Chapter

Hematopoietic Development of Human Pluripotent Stem Cells

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Abstract

Blood development proceeds through several waves of hematopoietic progenitors with unclear lineage relationships, which convolute the understanding of the process. Thinking of the hematopoietic precursors as the "blood germ layer" can integrate these waves into a unified hematopoietic lineage that originates in the yolk sac, the earliest site of blood development. Hematopoietic differentiation of pluripotent stem cells (PSCs) reflects to a certain extent the complexities of the yolk sac hematopoiesis. In the unified version of blood issue development, the PSC-derived hematopoiesis can also generate post-yolk sac hematopoietic progenitors. To do this, the differentiation has to be arranged for the reproduction of the intraembryonic hematopoiesis. Inflammatory signaling was recently shown to be actively engaged in blood ontogenesis. In addition, a highly recapitulative differentiation of human PSCs was found to spontaneously ignite intense sterile inflammation that has both instructive and destructive roles in the hPSC-hematopoiesis. Inflammatory induction of blood progenitors during hPSC-derived hematopoietic development has to be properly contained. A possible explanation of problems associated with *in vitro* blood development is the failure of inflammation containment and resolution.

Keywords: pluripotent stem cells, hematopoietic differentiation, inflammation, hematopoietic stem cells, tissue macrophages

1. Introduction

Hematopoietic differentiation initiated by human pluripotent stem cells (hPSCs) is a surrogate model for studying early human hematopoietic development. It is generally accepted that the transition of PSCs into blood cells essentially recapitulates the yolk sac stage of hematopoiesis [1, 2], which turns out to be significantly more complicated than previously thought. Most of the information on blood origin came from mouse studies despite the fact that mouse development is highly specialized and adapted to specific living conditions of the species. The mouse hematopoietic system emerges in the early ontogenesis to support embryo development after the initiation of the heartbeat. The first blood cells arise directly from extraembryonic mesoderm at murine embryonic day 7.0 (E7.0) and consist of primitive erythrocytes or erythroblasts, primitive megakaryocytes, and macrophages that perform the tissue oxygenation, preventing embryonic blood loss, and clearing of apoptotic cells, respectively [3]. The role of progenitors for these cell types is taken by mesodermal precursors of the yolk sac.

A second wave of erythromyeloid progenitors (EMPs) initiates in the yolk sac at E8.0–8.5, and progenitors with lymphoid or lymphomyeloid potential emerge in the yolk sac and embryo proper at around E9.5 [3–7]. These data picture an obvious trend of yolk sac hematopoiesis from primitive monopotential and bipotential progenitors [8–10] through EMPs toward the lymphoid-primed multipotent progenitors (LMPPs). This fast evolution of hematopoietic potentials does not culminate in the self-renewing multipotential progenitors, although it does not mean that precursors of these progenitors do not emerge in the yolk sac. Hematopoietic stem cells (HSCs) that can self-renew and repopulate the hematopoietic system of a conditioned recipient are first observed at E10.5, emerging in the aorta–gonad–mesonephros (AGM) region [11]. At this stage, very few HSCs arise alongside numerous HSC-independent hematopoietic progenitors from the endothelium of the dorsal aorta in an extremely peculiar process designated as endothelial to hematopoietic transition (EHT) [12, 13]. EHT is thought to be also involved in the formation of EMPs and possibly LMPPs in the yolk sac [3, 14]. Circulating HSCs and their precursors colonize the extravascular territories of the fetal liver (FL) at E12.0–12.5, where they undergo massive expansion before migrating to incipient bone marrow at E17.5, the main site of hematopoiesis during the adulthood [15, 16]. Of note, the FL colonization looks very similar to leukocyte extravasation during inflammation [17], and the FL niche harbors and cultivates both HSC-dependent as well as HSC-independent progenitors during development.

Human hematopoiesis development is far less amenable for systemic molecular and cellular studies but, based on several lines of evidence, follows a similar logic. Primordial angioblastic cords appear in the human yolk sac around Day 16 of gestation [18] whereas the formation of blood islands, observed starting from Day 19 [19], leads to the production of primitive erythrocytes and macrophages [20], as well as primitive megakaryocytes [21]. Clonogenic multilineage hematopoietic progenitors, the analogs of murine EMPs, are functionally identified in the human yolk sac at four to five weeks of gestation [22, 23] when systemic circulation already started. Under the influence of the discovery that the AGM region is a niche of the first mouse HSCs, early human studies had also presumed the intraembryonic origin of adult hematopoiesis and HSCs [24], although the autonomous generation of HSCs in the human AGM region was not confirmed [25].

Human PSC-hematopoiesis struggles to generate authentic HSCs [26] perhaps due to the yolk sac mode of the differentiation. The yolk sac-like concept of hPSC-hematopoiesis, however, does not overrule the possibility of HSC generation *in vitro*. There are two main reasons for the continuation of the efforts: (1) no solid data prove that the yolk sac does not produce precursors of HSCs, while the opposite may be correct [27]; (2) only primary hPSC differentiation is similar to the yolk sac hematopoiesis, and additional steps with selected cell populations may improve the HSC chances.

This chapter will discuss the role of archetypal cell interactions of sterile inflammation in hPSC-hematopoiesis. A recapitulative hPSC differentiation reproduces the inflammatory mechanisms that participate in the *in vivo* hematopoietic development. The spontaneous sterile inflammation and inefficient resolution can be important factors contributing to the difficulties in the derivation of HSCs from hPSCs.

2. Induction of hematopoiesis in the human conceptus

Early human development is drastically and conceptually different from the development of the mouse embryo, the standard developmental model. In mice, a

number of fate mapping and molecular studies have established that during the blastocyst formation ICM cells lose their potential for the trophectoderm specification. In contrast, more ontogenically advanced epiblast cells in human preimplantation embryos still showed outstanding developmental plasticity. Pre-gastrulation human epiblast was demonstrated to give rise not only to all three germ layers but also to the trophectoderm [28]. These findings provided compelling experimental evidence of the regulative embryonic development in humans and showed that the regulative development, inherent to all mammals and birds, is more prominently manifested in humans compared to mice. The increased developmental plasticity may be an adaptation to the long and relatively slow gestation so that a majority of developmental deviations or failures can be effectively fixed by other embryonic cells. Alternatively, stricter lineage bifurcation in the mouse embryo suits better to fast and short-term ontogenesis. The outstanding human plasticity may not be limited to epiblast cells. Later conceptal locations, including extraembryonic mesoderm with the emerging hematopoietic system, are likely to possess the increased regulative potential, although experimental proof of the postimplantation plasticity is difficult to obtain.

Due to the limited access to the tissues of the early human conceptus, the initiation and structure of the human hematopoietic development is less well-known compared to that of the mouse. In mice, the founding members of the blood germ layer are located in the pre-gastrulation epiblast giving rise to the primitive blood and hemogenic endothelial cells (HECs) within the yolk sac vascular endothelium [29]. A body of evidence [18, 30–32] indicates that human hypoblast or primitive endoderm rather than epiblast gives rise to the extraembryonic mesoderm (EXM) of the primary yolk sac. The anatomical origin of EXM had been debated and other anatomical regions of the pre-gastrulation embryo were thought to contribute to its emergence [18, 33]. Nevertheless, the most compelling cell tracing evidence points to the hypoblast origin [32]. Close alignment of EXM and epiblast transcriptomes in monkey pre-streak embryo [34] is an indication of the early commitment of epiblast cells to mesodermal specification before gastrulation. It is also possible that the secondary mesoderm arising from the primitive streak during gastrulation also contributes to expanding EXM [35], although the extent of this contribution is unknown. The major role of EXM is the generation of first blood cells and vasculature in primordial structures traditionally called blood islands. These tissues sustain the developing human embryo for a period, but this is not the only function of the EXM derivatives. Mouse studies demonstrate the long-term contribution of the yolk sac hematopoiesis into various cohorts of adult cells [36–38], including adult HSCs [27, 39].

The hypoblast origin of the early hematopoiesis implies that the human blood tissue develops in parallel with primitive streak-derived secondary mesoderm. The origin of the yolk sac blood islands is polyclonal [40], otherwise, there would be not enough cells to sustain embryonic development by providing erythroblast-based tissue oxygenation and timely clearing of cell debris by primitive macrophages. This polyclonal, definitive mesoderm-independent development means that blood induction is a morphogenic event analogous to the formation of ectoderm, trophectoderm, and gastrulation. Then, EXM and its progeny—blood can be considered a cryptic or atavistic germ layer containing all elements required for its development and function.

The concept of the blood germ layer helps to alleviate an obvious problem of the strange mode of hematopoietic ontogenesis with two seemingly separate developmental lineages, the primitive and the definitive hematopoiesis. In the blood germ layer hypothesis, EXM is a founder conceptus location for the whole of the hematopoietic lineage developing as a special germ layer. As in the classical germ layers, blood has

transitory primordial tissue, the primitive blood, within primordial organs, blood islands, that contain precursors of definitive tissue. These precursors represented by HECs use the vascular niche for their survival and maintenance of the undifferentiated state to serve as an origin of definitive tissues and organs. During ontogenesis, the derivatives of the definitive HECs migrate throughout the vascular system probing the potential niches for settling down and being sometimes or somewhere retained on vascular beds due to the affinity of their surface molecules to cognate receptors expressed on endothelial cells. Later on, the blood germ layer, in collaboration with the derivatives of other germ layers, develops into the immune system with its multiple organs and hematopoietic system settled in the bone marrow. In mice, under strong selective pressure to minimize the gestation period, the regulative development canceled the primary mesoderm and fused the blood layer to the secondary mesoderm.

Supporting the blood germ layer concept, recent studies show that the yolk sac hematopoiesis is more complicated than was previously thought. Early evidence of the key role of the yolk sac in the initiation of fetal and adult hematopoiesis [41–43] was dismissed based on experimental data supporting the intraembryonic origin of definitive hematopoiesis, which was located in the pre-circulation para-aortic splanchnopleura and its derivative—the AGM region. The presence of definitive EMPs and LMPPs in the yolk sac was rediscovered by the new post-AGM generation of researchers using modern approaches and techniques. The lineage tracing experiments in mice demonstrated that populations of tissue-resident macrophages in the adult, including Kupffer cells, alveolar macrophages, and microglia develop from the progenitors originating in the yolk sac [36–38, 44]. The role of the yolk sac in the establishment of adult cell populations became even more apparent after it was found that subpopulations of mast and T cells descend from hematopoietic programs localized in the yolk sac [45–47]. The unifying feature of the abovementioned adult, yolk sac-derived cell populations is their capacity to maintain themselves within the tissues by lifelong self-renewal. Taken together, these data indicate that at least some yolk sac progenitors can acquire self-renewal potential during development. In other words, the ability to self-renew is not something impossible for yolk sac cells to gain on their way toward adulthood. It is then not too untrustworthy that cell tracing and gene reactivation studies indicate the yolk sac origin of the pre-HSCs [27, 39].

The signaling events participating in the induction of human blood are still poorly understood. In the mESC differentiation model, Wnt/β-catenin signaling promotes, whereas Notch signaling suppresses primitive erythroblast development [48]. It was also found that the transient expression of Numb in mesodermal precursors led to the inhibition of the Notch signaling. BMP4 promotes the generation of $VEGFR2^+$ cells within the mESC-derived lateral mesoderm and VEGF supports the subsequent specification and expansion of hematopoietic and endothelial cells [49–51]. Short-term exposure to BMP4 was also instrumental in driving the mesodermal specification of human ESCs [52]. It is highly likely that BMP4 is among the human conceptal factors that induce EXM and help to initiate the hematopoiesis transition. Recent hPSC differentiation data showed that FGF2, BMP4, VEGF, and possibly signals evoked by the collagen IV adherence are sufficient to induce efficient primitive and definitive human hematopoiesis [53].

3. Hematopoietic differentiation of hPSCs

Since their derivation in 1998, human embryonic stem cells (hESCs) have become a useful model for studying human development and molecular mechanisms of

lineage specification *in vitro*. Reprogramming human somatic cells into embryonic-like stem cells [54], named human induced pluripotent stem cells (hiPSCs), opened realistic perspectives for generating various therapeutic cell populations, disease modeling, and drug discovery [55]. The hope is boosted by experiments, in which mouse iPSC-derived primitive macrophages differentiated into tissue-resident macrophages and microglia upon injection into recipient animals [56]. Nevertheless, common usage of instructive hematopoietic cytokines shifts the ontogenic program of PSC-derived mesodermal precursors into the development of cells with a limited functional diversity [57].

Conventional, not naïve, hPSCs that possess so-called primed pluripotency are considered to represent postimplantation epiblast. In order to begin their way to hematopoiesis these cells have to transit from epiblast-like though hypoblast-like to mesodermal epigenetic state under the influence of internal and external effector molecules. This transition is facilitated by a standard variety of factors, and over the decades, there were no significant changes in basic approaches to the hematopoietic differentiation of hPSCs. The traditional methods include coculture with supportive stromal cells, most prominently the M-CSF-negative OP9 cell line [58, 59]; various modifications of the planar differentiation [60, 61]; 3D cultures through the formation of embryoid bodies (EBs) [62, 63], or a combination of 2D and 3D differentiation [53, 64]. In the well-substantiated trend, the protocols are modified in order to remove the fetal calf serum from the culture medium. The *in vitro* analog of the primary mesoderm is generally induced through the BMP signaling [52, 65, 66], followed by hemogenic endothelium induction by VEGF, SCF, FLT3L, IL3, and FGF2 [67], or similar recipes of exogenous growth factors and hematopoietic cytokines. The shift toward hematopoiesis is first manifested by the expression of CD235a, CD43, and CD34, a marker of hematopoietic progenitors and vascular endothelial cells. The endothelium is always present in the hematopoietic differentiation of PSCs since both lineages have common ancestor cells: the hemangioblasts—a mouse primitive streak mesendodermal entity [68], and hemogenic endothelial cells, VEGFR2-positive mesodermal precursors expressing CD34 and the endothelial marker VE-cadherin [69, 70]. CD34 is expressed at a substantially higher level on endothelial cells compared to emerging $CD43^+$ hematopoietic progenitors. The objective of the first step of differentiation is usually to obtain $CD34^+CD45^+$ cells that can be used as hematopoietic progenitors in downstream cultures or applications. Omitting the hematopoietic cytokines and minimizing the use of the mesodermal/endothelial growth factors can strongly improve the recovery of the clonogenic hematopoietic progenitors, including the multilineage ones [53, 64]. The hPSC-derived progenitors can be used to generate clinically relevant blood and immune cell populations [71, 72]. Nonetheless, a practical biotechnological design for getting large numbers of safe functional cells is missing. There is also a serious concern about the genomic stability of ethically acceptable induced pluripotent stem cells, which threaten to prevent the efficient use of hPSC differentiation for therapeutical purposes [73].

The search for a powerful inducer of hematopoiesis led to the conclusion that BMP4 signaling plays a key role in the induction of blood-competent mesoderm upon hPSC differentiation. In mice, there is compelling evidence that Bmp4 signaling is critically required for mesodermal induction [50, 74–79]. In the recently developed model of the early human peri-implantation development, BMP4 was shown to participate in the maintenance of EXM [80]. Induction of EXM was achieved by inhibition of Nodal signaling and GSK3β. This suppression only indirectly reflects the

induction events in the developing human embryo and actual signals leading to the suppression are unknown. We, therefore, can only guess which factors induce EXM in the peri-implantation embryo. It seems that the BMP4 signaling does not only participate in the maintenance of EXM *in vitro* but also may induce the emergence of the tissue. Indeed, previous studies showed that Nodal inhibition results in enhanced BMP4 signaling in the pluripotent stem cell context [81]. Activin/Nodal/TGF-β pathway was not active in the naïve hPSC-derived EXM cells, and no data were available on the role of the FGF2 signaling.

In addition to BMP4, hematopoietic induction in hPSC-derived extraembryonic mesoderm is strongly dependent on VEGF, a growth factor of outstanding pleiotropy. In human development, VEGF treatment enhances blastocyst outgrowth and stimulates embryo implantation [82]. In postnatal development, accumulating evidence indicates the crucial role of VEGF in body growth and organ development [83]. Other findings demonstrate that VEGF also promotes neurogenesis, neuronal patterning, neuroprotection, and glial growth independently of the angiogenic function of the growth factor [84]. The major developmental function of VEGF is organizing and stimulating embryonic vasculogenesis and angiogenesis [85, 86]. In inflammation, VEGF and one of its receptors, VEGFR1, previously identified as a decoy receptor, were found to play a role in the recruitment and activation of monocytes and macrophages [87, 88]. In hPSC biology, VEGF signaling participates in the mesendodermal induction of hESCs [89] and selectively promotes erythropoietic development from hESCs, which have been strongly augmented by BMP4 [90]. Together with FGF2, VEGF is crucial for the progression of mesoderm to hemogenic endothelium [91], which was identified as $CD31^+CD34^+$VE-CADHERIN$^+KDR^+$ cell population [92]. These hPSC-HECs can initiate both primitive and broadly defined definitive hematopoiesis.

Strictly defined definitive hematopoiesis arises only from fetal or adult-type hematopoietic stem cells (HSCs). Generation of lymphoid cells from PSCs was previously considered proof of the definitive lineage potential, although most likely it recapitulates the emergence of pre-HSC hematopoiesis of the yolk sac type. The derivation of HSCs of adult or fetal type remains elusive despite many efforts, including the most recent one [93]. One possible reason for the HSC failure is a low recapitulative quality of hPSC hematopoietic differentiation *in vitro*. HSC precursors change several locations within developing conceptus until they land in the bone marrow [94]. During their development, the HSC lineage cells seem to focus on segregation from progenitor-driven embryonic hematopoiesis and settling in a safe haven of fetal liver, spleen, and bone marrow sinusoids. Therefore, a good recapitulation of HSC development should include a second leg of differentiation so that pre-HSCs are isolated from active hPSC-hematopoiesis. The secondary differentiation most likely should include a stromal culture supported by selected cytokines and small molecules such as stable derivatives of ascorbic acid.

Excessive use of growth factors and cytokines at a nonphysiological concentration to induce the emergence of EXMCs and HECs is another reason for the poor generation of multipotent hematopoietic progenitors and HSCs by differentiating hPSCs. We do not know the exact makeup of the signaling factors participating in the hematopoietic induction within the developing human conceptus. Moreover, inductive events in early mammalian embryos are performed by short-range signaling proteins and growth factors [65]. Therefore, any use of exogenous hematopoietic cytokines to initiate hPSC-derived hematopoiesis may *a priori* disturb the developmental pathway of HSC precursors due to the strong instructive influence of these cytokines [95].

The published protocols use complex compositions and excessive concentrations of cytokines, growth factors, and signaling molecules to ensure efficient conversion of hPSCs into hematopoietic cells at the cost of a proper recapitulation of hematopoietic development in the conceptus. Such an approach decreases the value of the hPSC differentiation as a model of early hematopoietic ontogenesis. Furthermore, the use of external cytokines accelerates terminal differentiation of hematopoietic progenitors that negatively influences the length of the proliferative stage and the yield of clinically relevant cellular material.

In the structural aspect of hPSC-hematopoiesis, planar, 3D, or stromal differentiations do not reproduce to any acceptable extent the anatomy of the peri-implantation human embryo. In the early postimplantation human embryo, EXM cells spread from the 3D embryo proper part over the distal trophoblast in an essentially planar fashion

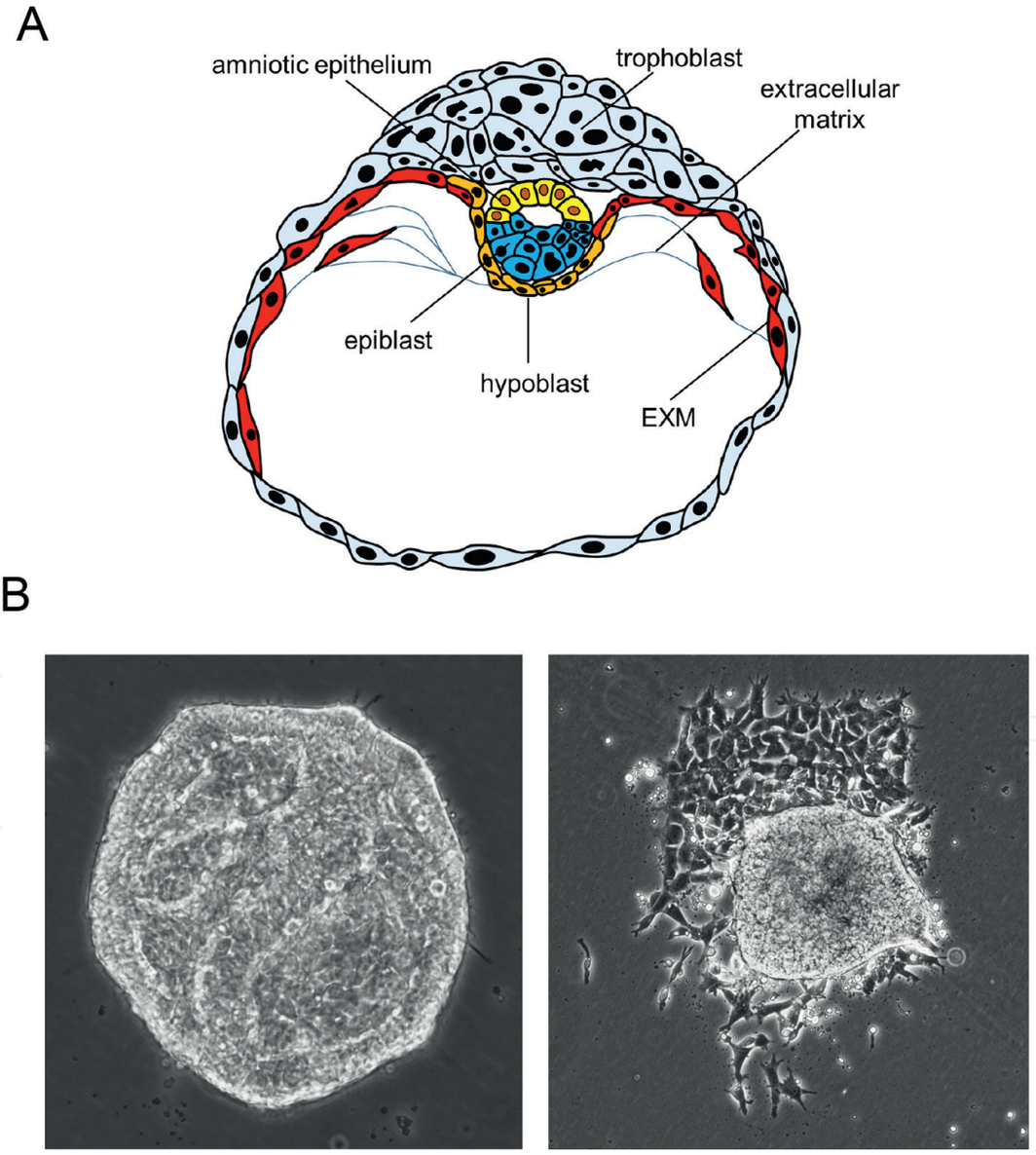

Figure 1.
Forced attachment of hPSC-EBs reproduces the early EXM development. (A) Day 8–9 human peri-implantation embryo. The induction of EXM. (B) Early stages of hPSC differentiation. Shortly after attachment to the collagen surface, EB undergoes epithelialization (left panel). Next day, the EB-derived EXM starts to spread over the surface.

(**Figure 1A**). Therefore, the optimal recapitulation of mesodermal induction in peri-implantation embryos should include the spreading of differentiating hPSCs from a central 3D cell mass in a planar circumferential fashion. Stromal support from adult sources, the fetal liver, and the AGM region creates artificial differentiation conditions that are not encountered at the start of hematopoietic development.

In the recently published protocol for recapitulative differentiation of hPSCs, no external cytokines were used [53]. The onset of EXM development was reproduced by forced attachment of hPSC-EBs to collagen-covered surfaces in the presence of BMP4, VEGF, and mTeSR1-derived FGF2. The attachment initiated active planar spreading of mesenchymal cells (**Figure 1B**) followed by spontaneous formation of blood island-like aggregates (**Figure 2**) in which hematopoietic induction occurs. Differentiating cultures produced a variety of endogenous cytokines that supported hematopoietic development and the production of large numbers of progenitors.

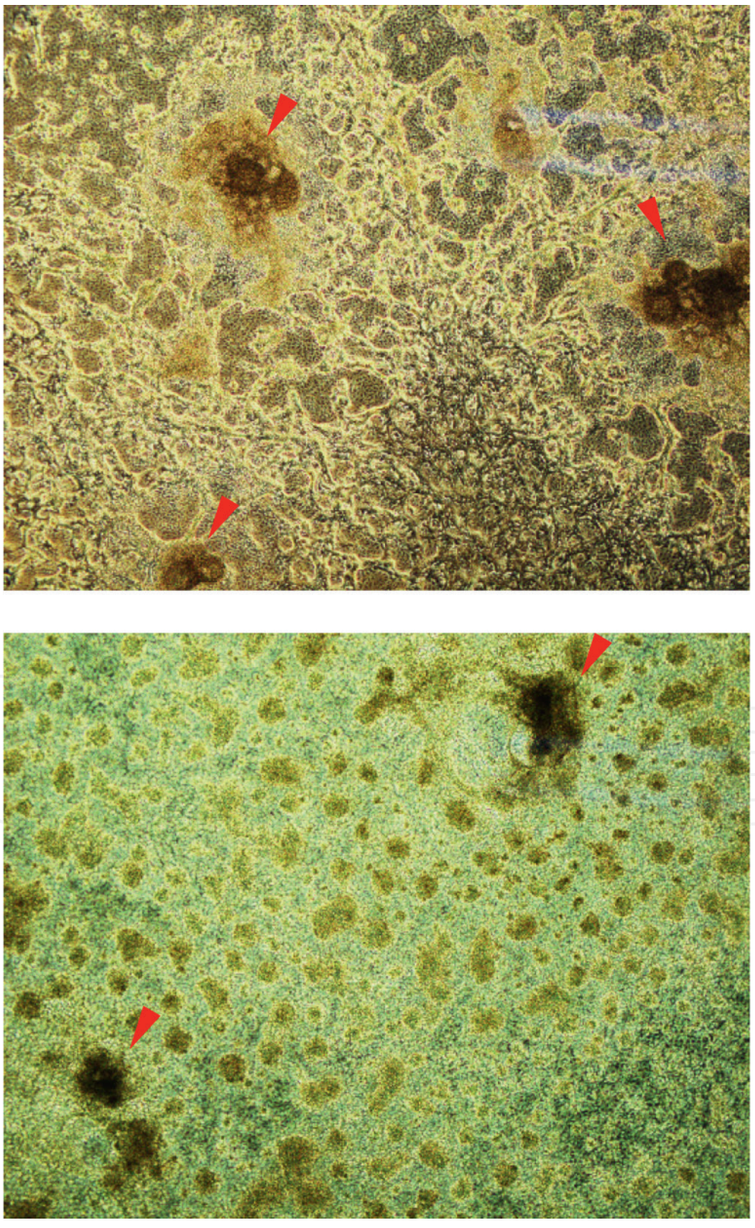

Figure 2.
After attachment to collagen-coated surface, hPSC-derived embryonic bodies form angioblastic cords (upper panel) that transform later on into blood island-like structures (lower panel).

Among others, M-CSF, a pro-inflammatory cytokine [96], was secreted at an exceptionally high level. After 1 week of the differentiation, emerging myeloid cells activate genetic programs that induce and control spontaneous sterile inflammation.

4. Inflammation

Inflammation is a complex, local and systemic, defensive, and adaptive response of the immune system triggered by a variety of agents that disturb homeostasis on an organismal or cellular level. The inflammatory factors include pathogens, damaged cells, toxic compounds, irradiation, and irritation. The variety of the factors boils down to the intrusion of external organisms and the noninfectious damage to tissue cells. Cell damage and infectious agents activate inflammatory cells and trigger inflammatory signaling pathways. In most cases, these are the NF-κB, MAPK, and JAK-STAT signaling [97]. The cellular challenges are sensed by special sentinel receptor molecules that activate the immune system.

Damage-associated molecular patterns (DAMPs) such as oxidized lipoproteins, HMGB1, S100 calcium-binding proteins, heat-shock proteins, or pathogen-associated molecular patterns (PAMPs) such as uncapped viral RNA, lipopeptides, flagellin, and lipopolysaccharides are identified by pattern recognition receptors (PRRs) on the cell surface or endosomes in the cytoplasm. These PRRs are represented by a vast variety of cell surface and cytoplasmic molecules, including the TLRs (Toll-like receptors), the CLRs (C-type lectin receptors), NLRs (NOD-like receptors), the RLRs (RIG1-like receptors), and the RAGE (receptor for advanced glycosylation endproducts). PRRs are preferentially expressed on sentinel immune cells, including mast cells, macrophages, dendritic cells, innate lymphoid cells, and basophils. In addition, many tissues have nonimmune-tissue sentinel cells [98, 99]. Binding the DAMPs/PAMPs ligands to cognate PRRs activates the NF-κB transcriptional effector complex, which then induces the expression of inflammatory cytokines. These cytokines then initiate the cellular phase of the inflammatory reaction. The key step is upregulating cell adhesion molecules on endothelial cells. VCAM-1, ICAM-1, and E-selectin, all inducible by inflammatory cytokines, promote, in cooperation with chemokines and other endothelial adhesion molecules, the adherence and extravasation, or diapedesis, of neutrophils and monocytes into damaged tissue. In sterile inflammation, the major post-diapedesis role belongs to monocytes, which differentiate into tissue macrophages that clean up cellular and extracellular matrix debris.

To preserve tissue homeostasis, acute inflammation has to be suppressed to avoid persistent, chronic inflammation that leads to additional and broader tissue damage. Inflammatory neutrophils are major culprits in collateral tissue and cell breakdown [100]. Stable chemokine gradients may attract an excess of neutrophils even when the microbial infection is already contained. These granulocytes discharge their immense cytotoxic arsenal into the extracellular space of surrounding tissues even if they fail to encounter a microbial agent for a short period of time. Similar cell damage occurs upon neutrophil activation and degranulation in sterile inflammation conditions [101]. Neutrophils release their own set of proteases, activate proteases that are expressed in a latent form by cells resident in the tissues, and inactivate anti-proteases by oxidation [102, 103] using reactive oxygen species (ROS). In addition to neutrophils, activated macrophages initiate nitric oxide-dependent killing of resident cells [104].

Inflammation resolution is an active, well-coordinated process that normally initiates shortly after the start of an inflammatory reaction. It involves the spatially- and

temporally-controlled production of specialized pro-resolving mediators (SPMs) [105], which coincide with a gradual dilution of chemokine gradients across an inflamed tissue. In consequence, neutrophil recruitment becomes attenuated and eventually stops, and then programmed death by apoptosis is engaged. Apoptotic neutrophils are cleared by macrophage phagocytosis followed by the release of anti-inflammatory and reparative cytokines such as IL-10 and TGFβ1, which can suppress pro-inflammatory signaling from Toll-like receptors [106, 107]. The inflammation resolution sequence ends with the conversion of macrophages into the M2 reparative type and/or the departure of macrophages through the lymphatic vessels. Phagocytosis of apoptotic cells inhibits activated macrophage killing of resident tissue cells and triggers the secretion of VEGF, which participates in the repair of endothelial and epithelial injury.

5. Inflammatory hematopoietic development

Most of the research on the role of inflammatory signaling in hematopoiesis is concentrated on the emergence and regulation of HSCs. Adult HSCs are capable not just to respond to inflammatory signals but also to secrete pro-inflammatory/anti-inflammatory cytokines and chemokines [108, 109], including IFN-α [110, 111], IFN-γ [112], TNF-α [113], TGF-β [114], IL-1, and IL-6 [115]. All these cytokines are pleiotropic, and their expression in the blood stem cells may not translate into classical inflammatory cell interactions. However, the expression of PRRs and their co-receptors in HSCs and hematopoietic progenitors [116] directly indicates the involvement of the progenitor domain in sensing stress situations in the hematopoietic system. Hematopoietic progenitors respond to the ligation of TLRs by entering cell cycling, proliferation, and differentiation, and, therefore, can be considered as a part of the innate immune system. In the developmental aspect, hematopoietic progenitors, including HSCs, may arise as highly specialized innate immune cells possessing substantial proliferation and differentiation potential. Indirectly, this notion is supported by another mouse study, which has shown that HSCs in the AGM region exhibit lower levels of IFN-α expression compared to fetal liver HSCs, and this trait can contribute to the lower engraftment potential of the AGM HSCs. Similar to innate immune cells, these HSCs strongly reacted to IFN-α treatment by improvement of their proliferation capacity upon transplantation [117].

Homeostatic expression of another interferon, IFN-γ, a powerful anti-viral cytokine, also activates the proliferation of HSCs *in vivo* during normal hematopoiesis [112]. However, in the hPSC differentiation model, exogenous IFN-γ failed to stimulate the emergence of $CD34^+$ HECs and $CD34^+CD43^+CD45^+CD235a^-$ definitive hematopoietic cells [118]. The iconic pro-inflammatory cytokine IL-1β affects murine adult hematopoiesis by accelerating HSC proliferation and myeloid differentiation through activation of the PU.1 gene program [119]. TNF-α has been implied to play an important role in hematopoietic development based on its abundant expression in the murine yolk sac and fetal liver [120]. Zebrafish studies strongly indicate TNF-α importance for HSC emergence and specification [121]. The data is especially interesting because it implies the involvement of primitive neutrophils in the maintenance and emergence of HSCs in the zebrafish AGM region. Nevertheless, IL-1β and TNF-α signaling did not improve the hematopoietic differentiation of hPSCs [118]. The most straightforward explanation of IFN-γ, IL-1β, and TNF-α failure to influence hPSC-derived hematopoiesis is a non-recapitulative differentiation protocol, although, in

the more positive attitude, it is possible that strong and instructive inflammatory signaling was spontaneously activated during the hematopoietic transition of hPSCs and additional external stimulation could not have any visible effect.

The notion of spontaneous sterile inflammation is supported by recent bioinformatics studies of highly recapitulative hematopoietic differentiation of hPSCs [53]. In the study, efficient hematopoiesis was induced and supported by hematopoietic cytokines produced endogenously in differentiated cultures. Many of the secreted cytokines, such as IL-8, IL-11, IL-16, and M-CSF, were pro-inflammatory, some of them were detected before the emergence of hematopoietic cells. These cytokines programmed the early hematopoietic cells to create an inflammatory milieu that included neutrophil activation, T cell activation, response to bacterial molecular patterns, activation and regulation of innate immune response, phagocytosis, viral response, and others. These observations strongly suggest the instructive role of inflammation in the recapitulative hPSC-hematopoiesis. Such hematopoiesis, however, cannot be sustained long-term in the primary hPSC differentiation probably due to the failure of inflammation resolution, so poorly controlled neutrophil activation leads to the gradual destruction of generated hematopoietic progenitors and blood cells. The optimal solution is to recapitulate the post-yolk sac phase of embryonic hematopoiesis by transferring early hPSC-derived hematopoietic progenitors into secondary differentiating cultures supported by stromal cells. The transfer allows the progenitors to escape from the inflammatory environment before the neutrophil activation. In the secondary differentiation, the escapees undergo extensive proliferation and can develop into lymphoid cells and HSCs in proper culture conditions.

The induction of sterile inflammatory programs in the hPSC differentiation is mediated by DAMPs released from dead or dying hPSCs. Many hPSC cannot easily enter the commitment sequence and have to undergo apoptosis. The major effector of the sterile inflammation is possibly BMP4, the growth factor that is required for the initiation of hematopoietic development. Corroborating evidence [122–124] shows that BMP4 signaling is linked to the induction of inflammatory nuclear factor-κB (NF-κB), nicotinamide adenine dinucleotide phosphate oxidase-1 (NOX1), and intracellular adhesions molecule-1 (ICAM-1), which are the key factors of inflammation initiation. This inflammatory role of BMP4 was described in the adult context, but it demonstrated that there exist molecular mechanisms involving BMP4 in the inflammatory response. It is unknown whether BMP4 induces inflammation at early stages of embryo development, but it is safer to minimize both the concentration and duration of the BMP4 treatment to avoid its participation in the ignition of a potent inflammatory reaction, including neutrophil activation and degranulation.

6. Conclusions

Accumulating evidence suggests that hematopoiesis develops as a single germ layer. Emerging hematopoietic progenitor cells adapt to the constantly changing conceptal environment by modulating their proliferative and self-renewal potential. They have to use inflammatory mechanisms to penetrate endothelial barriers and settle into transitory or permanent niches. Human PSC-derived hematopoiesis, despite evident problems, has the capacity to develop the self-renewal potential but has to reproduce the *in vivo* development as close as possible.

Inflammatory signaling can play an instructive role in hPSC-derived hematopoiesis. It is not just an artifact of the culture and may reflect some aspects of the

hematopoietic development in the conceptus. Inflammation can also negatively influence the expansion of the hematopoietic populations due to the failure of regulated resolution. For successful cell engineering, the early hematopoietic progenitors have to be removed from the primary differentiation culture and used in the secondary differentiation with the immunosuppressive environment.

Conflict of interest

The authors declare no conflict of interest.

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