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Review Article

Modeling of human T cell development *in vitro* as a read-out for hematopoietic stem cell multipotency

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Hematopoietic stem cells (HSCs) reside in distinct sites throughout fetal and adult life and give rise to all cells of the hematopoietic system. Because of their multipotency, HSCs are capable of curing a wide variety of blood disorders through hematopoietic stem cell transplantation (HSCT). However, due to HSC heterogeneity, site-specific ontogeny and current limitations in generating and expanding HSCs *in vitro*, their broad use in clinical practice remains challenging. To assess HSC multipotency, evaluation of their capacity to generate T lymphocytes has been regarded as a valid read-out. Several *in vitro* models of T cell development have been established which are able to induce T-lineage differentiation from different hematopoietic precursors, although with variable efficiency. Here, we review the potential of human HSCs from various sources to generate T-lineage cells using these different models in order to address the use of both HSCs and T cell precursors in the clinic.

Introduction

Human hematopoiesis *in vivo*

Hematopoiesis is a strictly regulated process in which hematopoietic stem cells (HSCs) gradually lose multipotency in order to generate the full repertoire of blood cells that comprise the hematopoietic system. These multipotent HSCs originate or reside in specific niches that are located at different sites throughout life [1–4]. In the human embryo, hematopoiesis is characterized by a primitive and a definitive wave which are spatiotemporally separated and have different characteristics [5–7]. The primitive program of hematopoiesis occurs after 3 weeks of gestation in the blood islands of the extra-embryonic yolk sac (YS) and generates a transient wave of mainly primitive erythroblasts, megakaryocytes and macrophages [8]. Following primitive hematopoiesis, the definitive program is initiated, at day 32 of gestation, within the dorsal aorta of the aorta-gonad-mesonephros (AGM) region which originates from the embryonic mesoderm [9]. The definitive wave of embryonic hematopoiesis is characterized by the generation of the first self-renewing HSCs that have multilineage hematopoietic potential. These HSCs will subsequently migrate to the fetal liver (FL) where they expand and further support hematopoiesis during fetal life [10,11]. Finally, HSCs will colonize the developing bone marrow (BM) which is the most dominant site of hematopoiesis starting from 20 weeks of gestation [12]. Upon birth, these HSCs will cease to proliferate and acquire a quiescent state in order to maintain lifelong postnatal hematopoiesis [13,14]. Understanding the nature of HSCs has important implications for their *in vitro* generation and use in the clinic.

Human T cell development *in vivo*

T-lineage potential has been regarded as a hallmark of definitive HSCs [15]. T cell development occurs in the thymus, a small organ located in the thoracic cavity that instructs HSC-derived thymus seeding precursors (TSPs) to differentiate along the T cell lineage in response to the micro-environmental stimuli that it provides (Figure 1) [16–18]. Because of its distinct anatomical site compared with where HSCs reside, immature precursors need to egress from the BM in order to colonize
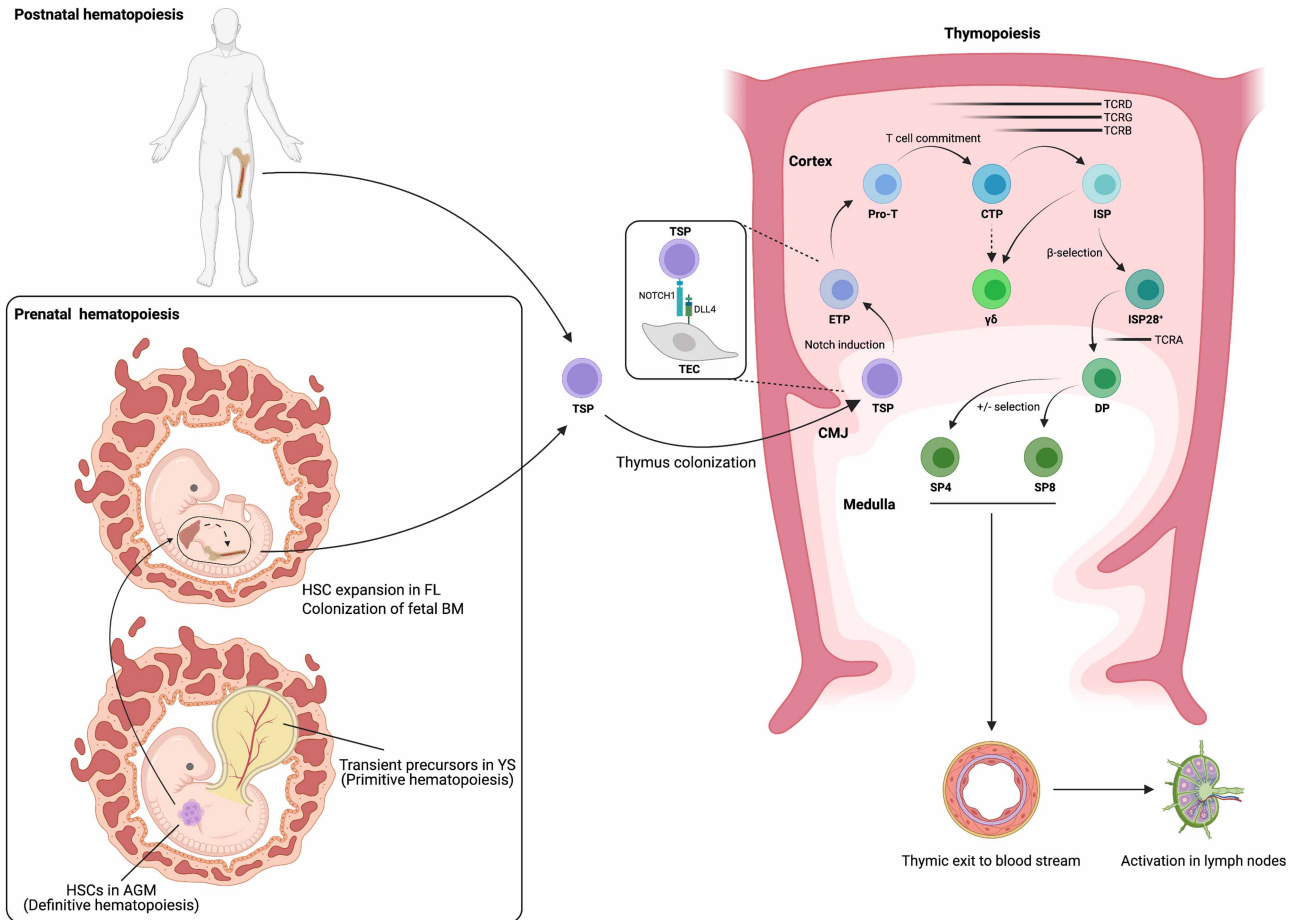
Figure 1. Human hematopoiesis and thymopoiesis *in vivo*.
Human embryonal hematopoiesis is characterized by a primitive program capable of generating transient precursors after which true HSCs are generated in a definitive wave. These HSCs further expand in the FL before migrating to the fetal BM which supports hematopoiesis throughout postnatal life. HSCs then egress as TSPs into the bloodstream in order to colonize the thymus where the NOTCH1/DLL4 axis induces the T cell program. T cell development occurs through sequential progression through distinct intermediate stages in which germline rearrangements of TCR loci primarily instructs the bifurcation of the $\alpha\beta$ and $\gamma\delta$ T cell lineages. HSC: hematopoietic stem cell; AGM: aorta-gonad-mesonephros; YS: yolk sac; FL: fetal liver; BM: bone marrow; CMJ: cortico-medullary junction; TSP: thymus seeding precursor; TEC: thymic epithelial cell; ETP: early T cell progenitor; pro-T: progenitor T cell; CTP: committed T cell precursor; ISP: immature single positive; ISP28$^+$: β-selected ISPs; DP: double positive; SP4/8: CD4/CD8 single positive; $\gamma\delta$: $\gamma\delta$ T cell. (Created with BioRender.com).

the thymus, starting from week 8 of gestation [19–21]. Upon intra-thymic Notch signaling, predominantly initiated by the NOTCH1–DLL4 axis, the expression of CD7 is highly up-regulated in the TSPs as they differentiate into early T cell progenitors (ETPs). Subsequently, upon induction of IL-7 signaling and continuous Notch stimulation, these ETPs develop into progenitor T cells (pro-T cells) and display a decreased potential to develop into other hematopoietic lineages [22–25]. These pro-T cells will then fully lose the capacity to differentiate into non-T cells by committing to the T cell lineage which is generally characterized by the up-regulation of CD1a [26,27], although more recently the loss of CD44 was found to more accurately define human T cell commitment [28]. During the induction of T cell development, rearrangements of the *TCRD*, *TCRG* and *TCRB* loci are initiated in a process called V(D)J recombination which leads to the generation of functional T cell receptors (TCRs). The outcome of these TCR rearrangement events is the main driver of the developmental bifurcation of the $\alpha\beta$- and $\gamma\delta$-lineages which mainly occurs at the ISP stage, and this allows both lineages to mature into distinct T cell subsets that have unique functions in the periphery [29]. During $\alpha\beta$-lineage development, an in-frame rearranged TCRβ chain associates with the invariant pre-T cell receptor α

chain (pTα) in order to generate the pre-TCR that induces β-selection, an event that mainly occurs at the CD4^{+} immature single positive (ISP) stage [30–33]. Upon pre-TCR signaling, developing thymocytes receive proliferation and survival signals and up-regulate CD8β to differentiate into CD4^{+}CD8β^{+} double positive (DP) T cells [34]. These DP T cells then enter a quiescent state to permit V(D)J recombination of the *TCRA* locus [35]. Upon replacement of the pTα by an in-frame rearranged TCRα chain, a functional TCRαβ is expressed on the cell surface together with the CD3 co-receptor [36]. Subsequently, these αβ-lineage T cells undergo positive and negative selection in the thymus in order to establish major histocompatibility complex (MHC)-restricted pathogen recognition and central tolerance, respectively [37,38]. During positive selection, the thymocytes develop into either CD4^{+} or CD8^{+} single positive (SP) T cells that will eventually acquire an immunomodulatory or cytotoxic function, respectively. Finally, these mature naïve CD4^{+} and CD8^{+} SP T cells exit the human thymus and migrate to the lymph nodes where they can be activated by MHC:antigen complexes on antigen presenting cells (APCs) to enable the elimination of both pathogens and cancer cells (Figure 1).

The clinical application of various sources of HSCs

The access to HSCs is crucial to cure a wide range of blood disorders via hematopoietic stem cell transplantation (HSCT). HSCs are mainly characterized by their self-renewal capacity while preserving multilineage potential. However, our understanding of the biology and immunophenotypic landscape of true HSCs remains rather elusive due to their heterogeneous nature and origin. Substantial efforts have been made to characterize the immunophenotype and multilineage potential of HSCs in more detail using serial transplantation studies in immunocompromised mice, clonal lineage tracing experiments and single cell approaches [39–43]. Several populations within the CD34^{+}CD38^{-}CD45RA^{-} multipotent compartment were found to be enriched for true HSCs, such as CD90^{+} and CD49f^{+} fractions [44,45]. Next to their immunophenotypic heterogeneity, HSCs gradually display altered functional properties upon aging since adult HSCs often show a myeloid bias because of changing epigenetic and transcriptional programs [46]. In addition, impaired DNA damage repair and increased production of reactive oxygen species (ROS) in aged HSCs results in lower reconstitution activity upon HSCT [47,48]. Furthermore, the yield of HSCs from different sources can be limited and the search for human leukocyte antigen (HLA)-matching donors in order to avoid graft failure and graft-versus-host disease (GvHD) remains challenging [49]. Therefore, providing larger numbers of HLA-matching HSCs in these transplantations would substantially improve hematopoietic reconstitution and broaden its usage. Only recently, the small molecules StemRegenin 1 (SR1) and UM171 have been identified to promote HSC self-renewal in long-term *ex vivo* cultures, while maintaining engraftment potential [50–53]. By antagonizing the aryl hydrocarbon receptor (AHR), SR1 prevents HSC differentiation while UM171 promotes HSC self-renewal by indirectly depositing H3K4me2 and H3K27Ac activation marks in the proximity of stem cell genes [54]. Although the retained long-term lymphoid potential of these *ex vivo* expanded HSCs still remains to be verified *in vivo*, these small molecules have important therapeutical implications. In addition to the *ex vivo* expansion of HSCs, pluripotent stem cells (PSCs), such as embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), are actively being studied to exploit their potential for generating HSCs *in vitro*. As such, iPSCs may provide an inexhaustive source of patient-specific HSCs that can be used for autologous HSCT or to model disease [55,56]. The *ex vivo* expansion and *in vitro* generation of HSCs might furthermore improve fundamental studies since larger numbers of HSCs could be generated to perform perturbation studies using gene editing approaches such as CRISPR/Cas9 [57]. Since assessment of the T-lineage potential of HSCs has been regarded as a valid evaluation strategy of their multilineage capacity, modeling of human T cell development can serve as a valuable read-out to address HSC multipotency after *in vitro* generation or *ex vivo* expansion, and thus to evaluate their therapeutic use.

In vitro modeling of human T cell development

T cell development can be studied by multiple *in vitro* approaches. While the establishment of fetal thymic organ cultures (FTOCs) provided the first *in vitro* model to study human T cell development around 30 years ago, the OP9-Delta-like ligand 1/4 (OP9-DLL1/4) coculture system is generally more accessible and technically less challenging. Recently, the artificial thymic organoid (ATO) model has shown to be a reliable 3D format to study the more mature stages of T cell development from different hematopoietic stem and precursor cell (HSPC) sources. In addition, feeder-free approaches have been designed for the generation of clinical-grade T cell precursors (Figure 2).
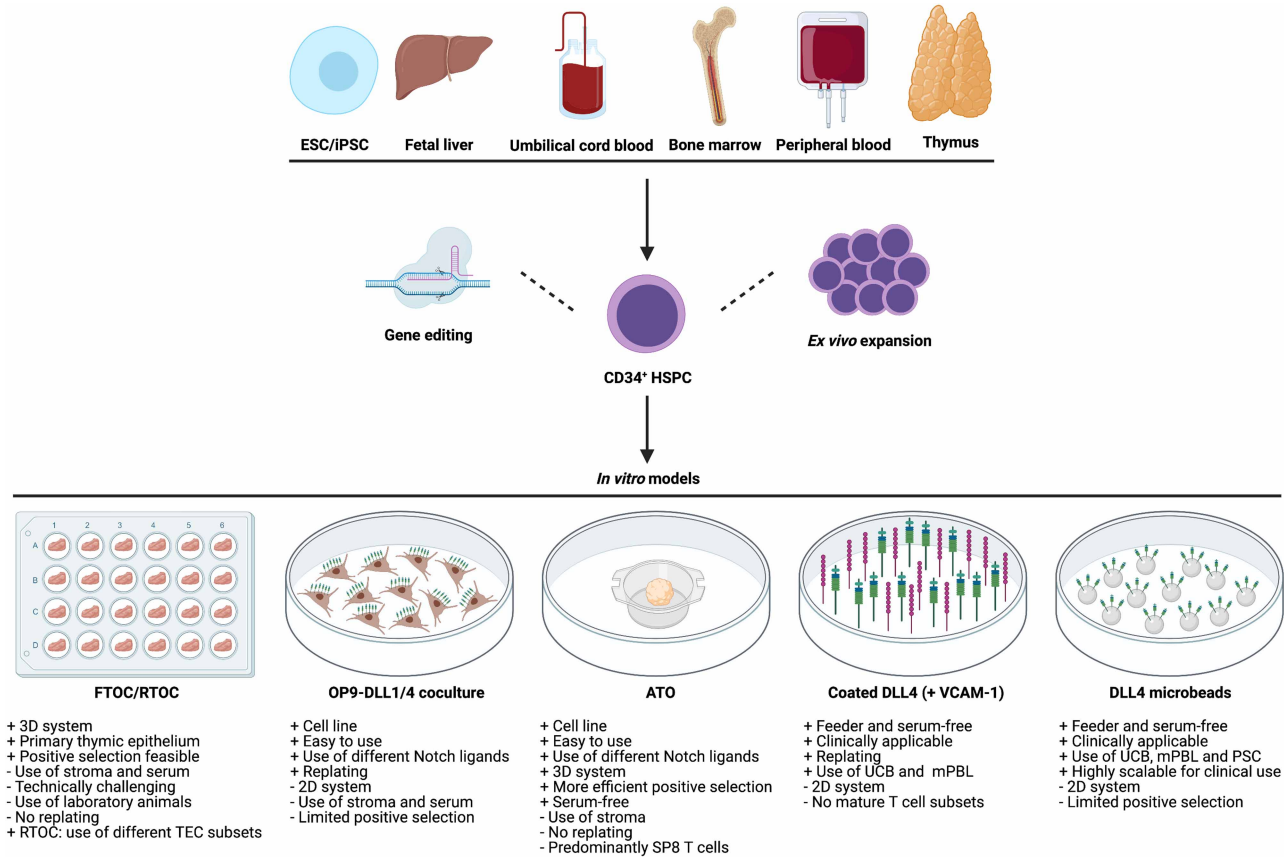
Figure 2. HSPCs from various sources can be exploited to model human T cell development *in vitro*.

CD34$^+$ HSPCs can be isolated from either bone marrow, thymus, fetal liver, umbilical cord blood, peripheral blood or *in vitro* differentiated from ESCs and iPSCs. These HSPCs can be expanded and manipulated *ex vivo* before subsequent application in *in vitro* T cell differentiation models. Green and purple sticks represent DLL1/4 Notch ligand and VCAM-1, respectively. The OP9 stromal cells and microbeads are depicted as brown cells and silver spheres, respectively. ESC: embryonic stem cell; iPSC: induced pluripotent stem cell; HSPC: hematopoietic stem and precursor cell; FTOC: fetal thymic organ culture; RTOC: reaggregated thymic organ culture; TEC: thymic epithelial cell; OP9-DLL1/4: OP9-Delta-like ligand 1/4; ATO: artificial thymic organoid; SP8: CD8 single positive; VCAM-1: vascular cell adhesion molecule 1; UCB: umbilical cord blood; mPBL: mobilized peripheral blood; PSC: pluripotent stem cell. (Created with BioRender.com).

Fetal thymic organ cultures

FTOCs were the first approach to study human T cell development *in vitro*. Here, thymic lobes from E14-E15 immunodeficient mice are isolated and reconstituted with human HSPCs by the hanging drop method [58]. Initially, thymic lobes from wildtype mice were used and these were depleted from endogenous thymocytes using deoxyguanosine to allow more efficient engraftment of human precursors. Since these FTOCs do not permit to study the specific impact of different subsets of thymic epithelial cells (TECs) or other stromal cells, reaggregated FTOCs (RTOCs) were developed in which HSPCs and murine TECs were combined in small organoids [58]. FTOCs provided the first insights into the developmental potential of human HSPCs that were derived from different sources. Precursors from fetal BM, FL and umbilical cord blood (UCB) differentiated more efficiently towards mature T cell subsets compared with those from adult BM, which highlights important differences in T cell potential of fetal versus adult hematopoietic programs [12,59–61]. Particularly aging affects T lymphoid potential since CD34$^+$ precursors from older BM donors displayed decreased T cell potential compared with younger counterparts as shown by the low efficiency of aged HSPCs to generate CD4$^+$CD8β$^+$ DP T cells in FTOCs [62]. In addition, it also has been shown that aging predominantly affects lymphoid progenitors as illustrated by the decreased growth and survival of aged lymphoid precursors, mediated by elevated levels of the cell cycle regulators p16^{INK4a} and p14^{ARF} [63]. To further underscore that HSPCs are highly heterogeneous,

the expression of CD38 within bulk CD34$^+$ BM-derived HSPCs was shown to be inversely correlated with T lymphoid potential in FTOCs [64]. Although these 3D *in vitro* organ cultures provided valuable insights in HSC/HSPC heterogeneity and allowed to study human T cell development, their use might raise ethical concerns and the assembly of FTOCs is technically challenging which can lead to variable results.

OP9-DLL1/4 coculture models

Because of these issues, the OP9-DLL1 system was developed as an alternative *in vitro* model to improve the study of human T cell development. OP9 cells are derived from the BM stroma of op/op mice, a strain that has defects in myeloid development since these mice do not express macrophage colony-stimulating factor (M-CSF). These OP9 cells were transduced with a DLL1 Notch ligand to support the generation of T cells since Notch signaling is indispensable for T cell development *in vivo* [65]. In addition, the OP9-DLL4 variant was developed since it has been shown that the NOTCH1/DLL4 axis is the physiological driving force of early T cell development *in vivo* [66,67]. In these models, HSPCs are seeded onto confluent layers of OP9-DLL1/4 stroma in the presence of SCF, FLT3-L and IL-7. Upon detaching of the exhausted OP9 monolayer, the coculture can easily be replated onto new OP9-DLL1/4 stromal cells which allows to continuously and kinetically track the development of the initial HSPC population, even from single cells [68,69]. This avoids the need for multiple parallel cultures, in contrast with when using FTOCs. Similar to FTOCs, however, HSPCs from various sources display variable T lymphoid potential in OP9-DLL1/4 cocultures as shown by the inefficient generation of mature T cell subsets from BM-derived CD34$^+$CD38$^{-/low}$ HSPCs compared with from their UCB-derived counterparts. This phenomenon, which is cell-autonomous as demonstrated by mixed cocultures, is probably caused by a bias towards the myeloid program in BM-derived HSPCs [69]. Interestingly, pro-T cells that were generated on OP9-DLL1 cocultures from CD34$^+$CD38$^{-/low}$ UCB-derived HSPCs are capable of reconstituting the thymus of immunodeficient mice, illustrating their clinical potential [70,71]. While this model has revolutionized the study of early human T cell development, OP9-DLL1/4 cocultures unfortunately do not very efficiently support the generation of mature T cells.

OP9-DLL1/4 models have also been used to evaluate the T cell potential of HSPCs that are derived from PSCs. In order to generate HSCs, PSCs first need to differentiate into mesoderm which then gives rise to hemogenic endothelium (HE) from which HSCs originate during definitive hematopoiesis [6]. The first protocol described by Vodyanik et al. [72] seeded single ESCs onto a confluent monolayer of OP9 stroma in the presence of serum, without the addition of growth factors. This approach resulted in the generation of CD34$^+$KDR$^+$ HE-derived CD43$^+$ HSPCs with multilineage capacity although T cell development was not addressed [73]. As a modification of this pioneering approach, Timmermans et al. [74] initiated OP9 cocultures by seeding ESC clumps in the presence of both serum and hematopoietic growth factors. After 10–12 days of coculture, hematopoietic zones (HZs) could be identified in which both CD34$^+$ HSPCs and surrounding CD34$^+$ endothelial cells were present. Upon transfer of these HZs onto OP9-DLL1 stromal cells, CD34$^+$ HSPCs gave rise to T-lineage cells after 4–5 weeks. Since these approaches are serum-based and use xenogeneic OP9 stroma, Kennedy et al. [15] designed a protocol to generate embryoid bodies (EBs) in a well-defined medium and low oxygen environment in order to better mimic the *in vivo* situation [75]. During mesoderm induction, the GSK3-β antagonist CHIR99021 is added to stimulate Wnt signaling which promotes definitive hematopoiesis [76]. After 8 days, CD34$^+$CD43$^-$CD84$^-$CD173$^-$ HE cells can be sorted to generate definitive CD34$^+$CD45$^+$ HSPCs in a cytokine-rich medium. Finally, these HSPCs can be isolated and seeded onto OP9-DLL1 stroma to induce T cell development *in vitro*. This EB approach was further employed by Themeli et al. [77,78] where a less complex defined medium was used to either generate iPSC-derived T cells having anti-tumor activity or to model severe combined immune deficiency (SCID) using patient-specific iPSCs. Overall, the OP9-DLL1/4 coculture model has significantly boosted the study of early human T cell development from a large variety of HSPC sources and this model can easily be applied, also from a limited amount of precursor cells.

Artificial thymic organoids

While the OP9-DLL1/4 coculture model has been instrumental to unravel the molecular mechanisms that drive early human T cell development, this 2D model does not efficiently support the generation of SP4 and SP8 T cells. Therefore, the 3D ATO *in vitro* model was recently developed in which CD34$^+$ HSPCs and MS5-DLL1/4 stromal cells are combined at defined ratios, similar to RTOCs, and incubated on a transwell insert that is in contact with serum-free T cell induction medium [79]. This system has been shown to support T cell differentiation using CD34$^+$ HSPCs from different sources, including UCB, peripheral blood (PBL), thymus and BM,

highlighting the high T-lineage potential of ATOs. However, HSPCs derived from either BM or PBL show slower kinetics during T cell maturation, again underscoring the heterogeneous nature of HSPCs. Although no primary thymic epithelium is used, the spatiotemporal network of ATOs might provide thymic-like niches that allow T cell progenitors to migrate through these niches which may facilitate the generation of more mature stages of T cell development. This might be further enhanced by the altered oxygen levels in these ATOs which are cultured at the air/liquid interface, compared with OP9-DLL1/4 cocultures in which the T cell precursors are submerged in the culture medium. In addition, the prolonged cross-talk between stromal cells and T cell precursors, in combination with the high density of these small organoids that enables reciprocal stimulation and selection of progenitors, may also enhance thymocyte maturation. The development of SP8 T cells is favored in ATOs because of the ubiquitous expression of MHC-I molecules on the developing thymocytes while the low abundance of MHC-II-expressing dendritic cells complicates the maturation of SP4 T cells. Because of the technical simplicity of ATOs and the efficient generation of the more mature stages of T cell development, ATOs may become increasingly important to assess the multilineage potential of HSPCs. Indeed, ATOs have been recently used to model SCID using patient-derived HSPCs [80]. However, as opposed to OP9-DLL1/4 cocultures but similar to FTOCs, well-to-well variation might be observed since ATOs cannot be reaggregated after analysis for kinetic studies within the same organoid. Nevertheless, as with OP9-DLL1/4 cocultures, ATOs can serve as a suitable preclinical model but will need to be further optimized to enable the generation of clinical-grade T cells since murine stromal cells are used.

Importantly, ATOs are also capable of generating human ESC- and iPSC-derived SP T cells. Here, following the initial generation of a $CD326^+CD56^+$ embryonic mesodermal progenitors (EMPs) from PSCs, the emergence of HSPCs is induced in embryonic mesodermal organoids (EMOs) by mixing these EMPs together with MS5-DLL4 stromal cells. Subsequently, the medium is switched to the T cell induction medium to initiate T cell development in the same initial organoid [81]. This serum-free PSC-ATO protocol is capable of generating PSC-derived T cells at a higher efficiency compared with the 2D OP9 model. This presumably not only results from the 3D architecture that is provided by the organoids during T cell development, but may also be facilitated by the presence of Notch signaling during the hematopoietic specification step in the EMOs since definitive hematopoiesis is indeed dependent on Notch signaling in vivo [82,83].

Feeder-free cultures

Since the stroma-based in vitro models rely on the use of murine cells, they are not yet suitable for the generation of HSPC-derived T cells or T cell precursors for clinical application. Therefore, a feeder-free model was developed in which coated DLL4 and vascular cell adhesion molecule 1 (VCAM-1) provided a fully defined thymic-like niche that is capable of synergistically inducing early human T cell development from UCB-derived HSPCs [84]. Here, VCAM-1 engages with α4-integrin on HSPCs to enhance Notch signaling for the generation of $CD7^+CD5^+CD45RA^+$ pro-T cells. Upon injection in immunodeficient mice, these pro-T cells home to the thymus and differentiate into functional SP8 T cells. Also specific cytokines such as TNFα, combined with coated DLL4, have been shown to promote the development of early human T cell precursors from both UCB and mobilized PBL (mPBL) sources [85,86]. More recently, a novel method has been designed for the generation of clinical-grade T cells by incubating HSPCs with DLL4-coated microbeads in suspension which significantly enhances the scalability of T cell precursor generation out of HSPCs from either ESCs, UCB and mobilized peripheral blood (mPBL) and the therapeutic applicability [87]. Importantly, the impact of thymic involution and injury on T cell reconstitution upon infusion of these clinical-grade T cell progenitors remains to be addressed. Nonetheless, these results show that off-the-shelf pro-T cells can easily be generated in vitro in a fully defined serum-free and stroma-free environment. As such, these feeder-free approaches might become increasingly important in the clinic to improve T cell reconstitution after myeloablative conditioning and HSCT.

Concluding remarks

Investigating the multilineage potential of human HSCs that are isolated from different sources, or that are expanded or generated in vitro, is instrumental to evaluate their clinical potential. Studying the T cell developmental capacity of these highly diverse sources of HSCs has been very useful in this context since T lymphoid potential has been regarded as a valid read-out of HSC multilineage potential. In addition, the generation of off-the-shelf T cell precursors has important implications in the clinic to improve and accelerate reconstitution of the T-cell lineage which can take months or even years following classical HSCT. However, their application

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is still suboptimal and requires further optimization of existing protocols or the development of novel innovative tools in order to bring such patient-tailored clinical applications from bench to bedside. Fortunately, significant advancements have been made over the past years that facilitate the evaluation of T-lineage potential which will significantly accelerate the functional evaluation of these novel approaches.

Perspectives

- **Importance of the field:** The *in vitro* generation and expansion of clinical-grade HSPCs, T cell precursors and functional mature T cells holds enormous therapeutic potential for treating hematological disorders through HSCT, and for improving and broadening immune therapeutic approaches.

- **Current state of the research:** While *in vitro* models allow to study human T cell development and can be used to evaluate the multilineage potential of *ex vivo* isolated or *in vitro* generated and expanded HSC/HSPCs, they are not fully suitable yet to generate mature functional T cells that can be used in the clinic. Although clinical-grade human T cell precursors can be generated *in vitro* out of HSPCs, the *in vitro* expansion and generation of human HSCs requires further research.

- **Future directions:** By optimizing current *in vitro* models, or by designing novel innovative tools, the generation of off-the-shelf HSPCs and T cells might become a revolutionary curative strategy to improve both HSCT and immune cell therapy.

Author Contributions
S.S. and T.T. wrote the manuscript.

Abbreviations
AGM, aorta-gonad-mesonephros; ATO, artificial thymic organoid; BM, bone marrow; DP, double positive; EMOs, embryonic mesodermal organoids; EMPs, embryonic mesodermal progenitors; ESCs, embryonic stem cells; ETPs, early T cell progenitors; FL, fetal liver; FTOCs, fetal thymic organ cultures; HE, hemogenic endothelium; HLA, human leukocyte antigen; HSCT, hematopoietic stem cell transplantation; HSCs, hematopoietic stem cells; HSPC, hematopoietic stem and precursor cell; HZs, hematopoietic zones; ISP, immature single positive; MHC, major histocompatibility complex; PBL, peripheral blood; PSCs, pluripotent stem cells; SCID, severe combined immune deficiency; SP, single positive; SR1, StemRegenin 1; TEC, thymic epithelial cell; TSPs, thymus seeding precursors; UCB, umbilical cord blood; VCAM-1, vascular cell adhesion molecule 1; YS, yolk sac.

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