pre., precursors.

[a]Total cell number estimated considering 2×10^8 total BM cells.

[b]Total cell number estimated using the total cell number per FL from (Ema & Nakauchi, 2000).

[c]ESLAM ($CD45^+$ $EPCR^+$ $CD48^-$ $CD150^+$) or SLAM (LSK $CD48^-$ $CD150^+$) cells with LTR potential.

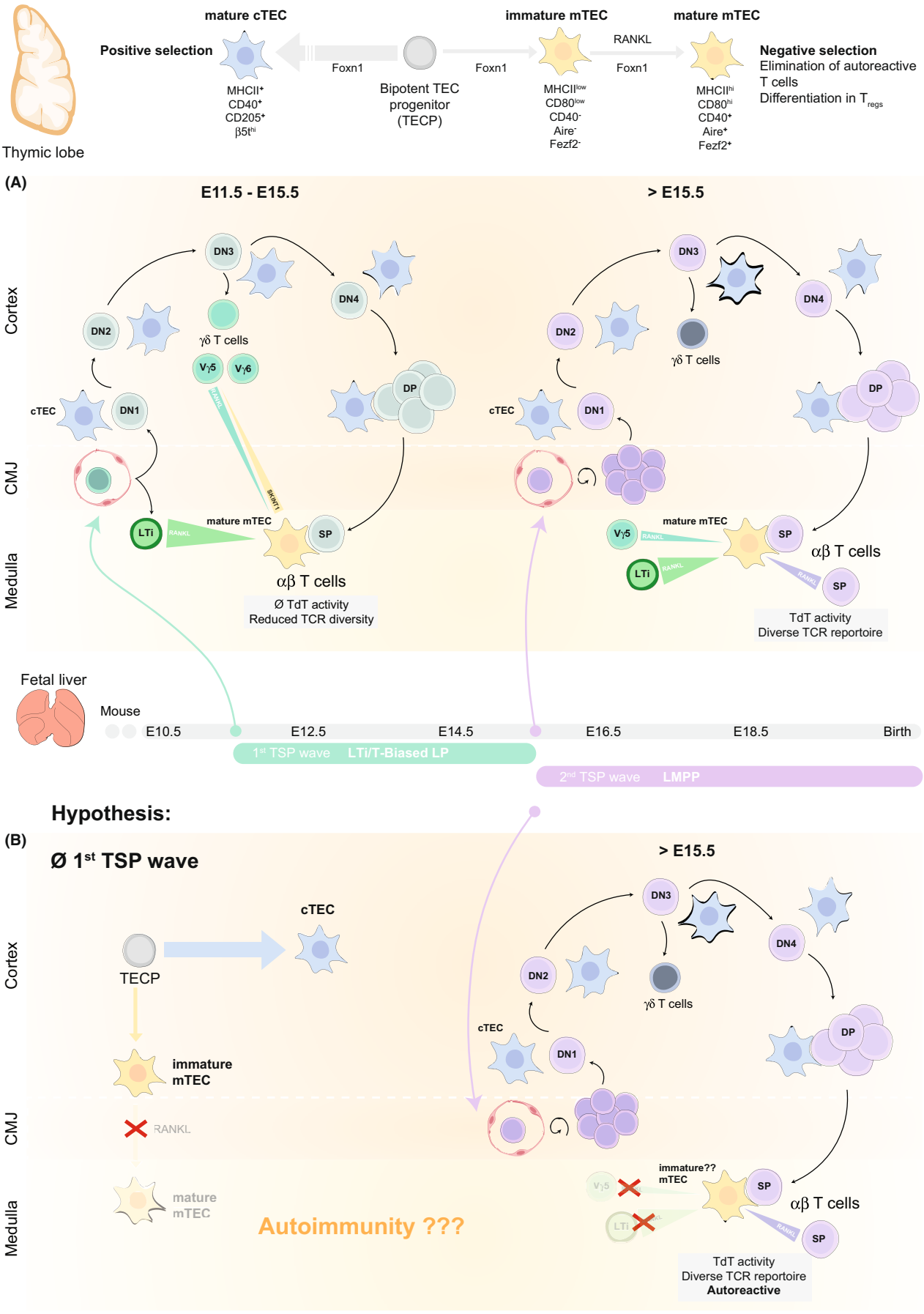

FIGURE 2 Importance of an orchestrated TSP colonization for thymocyte selection and thymic architecture.

3.3.1 | Is HSC embryonic expansion in FL a dogma on the edge?

Although there is some variability in the estimations of fetal HSC numbers (Table 1), the most conflicting value that impacts the extent of embryonic HSC expansion is the starting number of AGM progenitors: ~60 immature HSC precursors[79] vs. ~600 precursors fated to become HSC.[93] Numbers based on transplantation systems are likely prone to underestimations. Also, it should be noted that the work of Ema and Nakauchi[28] is not informative as to whether the increase in RU they observed was due to expansion of HSC or to maturation of multipotent progenitors into cells with LTR activity. While the strategy of Ganuza et al[94] is of considerable interest, they assume that the linear correlation they found between sample-to-sample variance and numbers of precursors established with fibroblastic cell lines in vitro also applies to hematopoietic progenitors in vivo. Their system is prone to overestimations because newly generated progenitors are actively cycling and the progeny of single cells can be marked more than once during the period of TAM activity. Furthermore, their view does not easily accommodate increasing evidence suggesting that, similar to adult HSC, the bulk of embryonic HSC are not actively contributing to blood production, which is essentially ensured by other multipotent progenitors (originated from HSC-independent AGM or YS waves).[71,83,108]

A precise quantification of the stem/progenitor cell compartments throughout ontogeny, contemplating the layered organization, is still missing. Understanding the kinetics of the different waves and progenitors in the FL and fetal and neonatal BM will be fundamental to comprehending the establishment of the adult hematopoietic system and the impact of different niches in hematopoietic development.

Many questions remain unresolved: What is the fraction of multipotent cells generated in the intra-embryonic vessels that evolve to become HSC? Does the FL microenvironment have a role in HSC proliferation? Does the neonatal BM also sustain HSC expansion? Is HSC expansion niche-dependent or a cell-intrinsic property? What contributes to the HSC shift into quiescence? Knowing that HSC expansion only occurs during the initial stages of development, when the whole organism is growing, the possibility that a general mechanism, rather than a local one is the key to the conundrum, cannot be disregarded.

4 | LAYERED THYMOPOIESIS AND THE THYMIC ARCHITECTURE

4.1 | The thymus

Within the thymus, thymic epithelial cells (TEC) provide the unique microenvironment required for developing functionally diverse T cells expressing TCR reactive to foreign antigens while tolerant to self. TEC coordinate virtually all stages of T cell development, providing key cytokines, chemokines, and ligands for the commitment, survival, proliferation, and selection of T cell precursors (thymocytes).[109] Deficits in thymic function arise with aging, cytoablative protocols or during pregnancy and infection, contributing to poor T-cell responses to newly encountered pathogens and predisposing to autoimmunity and cancer. This deficit is due to an involution of the organ that is characterized by a progressive loss of architectural integrity, size, as well as cellularity that, in turn, results in reduced T lymphopoiesis. While the involution observed with aging appears to be irreversible, transient involution, such as the one occurring in pregnancy, after irradiation, cytostatic drugs, or infection, is reversible indicating that the adult epithelial compartment has regenerative capacity (reviewed in 110).

The TEC compartment is classically subdivided into functionally distinct cortical (c) and medullary (m) TEC and develops from the endoderm of the third pharyngeal pouch.[111] The use of specific surface markers[112,113] and clonal analysis[114,115] established that cTEC and mTEC derive from common bipotent TEC progenitors (TECP). Bipotent TECP have also been identified in the adult thymus up to 9 months of age.[116] These cells resemble cTEC by phenotype.[117,118] Interestingly, the bipotent compartment has recently been characterized as biased toward cTEC differentiation during neonatal life, shifting to biased toward mTEC differentiation thereafter.[119]

Whereas cTEC regulate T cell lineage commitment and positive selection, mTEC contribute to the elimination of autoreactive T cells and to the differentiation of regulatory T cells. The key role of mTEC in tolerance induction depends on their capacity to express tissue-restricted antigens (TRA), a process that is determined in part by *Aire*[120] and *Fezf2*.[121] TRA expression by mTEC anticipates the presentation of peripheral self-antigens to mature T cells,[122] such that potentially autoreactive thymocytes are eliminated or deviated to the T regulatory cell lineage. Thus, genetic defects that impair TEC function will result in a broad range of life-threatening pathologies that range from immunodeficiency to autoimmunity.

The developmental pathway of cTEC remains elusive, whereas our understanding of the molecular events that regulate mTEC differentiation is far more detailed. The development of mTEC depends on instructive signals provided by distinct hematopoietic cells, including lymphoid tissue inducer (LTi) cells, γδT cells, and $CD4^+$ T cells.[53] These lymphoepithelial interactions, commonly termed "thymic crosstalk", engage specific members of the tumor necrosis factor receptor superfamily (TNFRSF), including receptor activator of NF-kB (RANK) and lymphotoxin a receptor (LTaR) in mTEC and their progenitors, and CD40 and lymphotoxin a in thymocytes, which, in turn, activate nuclear factor kappa B (NF-kB) signaling pathways required for mTEC maturation.

4.2 | Thymic epithelial regeneration

The thymus is a highly dynamic organ that increases in size for the first 3–4 weeks of post-natal life, in the mouse. After that, the thymus starts a long involution process with decreased numbers of

thymocytes and of epithelial cells such that, in aging mice and humans, few naïve T cells are produced. *Foxn1* is a transcription factor required for the formation of a functional epithelium. Loss of function mutations in *Foxn1* (like the one found in the nude mouse) results in the absence of thymus and hair follicles, although TECP are still present.[123] Most TEC express *Foxn1* during embryonic development and this expression is reduced in post-natal mice.[124] Reduced Foxn1 expression results in decreased levels of the target genes *Dll4*, *Kitl*, and *Cxcl12*, that are important for the earlier stages of thymocyte development. Delayed involution and regeneration of aging thymus function and architecture are observed when TEC are forced to upregulate *Foxn1* expression. Aging mice with increased *Foxn1* expression have an augmented TEC compartment that comprises MHCII^{low} TECP, and mTEC with an increase in *Aire* and TRA expression.[125]

Other environmental factors impact the cellularity of the thymus and its architecture. Possibly the most relevant is the myeloablative treatment required for BM transplantation. IL-22 is an important factor produced by ILC3, that is triggered by a decrease in double-positive thymocytes that follows total body irradiation (TBI). In the absence of IL-22 or ILC3, the TEC compartment does not recover as assessed up to 28 days after TBI.[126] RANK ligand (RANKL), that is produced by LTi and CD4$^+$ T cells, appears to be upregulated after myeloablative treatment and induces thymic regeneration.[127] Increased levels of lymphotoxin α in LTi cells upon RANKL upregulation take part in epithelial regeneration, raising the possibility that their clinical administration could improve thymic regeneration in transplanted patients. Two other molecules are upregulated in the thymus after irradiation. One is BMP4[128] that is produced by endothelial cells and regulates the expression of *Foxn1* and its targets and the other is *Fgf7* or keratinocyte growth factor (KGF). KGF treatment in vivo was shown to expand a population of Ly51$^+$Foxn1$^+$ cTEC[129] and to improve thymocyte recovery after myeloablation or BM transplantation (reviewed in 110). However, treatment with KGF after BM transplantation failed to improve T cell production in humans,[130] indicating species-specific variations in the regulation of factors that determine the thymus epithelial compartment.

Several TEC subsets were shown to be in cycle in the adult thymus and more precise BrdU incorporation experiments have shown that the *Aire*$^+$ mTEC cells are continuously renewed with a half-life of 2 weeks.[131]

Altogether the observations summarized above indicated that TEC respond to direct injury or massive thymocyte depletion by activating several mechanisms that restore their numbers indicating that it is a highly dynamic compartment. It is interesting to notice that some of these mechanisms of regenerative process specifically involve ILC3/LTi cells, highlighting their fundamental role in the homeostasis of the adult TEC compartment. It is intriguing to observe that these cells are very infrequent in the adult thymus.[132]

Thymomas are malignant hyperplasias of TEC, frequently associated with a decreased expression of *Aire* and a disorganized thymic architecture. This disease is also frequently associated with multiorgan autoimmune disorders,[133] including myasthenia gravis characterized by the presence of autoantibodies against acetylcholine receptors. This association of pathologies indicates that loss of *Aire* expression in the adult thymus results in a loss of self-tolerance.

4.3 | Embryonic thymus colonization: two waves of TSP

Continuous colonization of the thymus by hematopoietic progenitors is required for normal T cell production. The thymus anlage is initially colonized after E11 of mouse gestation[111] and fetal thymopoiesis occurs in two phases, initiated by two distinct waves of hematopoietic TSP, corresponding to the most immature subset of ETP[134] (Figure 2A). TSP of the first wave bear surface markers of common lymphoid progenitors (CLP) but have lost B cell differentiation potential. They have limited proliferative capacity and rapidly differentiate into mature T cells.[135] First wave TSP are the only progenitors that generate LTi cells and invariant Vγ5- or Vγ6-expressing T cells, which are exclusively produced before birth. Of note, thymic LTi appear to derive from a common T/ILC progenitor, in contrast to all other LTi that are generated in the FL and exit the organ to form the lymph nodes and Payer's patches anlage.[58,136] Of interest, bipotent ILC/Vγ5 progenitors were detected among first wave TSP (10% of all T/ILC progenitors).[135]

LTi and invariant Vγ5$^+$ T cells are the first mature cells to be produced in the embryonic thymus and both were shown to play a role in the maturation of mTEC through the expression of RANKL.[53,109] Although many mature T cells express RANKL, it is tempting to speculate that embryonic restricted cells operate in a time-window that ensures early maturation of the epithelial compartment. Loss of both populations leads to a transient impairment in mTEC maturation and to an altered thymic architecture.[53] The impact that a defective progeny of the first wave TSP has in the thymic epithelial compartment throughout adult life has not been directly assessed although *Aire* deficiency in the first days of post-natal life-induced autoimmunity.[137] This observation raises the possibility that Vγ5 T cells and LTi cells generated from the first wave TSP are essential for proper adult immune function. Thus, the first wave of TSP would fulfill the essential function of inducing TEC maturation before TCRαβ T cells develop, thereby shaping the thymic architecture required for thymocyte selection (Figure 2B). It is also conceivable that the TEC compartment has a window of opportunity for adequate maturation and that the equilibrium between mature TEC and TECP determines, at this stage, the proper response to disturbances in the adult TEC compartment. If that were the case, a late or inadequate crosstalk between thymocytes and TEC would result in a life-long defective compartment with potential consequences for development of autoimmunity and premature thymic involution.

After birth, invariant Vγ5$^+$ T cells migrate to the skin where they expand and contribute to the integrity of the epithelial barrier, and to

the tissue repair upon injury.[48,138] Thymic LTi are also gradually lost although their fate is presently unknown.[132] To date, the role of invariant Vγ6$^+$ T cells in thymic organogenesis has not been addressed. These cells are also of exclusive embryonic origin, and their development is coupled with their capacity to produce high levels of IL-17. While some stay resident in the thymus, the vast majority leave to the periphery in the perinatal period and colonize the mucosa of the genitourinary tract as well as the airway epithelia where they play an important role in acute inflammation, and tissue repair.[139] Although the first TSP also produce αβ T cells, the absence of terminal deoxynucleotidyltransferase (TdT) and, therefore, of N sequence additions at that stage limits the diversity of their TCR.[135] They are thought to be rapidly replaced by the highly diverse T cells produced from the second wave TSP that colonize the thymus after E15.5. These progenitors are endowed with high proliferative capacity and generate large numbers of TCR-diverse, αβ- and γδ-T cells (Figure 2A). Second wave TSP share essentially all key properties with adult TSP.[134]

4.4 | The origin of fetal TSP

The first TSP display general hallmarks of CLP but also express high levels of *Ccr7*, *Ccr9*, *Cd7*, and *Glis3*, typical of FL CLP that have lost B-cell potential. Indeed, in vitro and in vivo studies demonstrated that they have restricted T/ILC potential.[73] Of interest, these cells were identified by the surface expression of paired immunoglobulin-like receptors (PIR).[73]

A subset of CLP expressing PIR A/B was identified in FL exclusively between E11 and E14.[73] This subset had the same transcriptional signature of first-wave TSP, suggesting a lineage relationship. Similarly, again exclusively in the referred time window, the FL also harbors a subset of lymphomyeloid-primed progenitors (LMPP) that are PIR$^+$, whose transcription signature is already biased toward restricted T/ILC potential.[73] However, the capacity of PIR$^+$ LMPP and PIR$^+$ CLP to generate LTi and invariant Vγ5$^+$ T-cells has not been addressed experimentally. The second wave of TSP is made of cells with the hallmarks of LMPP (PIR-), that is, still retaining full lymphoid and limited myeloid potentials. It is worth noting that they only appear in the thymus after E15.5 despite the fact that their LMPP (PIR$^-$) FL equivalents largely outnumber FL PIR$^+$ LMPP and PIR$^+$ CLP between E11 and E14.[73,134]

4.4.1 | First wave of TSP may be HSC-independent

As mentioned above, HSC are first detected in E12 FL where they increase in numbers up to E16.[28] The fact that PIR$^+$ LMPP and PIR$^+$ CLP are already detected in FL and in circulation at E11, coincident with the onset of thymic colonization, implies that first-wave TSP must be HSC-independent.

Whether the first wave of TSP originates from YS or intra-embryonic multilineage progenitors that emerge from the dorsal aorta has been a matter of debate due to the fact that, in mice, the time window of hematopoiesis at both sites largely overlaps. It has been proposed that YS-derived cells have the potential to generate lymphoid cells.[140] These results were based on the observation that lymphoid-associated transcripts (namely Il7Ra, Rag1, and Rag2) were detected in YS cells, prior to the emergence of HSC.[140] Along the same line, it was also suggested that YS progenitors were the source of first wave TSP, as assessed by the emergence of invariant Vγ5$^+$ T cells, in experiments using a temporally-controlled lineage tracing model.[141] However, as the authors themselves acknowledged, the results were as well compatible with an intra-embryonic, HSC-independent, origin of these cells.

Recently, Elsaid et al[135] using a fate-mapping mouse line allowing the identification of the first emerging Il7Rα-expressing cells in E9.5 embryos, demonstrated that the expression of lymphoid-associated genes by YS progenitors, including EMP, was transient, and that IL7Rα-expressing YS cells had no T or B cell potential, as assessed in highly sensitive in vitro assays and in FTOC. Although Vγ5$^+$ T cells and LTi do not appear to derive from EMP, it has been difficult to determine the exact origin of these cells. These cells are labeled to the same extent as HSC in the Csf1R^{MERCreMER} mouse model[135] but not in the Cdh5^{CreERT2} model[141] when induced at E10.5. The former targets all hematopoietic and myeloid progenitors whereas the latter targets cells at their origin in the HE, in a shorter time window. Taken together, the results are compatible with the first TSP originating from a late YS multipotent progenitor independent from EMP, or from early intra-embryonic multipotent progenitors that have low probability to integrate the HSC compartment. Heterogeneity in the capacity of multipotent progenitors to integrate the HSC compartment has previously been reported.[142] A population of Flk2-expressing cells, detected in the FL prior to E12.5 that appear to preferentially differentiate into lymphocytes, particularly of the embryonic type.[142] Furthermore, intra-embryonic, HSC-independent progenitors with T-cell potential revealed by in vitro clonal assays and single-cell transcriptomics have also been observed.[84] All these experiments point to a fourth generation of transient progenitors biased for differentiation that generate embryonic-type lymphoid cells independently from HSC.

4.4.2 | Zebrafish and humans

Using high-resolution spatiotemporal laser-induced lineage tracing technology in zebrafish Tian et al[143] showed the emergence of HSC-independent and HSC-dependent progenitors emerge at non-overlapping time windows. Specifically, they showed that the first T-cells identified in the embryonic thymus arise from HSC-independent progenitors. T cells generated from this first wave were no longer detectable at the juvenile stage. These results were recently confirmed by Ulloa et al[144] using different experimental strategies.

In humans, evidence also exists supporting the concept of a layered organization of thymopoiesis during development. It has

been proposed that fetal and adult HSC give rise to distinct T-cell lineages.[145] This study adds to a long series of publications demonstrating that the fetal and the adult T-cell immune systems are quite distinct, in line with the very old concept that the fetal environment is prone to tolerance induction. However, it does not directly inform on whether fetal and adult HSC are intrinsically distinct.

Vermijlen et al.[146] identified human semi-invariant and invariant γδ T-cell populations exclusively produced in the fetus, whose development was coupled to an effector program, namely the production of IFNγ and granzymes. These developmental properties appear analogous to those of first wave TSP-dependent invariant Vγ5-expressing T cells in the mouse thymus. Recently the same group demonstrated that the above-cited properties of fetal γδ T-cells were due to the expression of high levels of the RNA-binding protein Lin28b, and that this type of cells could only be generated in vitro from fetal HSC precursors or Lin28b-transduced post-natal HSC.[147] Whether expression levels of Lin28b might distinguish waves of TSP, or HSC-dependent from HSC-independent multipotent progenitors, remains at this stage speculative. Interestingly, forced expression of Lin28b in mice confers adult BM progenitors with the capacity to generate fetal-type cells like B1 B and NKTγδ cells, in mice.[148]

Highlights of current knowledge on fetal thymopoiesis, as here summarized, add to the many studies in recent years that converged to the concept that, in the mouse embryo, hematopoiesis is essentially driven by YS-derived and intra-embryonic HSC-independent progenitors. A contribution of HSC to late fetal thymopoiesis is likely to occur in species with long gestation periods. However, as discussed elsewhere,[149] even in these cases the evolutionary strategy of a layered organization of thymopoiesis is, in all likelihood, conserved

5 | A NEW HSC-INDEPENDENT INTRA-EMBRYONIC WAVE OF HEMATOPOIETIC GENERATION

Considerations relevant when studying the embryonic origin of the hematopoietic populations are 1. In the mouse, the three generations of hematopoietic progenitors occur sequentially but overlap 2. It has been challenging to find specific markers that are uniquely expressed in YS or intra-embryonic HE; 3. After circulation is established (E8.5) it is difficult to define the intra- or extra-embryonic origin of the cells because blood vessels connect YS and embryo proper 4. Lymphoid potential does not equate with HSC generation because some lymphoid progenitors could be HSC-independent[141,142]; 5. However, the absence of LTR activity in embryonic cells does not preclude the presence of pre- or immature HSC; 6. A third event of EHT occurs in the omphalo-mesenteric and vitelline arteries that connect the embryo proper with the placenta and YS. Therefore, isolation of extra-embryonic tissue including these vessels will exhibit multipotent hematopoietic activity. Ultimately, given these considerations, the YS versus AGM origin of the progenitors becomes of limited functional relevance. Instead, it is their differentiation potential and their capacity to contribute to adult hematopoiesis that are of key significance.

The fundamentally different properties of embryonic restricted lymphocytes compared with their adult counterparts raise the problem of their origin. If all lymphoid cells originate from HSC, it is not obvious why a cell that is in nature multipotent becomes unable to generate a very specific subset of cells as they transit from the FL to the BM. As discussed above, however, FL and BM are very different environments and could exert selective pressures on the progeny of HSC. FL exhibit low levels of Epo, but also possibly of most hematopoietic cytokines including interleukin (IL)-7, a potent regulator of lymphoid development. Low levels of IL-7 favor the accumulation of T/ILC versus B progenitors in the FL[73] and modulate the repertoire of B1 B cells.[44] It could be argued that irrespective of their site of origin, the first layer of lymphoid cells exhibits properties that were primarily selected as an adaptation to environmental cues, determining different cell fates. The transition from FL to the BM could also induce epigenetic modifications modulating the differentiation potential and rendering some *loci* inaccessible in HSC.

However, functional properties of embryonic lymphoid cells appear to be specific of the developmental stage and to be cell autonomous, and, therefore, it is tempting to consider the possibility that they derive from different progenitors that are developmentally restricted (Figure 2). These cells would derive from an independent wave of hematopoietic generation between the emergence of YS EMP and that of the dorsal aorta HSC. They would have multipotent differentiation properties but would have low probability to integrate the adult HSC pool. They would rather devote to differentiate into embryonic type lymphocytes that have fundamental roles in organ development.

Several lines of evidence support this notion and are outlined below.

The possibility to follow the progeny of cells by permanently inducing the expression of a fluorescent protein (lineage tracing, fate map) revolutionized developmental hematology. Because it has been difficult to identify specific markers to distinguish the hemogenic endothelium that generates EMP and multipotent progenitors, experiments that fate map the second and third layer of hematopoietic generation relied in an induction of fluorescence at different developmental stages:

1. Lineage tracing studies[141] show low frequency of fluorescent-labeled invariant γδ T cells and LTi cells were observed in conditions that label YS-EMP, indicating that they originate from different progenitors also found by Elsaid et al.[135] However, also conditions that label a majority of HSC yield low frequency of YFP labeled embryonic lymphoid cells, suggesting that they do not originate from HSC or their immediate progenitors.
2. A transient population of multipotent progenitors appears biased to the production of B1 cells and invariant γδ T cells in vivo,

and these cells emerge in the mouse embryo before HSC are detected.[142]

3. Human fetal hematopoietic progenitors were shown to generate T cells with different properties than their adult BM counterparts.[150]
4. CXCR4 expression in the dorsal aorta HE distinguishes an early and a late hematopoietic generation that differ in their capacity to integrate the HSC compartment.[84]

Another compelling argument is the analysis of the incidence of childhood leukemias:

1. The higher incidence of myeloid leukemias in aged individuals contrasts with the higher incidence of acute lymphoid leukemias (ALL) in infants.[151] It was recently suggested that the target of childhood ALL is a progenitor cell that is lymphoid biased and can only be found during embryonic development.
2. Another interesting observation is the high susceptibility of children with Down syndrome (DS) to develop leukemias, not found in adult DS patients.[152] It is, therefore, tempting to speculate that the target cell for the infant DS leukemia is no longer present in the adult BM thus accounting for the discrepancy between young and adult DS patients in developing the disease.[153]
3. The common ALL translocation, MLL-AF9, induces different diseases depending on whether it is induced in neonatal human progenitors or in adult $CD34^+$ cells. It is therefore tempting to speculate that, in humans, lymphoid-biased transient multipotent cells, equivalent to the ones we propose here, are targets of the translocations that lead to ALL.[154]

The first wave of TSP induces the maturation of the thymic architecture, essential to the selection of conventional T cell.[53,135] Genetic or environmentally induced variations in the maturation of the thymic epithelial compartment might impact immunity for life, increasing risks of autoimmune disorders or premature thymic involution. The invariant γδ T cells also have roles in the periphery by improving wound healing, in the skin,[155] and epithelial regeneration, in the lungs.[139] Likewise, it could be speculated that altering the dynamic of secondary lymphoid organ formation initiated by LTi could modulate memory immune responses and even cancer development. The natural antibody repertoire of B1 cells appears to be selected by early exposure to bacterial products and might be essential as first line of defense specially in newborns where the adaptive immune system is immature.[38] All these properties would have conferred a selective pressure to maintain this new, yet transient, generation of progenitors that do not contribute to the HSC compartment and possibly also not to adult hematopoiesis.

Further studies are required to assess how long these hematopoietic progenitors persist, whether they can reach the BM, how to distinguish them from other multipotent cells and what are the molecular basis for their different behavior.

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CONFLICT OF INTEREST

All authors declare no have a conflict of interest.

DATA AVAILABILITY STATEMENT

Data available on request from the authors.

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