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Heideveld, E.

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CHAPTER 1

General introduction

Erythropoiesis during development

Red blood cells have a unique function as oxygen transporter and are required during the entire lifespan of an individual. In human adults, red blood cells are formed in the bone marrow. However, during development there are multiple waves of erythropoiesis at several different anatomic sites (Figure 1). After gastrulation, primitive red blood cells are derived from the hemangioblast, a mesodermal precursor cell in the primitive streak that migrates to the yolk sac[1-3]. Here, the mesodermal cells adhere to form blood islands, which are the origin of primitive hematopoiesis[1, 4]. The human yolk sac produces mainly erythroid cells that are relatively large, short-lived, nucleated erythrocytes and can be found in the embryo at about day 16-20 of development[5-10]. Macrophages and primitive megakaryocytes have been described in the yolk sac as early as day 25 of development. However, at 8.5 weeks of development, hematopoiesis in the yolk sac entirely disappears[11, 12]. Although human yolk sac hematopoietic development is difficult to study due to ethical issues and availability of tissue[13], it has been proposed that part of the tissue macrophages in adults are derived from the yolk sac and are not replenished by monocyte-derived macrophages, which we will discuss in the next section[14].

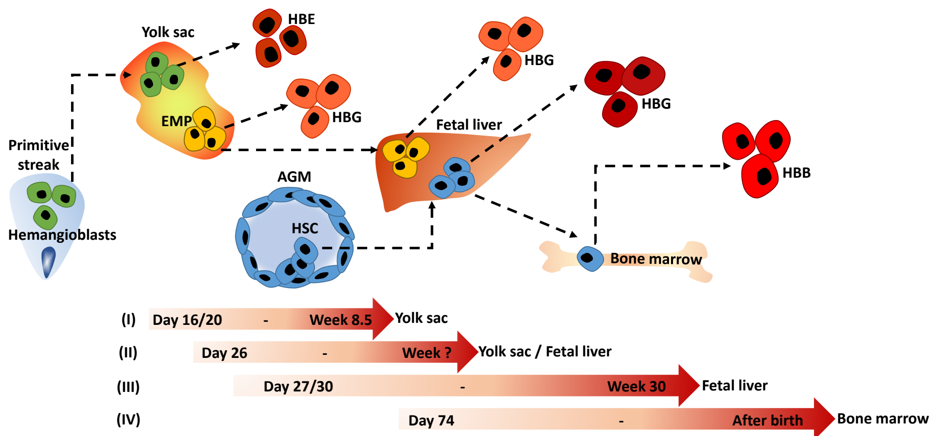

Figure 1. Hematopoietic development in the human embryo. (I) Hemangioblasts from the primitive streak migrate to the yolk sac and form blood islands, which produce HBE-expressing primitive erythrocytes from day 16-20 up to 8.5 weeks. (II) In parallel, EHT-derived EMP arise in the yolk sac around day 26 and develop into HBG-expressing definitive erythrocytes. In addition, yolk sac EHT-derived EMP migrate to the fetal liver and differentiate towards erythrocytes. (III) From day 27-30 until the 7th month of gestation, the first AGM EHT-derived HSC migrate to the fetal liver and develop into definitive erythrocytes, also expressing fetal hemoglobin HBG. (IV) At week 10.5 (day 74), HSC from the fetal liver colonize the bone marrow and differentiate towards definitive erythrocytes expressing adult hemoglobin HBB.

Shortly after the initial wave of primitive yolk sac-derived erythropoiesis, a second intermediate definitive wave has been identified around day 26 of human development to putatively bridge the gap between primitive and definitive hematopoietic stem cell (HSC)-derived cells. This transient wave

in the yolk sac derives from transient multipotent erythromyeloid progenitors (EMP) that are generated from hemogenic endothelium in a process called endothelial-to-hematopoietic transition (EHT). EMP are the first cells to colonize the fetal liver[15, 16]. Interestingly, this wave produces definitive erythroblasts with a remarkable *in vitro* expansion potential in mice (>1 year)[17]. The first CD34$^+$ HSC are formed from endothelial cells in the aorta-gonad-mesophrenos (AGM) region through EHT and start to colonize the liver at day 27 to 30 of development[8, 18-24]. During this high flux of definitive fetal liver erythropoiesis from the 9th to the 24th week of gestation[25, 26], HSC localize to the bone marrow at 10.5 weeks of development, which is at time of birth the main organ for erythropoiesis. Erythroid cells from the different developmental waves can be readily identified due to switching of hemoglobin subunit expression[27]. The hemoglobin molecule is a tetramer and consists of two α-like globin peptide chains and two subunits of the β-like globin peptides. During primitive erythropoiesis in the yolk sac of the early embryo, abundant expression of the β-like globin epsilon (HBE) and α-like globin zeta (HBZ) is found in erythroid cells. Thereafter, during production of definitive erythroid cells in the fetal liver the β-like globin gamma (HBG) is expressed[28]. However, it must be noted that both EMP-derived erythroid cells from yolk sac and fetal liver express HBG and thus this cannot be used to discriminate between the second and third definitive wave. Markers that discriminate between these two definitive waves or their progeny are in need. After birth, the gamma chain is exchanged for beta-globin (HBB >97%) or delta-globin (HBD ~3%) and pairs with the alpha-globin (HBA) to form adult hemoglobin[29, 30]. During aging, the red marrow gets replaced by yellow marrow, and hematopoiesis shifts from the long bones, to the pelvis, sternum, cranium, and vertebrae.

Within the human bone marrow, the differentiation from stem cell to erythrocyte follows several stages based on cell morphology, colony forming capacity or marker expression by flow cytometry (Figure 2). CD34$^+$ HSC first differentiate to multipotent progenitors (MPP), which give rise to either common lymphoid progenitors (CLP) or common myeloid progenitors (CMP). CLP can generate all lymphoid cells including T-cell, B-cell and NK-cells[31], while CMP can generate all myeloid cells but not lymphoid cells[32, 33]. CMP develop into granulocyte-macrophage progenitors (GMP) or differentiate to megakaryocyte-erythroid progenitors (MEP). All three populations express CD34, however, we can discriminate between the populations using CD45RA and CD123 in the Lin$^-$CD34$^+$CD38$^+$ cells: CD123^{low}CD45RA$^-$ CMP, CD123^{low}CD45RA$^+$ GMP and CD123$^-$CD45RA$^-$ MEP[32]. Another report has been published using BAH1.1 and CD45RA and described BAH1.1$^-$CD45RA$^-$ CMP, BAH1.1$^-$CD45RA$^+$ GMP and BAH1.1$^+$CD45RA$^-$ MEP[34]. Colony assays also allow to follow commitment of cells to the hematopoietic lineages. CMP can generate colony forming unit granulocyte, erythrocyte, monocyte (CFU-GEMM) including all types of myeloid colonies, whereas GMP give granulocyte and macrophage colonies (CFU-GM) and MEP give rise to the earliest erythroid colonies identified as erythroid burst forming units or BFU-E. MEP will further develop into CD34$^-$CD71^{high}CD235a^{low} pro-erythroblasts while gradually losing CD34 expression and acquiring high expression of CD71[32, 33, 35]. Cells between the MEP and pro-erythroblast stage give rise to colonies of the late-stage erythroid lineage (CFU-E). In addition, variations to the general hematopoietic differentiation scheme have been emerging lately with the

availability of single cell RNA-sequencing techniques. In these novel hematopoietic trees, the MEP population plays a less prominent role, because HSC may directly differentiate into megakaryoid cells without any precursors *in vivo*[36]. Pro-erythroblasts subsequently differentiate into basophilic, polychromatophilic, and orthochromatic erythroblasts and gradually acquire expression of CD235a (GPA)[6, 37-39]. During this process cells become smaller, the nucleus condensates and will be expelled resulting in an enucleated reticulocyte that loses CD71 and enters the blood stream to mature into an erythrocyte[6]. The enucleation process can be followed using the DNA-associated cell permeable DRAQ5 as a marker for nucleated cells.

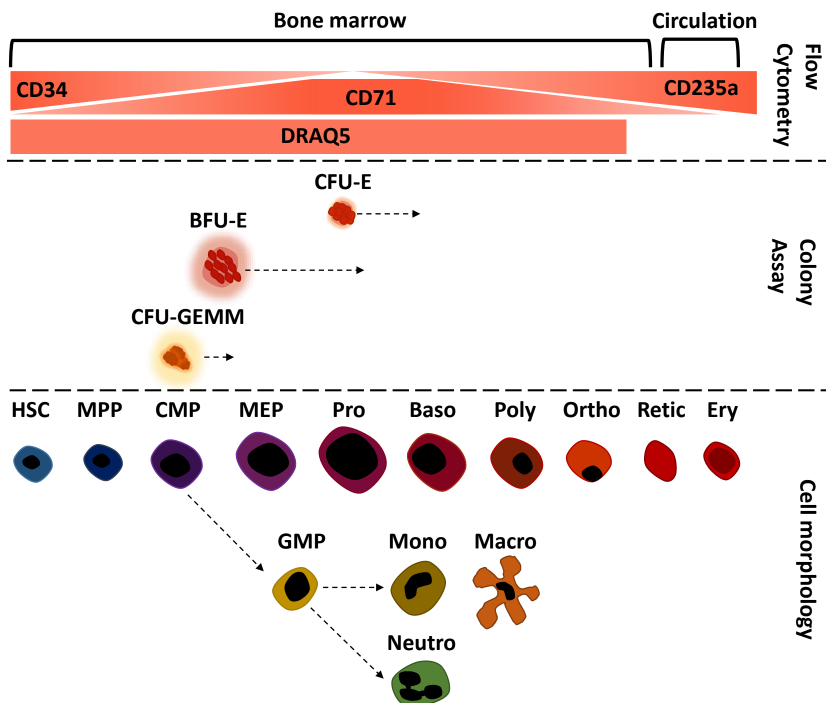

Figure 2. Differentiation of HSC to erythrocytes based on cell morphology, colony assay and flow cytometry. CD34⁺ HSC develop into CFU-GEMM in the adult bone marrow. Here, the decision is made if cells follow an erythroid differentiation program and differentiate to MEP, or a myeloid program and differentiate to GMP. GMP will further develop into monocytes (Mono) and macrophages (Macro) or neutrophils (Neutro), while MEP further develop into CD71⁺ pro-erythroblasts (Pro) and differentiate towards basophilic (Baso), polychromatophilic (Poly), and orthochromatic (Ortho) erythroblasts. During erythroid differentiation cells loose CD71 expression, while acquiring expression of CD235a. Orthochromatic erythroblasts enucleate and thereby loose the expression of DRAQ5 and reticulocytes (Retic) enter the blood stream to develop into an erythrocyte (Ery).

After the release of reticulocytes from the bone marrow, the spleen acts as a quality control during the entire lifespan of the red cell. A small amount of the circulating human reticulocytes have large autophagic vacuoles[40]. The amount of vacuoles was increased in splenectomized individuals and patients

with sickle cell disease, however, these specific reticulocytes were cleared in a healthy recipient[41-43]. This suggests that final remodeling of reticulocytes by removal of the autophagic vacuoles most likely occurs in the spleen. Extrusion of the autophagic vesicles allows reticulocytes to reduce the surface area and volume of the cell. It is believed that the autophagic vesicles are phagocytosed by red pulp macrophages, as clodronate depletion of macrophages inhibits reticulocyte maturation in the circulation[44-46]. However, Griffiths et al. observed the presence of mature erythrocytes in splenectomized indivuals, which suggests that the final maturation of reticulocytes could occur independent of splenic macrophages[47]. Besides this ill-defined role of splenic macrophages in the removal of autophagic vesicles from early reticulocytes, splenic macrophages are also known for the clearance of intracellular inclusions from the red cell membrane and removal of aged red blood cells from the circulation[48-53]. In addition, clearance of old erythrocytes and iron recycling has also been observed in the liver[54, 55].

During steady-state erythropoiesis, red blood cells are produced at a constant rate of two million erythrocytes per second and this is restricted to the adult bone marrow in both human and mouse. However, erythropoiesis in human and mouse is differently regulated during stress erythropoiesis. Stress-induced erythropoiesis is distinct from basal steady-state erythropoiesis as various types of stress like hypoxia, hemorrhage or (chronic) anemia induce a rapid production of new erythrocytes, resulting in restored oxygenation. In humans, both steady-state and stress-induced erythropoiesis are restricted to the bone marrow. Although some papers suggest that human stress erythropoiesis also occurs in the spleen[56], this has never been confirmed. Splenomegaly that has been observed during hypoxia is not the result of *de novo* erythropoiesis, but the release of stored erythrocytes in the spleen, which has been observed during normobaric hypoxia in humans that showed increased spleen volume, spleen contraction and erythrocyte release into the bloodstream[57]. In contrast, stress-induced erythropoiesis in mice is localized to the extramedullary sites and occurs outside the medulla of the bone, mainly in the spleen but also the liver is involved[58, 59].

Signaling during erythropoiesis

The mechanisms defining the “decision” to differentiate into either one or the other hematopoietic lineages are not completely known. Currently, it is generally accepted that lineage specific growth factors allow the survival of specific lineages through concomitant receptor expression. This “permits” lineage commitment and hence is termed the permissive model. Erythropoietin (EPO) is produced by the fetal liver and shifts to the kidney after birth and although the kidney produces most EPO (80%) in adults, the liver retains its ability to produce 10-15% of total EPO production in human, which can be even further increased to 30% upon stress due to low oxygen levels[60, 61]. EPO binds and activates the EPO-receptor (EPOR) on immature erythroid cells and functions as a survival factor during erythroid differentiation[62, 63]. The importance of EPO has also been shown by *Epo*$^{-/-}$ or *EpoR*$^{-/-}$ mice, which are both embryonically lethal due to lack of mature erythrocytes[64, 65]. Another key regulator during erythropoiesis is stem cell factor (SCF), which signals via the mast/stem cell growth factor receptor KIT (CD117) to enhance growth and survival of erythroid progenitors. Interestingly, SCF in combina-

tion with EPO has a synergistic effect on erythroid cell proliferation, which relies on synergistic signal transduction activation[64, 66-68]. SCF is provided by the stromal niche cells in the bone marrow as a membrane bound protein. Mutations that allow only soluble SCF display a severe erythroid deficiency and *Kit* knockout mice are non-viable at embryonic day 14 due to reduced numbers of erythroid cells in the fetal liver[69]. Furthermore, it has been shown that KIT plays a role in EPOR maintenance, which results in erythroid progenitor survival upon EPO stimulation[63]. During stress-induced erythropoiesis, the glucocorticoid receptor (GR) cooperates with KIT and EPOR and induces long-term proliferation of immature erythroid cells without differentiation[68, 70-78]. Glucocorticoids, which are the ligands of the GR, are produced in the adrenal gland. In absence of ligands, the GR is bound to HSP90 and its co-chaperones and localizes outside the nucleus in the cytoplasm[79]. HSP90-independent modes of GR extranuclear localization have been reported and involve direct interaction of the GR with EPOR[80]. Binding of glucocorticoids release the GR from HSP90 and enable nuclear translocation. Binding of full agonists such as hydrocortisone or dexamethasone also enable binding of transcriptional activators to the C-terminal transactivation domain, while partial (ant)agonists do not[81]. These latter still enable nuclear localization and transcriptional activation via the hormone independent N-terminal transactivation domain and enable the repressive function of the GR. The GR requires homodimerization to be active as a transcription factor that binds the glucocorticoid transactivation element. However, the GR can also heterodimerise with transcription factors such as STAT5 to bind a compound site consisting of a half-mer GR site and half-mer STAT5 site[81]. Although STAT5 plays a prominent role in erythropoiesis, there is currently no evidence that the GR acts as a GR-STAT5 dimer as it does in the mammary gland[68]. Transcriptional repressive and active roles of STAT5 are important in macrophage functionality, but also here it is unknown if GR-STAT5 oligomers are formed or involved. In addition, DNA-binding domain independent monomeric GR association with chromatin was found in complex with AP1 or NFκB[81]. This interaction represses the activity of AP1 and NFκB and is important in repression of the immune function of macrophages. Stress erythropoiesis requires the activity of the dimerized GR, because *Gr* knockout mice or mice expressing a mutant incapable of dimerization (*Gr$^{dim/dim}$*) lack any form of stress erythropoiesis and display severely decreased fetal liver erythropoiesis[74, 75].

Direction to specific lineages is generally believed to occur via stochastic variations in the concentration of specific transcription factors. A well-known example is the “decision” of CMP to become GMP or MEP, which depends on the relative levels of PU.1 (GMP direction) and GATA1 (MEP direction). The transcription factor GATA1 plays a critical role in erythroid development, both in primitive and definitive erythropoiesis, as disruption of *Gata1* in mice results in embryonic lethality due to a defect in primitive erythropoiesis, while *GATA1$^{-/-}$* embryonic stem cell-derived pro-erythroblasts *in vitro* develop a block in differentiation[82-86]. Furthermore, during maturation, it has been shown that both EPOR and GATA1 levels are increased, especially when cells differentiate from pro-erythroblast into basophilic erythroblasts. During terminal differentiation from basophilic erythroblasts to erythrocytes, both GATA1 and EPOR are reduced[87-90]. Failure to reduce GATA1 impairs erythroid maturation[91]. In contrast, PU.1 is a positive regulator of lymphoid and myeloid differentiation and prevents differen-

tiation into the erythroid lineage. PU.1 is still expressed in early erythroid cells, but downregulated when cells mature[92]. Ectopic overexpression of PU.1 in erythroblasts inhibits the differentiation and can result in immortalized erythroblasts[93, 94]. Another example is the transition of a MEP towards the erythroid or megakaryoid lineage, which depends on the relative abundance of KLF1 (erythroid) and FLI1 (megakaryoid). KLF1, the erythroid Krüppel-like factor (or EKLF), is crucial for erythropoiesis as *Klf1*$^{-/-}$ mice show defects in hemoglobin metabolism and die around embryonic day 14[95, 96]. In addition, FLI1 suppresses erythroid differentiation and direct cells towards the megakaryoid lineage[97-100]. Interestingly, recently this transcription factor driven directive model was challenged, as single cell HSC tracking through differentiation revealed that PU.1 and GATA1 are never expressed at the same time points during HSC differentiation[101].

Erythropoiesis is not only regulated by intrinsic and soluble extrinsic factors, but also by cell-cell interactions. During the development from HSC to erythrocyte, the microenvironment plays a crucial role as macrophages in the stroma, which are located near the HSC in the bone marrow, affect HSC and erythroid cells through ill-defined mechanisms. It is important to know the underlying mechanisms of erythroid-macrophage interactions, as this could help optimizing red blood cell cultures. In the next sections, we focus on these supporting macrophages and give an overview of their origin, characterization in mice and human and their role in the HSC and erythroid niche in the bone marrow.

Tissue resident macrophages and their origin

The bone marrow is a complex organ devoted to the generation of all blood cells. It is divided in various compartments or niches, presumably dependent on the presence of different cell populations, secreted cytokines and chemokines. Within the bone marrow, hematopoietic stem and progenitor cell (HSPC) homeostasis and erythropoiesis are co-regulated by macrophages. Macrophages are key regulators of both innate and adaptive immunity, however, they are also known for their role in tissue homeostasis, development and malignancy. Dependent on cues in the microenvironment, monocytic cells differentiate into macrophages with various phenotypes and functions, and migrate to different tissues. In 2000, Mills et al. described a model for macrophage activation in which two major opposing macrophage activities were classified into subtypes: classical M1 or alternative M2 macrophages[102]. M1 macrophages inhibit cell proliferation and induce a pro-inflammatory response, while M2 macrophages are anti-inflammatory, promote cell proliferation and are known to be involved in tissue repair and wound healing[103-107]. The M1/M2 model has been used predominantly as it is a simple way to distinguish between the two functional properties of macrophages. However, it depicts M1 and M2 activation as clearly distinct processes, while macrophage polarization is more complex[108]. For instance, the M2 population has been further divided into M2a-d macrophages based on inducing agents, marker expression and functionality irrespective of tissue residence[109-111]. This sub-classification becomes even more complex upon describing resident macrophages in different tissues as marker expression and functionality can be influenced by the specific niche in which these macrophages reside. This results in an array of different notifications and classifications for tissue resident

macrophages making generalizations like the M2 sub-classification rather limited and oversimplified. In the following sections, we will describe the different macrophages based on marker expression and functionality and will refrain from classical definitions like M1 and M2.

Based on their origin, tissue resident macrophages can be divided into two subsets. One derives from the yolk sac and is maintained by self-renewal and proliferation. Another population originates from bone marrow myelopoiesis and from resulting circulating monocytes[112]. These monocytes are a heterogeneous group consisting of three subsets, the classical $CD14^{++}CD16^{-}$, intermediate $CD14^{++}CD16^{+}$ and non-classical $CD14^{+}CD16^{+}$ monocytes[113, 114]. The non-classical monocytes are known for their patrolling function in the tissues and are therefore suggested to differentiate into resident macrophage populations that function in wound healing and tissue repair[115]. Until recently, it was believed that all macrophages including tissue resident macrophages derived from monocytes. However, the complexity and heterogeneity of macrophages have been underestimated. In the last decade, interest has been grown to understand the development, relationship, function and origin of the different macrophage subsets within the different tissues. The dogma, which came into existence during the 1960s and 1970s, is increasingly challenged. This dogma dictates that tissue resident macrophages derive from *de novo* monocytes produced during myelopoiesis from definitive HSC in the bone marrow[116], although the same researchers already in 1984 showed that macrophages in the spleen had dual origins, some were derived from monocytes while others were dependent on self-renewal[117]. The general 20th century simplistic view of HSC differentiating to a CMP which further matures to a GMP and subsequently monocytes that exit the bone marrow has gained in resolution with the discovery of a clonogenic progenitor[118]. This progenitor gives rise to monocytes, macrophages, and dendritic cells[119], and more recently to a monocyte-restricted bone marrow precursor termed common monocyte progenitor (cMOP)[120]. In any case, *de novo* monocytes may then home to their respective tissues and further differentiate into tissue resident macrophages or pro-inflammatory macrophages depending on the systemic need[116, 121, 122]. Nevertheless, several lacunae concerning the presumed bone marrow origin of tissue resident macrophages within this dogma remained. Hashimoto et al. reported that recovery of specific tissue resident macrophages (e.g., microglia or Langerhans cells) after tissue damage did not involve donor cells but appeared to be of host origin[112]. Interestingly, monocytopenic mice present with normal macrophage distributions in the tissues[123]. Several other authors showed persistent and maintained macrophage populations independent of monocyte production[112, 124-134]. These results suggested that certain macrophage populations did not arise from *de novo* generated bone marrow monocytes. Further investigation using i) parabiotic mice, ii) selective ablation using specific macrophage markers, iii) gene expression analysis, and iv) single population tracing led to the notion that a selection of tissue macrophages are derived independently from bone marrow myelopoiesis[112]. Surprisingly, these cells are able to undergo renewal divisions in order to repopulate the tissue after injury or insult to the tissue. Phenotypically these cells are completely different from the inflammatory macrophage[108]. Interestingly, host-derived recovery of macrophages in specific tissues after whole body irradiation prior to bone marrow transplantations

of mice can arise independently which suggests the presence of bone marrow myeolopoiesis-independent macrophages. The origin of these specific macrophages, their renewal capacity, signaling cues that maintain these cells and whether the tissue resident effector cell and renewal population are different entities are currently vividly pursuited within the field. Recently, it has been shown that minor populations of macrophages in specific tissues are originating from yolk sac myelopoiesis and derive prior to the establishment of definitive HSC-dependent hematopoiesis[14, 112]. It suggests that a subset of macrophages finds their origin in early embryogenesis and maintains their tissue presence, functionality and renewal capacity throughout adulthood. Indeed, fate-mapping studies now show that macrophages are present in skin, brain and other tissues before the onset of monocytes in the bone marrow[112, 129, 131, 135]. Arterial macrophages are mainly generated from early and late EMP derived from the yolk sac[126], while intestinal macrophages are maintained by constant replenishment of monocytes[136]. In addition, the first Langerhans cells are thought to derive from yolk sac EMP-derived macrophages that migrate to the skin and are exchanged by fetal liver monocytes after the second wave of hematopoiesis[124, 137]. This suggests that there could be even three sources of macrophages: yolk sac-derived macrophages, and fetal liver or adult bone marrow-derived monocytes that differentiate into macrophages in the tissue[138, 139]. The contribution of these embryo-derived macrophages to tissue functionality and regeneration in particularly must be resolved in order to understand their role in tissue homeostasis as well as in pathological conditions. Indeed, the importance of host-tissue resident macrophages persistence can be clinically important[140]. One of these studies indicated that host Langerhans cells in the skin may remain after bone marrow transplantation in patients, the persistence of these cells is highly correlated with severity of graft-versus-host disease[140, 141]. In addition, the bone marrow-independent origin of these macrophages places serious constraints on the ability of *in vitro* differentiation model systems to generate and study tissue resident macrophages. To establish if other tissues are equally affected, the different macrophage populations in human tissues must be characterized phenotypically, functionally and upon tissue insult. In addition, it is unknown if these specific macrophages are able to transmigrate to different tissues. This is difficult to assess as macrophage identity depends on the tissue niche and markers may differ from tissue to tissue but also during transit from one tissue to the other. However, Hashimoto et al. showed that exchange of macrophages between tissue is limited in parabiotic mice[112]. In conclusion, the monocyte/macrophage system needs to be overhauled and hierarchically reclassified to include the novel class of bone marrow hematopoietic-independent tissue resident (embryonic) macrophage populations. Hematopoiesis and specifically erythropoiesis is inseparably connected with nursing macrophages and the total erythroid flux may thus also dependent on the availability and regulation of these specific macrophages. Next, we will review two important niches in which macrophages have been observed.

Macrophages within the stem cell niche

HSC reside in specific distinct microenvironments in the bone marrow, however these niches are poorly understood as characterization of the specific locations within the bone marrow is difficult to visualize and study *in vivo* as well as *ex vivo*. Although the markers that define HSC have been

relatively well characterized in mice[142], the markers that define human long-term repopulating HSC have not been properly identified and these cells are currently mostly defined as lineage-negative $CD34^{+}CD38^{-}$ cells with inclusion of several other markers like CD49d, CD117, CD133 and the ability to expel Hoechst through multi-drug resistance receptors[143, 144]. Some HSC have been found as single cells in the trabecular cavities of the long bones. Others have been found in a highly vascularized area towards the center of the bone marrow[145-147]. The bone marrow contains a set of bone marrow resident macrophages of which some reside within the main HSC niches: the endosteal and perivascular niche. Within these niches stroma provide the optimal environment for the bone marrow and support the maintenance of HSC. These stromal cells contain osteoblasts, CXCL12-abundant reticular cells (CAR cells), endothelial cells and macrophages. 60% of the long-term repopulating HSC are localized near the vasculature, close to the endothelium lining the sinusoids[148]. The sinusoids can be found close to the endosteal niche, but are more likely to be located at greater distances and contain CAR cells that secrete factors to promote self-renewal of HSC. This perivascular niche includes the sinusoids in the bone marrow and contains a specific subset of macrophages that support maintenance and proliferation of mesenchymal stem cells (MSC). In addition, they instruct HSC to reside in the bone marrow[149].

The endosteal niche contains quiescent HSC and is located in the proximity of the bone surface, where osteoblasts line the membrane. This niche contains a specific subset of tissue resident macrophages referred to as osteal macrophages or osteomacs which play diverse roles in bone biology. Osteomacs can be found immediately adjacent to osteoblasts and regulate bone formation and homeostasis. Furthermore, they regulate maintenance and proliferation of $Nestin^{+}$ MSC. These MSC express a variety of HSC retention factors and it is thought that macrophages 'talk' to MSC via unknown secreted factors, excluding IL1, IL10, TNFα, and insulin-like growth factor 1 (IGF1), resulting in HSC retention in the bone marrow[150-152]. Indeed, macrophage Fas-induced apoptosis (MAFIA) mice lack osteomacs and as a consequence have increased mobilized HSC[151, 153]. Ablation of $CD169^{+}$ macrophages leads to HSC mobilization and defects in erythropoiesis[154, 155]. In mice, osteomacs have been characterized by the expression of a variety of markers and it has been suggested to have a counterpart in humans. Osteomacs are positive for F4/80, CD169, vascular cell adhesion molecule 1 (VCAM1), CD11b, CD68, CD115, macrophage-3 antigen (MAC3) and are negative for tartarate-resistant acid phosphatase (TRAP)[151, 154, 156]. The commonly used marker F4/80 for murine macrophages has a human ortholog, an EGF-like module containing mucin-like hormone receptor 1 (EMR1). However, expression of EMR1 is absent on mononuclear phagocytic cells and is only restricted to eosinophilic granulocytes[157]. EMR1 can therefore not be used to define human macrophages. In human bone marrow, Chow et al. describes a macrophage population that shares characteristics with murine bone marrow macrophages. The human ortholog for $CD169^{+}VCAM1^{+}$ murine macrophages was identified as $CD15^{-}CD163^{+}VCAM1^{+}CD169^{+}$ human bone marrow macrophages[155]. It was believed that the murine macrophages described by Winkler et al.[151] were different from the murine $CD169^{+}$ macrophages by Chow et al.[158], however, as both macrophages have a similar function, share a sim-

ilar marker expression profile, and are both located close to the bone and osteoblasts, we suggest CD169^{+}VCAM1^{+} macrophages described by Chow et al. could in fact be osteomacs. Haldar et al. described a bone marrow macrophage population in mice with a similar molecular expression profile compared to osteomacs (expression of F4/80^{+}VCAM1^{+}CD68^{+}CD169^{+}CD11b^{low}) of which the development is dependent on SPI-C transcription factor[159]. SPI-C was highly expressed in these specific macrophages as well as red pulp macrophages in the spleen and mice deficient for *Spi-c* lack both bone marrow and spleen macrophages[159, 160].

Table 1. The different bone marrow macrophage populations present in human and mice based on key cell surface markers and functionality.

Mouse bone marrow macrophages

Name	Phenotype	Function
Bone marrow macrophages	Gr1^{+} CD115^{int} F4/80^{+} CD169^{+} MHCII^{int} CD11c^{int} CD68^{int} CD11b^{low} CX3CR1^{-}	Maintenance and retention of HSC in bone marrow[154]
Tissue resident macrophages	CD169^{+} VCAM1^{+} F4/80^{+} TRAP^{-}	Support erythropoiesis[155]
Osteomacs	F4/80^{+} CD115^{+} MAC3^{+} CD68^{+}	Lining endosteal/periosteal bone surfaces, regulate osteoblast function[150, 152]
	F4/80^{+} CD11b^{+} Ly6G^{+}	Maintenance of HSC niches and HSC retention[151, 153]
	G-CSFR^{+} CD68^{+}	Support growth/survival of osteoblasts and inhibit HSPC mobilization[156]
Tissue resident SPI-C expressing macrophages	F4/80^{+} VCAM1^{+} CD68^{+} CD169^{+} CD11b^{low} MHCII^{int} TREML4^{+} Zbtb46^{-}	Heme degradation and iron recycling[159]
Bone marrow resident macrophages and monocytes	CD11b^{+} CX3CR1^{+} COX2^{+} a-SMA^{+} Ly6C$^{int/low}$ CD115^{+} CD150^{+}	Adjacent to HSPC and support maintenance and protection of HSPC from exhaustion during stress[161]
Central macrophages	F4/80^{+} VCAM1^{+} CD169^{+} CD163^{+}	Promote pro-erythroblast proliferation in erythroblastic islands[155, 162-164]

Human bone marrow macrophages

Name	Phenotype	Function
Osteomacs	CD68^{+}	Support osteoblast-mediated bone formation[150]
Tissue resident macrophages	CD45^{+} CD169^{+} VCAM1^{+} CD163^{+} CD15^{-}	Unknown, might play a similar role as tissue resident macrophages in mice[155]
Central macrophages	FcRI-III^{+} CD4^{+} CD31^{+} CD11a^{+} CD11c^{+} CD18^{+} HLA-DR^{+}	Support pro-erythroblast proliferation in erythroblastic islands[163]

In monocytes, *Spi-c* is repressed by BACH1, however, when heme levels increase BACH1 protein in monocytes is degraded. *Spi-c* is no longer repressed and monocytes differentiate to either bone marrow or red pulp macrophages. When these macrophage numbers increase, heme will be removed and *Spi-c* will be repressed by BACH1 which will stop monocyte differentiation[159, 160]. Whether this mechanism is similar in all SPI-C expressing monocytes or whether it is restricted to a particular

monocyte subset is still unclear. Ludin et al. identified a rare activated bone marrow monocyte and macrophage subset in mice expressing the α-smooth muscle actin and cyclooxyrgenase 2 (COX2)[161]. These cells were adjacent to HSC and have been shown to promote the expression of CXCL12 in perivascular Nestin-positive cells and possibly the maintenance and protection of HSC from exhaustion during stress. The different bone marrow macrophages identified in human and mice have been summarized in Table 1 and show the complexity and lack of uniform descriptions. There is a need for an in depth characterization of (bone marrow) macrophages with the use of cell surface markers, flow cytometry and/or proteomics to allow further research into the roles of these putative different macrophage population within the tissues and specifically the hematopoietic tissues as studied in this thesis.

Macrophages within erythroblastic islands

The erythroblast island is a specific structure in the bone marrow comprising of a central macrophage surrounded by erythroid precursors at different stages of terminal differentiation. Bessis et al. first described erythroblastic islands as the specialized microenvironmental compartments in which mammalian erythroblasts proliferate and differentiate during their second stage of maturation[165]. These islands consist of a central macrophage that extends cytoplasmic protrusions to a ring of surrounding erythroblasts[166]. Erythroblastic islands have been demonstrated *in vivo* at all sites of human definitive erythropoiesis (e.g., in the fetal liver and bone marrow). In addition, erythroblastic islands may be reconstituted *ex vivo* upon culturing bone marrow or peripheral blood mononuclear cells (PBMC) resulting in simultaneous differentiation of HSC into erythroblasts and monocytes into specific macrophages[162, 167]. This latter process is dependent on glucocorticoid directed differentiation of $CD34^+$ cells. Bone marrow central macrophages support erythropoiesis by regulating erythroid proliferation, differentiation, and enucleation and are believed to clear the expelled erythroid nuclei surrounded by plasma membrane (or pyrenocytes) resulting from the enucleation process[168, 169]. These islands, although one would expect them to reside near the sinusoidal capillary lumen as reticulocytes will be released into the blood stream, are actually evenly distributed across the bone marrow[170]. Quantitative light and electron microscopy of rat bone marrow indeed showed a difference in the composition of islands adjacent and nonadjacent to the sinusoids. Nonadjacent islands contain more pro-erythroblasts, adjacent islands are rich in orthochromatophilic erythroblasts, while the numbers of basophilic and polychromatophilic erythroblasts are comparable in both[171]. This remarkable finding suggests dynamic islands which are able to migrate to the sinusoids when cells within the island maturate. Islands harvested from human bone marrow contain 5-30 erythroblasts per island. Morphologically, central macrophages are relatively large with many extrusion to facilitate 15-25 erythroid cells[172]. Phenotypically, these macrophages are relatively well-described in humans and seem to express a set of M2 macrophage markers like CD163, CD169 and CD206[112, 173-175]. Furthermore, they have been described to express Fc-receptor I-III, CD4, CD11a,b^{dim},c, CD18, CD31, CD36, and HLA-DR (Table 1), however, there is no respiratory burst activity reminiscent of pro-inflammatory macrophages and they do not express C2b or CD35[163, 172].

It has been shown that central macrophages in the bone marrow can bind erythroblasts through various interactions. Adhesion molecules are the logical proteins to mediate the association between erythroid cells and central macrophages[176-179]. Indeed, several have been reported to play a role in binding of erythroid cells to macrophages, while others have been described to be involved in the uptake of the pyrenocytes (Figure 3). In mice, CD169+ erythroid-supporting macrophages express both the erythroblast adhesion receptor CD163 and VCAM1. VCAM1 bind to integrin-α4β1 (VLA4) on the erythroid cells and blocking these molecules disrupts erythroblastic islands[155, 175, 180, 181]. CD163 scavenge hemoglobin-haptoglobin complexes, however, future studies are required to unravel how the interaction between macrophages and erythroblasts via CD163 occurs[175]. Another integrin involved in erythroblastic-island formation is ICAM4. This adhesion molecule is present on erythroid cells during terminal differentiation and mediates binding between erythroid cells but also binding to integrin-α5β1 (VLA5) on macrophages[176, 180, 182-185]. Interestingly, VLA5 is not only expressed by macrophages but is also present on erythroid cells and binds to fibronectin (FN)-expressing stromal cells, including macrophages[186].

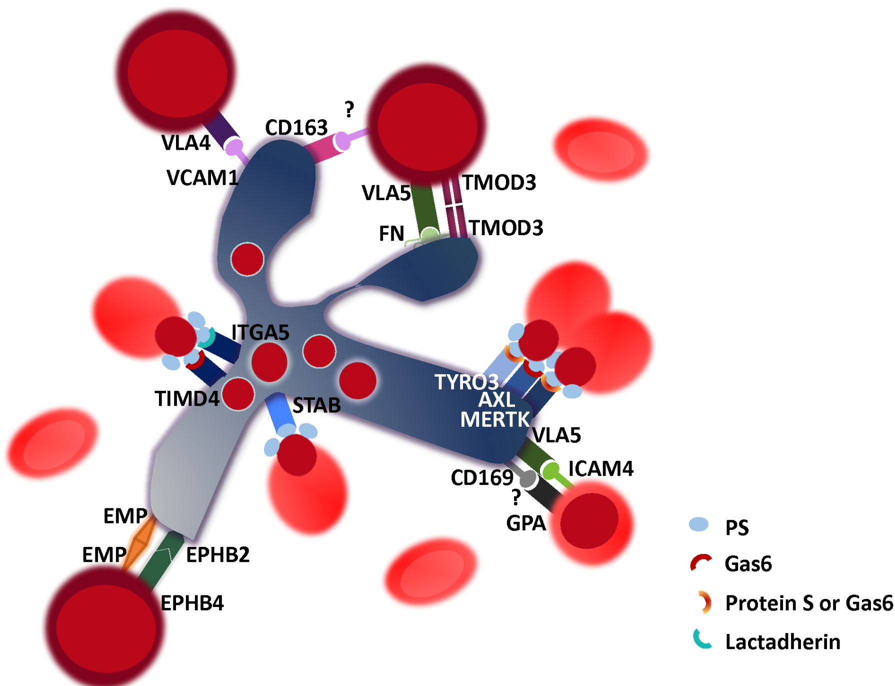

Figure 3. Erythroid cells bind to central macrophages and pyrenocytes can be phagocytosed via several mechanisms. Central macrophages express several adhesion molecules and other receptors that enhance the binding of pro-erythroblasts, and more differentiated erythroid cells to macrophages and support the recognition and phagocytosis of phosphatidylserine-exposed pyrenocytes (PS) via Gas6, Protein S or lactadherin.

Other interactions are facilitated by the macrophage erythroblast attacher (MAEA), also known as erythroblast macrophage protein (EMP), which is present on both macrophages and erythroid cells. EMP-EMP interactions are involved in the tight binding of erythroid cells to macrophages and *Emp*-deficient mice die perinatally due to a reduction in erythroblastic islands[176, 187, 188]. Recently, Sui et al. reported that tropomodulin 3 (TMOD3) is present on both macrophages and erythroid cells in the fetal liver and plays an important role in erythroblastic island formation. *Tmod3*$^{-/-}$ mice have defective adhesive interactions in erythroblastic islands in the fetal liver, resulting in impaired terminal erythroid differentiation, survival, cell-cycle progression and reduced enucleation in erythroblasts[189]. It is also thought that ephrins play a role in erythroblastic islands. Ephrin type-B receptor 4 (EPHB4) is expressed on the early CD34$^+$CD117$^+$ HSPC cells from cord blood and bind to EPHB2 ligand-expressing macrophages. This results in a fast differentiation towards mature erythroid cells, while the cells immediately downregulate EPHB4 and detach from the EPHB2 expressing cells[190]. Interestingly, EPHB4-positive erythroid cells can also bind EPHB2-negative stromal cells, however, the erythroid cells remained positive for EPHB4. Another study showed that the EPHB4-EPHB2 interaction is required for bone marrow HSPC mobilization to the blood[191]. In addition, EPHB4 has been described as an alternative candidate for EPOR, which stimulated by EPO increase human ovarian and breast tumor growth[192].

As described in the first section, erythroid cells gain expression of CD235a during terminal maturation. It has been shown that CD235a$^+$ erythroid cells engage via SIGLEC9 on neutrophils. A similar mechanism may be involved in the binding of GPA-expressing erythroid cells to CD169 (SIGLEC1) on macrophages[193]. Furthermore, in this late stage of erythropoiesis VLA4 and VLA5 expression on erythroid cells is downregulated, presumably to facilitate reticulocyte release from the islands and stroma to enter the blood stream where reticulocytes further mature into biconcave erythrocytes[194, 195]. Late in human erythroid differentiation Lutheran (BCAM) is expressed, which can bind laminin 10/11 localized at the bone marrow sinusoids to facilitate entrance into the peripheral circulation[196, 197]. Besides regulating erythroid output, another function of the central macrophage is pyrenocyte phagocytosis during enucleation. In mice, clearance of pyrenocytes occurs via the TAM-receptor family of tyrosine kinases TYRO3, AXL, and MERTK on central macrophages. Pyrenocytes expose phosphatidylserine (PS) and will be recognized via MERTK or TYRO3 in a protein S or GAS6-dependent manner, whereas AXL only recognizes GAS6[168, 169]. The TAM-receptors play an important role in the phagocytic ability of macrophages, however, *MerTK*-deficient mice do not show a defect in pyrenocyte clearance suggesting that TAM-receptor family members AXL and TYRO3 may cause redundancy or other compensatory *in vivo* mechanisms are present that have not been elucidated yet[198]. Indeed, mice with a triple knock-out of all TAM-receptors fail to clear apoptotic cells in multiple tissues. These mice develop normally, but eventually develop autoimmunity like systemic lupus erythematosus (SLE)[199]. This is in line with studies that showed that SLE has been associated with failure of macrophages to phagocytose apoptotic cells and pyrenocytes in both human and mice[200-203]. In addition, anemia is found in about 50% of SLE patients and Toda et al. showed that embryos suffer from severe anemia

caused by failure of macrophages to phagocytose pyrenocytes[204]. Besides the TAM-receptors, other receptors have been shown to play a role in clearance of apoptotic bodies, such as TIMD4 which binds phosphatidylserine-exposed pyrenocytes via GAS6 and stabilin (STAB), which directly binds to phosphatidylserine[205-207]. In addition, integrin-α5 (ITGA5) expressed on macrophages can also recognize PS-exposed pyrenocytes via lactadherin[208].

Although the origin of erythroblastic island macrophages is unknown, they already appear in the fetal liver prior to HSC homing. This suggests that these fetal liver macrophages may originate from the first wave of HSC-independent hematopoiesis in the yolk sac[14 209]. However, studies with adult *Mcsf*-deficient$^{op/op}$ mice showed a reduction in the amount of bone marrow resident macrophages suggesting an HSC-dependent origin of tissue resident macrophages in adults[210]. The cues that drive central macrophage differentiation from the HSC are still unknown. Ramos et al. described the necessity of retinoblastoma (RB) mediated downregulation of PU.1 target gene inhibition through inhibitor-of-differentiation 2 (ID2) for terminal differentiation of central macrophages[211]. However, the origin of central macrophages, cues that regulate differentiation towards central macrophages and the regulation of central macrophage numbers in the bone marrow remain ill-defined, and is in need of more directed research. In fact, a general characterization of human fetal and bone marrow erythroid island macrophages has not been performed and would be an excellent starting point for further studies.

Do central macrophages primarily regulate erythropoiesis in response to anemia?

Interestingly, specific diphtheria toxin (DTX) mediated ablation of CD169$^+$ macrophages, thus also ablating central macrophages, results in perturbed erythropoiesis albeit without anemia. Lack of anemia may be partly attributed to decreased clearance of aged erythrocytes in the spleen as splenic macrophages responsible for clearance and reticulocyte quality control will also be ablated[112, 212, 213]. Although macrophages are implicated in the process of erythropoiesis, the absence of anemia after CD169$^+$ macrophage ablation clearly shows that erythropoiesis can exist without a central macrophage. Indeed, *in vitro* erythropoiesis can be performed from CD34$^+$ cells from HS(P)C isolated from PBMC resulting in enucleated reticulocytes that can be used for transfusion purposes[75, 77, 214, 215]. In addition, not all erythroid cells in the bone marrow interact with a macrophage[216]. In agreement with this, Choi et al. showed that physical interactions between erythroid cells mediated through the association of ICAM4 with Rho GTPase activating protein DLC1 during high density cultures increased the enucleation and survival rate and thus ensures autonomous erythroid differentiation[217]. These data indicate that central macrophages are largely dispensable for steady-state erythroblast differentiation towards reticulocytes which includes the process of enucleation. However, induction of anemia through e.g., phenylhydrazide treatments shows impaired erythroid recovery upon macrophage ablation, indicating that fast responses to alter bone marrow erythroid output is regulated by and dependent on the presence of the central macrophages[112, 211]. Interestingly, *Rb*-deficient mice die perinatally due to defective erythropoiesis[218] caused by a perturbed differentiation of fetal liver

central macrophages leading to absence of erythroid islands. Deficiency in *Id2*, directing the inhibitory function of PU.1 on specific target genes, rescued the defect observed in *Rb*-deficient fetal liver macrophages. In addition, in *Emp*-deficient mice the interaction between macrophages and erythroid cells is also perturbed. These mice also die perinatally, however, this is also partly attributed to an intrinsic defect in enucleation[176]. These studies show that erythroid macrophage interactions are crucial during ontogeny. At first glance these results look contradictory to adult erythropoiesis in which the contribution of central macrophages to erythropoiesis is limited during steady-state. However, as indicated the necessity of the central macrophage is clearly shown during stress erythropoiesis, a time of severely increased erythroid output. Parallels to the enormous erythroid flux in fetal liver necessary to produce sufficient erythrocytes demanded by the developing fetus and stress erythropoiesis in adults can be made and suggests that central macrophages are crucially important for erythroid regenerative capacity. Indeed, recently it was shown that the erythrocytosis phenotype in Polycythemia Vera (PV) can be reversed upon clodronate-containing liposomes mediated ablation of macrophages carrying the *Jak2*^{V617F} mutation, clearly indicating a role for macrophages in the pathophysiology of PV[211]. Furthermore, the data showed that the proliferation of cells from patients with this *JAK2*^{V617F} mutation was increased upon co-culturing with macrophages. This notion is strengthened by the observation that mice with these aberrant central macrophages have a PV-like phenotype[211].

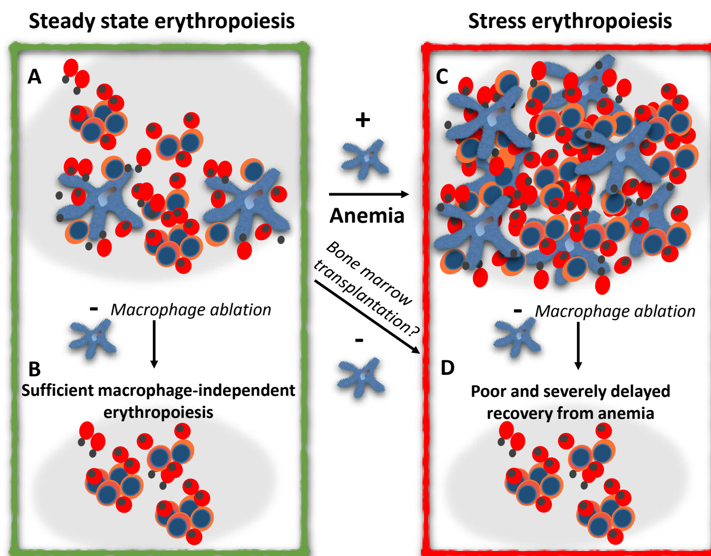

Figure 4. Normal steady-state erythropoiesis consists of macrophage-dependent and independent erythropoiesis. (A) Upon ablation of macrophages (B), macrophage-independent erythroid flux produces sufficient erythrocytes. However, upon anemia or stress, macrophage-dependent erythropoiesis is needed to adequately respond to produce enough erythrocytes to alleviate the shortage (C). Myeloablation, but also bone marrow transplantations that eliminate central macrophages which leads to the inability to respond to anemia and results in (severe) delay in recovery from anemia (D). Of note, we hypothesize the increase in macrophages in panel C.

In addition, macrophage depletion in β-thalassemia mice lead to reduced erythroid proliferation and reduced reticulocytosis, while erythroid differentiation was increased resulting in increased red blood cells because of increased red blood cell survival and lifespan. Combined, these studies suggest that central macrophage numbers and functionality regulates the response to anemia and puts the central macrophage in the center spot of stress-induced erythropoiesis (Figure 4). However, it remains unclear if erythroid cells dictate the number of central macrophages or if the number of central macrophages dictate the erythroid flux. In addition, what regulates the number of central macrophages in the bone marrow and can their number be pharmacologically influenced? These are important but unaddressed questions to which the answers may have implications in disease treatments. Models to facilitate investigating erythroid-macrophage interactions are not readily available as to date, central, particularly human, macrophages are ill-defined. This results in a lack of studies on culturing central macrophage-like cells in order to unravel the underlying mechanisms involved in the erythroid-supportive function of central macrophages.

Macrophages after bone marrow transplantation

Macrophages have a prominent role during hematopoietic homeostasis within the bone marrow, which also suggests that they control hematopoietic regeneration after bone marrow transplantation. Macrophages may play a decisive role in hematopoietic recovery as conditioning before bone marrow transplantation almost always involves myeloablative treatments. The different origins of tissue macrophages may also define specific macrophage populations within the adult bone marrow. It is expected that myeloablation leads to the destruction of bone marrow resident macrophages, however, limited data is present on the survival of the specific subsets or the recovery of macrophages within the bone marrow after transplantation. In addition, it is unknown if specific subsets are derived from host or donor. Langerhans cells were shown to resist high doses of irradiation and repopulate from the host after congenic bone marrow transplantation, whereas monocytes were all of donor origin[219]. Similar results have been observed for microglia[220, 221], suggesting that both populations maintain themselves independently of the contribution of bone marrow-derived circulating precursors, even after exposure to lethal doses of irradiation. However, microglia can repopulate from bone marrow-derived myelopoiesis as donor microglia were found in the brain of transplanted mice indicating that at least a portion of the microglia in the brain can be repopulated from bone marrow-derived hematopoiesis[222]. Interestingly, treatment with EPO in the first weeks after transplantation does not result in faster recovery from anemia[223, 224]. Indeed, in the first weeks after transplantation, high endogenous EPO levels in plasma do not induce fast recovery indicating that erythropoiesis is severely perturbed in the first month after transplantation. On the other hand, EPO treatment after this first month does lead to increased erythroid recovery compared to non-treated patients[225] which suggests that the erythroid system is only able to respond to EPO after an initial period of regeneration. One study suggested that the number of $CD68^+$ macrophages after bone marrow transplantation correlates with erythroid flux in the bone marrow[226]. Taken into account that the crucial role of the central macrophage in the regulation of stress erythropoiesis facilitates rapid responses to anemia, it would be interesting to evaluate the number

of central macrophages within the bone marrow after transplantation as the regeneration of central macrophages and the response to EPO may be connected. Besides this tentative connection, these data also suggest that central macrophages are lost during bone marrow ablation therapies and may be *de novo* generated[226]. This makes the prospect of differentiating these cells from CD34$^+$ HSC a possibility, if the cues that dictate differentiation to these macrophage subsets are known, which is presently not the case. In addition, increasing evidence suggests that besides central macrophages also osteomacs within the HSC niche are ablated after chemotherapy resulting in a decreased functionality of the niche. Therefore, it would be reasonable to conclude that homing of HSC to the bone marrow is affected due to the absence of specific macrophages and thus selective protection or co-transplantation of these macrophages may increase the transplantation efficiency and hematopoietic recovery. Of note, granulocyte colony stimulating factor (G-CSF) also efficiently mobilizes the macrophages that are responsible for HSC retention in mice. These macrophages have been characterized by the expression of CX3CR1 (the fractalkine receptor), CSF-1R, or CD11b[156, 227]. This enables purification and isolation of these specific macrophages from peripheral blood[112, 151, 156] which can be used to understand the interaction between macrophages and HSC. In addition, inhibitory measures can be developed that disrupt the interaction, thereby only mobilize HSC and keep part of the HSC niche intact to receive the transplanted HSC.

Scope of this thesis

Annually, nearly 400.000 volunteer blood donations are made in the Netherlands[228], however, in future shortage of blood products may develop due to an increased need or decline in donor numbers in an aging population[229-232]. In addition, safe products for transfusion-dependent alloimmunized patients, for which compatible donor blood is missing, are needed. In particular for patients with sickle cell disease, that have been alloimmunized against different alloantigens by previous blood transfusions, it can be difficult to find compatible blood. Therefore, research to *in vitro* generated, specific matched blood group units of erythrocytes is essential to obtain a degree of donor independency and to minimize donor-patient blood type variation. However, it is technically challenging to reach red cell quantities that make up a unit of red blood cells given for transfusion.

Erythroid development *in vivo* requires support from surrounding cells, such as macrophages. However, the stage during which support cells influence erythropoiesis is not clearly defined and molecular events that underlie these support functions are ill-defined. We have previously shown that the erythroid yield from total PBMC is 10-15 fold increased compared to CD34$^+$ cells isolated from a similar amount of PBMC[77]. PBMC consist of several effector cells of which mainly CD3$^+$ T cells, CD14$^+$ monocytes/macrophages and CD19$^+$ B cells. It has been shown that macrophages play an important role during erythropoiesis, as central macrophages in erythroblastic islands support the erythroid proliferation, differentiation and enucleation. In order to further optimize erythroid cultures, we aim to examine if CD14$^+$ monocytes/macrophages from PBMC could increase the erythroid yield and if so, how these cells inflict their effect. As the erythroid cultures start from PBMC, CD34$^+$ HSC in PBMC will differentiate towards CD71$^+$CD235a$^+$ erythroblasts after 8 days of culture. This indicates that effector

cells could affect the early HSC and/or more differentiated cells. We therefore aim to provide a new flow cytometry panel in order to track the differentiation from HSC to erythroblast and investigate in which stage effector cells inflect their effect (**chapter 2**).

In order to obtain massive amounts of red blood cells for routine red blood cell production, we aim to further optimize erythroid culture systems to increase erythroid expansion, differentiation and enucleation. We therefore divided the culture system in three phases: i) from HSPC to erythroblasts, ii) expansion of erythroblasts and iii) differentiation of erythroblasts to erythrocytes. Within these stages we examined when and which supplements had to be added to the culture for optimal erythroid outgrowth. Furthermore, we explored alternative culture systems (bioreactors) as culturing large numbers of red cells from culture dishes is infeasible (**chapter 3**).

Although the importance of central macrophages during erythropoiesis has been reported since the 80s, to date, these macrophages are still ill-defined in humans. In addition, there is no convenient human model to study the interaction between macrophages and erythroid cells in erythroblastic islands. As *ex vivo* central macrophages are difficult to obtain and culture conditions could affect the macrophage phenotype, we investigated if human $CD14^+$ monocytes could be differentiated towards erythroid-supporting macrophages that function as erythroblastic island macrophages. Erythroid intrinsic effects of glucocorticoids have been well documented, but it is also known that glucocorticoids affect monocyte differentiation and macrophage function. As a dual role for glucocorticoids on the process of erythropoiesis may be present, we investigated the role of glucocorticoids on monocyte differentiation to macrophages in comparison to cells that have been cultured in the absence of glucocorticoids. In order to relate these cells to their *in vivo* counterparts, these macrophages were phenotypically characterized and compared to fetal liver and adult bone marrow macrophages (**chapter 4**).

Most studies on erythroblastic island macrophages in the bone marrow or fetal liver have only been described in mice. As a result, the human counterparts are still ill-characterized. In addition, it is important to make a comparison between erythroblastic island macrophages from the fetal liver (week 17-22), adult bone marrow and *in vitro* cultures, as these macrophages all share an erythroid-support function. This data will give new insights which are relevant for both the erythroid and the macrophage field. Therefore, we aimed to start an unbiased analysis of the proteome of human macrophage populations from fetal liver and bone marrow and compare this to *in vitro* cultured erythroid-supporting macrophages. This will give clues about the possible underlying mechanisms and processes by which macrophages interact with erythroid cells and facilitate erythropoiesis. In addition, novel markers may be identified to discriminate between macrophages from different origins, as there is a lack of uniform descriptions in the macrophage field (**chapter 5**).

Finally, we summarize the work described in this thesis and discuss our findings in the light of current knowledge (**chapter 6**).

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