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SPOTLIGHT REVIEW

Development and trafficking function of haematopoietic stem cells and myeloid cells during fetal ontogeny

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Abstract Fetal haematopoiesis is a highly regulated process in terms of time and location. It is characterized by the emergence of specific cell populations at different extra- and intraembryonic anatomical sites. Trafficking of haematopoietic stem cells (HSCs) between these supportive niches is regulated by a set of molecules, i.e. integrins and chemokine receptors, which are also described for the recruitment of differentiated innate immune cells. In this review, an overview will be given on fetal haematopoiesis as well as trafficking of HSCs during fetal life. In addition, we will focus on the appearance of the first differentiated neutrophils and monocytes in the fetal circulation and describe how they acquire the ability to roll, adhere, and transmigrate into inflamed fetal tissue. Furthermore, we will discuss other effector functions of innate immune cells evolving during fetal ontogeny.

Keywords Leucocyte recruitment • Fetal • Haematopoiesis • Innate immunity

This article is part of the Spotlight Issue on Leucocyte Trafficking.

1. Introduction

On the daily basis, various types of haematopoietic cells ensure proper transport of oxygen and nutrients through the body as well as protection against infections and other foreign threads. Most haematopoietic cells die or have a limited lifetime after they performed their function. Therefore, a massive and constant haematopoietic production is needed throughout life. This is possible due to the presence of haematopoietic stem cells (HSCs) that have two major properties, multipotency and self-renewal. The concept of stem cell and haematopoietic cell hierarchy was first postulated at the beginning of the 21st century (reviewed in Ramalho-Santos and Willenbring[1]). Pappenheim[2,3] was pioneer in suggesting the existence of precursor cells capable of differentiation into both red and white blood cells, and Maximow[4] thereafter popularized the term of 'stem cell'. HSCs can indeed differentiate at the clonal level into more committed progenitors and precursors that massively proliferate to produce all blood cell types from the lymphoid and myeloid lineages. HSCs can also self-renew to maintain a constant HSC pool throughout the life of an organism. In adults, most HSCs are located in the bone marrow (BM) where they are mainly slow cycling/quiescent.[5,6] Interestingly, HSCs have the ability to traffic between tissues. This biological process is also a crucial feature during embryonic development, a time when the first HSCs are generated and then colonize different and sequential haematopoietic sites before finally relocating to the BM before birth. At the same time, the first immune cells arise from haematopoietic precursor cells and circulate in the fetal vasculature in growing numbers. To date, our understanding of fetal blood cell trafficking is still limited. This will most likely change with the recent development of appropriate new *in vivo* models in the mouse foetus, which provide interesting new opportunities to expand our knowledge about cell trafficking in the fetal vasculature.

This review will provide the reader with an overview on the current understanding of haematopoietic stem/progenitor cell and innate immune cell (neutrophils and monocytes) trafficking during murine and human fetal life. In addition, we will discuss future challenges and perspectives in this field.

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2. Development of the haematopoietic system

2.1 Primitive and definitive haematopoiesis

Sabin[7] brought to light the existence of progenitors for both blood and endothelial lineages in vertebrate yolk sac (YS). The establishment of *in vitro* explant culture systems and *in vivo* analysis elucidated the differentiation potential of these cells referred to as the haemangioblasts,[8–14] and it was long suggested that the YS might be the site of HSC production.[15] The first haematopoietic cells produced in the YS are mostly erythroblasts (large nucleated erythrocytes).[16] They are formed in the extra-embryonic mesoderm of the YS in structures called 'blood islands' at an early embryonic stage (embryonic day (E)7.25 post coitus in the mouse, the animal model that we mainly describe in this review)[17] (and reviewed in Ferkowicz and Yoder[10]). To a lower extent, macrophages and megakaryocytes are also produced in the YS.[18] Slightly later during development (after E7.5), various committed progenitors from the lymphoid, erythroid, and/or myeloid lineages are produced in the YS,[15,16,19–22] the allantois and chorion (which will fuse later on to form the placenta and the umbilical cord),[16,23–26] and the caudal part of the embryo.[27,28] The intraembryonic region including the dorsal aorta, vitelline artery, gut, and lining splanchnopleura is referred to as the para-aortic splanchnopleura (P-Sp) region. Because these mature cells and progenitors are transiently present during embryonic life and do not last into adulthood, their production is referred to as the 'primitive' or embryonic haematopoietic wave (*Figure 1*).

Since the first blood cells are produced in the YS, it was hypothesized in the 1970s that the first HSCs were also generated in the YS.[15] However, grafting experiments conducted in the avian model (i.e. quail–chick and then chick–chick grafting approach)[29,30] and thereafter in the amphibian model (*Xenopus laevis*)[31–33] have challenged this dogma. Indeed, the haematopoietic production from the YS appeared to be transient while a sustained haematopoiesis leading to adult haematopoietic cell production was, in fact, derived from cells produced in the region of the aorta. Therefore, a second wave of haematopoietic production referred to as 'definitive' or adult occurs within the embryo. This wave is characterized by the production of HSCs that are needed for the long-term establishment of the adult haematopoietic system (*Figure 1*). Of note, the resident macrophage population of the adult central nervous system, named microglia, derives from myeloid progenitors that were initially generated in the YS (before E8).[34] Very recently, it was also shown that, in fact, the majority of adult tissue-resident macrophages (in liver, brain, epidermis, and lung) originates from erythro-myeloid progenitors that develop in E8.5 YS.[35] Such cells therefore constitute an exception to the transient YS haematopoietic wave described so far.

Tissue grafting experiments and cell tracing in chimeras are impossible experiments to perform in mammals since embryos develop inside the mother uterus. Therefore, other assays were developed to detect haematopoietic stem/progenitor cells (HSPCs) in tissues. McCulloch and Till developed the first functional assay to explore *in vivo* stemness in mammals. In this transplantation assay, donor BM cells were injected into irradiated mice recipients where they gave rise to myeloid multilineage colonies in the spleen of the transplanted animals.[36,37] It proved the multilineage potential of single BM cells, so-called colony forming unit in the spleen (CFU-S).[38] Similar transplantations performed with mouse embryonic tissues revealed that the aorta-gonad-mesonephros (AGM, derivative of the developing P-Sp region) of E10 embryos contain a high proportion of CFU-S (compared with YS and fetal liver).[39] The gold standard assay to identify HSCs remains to date the long-term *in vivo* transplantation where cells are tested for their capacity to restore the haematopoietic system of irradiated adult wild-type recipients up to 4 months after transplantation.[40] Using this assay, HSCs were first detected in the AGM region starting at E10.5.[41] The HSC activity within the AGM was remarkably increased after a 3-day organ culture of the AGM (as explant) prior to cell transplantation.[42] Such studies have ascertained the embryo as the first site of HSC detection in mammals, therefore confirming the findings obtained in the avian and amphibian embryo models. The location of the first HSCs was later on refined and shown to be at E10.5 in the major arteries such as the aorta (of the AGM region) and the vitelline and umbilical arteries.[43] Surprisingly, HSCs are also present at that stage in the vasculature of the head.[44]

Clusters of cells tightly attached to the main arteries were observed for the first time more than a century ago[45,46] and were since found in most vertebrate species that were looked at.[47] HSCs are believed to reside in these clusters (also referred as intra-aortic haematopoietic clusters or IAHCs) because Runx1$^{-/-}$ haematopoietic mutant embryos that lack HSCs have also no IAHCs.[48,49] The transcription factor RUNX1 is essential for HSC production.[50–52] IAHCs appear first in the vitelline and umbilical arteries at E9 and then in the dorsal aorta at E9.5.[53] The number of IAHC cells peaks at E10.5 (≈700 IAHC cells/aorta) and progressively decreases till E14.5.[53] It is interesting to note that IAHCs are located in the ventral aspect of the aorta and are thus polarized in most vertebrate species. The mouse embryo is an exception since IAHCs are also present in the dorsal aspect of the aorta (although to a lesser extent).[53,54] However, the HSC activity is restricted to the ventral part of the aorta as shown by long-term transplantation of either the sub-dissected ventral or dorsal part of the aorta.[54] Clusters were also observed in the head vasculature and the vascular labyrinth of the placenta.[44,55,56] Our group has recently shown that beside very few HSCs,[41,57–59] IAHCs also contain very few committed progenitors and very few pre-HSCs.[60] Pre-HSCs or HSC precursors are incapable to reconstitute the haematopoietic system of wild-type adult recipients upon transplantation, but they are capable to reconstitute more permissive recipients such as neonates or immunocompromised adult recipients.[61–63] The exact and complete cell composition of IAHCs remains an open question to date.

Despite decades of uncertainty, the endothelial origin of HSCs is now well documented. First hypothesized more than a century ago,[45,46] IAHCs and therefore HSCs indeed derive from a specialized endothelium named haemogenic endothelium due to its ability to generate haematopoietic cells. It appears that IAHC cells co-express haematopoietic and endothelial surface markers, indicating a close relationship between endothelial and haematopoietic lineages.[64] The endothelial origin of IAHCs was first demonstrated in the chicken embryo by endothelial lineage tracing.[65,66] Various strategies including genetic lineage tracing experiments,[67] conditional *Runx1* deletion in endothelial cells,[68] and embryonic stem cells/embryoid bodies differentiation model[69,70] have been then used to confirm the endothelial origin of mouse HSCs. The direct visualization of the haemogenic endothelium into HSPC transition by time-lapse live confocal imaging has provided the most convincing evidence thus far of the existence of a haemogenic endothelium in the embryonic aorta. Imaging was performed *ex vivo* in non-fixed mouse embryo slices[71,72] and *in vivo* on zebrafish embryos.[73–75]

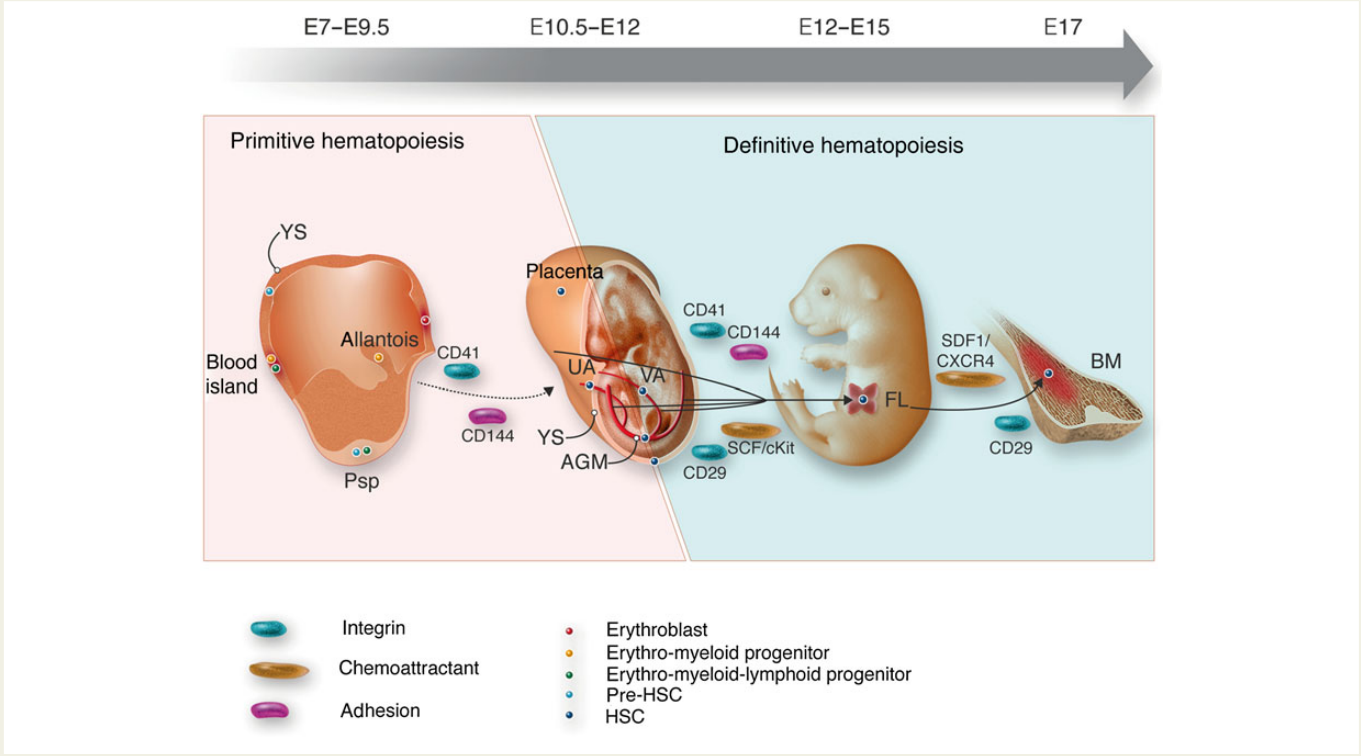

Figure 1 Molecules involved during embryonic/fetal HSPC trafficking. The main molecules involved in HSPC trafficking include: (i) the two integrin chains, αIIb (CD41) and β1 (CD29); (ii) VE-cadherin (CD144), the type I transmembrane protein of the cadherin superfamily, which is an endothelial cell-specific adhesion molecule; (iii) SCF, a dimeric molecule that binds to and activates the receptor tyrosine kinase c-kit. Activation of the SCF/c-kit axis mediates cell survival, migration, and/or proliferation; (iv) SDF-1, also known as C-X-C motif chemokine 12 (CXCL12), that binds primarily to CXC receptor 4 (CXCR4; CD184). Activation of the CXCL12/CXCR4 axis induces intracellular signalling leading to chemotaxis, cell survival, cell proliferation, and gene transcription. The first time points of appearance of the different HSPC types are indicated during primitive and definitive mouse haematopoiesis. YS: yolk sac; PsP: para-aortic splanchnopleura; UA: umbilical artery; VA: vitelline artery; AGM: aorta-gonad-mesonephros; FL: fetal liver; BM: bone marrow.

Slightly after their detection in the main arteries, HSCs start to be detected at E11–11.5 in highly vascularized tissues such as the YS, placenta, and fetal liver.[41,57,58,76] However, it is important to mention that the YS and placenta might generate cells with HSC potential, but this remains a matter of debate to date.[55,77–79] The mouse blood vessels are formed from E6.5 to E9.5, and the heart starts to beat around E8.5 leading to the beginning of blood circulation.[80] However, a functional circulatory system is not obtained until E10,[81] which occurs before HSC detection making it difficult to ascertain the anatomical origin(s) of HSCs (for review see Boisset and Robin[82]). As soon as the circulatory system is operative, HSCs and progenitors can freely migrate through the circulation to colonize the fetal liver and other organs around E10.5[83] (*Figure 1*).

2.2 Fetal sites of haematopoiesis

Limiting cell dilution transplantations and statistical analysis were performed to estimate the number of HSCs in the different haematopoietic organs throughout mouse embryonic development.[57,58] An average of 11 HSCs are present in the whole embryo conceptus at E11.5.[57,58] This number rapidly increases to reach 152 HSCs at E12.5.[57] They mainly locate in the fetal liver and placenta that constitute two very important HSC reservoirs at mid-gestation.[57,76] The mechanism by which HSCs migrate from the AGM to the fetal liver and placenta is still not well understood. In addition to HSCs, various types of progenitors mainly produced in the YS[84] start to colonize other haematopoietic tissues most likely via the blood circulation. By E10, the placenta is the largest reservoir of progenitors. However, from E15 on the number of progenitors in the fetal liver will exceed by far the number in the placenta (e.g. 1300 CFU in culture (CFU-C)/placenta and 11 200 CFU-C/fetal liver at E17).[23] Committed progenitors and/or HSCs will then further colonize the spleen to mainly differentiate into myeloid cells and some lymphoid cell subsets[85] and the thymus to differentiate in T-lymphoid cells.[86] The HSC pool, constituted at mid-gestation in the fetal liver, starts then to colonize the BM at E17[87] (*Figure 1*). Although HSCs are known to circulate and reside in multiple tissues, they mainly reside in the BM in adults.[88]

3. Trafficking of HSPCs throughout development

The trafficking of HSCs and haematopoietic progenitors via blood circulation from specific intra- and extra-embryonic sites is needed and most likely highly regulated throughout development to guarantee that these cells reach their final destination. The migration process of these cells is not yet fully understood due to major technical limitations such as the difficulty to access mammalian embryos or the lack of genetic tools for the avian model that has slowed down lineage tracing

approaches. However, several families of trafficking molecules involved were shown to be key players of HSC trafficking (*Figure 1*).

3.1 Two major synergistic regulatory axes: c-kit/SCF and CXCL12/CXCR4

HSCs have the ability to migrate from the circulation to the BM, which occurs physiologically when the first HSCs leave the fetal liver to colonize the BM, starting at E17.[87] This process called 'homing' involves important players. Chemotaxis is an important contributor of embryonic cell trafficking. The stem cell factor [SCF, encoded by the *Steel* (*Sl*) locus], also known as kit ligand (KitL), is an important factor among the numerous chemokines and chemoattractants. Throughout development, HSCs express high levels of the SCF receptor, the tyrosine kinase c-kit. In the fetal liver, SCF is an essential chemoattractant,[87] notably produced by the fetal hepatic progenitors known to support adult HSCs *in vitro*.[89] In adults, conditional deletion of SCF or CXCL12 (C-X-C motif-ligand 12 or stromal cell-derived factor-1α) in endothelial and perivascular stromal cells affects HSC frequency.[90,91] $Sl^{-/-}$ embryos dye at birth. They have less CFU-S than their wild-type littermates, but they have a similar fold increase in CFU-S numbers between E13 and E15.[92] Thus, SCF might be dispensable for the initiation of mouse haematopoiesis and HSC proliferation in the fetal liver,[92] but might be essential to maintain HSCs once they colonized the BM. c-kit signalling is in fact needed for the maintenance of quiescent HSCs in the BM.[93] SCF and CXCL12 synergize to enhance the migration of fetal liver HSCs to the BM.[87] Endothelial cells in the placenta express high levels of SCF mRNA and protein,[56] suggesting that SCF/c-kit signalling might also be important for HSC regulation.

BM cells such as osteoblasts, endothelial cells, and a subset of reticular cells continuously produce CXCL12, which creates a chemoattracting gradient for the circulating HSCs that express its receptor CXCR4 (C-X-C receptor 4).[90,94–96] The binding of CXCL12 to CXCR4 activates multiple signal transduction pathways (reviewed in Busillo and Benovic[97] and Kucia *et al.*[98]). Upon attraction, the circulating HSCs in proximity to the BM start to tether and slowly roll on the vascular endothelium until firm adhesion, followed by transmigration along the chemoattractant gradient into the BM occurs, a process reminiscent to leucocyte recruitment into inflamed tissue, which is described in the second part of the review. *Cxcr4*[99] and *Cxcl12*[100] knockout mice die at embryonic stage, with similar developmental defects such as the incapacity of HSPCs to migrate from the fetal liver to the BM. On the other hand, the conditional deletion of *Cxcr4*[101] or *Cxcl12*[90,102] in adult mice leads to the release of HSCs into the blood circulation. However, HSCs lacking functional CXCR4 conserve their homing and engraftment capabilities,[101,103] leading to the hypothesis that HSCs express other redundant adhesion molecules. For example, the HSC-specific adhesion molecule roundabout (ROBO) 4[104] and its ligand SLIT2, which are expressed by the BM microenvironment, were suggested to reinforce CXCL12/CXCR4 function.[105] CXCL12/CXCR4 thus form an important axis for both HSC colonization and retention in the BM by regulating cell adhesion, cell survival, and cell cycle status.[103]

3.2 Integrins

Integrin-mediated adhesion represents a crucial mediator for HSC movement throughout development. Several integrin heterodimers were described so far at different developmental stages and locations. β_1 integrin (CD29) is required on the surface of HSCs for proper fetal liver colonization.[106] Indeed, β_1-deficient haematopoietic cells in chimeric embryos were found in the circulation and YS, but not in the fetal liver, showing the incapacity of the cells to colonize the fetal liver in the absence of β_1 integrin.[106] Moreover, β_1-deficient cells exhibit an accumulation of primordial germ cells along their migration route and only very few cells manage to populate the gonads.[107]

α_{IIb} integrin subunit [CD41, Itga2b, and platelet (gp)IIb] is expressed on the surface of all HSCs in the AGM and only on a portion of HSCs in the YS. In contrast, HSCs from the placenta and fetal liver do not express α_{IIb}.[108] Besides being a good HSC marker, α_{IIb} is also essential for the maintenance of the HSC activity specifically in the AGM.[109] α_{IIb} expression on HSCs decreases throughout development and its expression regulates HSPC production in various embryonic sites, suggesting a potential role (yet to demonstrate) of α_{IIb} in maintaining HSPCs in their successive embryonic microenvironments.[83,110,111]

α_6 integrin subunit is implicated in the BM homing of fetal liver progenitors, while α_4 integrin subunit is involved in the homing of HSCs.[112]

3.3 VE-cadherin

Mouse HSCs and precursors in the YS, placenta, and AGM express VE-cadherin (CD144),[113,114] which is involved in adults in regulating endothelial barrier function and leucocyte transmigration. The surface expression level of VE-cadherin on HSCs starts to decrease in the fetal liver (by E16.5), which might allow the migration to and the colonization of the BM.[115,116]

4. Trafficking of myeloid cells during fetal development

During mouse fetal development, the onset of blood circulation is observed at around E8.5.[81] At that time, primitive haematopoiesis in the YS consists of erythro-myeloid progenitor cells, which give rise to erythroblasts, megakaryocytes, and cells of the myeloid lineage like monocytes and neutrophils. These cells are gradually replaced during fetal life by cells derived from HSCs originating from sites of definitive haematopoiesis like the fetal liver and BM.[117] Since monocytes and neutrophils constitute the first line of defence against invading bacteria and pathogens especially in early life, when adaptive immunity is not yet fully developed, in this part of the review, we will concentrate on the trafficking and function of myeloid cells during fetal ontogeny.

Mouse fetal and adult neutrophils and monocytes are characterized by surface expression of CD11b and Gr1. Within this population, neutrophils can be differentiated by their high expression of Ly6G. Neutrophils can only be found in very low numbers early during ontogeny (<2% of circulating nucleated cells at E14). At later fetal stages, they increase in number and reach ~20% at E18.[117] Neutrophils together with monocytes are the main cellular components of the innate immune system, and effective immune defence requires their recruitment to sites of inflammation.

In adults, leucocyte recruitment follows a well-described cascade of adhesion and activation events, which traditionally distinguishes three different steps: leucocyte rolling, firm adhesion, and transmigration (reviewed in Ley *et al.*[118] and Kolaczkowska and Kubes[119]). Over the years, the traditional concept of leucocyte recruitment has been further expanded, integrating new findings outlined below. Such progress was possible mostly by significant advances in bioimaging tools [i.e. multiphoton laser scanning microscopy, spinning disk microscopy, total internal reflection fluorescence (TIRF) microscopy] and a better understanding of intracellular activation events in leucocytes and

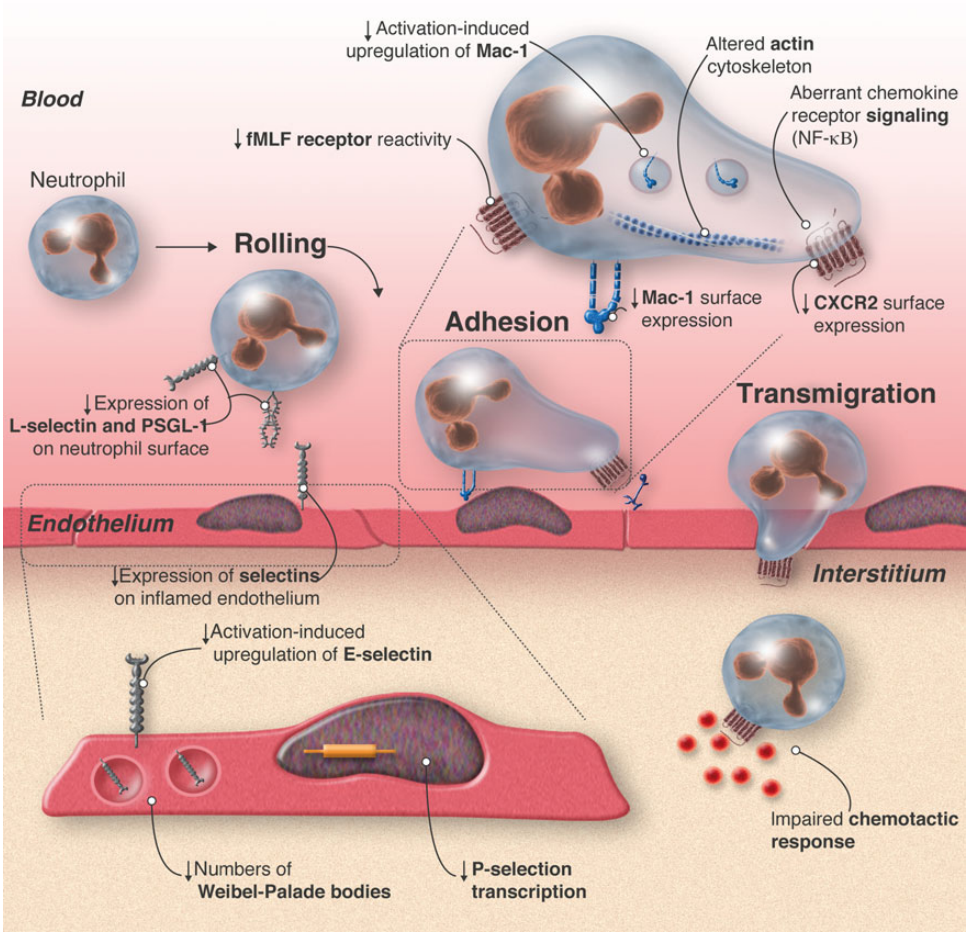

Figure 2 Potential causes of impaired leucocyte trafficking. Rolling of fetal neutrophils on inflamed endothelium is severely compromised due to lower surface expression of L-selectin and PSGL-1 on the neutrophil surface as well as lower expression and transcriptional up-regulation of P- and E-selectin on inflamed endothelium. For P-selectin, a lower number of P-selectin containing Weibel-Palade bodies has also been described in fetal endothelial cells. Diminished leucocyte adhesion in the foetus is associated with reduced Mac-1 surface expression as well as reduced activation-induced up-regulation of Mac-1 on neutrophils. Additional factors, which contribute to decreased adhesion, are reduced CXCR2 surface expression on neutrophils, aberrant chemokine receptor signalling, reduced fMLF-receptor activity, and an altered neutrophil actin cytoskeleton.

endothelial cells (*Figure 2*). In the following paragraph, we will shortly summarize the molecular events of rolling, adhesion, and extravasation of adult myeloid cells before pointing out the differences of leucocyte recruitment during fetal ontogeny.

In inflamed tissues, leucocytes are captured to and roll along the inflamed endothelial surface of postcapillary venules via binding to P- and/or E-selectin expressed on the inflamed endothelium. During rolling, leucocytes have ample of time to communicate with the endothelial compartment through interactions with different endothelial surface receptors and recognition of surface-associated molecules. Such molecules are either expressed by inflamed endothelial cells, deposited on the endothelial cell surface from sources within the vasculature, or transferred from the underlying inflamed tissue. Processing these signalling cues may lead to the activation of leucocyte-expressed integrins (β_2 integrins and $\alpha_4\beta_1$ integrin) with concomitant slowing down of rolling velocity and finally firm arrest on the inflamed endothelium. Subsequent integrin-dependent signalling events induce considerable changes in the actin cytoskeleton. It leads to postarrest adhesion, strengthening of leucocytes with spreading and flattening of the adherent neutrophil on the inflamed endothelium. Thereafter, neutrophils start to crawl along the endothelial surface in search for an appropriate extravasation site (preferentially at tricellular endothelial junctions) where neutrophils and monocytes eventually transmigrate into tissue. This process is not only dependent on integrins, but also on a whole variety of junctional molecules, including VE-cadherin, PECAM-1, JAM family members, CD99, CD99L, and others.[120]

While the different leucocyte recruitment steps were thoroughly investigated in the adult organism under *in vitro* and *in vivo* conditions (taking advantage of an ever-growing number of gene modified mice), the regulation of leucocyte recruitment during fetal ontogeny is still incompletely understood. However, recent progress in the development of suitable *in vivo* models in the mouse foetus has shed new light on the molecular mechanisms of leucocyte recruitment *in vivo*.[117] Neonates exert a high susceptibility to bacterial infections[121] with a strong correlation between gestational age and morbidity and mortality.[122,123] Therefore, understanding the intricacies of fetal leucocyte recruitment is instrumental for the development of new therapeutic approaches in the treatment of severe bacterial infections in the foetus and neonate.

Numerous studies have documented the high risk of bacterial infections in neonates,[121] and clinical studies report a strong correlation between morbidity and mortality and the degree of prematurity.[122,123] The increased risk for neonatal infections (which can reach up to 60% in extremely premature infants[123]) was attributed to the immaturity of the innate immune system, which gives rise to the presumption that both leucocyte recruitment and effector functions are ontogenetically regulated during early fetal life. Further below, we will describe what is currently known about the regulation of the leucocyte recruitment cascade during fetal ontogeny, mainly in mouse and man (*Figure* 2).

4.1 Leucocyte rolling is developmentally regulated

Several groups have investigated selectins and their ligands on fetal and neonatal myeloid cells using flow cytometry or functional *in vitro* assays. L-selectin expression in neutrophils isolated from cord blood samples of preterm and term neonates is severely compromised.[124–128] Functionally, reduced L-selectin expression could be correlated with the diminished attachment of neonatal neutrophils to human umbilical vein endothelial cells (HUVECs).[129] However, the mechanism remains unclear as acutely inflamed endothelial cells in peripheral tissue (excluding lymphatic tissue) do not express L-selectin ligands.[130] Besides reduced L-selectin expression on leucocytes, reduced P- and E-selectin expression was also reported on inflamed endothelial cells shortly after birth. *In vivo* studies in neonatal rats revealed reduced endothelial P-selectin expression. This was verified on human endothelial cells of premature infants and could be traced back to reduce numbers of P-selectin storage granules and P-selectin transcription.[131] Along the same line, lipopolysaccharide (LPS)-induced up-regulation of E-selectin on HUVECs of premature infants was severely reduced,[132] suggesting that the fetal endothelial compartment is impaired in its ability to support leucocyte rolling when compared with adult organism. In this context, it is important to note that such responses were investigated in primarily isolated HUVEC until Passage II. Using higher passages of HUVECs, the results might be different. For the blood compartment, two independent reports have found reduced expression of P-selectin glycoprotein ligand-1, the major selectin ligand on neonatal and fetal neutrophils, respectively.[132,133] Finally, using an intravital microscopy model in the mouse foetus, one of the authors (M.S.) recently demonstrated that neutrophil rolling in inflamed YS vessels *in vivo* is strongly reduced to absent in the mouse foetuses until E15 (of 21 days carriage in mice). For later fetal time points (E16–E18), rolling interactions

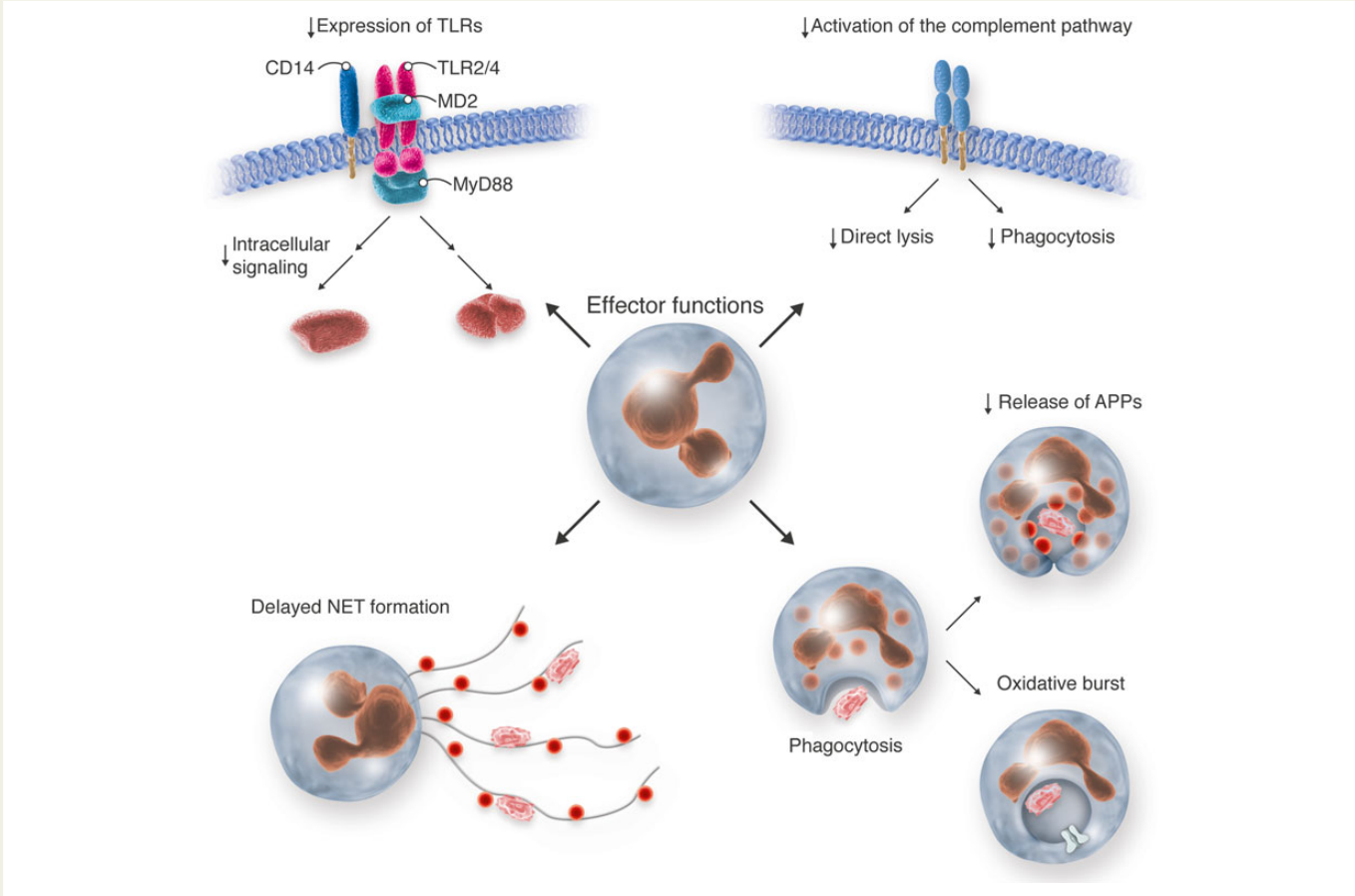

Figure 3 Effector functions of myeloid cells are compromised during fetal life. Expression of TLRs only rises to normal levels late during fetal ontogeny. Intracellular signalling events are compromised in the developing embryo. Furthermore, activation of the complement system is severely diminished resulting in lower direct pathogen lysis as well as reduced phagocytic capacity. In addition, concentrations of neutrophil-derived antimicrobial peptides and proteins (APPs) are significantly lower in cord blood plasma of both preterm and term infants. Similarly, the generation and release of reactive oxygen species (ROS) is compromised during fetal life. Finally, NET formation, although functional, is significantly delayed in neonates.

increased towards birth, suggesting an ontogenetic regulation of neutrophil rolling. To exclude the possibility that microvascular parameters like vessel diameter, blood flow velocity, and wall shear rates in YS vessels contribute to the observed rolling deficit, these parameters were quantified and no marked differences observed during the different stages of fetal development investigated. Furthermore, *in vitro* flow chamber experiments performed under steady shear stress conditions and using isolated human cord blood neutrophils confirmed the developmental regulation of neutrophil rolling on immobilized P- or E-selectin.[132]

4.2 Integrin-dependent adhesion in preterm and term neonates

As selectin-dependent interactions are not sufficient to arrest leucocytes on the endothelium, firm adhesion of leucocytes (similar to HSCs) requires the activation of leucocyte-expressed integrins which mediate firm leucocyte adhesion on the endothelium via binding to integrin ligands on the endothelium including ICAM-1 and the receptor for advanced glycation endproducts (RAGE). The necessary activation step can be provided by several pro-inflammatory mediators such as chemokines, formyl peptides, LTB4, and others. Integrin activation consists in conformational changes, redistribution of the integrin molecules on the surface (clustering), as well as increased integrin surface expression.[134] Studies on β_2 integrin expression and function in neonatal neutrophils revealed diminished total cell content of Mac-1 ($\alpha_M\beta_2$, CD11b/CD18)[135] and suggest a correlation of total and activation-induced Mac-1 expression and gestational age.[127,136] In contrast, other reports describe equal surface expression of Mac-1 under resting conditions in neonatal and adult neutrophils, but diminished Mac-1 up-regulation after chemotactic stimulation in neonatal neutrophils.[137] In contrast to Mac-1, LFA-1 ($\alpha_L\beta_2$, CD11a/CD18) expression and LFA-1-dependent adhesion are not reduced in neonates.[124,138]

To date, little is known about the adhesive properties of neutrophils during fetal development or in preterm infants. In 1990, Bektas *et al.*[139] reported decreased adherence to nylon fibres of neutrophils in preterm infants. This was attributed to diminished β2 integrin expression. Strunk *et al.* [28] detected diminished expression of the integrin subunits CD11a, CD11b, and CD18 on neutrophils and monocytes isolated from preterm infants (21–32 weeks of gestational age). However, McEvoy *et al.*[136] have shown severely diminished Mac-1 and a less pronounced reduction in LFA-1 expression in preterm compared with term neonates. Nussbaum *et al.* investigated cord blood neutrophils obtained from very premature to mature infants. They found no differences in LFA-1 expression on cord blood neutrophils from preterm and term infants, but reduced Mac-1 expression with decreasing gestational age.[132] In *ex vivo* microflow chambers coated with E-selectin, ICAM-1, and CXCL8, defective adhesion was observed in preterm neutrophils compared with neonatal and adult neutrophils.[132] The reduced expression of Mac-1 in neutrophils from preterm infants might contribute to the observed reduction in adhesion. However, the almost complete absence of neutrophil adhesion in the most immature infants investigated suggests additional factors contributing to this, including strongly reduced rolling and most likely also impaired intracellular signalling. Interestingly, fetal neutrophil adhesive function in humans was only correlated with gestational age, but not with postnatal life. This could be shown in flow chamber assays comparing adhesion of neutrophils isolated from premature and mature infants at different postnatal time points. These experiments revealed that postconceptional age, but not postnatal age, was the only determinant of neutrophil adhesive function, suggesting that postnatal exposure to the outside environment does not lead to a boosting of innate immunity. In addition, it implies that neutrophil function during fetal ontogeny is intrinsically regulated during ontogeny and not influenced by changes of the environment.[132]

4.3 Transmigration and chemotaxis in preterm and term neonates

The recognition of immobilized chemokines or other pro-inflammatory mediators such as leukotriene B4 on the luminal surface of inflamed endothelial cells is considered a critical step for the induction of firm adhesion of neutrophils and monocytes and subsequent extravasation into inflamed tissue.[140] Human neutrophils isolated from cord blood and adult peripheral blood had impaired chemotactic activity of neonatal neutrophils in response to activated serum[141–143] and to six neutrophil-specific chemokines *in vitro*.[144] Neutrophils isolated from stressed neonates migrated even less efficient compared with healthy neonates, emphasizing the problems neonates face with encountering pathogens.[145]

Locomotion of leucocytes is accompanied by an actin-dependent change of cellular shape, going from a round morphology in the resting state into an amoeboid shape after polarization.[146] Rigid cytoskeletal structures prevent both uropod formation and the redistribution of adhesion sites, and can thereby contribute to the impaired migratory response of neonatal neutrophils.[147] Decreased actin content and polymerization seems to relate to impaired chemotactic responsiveness[148] or impaired chemokine receptor signalling[149] in neonatal neutrophils. Additionally, aberrant NF-κB signalling downstream of chemokine receptors in neutrophils from newborns leads to their impaired migratory capacity.[150]

Impaired chemotactic capacity is also reported in neutrophils isolated from cord blood of preterm infants.[139] These findings were confirmed in murine YS vessels in our laboratory. Bacterial products like the formylated peptide fMLF represent a strong stimulus for neutrophils to adhere and subsequently extravasate into inflamed tissue. After fMLF superfusion of YS vessels, fetal neutrophils showed a significantly reduced responsiveness to fMLF stimulation, resulting in reduced adhesion and attenuated extravasation of fetal neutrophils. This was more pronounced in the younger foetuses (E13–E15) compared with older foetuses (E17–E18)[117] and the result of reduced binding of fMLF to its receptors on neutrophils. However, impaired fMLF-triggered intracellular signalling events in neutrophils cannot be excluded to contribute to reduced fetal neutrophil adhesion and extravasation following fMLF stimulation. Interestingly, reduced CXCR2 chemokine receptor expression was found on fetal neutrophils, which also may contribute to reduced neutrophil recruitment during fetal life.

5. Effector functions of fetal neutrophils and monocytes

Neutrophils and monocytes participate in innate immune defence through many different mechanisms. They share expression profiles for pattern recognition receptors such as Toll-like receptors (TLRs) or RAGE, but also express cell surface receptors binding complement components or antibodies. During fetal and neonatal life, several of these components are not fully functional yet, which explains in part the high susceptibility to severe bacterial infections in premature and mature infants (*Figure 3*).

5.1 Complement receptors

Monocytes and neutrophils express three complement receptors: CR1 (CD35), CR3 (Mac-1, $\alpha_M\beta_2$ CD11b/CD18), and CR4 ($\alpha_X\beta_2$, CD11c/CD18). While expression of these receptors is low in the resting state, they can rapidly be mobilized from intracellular stores after activation.[151,152] Complement receptors mediate binding and phagocytosis of complement-opsonized microbes and particles.

CR1 and CR3 expression is already detectable on fetal monocytes and neutrophils starting at 14 weeks post conception.[153] Activation-induced up-regulation seems to be functional on both premature[153] and mature leucocytes.[154] In contrast, investigations on the whole complement system revealed compromised activation of the complement pathway in term neonates. This is even more pronounced in preterm infants and results in impaired direct lysis of pathogens as well as in diminished phagocytosis of complement-opsonized pathogens.[155,156]

5.2 Toll-like receptors

Sensing of microbes can be the result of binding of conserved bacterial structures to TLRs.[157] Binding to specific TLRs (for instance, TLR4 recognizes bacteria-derived LPS) activates different signalling pathways, finally resulting in killing of microbes and the release of cytokines.[158] Therefore, age-dependent functional maturation of TLR and TLR-induced signalling pathways is of substantial interest.

TLR expression levels on cord blood leucocytes are severely diminished in extremely premature infants (<24 weeks of gestational age).[159] TLR expression positively correlates with gestational age, reaching normal expression levels in monocytes isolated from term neonates.[160,161] Consequently, diminished TLR expression seems not to be causative for higher susceptibility to bacterial infections, at least in term neonates. However, it is important to emphasize that despite normal expression levels, differences exist in the magnitude of intracellular signalling events like extracellular signal-regulated kinase 1/2 (ERK1/2) and p38 activation,[162] and expression of the TLR signalling adaptor molecule MyD88.[163] Interestingly, term newborns suffering from bacterial sepsis appropriately up-regulate both TLR surface expression levels and intracellular signalling pathways.[164]

5.3 Phagocytosis

There exist conflicting results on the phagocytic activity of leucocytes isolated from preterm and term neonates. Some studies report a diminished ability of premature leucocytes to engulf opsonized pathogens.[128,139,165,166] Interestingly, Prosser *et al.*[167] show that despite of lower absolute numbers of phagocytes, they retain a higher capacity for bacterial uptake. This finding is in accordance with Hallwirth *et al.*[168] who also reported higher phagocytic activity in monocytes of preterm infants.

5.4 Reactive oxygen species

Reduced respiratory burst activity is reported for monocytes[169] and neutrophils isolated from preterm infants,[170–172] and this correlates with gestational age.[173] In line with these findings, neutrophils from preterm infants show a lower capacity to up-regulate oxidative burst intensity upon stimulation with coagulase-negative staphylococci, which represents the major cause of neonatal septicaemia.[174] Contrasting results show that higher oxidative burst activity is detected in preterm infants compared with more mature infants and adults.[128] In addition, more reactive oxygen intermediates are also detected in phorbol-12-myristate 13-acetate (PMA)-stimulated cord blood neutrophils of term babies compared with adult neutrophils stimulated with PMA.[175] In respect to functional maturation of oxidative burst capacity of phagocytes after birth, it is only dependent on chronological age but not on gestational age.[176]

5.5 Antimicrobial proteins and peptides

The production of antimicrobial proteins and peptides (APPs) is a special characteristic of neutrophils. These proteins are scarce in monocytes and macrophages. APPs are stored in neutrophilic granules and they are released into the phagocytic vacuole after pathogen ingestion. Major families are defensins and cathelicidines, which act mainly through permeabilization of bacterial membranes.[177–180]

In neutrophils and cord blood plasma of term and preterm infants, lower concentrations of neutrophil-derived antimicrobial peptides can be found. In cord blood plasma of preterm infants with gestational age lower than 30 weeks, calprotectin (MRP8/14, S100A8/A9) and the acute phase reactant $sPLA_2$ were severely diminished.[181] With respect to cord blood leucocytes, significantly reduced levels of bactericidal/permeability increasing protein, which is a central effector molecule against gram-negative bacteria, can be detected in neutrophils of neonates.[182] Lactoferrin, another APP family member with bacteriostatic iron-binding activity, contributes to the initiation of the oxidative burst in neutrophils.[183,184] Neutrophils isolated from newborn infants show lower levels of lactoferrin production and this, accordingly, relates to their diminished antimicrobial activity.[185] Interestingly, preliminary results from clinical trials suggest a protective role for lactoferrin in neonatal sepsis.[186]

5.6 Neutrophil extracellular traps

In addition to intracellular killing of engulfed pathogens, neutrophils are also able to degranulate and release their content to the extracellular space. This phenomenon is described as neutrophil extracellular traps (NETs) consisting of DNA strands bound to neutrophil-derived antimicrobial peptides and proteins.[187] These NETs are released upon pro-inflammatory stimulation and capture and kill extracellular pathogens like bacteria,[188] viruses,[189] and fungi.[190] Even after stimulation, neutrophils isolated from preterm and term infants failed to form NETs in one study.[191] However, it turned out that NET formation in neonatal neutrophils is stimulus- and time-dependent, since equally potent NET formation can be observed after extended TLR2- and TLR4-stimulation in neonatal and adult neutrophils, suggesting that NET formation in neonatal neutrophils is functional, but significantly delayed.[192]

6. Conclusion and perspective

The first haematopoietic cells, including HSCs, are produced early during embryonic development in various anatomical sites. A great feature of these cells is their ability to traffic from successive supportive niches throughout development until they reach their final destination, where they will reside in adults. Understanding the mechanisms that underlie HSPC production and migration is crucial for the establishment of optimal transplantation protocols. Also knowing that leukaemic stem cells might use a trafficking machinery similar to HSCs, the field of cancer biology would certainly benefit from a better understanding of the mechanisms controlling stem cell trafficking.

Leucocytes are part of the cells produced during development that share trafficking capacities with HSCs. Recruitment and effector functions of neutrophils/monocytes in the growing foetus are still not fully

understood, but play a major role during fetal life. Indeed, there is accumulating evidence that impaired innate immune responses during fetal life critically contribute to a higher susceptibility to infections in premature infants.

Genome-wide association studies on fetal and adult myeloid cells have opened up new possibilities to elucidate the molecular basis for the observed differences between preterm, term, and adult leucocyte function. Transcriptome analysis of both resident tissue macrophages from newborn mice[193] and human fetal peripheral blood monocytes[193] revealed that both basal transcriptional profiles as well as changes in gene expression after stimulation are fundamentally different from their adult counterpart. Additionally, fate mapping techniques with transgenic mouse lines now allow to differentiate between cells originating from primitive vs. definitive haematopoiesis.[194] These findings have challenged the classical view on primitive haematopoietic cells being present only transiently during fetal life and will give new insights into the mechanisms of innate immune cell development. Complementing these approaches with novel microscopy techniques and fetal *in vivo* models will certainly further boost this research area and will offer an exciting new perspective for future studies in the foetus. This will not only advance our understanding of how HSPC trafficking and immune responses evolve during fetal ontogeny, but also open new opportunities to elucidate the interplay between maternal and fetal circulation, most notably in pregnant women with cardiovascular diseases including diabetes, atherosclerosis, and also pregnancy-associated disorders such as preeclampsia.

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