frontiers in Cell and Developmental Biology

MINI REVIEW
published: 18 February 2021
doi: 10.3389/fcell.2021.631699

Contributions of Embryonic HSC-Independent Hematopoiesis to Organogenesis and the Adult Hematopoietic System

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Specialty section:
This article was submitted to Stem Cell Research, a section of the journal Frontiers in Cell and Developmental Biology

Received: *20 November 2020*
Accepted: *22 January 2021*
Published: *18 February 2021*

Citation:
Neo WH, Lie-A-Ling M, Fadlullah MZH and Lacaud G (2021) Contributions of Embryonic HSC-Independent Hematopoiesis to Organogenesis and the Adult Hematopoietic System. Front. Cell Dev. Biol. 9:631699. doi: 10.3389/fcell.2021.631699

During ontogeny, the establishment of the hematopoietic system takes place in several phases, separated both in time and location. The process is initiated extra-embryonically in the yolk sac (YS) and concludes in the main arteries of the embryo with the formation of hematopoietic stem cells (HSC). Initially, it was thought that HSC-independent hematopoietic YS cells were transient, and only required to bridge the gap to HSC activity. However, in recent years it has become clear that these cells also contribute to embryonic organogenesis, including the emergence of HSCs. Furthermore, some of these early HSC-independent YS cells persist into adulthood as distinct hematopoietic populations. These previously unrecognized abilities of embryonic HSC-independent hematopoietic cells constitute a new field of interest. Here, we aim to provide a succinct overview of the current knowledge regarding the contribution of YS-derived hematopoietic cells to the development of the embryo and the adult hematopoietic system.

Keywords: yolk sac, lineage tracing, embryonic hematopoiesis, organogenesis, macrophage, hematopoietic waves, HSC-independent hematopoiesis

INTRODUCTION

In mammals, the hematopoietic system is established during embryogenesis in three consecutive overlapping waves (Dzierzak and Bigas, 2018). In mice, the first wave, also termed primitive hematopoiesis, is initiated around embryonic day (E)7 and produces unipotent precursors that give rise to either primitive-erythrocytes, -megakaryocytes, or -macrophages (Palis et al., 1999; Tober et al., 2007). The primitive macrophage precursors have also been named primitive myeloid precursors (pMP). Furthermore, it has also been reported that the first wave may give rise to c-Myb independent erythroid–myeloid progenitors (early EMP) (Hoeffel et al., 2015). However, so far, macrophage (microglia) but not erythrocyte potential has been experimental confirmed for these so-called early EMP (Wittamer and Bertrand, 2020). Henceforth, we will refer to wave 1 myeloid cells as pMP/early EMP. The second wave of hematopoiesis marks the onset of definitive (erythroid) hematopoiesis and sees the emergence of both EMPs around E8.25 (late-EMPs) (McGrath et al., 2015; Palis, 2016), and lymphoid–myeloid progenitors (LMPs) (Adolfsson et al., 2005; Boiers et al., 2013) around E9.5. Around E10.5 the third wave generates both hematopoietic stem and progenitor cells (HSPC) (**Figure 1A**). The hematopoietic stem cells (HSCs) subsequently play a central role in

maintaining the hematopoietic system for the lifetime of the organism (Medvinsky et al., 1993; Muller et al., 1994). Hematopoietic cells of all waves are generated from the mesoderm, which is known to give rise to both endothelial and hematopoietic lineages (Davidson and Zon, 2000; Dzierzak and Bigas, 2018). For the definitive hematopoietic waves (waves 2 and 3), it is now well established that hematopoiesis occurs via an endothelial-to-hematopoietic transition (EHT) from a specialized endothelial subpopulation known as hemogenic endothelium (HE) (Jaffredo et al., 1998; Zovein et al., 2008; Chen et al., 2009; Eilken et al., 2009; Lancrin et al., 2009; Boisset et al., 2010; Lacaud and Kouskoff, 2017; Garcia-Alegria et al., 2018; Ottersbach, 2019). The cellular origin of the first wave of primitive hematopoiesis is still disputed. It is unclear whether primitive hematopoiesis emerges directly from mesoderm, a hemangioblast, a HE, or another type of precursor (Amaya, 2013; Myers and Krieg, 2013). However, several recent studies suggest that primitive hematopoiesis (wave 1) is generated through a HE(-like) intermediate that has been termed hemogenic angioblast (Lancrin et al., 2009; Stefanska et al., 2017; Garcia-Alegria et al., 2018). Despite this potential common cellular origin, not all waves originate from the same anatomical site. The first two waves arise extra embryonically in the yolk sac (YS). In contrast, the third wave mainly takes place in the dorsal aorta within the aorta-gonad-mesonephros (AGM) region of the embryo, where HSC arise within so-called intra-aortic hematopoietic clusters (IAHC) (Boisset et al., 2010; Dzierzak and Bigas, 2018; Ottersbach, 2019). The HSC mature and amplify in the fetal liver (FL) before taking up residence in the bone marrow (BM).

Initially, the YS waves of hematopoiesis were thought to provide an essential but transient blood supply for the embryo destined to be replaced by HSC-derived cells. However, there is accumulating evidence that HSC-independent blood cells can make significant contributions to the adult hematopoietic system. Furthermore, it is also becoming evident that in the embryo, the HSC-independent hematopoietic waves can play essential roles beyond erythrocyte-mediated oxygen exchange and early immune surveillance. These roles have been mainly assigned to myeloid-derived populations that have been shown to play crucial roles in embryonic organogenesis.

Here we provide a concise overview of the recent findings obtained in mouse models regarding the contribution of HSC-independent hematopoiesis to embryonic organogenesis and the adult hematopoietic system.

RESOLVING THE ROLE AND ORIGIN OF HEMATOPOIETIC POPULATIONS

The overlapping and transient nature of the different hematopoietic waves makes it challenging to determine their individual contributions towards organogenesis and the adult hematopoietic system. Transgenic mouse knockout (KO) and fate mapping models have been instrumental in shaping our current understanding of the contribution of the different hematopoietic waves towards the hematopoietic system (**Table 1**). KO models (via gene deletion/mutation or lineage-specific activation of diphtheria toxin) provide functional information for specific populations, while lineage tracing models (typically using fluorescent proteins) highlight the contribution of specific populations. However, the perfect model to delineate the hematopoietic waves does not exist, and it is important to consider the target cell type, wave specificity and labeling efficiency of the models used when interpreting results.

Knockout mouse models have demonstrated specific dependencies of (wave-specific) hematopoietic populations on distinct transcription factors and signaling pathways. *Csf1r* (Colony Stimulating Factor 1 Receptor, cytokine receptor) KO mainly disrupts the early EMP differentiation (wave 1) and to a lesser extent the late-EMP differentiation (wave 2) (Dai et al., 2002). Although these mice are viable, they display drastically reduced levels of microglia and YS macrophages (Ginhoux et al., 2010; Hoeffel et al., 2012). *Csf1* (Marks and Lane, 1976) (Colony Stimulating Factor 1, cytokine) null mice display a similar but milder phenotype, with varying degrees of microglia and YS macrophages depletion, due to partial compensatory effects of the alternative CSF1R ligand IL-34 (Wiktor-Jedrzejczak et al., 1990; Cecchini et al., 1994; Kondo and Duncan, 2009; Greter et al., 2012; Wang et al., 2012; Easley-Neal et al., 2019). *Myb* (Mucenski et al., 1991) (MYB Proto-Oncogene, transcription factor) KO disrupts late-EMP and HSC differentiation (wave 2 and wave 3) and results in anemia and embryonic lethality around E15.5 (Schulz et al., 2012; Hoeffel et al., 2015). A similar phenotype is observed in KitL (KIT Ligand, cytokine) KO mice which die perinatally (Ding et al., 2012). *Nur77* (Lee et al., 1995) (Nuclear Receptor Subfamily 4 Group A Member 1, nuclear receptor) KO is viable but lacks circulating monocytes due to disrupted BM HSC differentiation (Hanna et al., 2011). Disrupting cell migration has also emerged as a useful strategy. *Plvap* (Rantakari et al., 2015) (Plasmalemma Vesicle Associated Protein, membrane protein) and *CCR2* (Boring et al., 1997) (C-C Motif Chemokine Receptor 2, chemokine receptor) KOs are viable but respectively show impaired FL and BM monocyte migration (Rantakari et al., 2016). *Cx3cr1* (Jung et al., 2000) (C-X3-C Motif Chemokine Receptor 1, chemokine receptor) KO is viable but shows impairment of leukocyte migration (Imai et al., 1997; Jacquelin et al., 2013) regardless of their wave of origin. There are several other models which disrupt all waves of hematopoietic development. *Pu.1* (Scott et al., 1994; McKercher et al., 1996) (Spi-1 Proto-Oncogene, transcription factor) KO mice have defective YS myelopoiesis and HSC maintenance and die shortly after birth (Olson et al., 1995; Kim et al., 2004; Kierdorf et al., 2013). Deletion of *Runx1* (Okuda et al., 1996; Wang et al., 1996a; North et al., 1999) (RUNX Family Transcription Factor 1, transcription factor) or its essential co-factor *Cbf*β (Sasaki et al., 1996; Wang et al., 1996b; Niki et al., 1997) (Core-Binding Factor Subunit Beta, transcription factor) is embryonically lethal and results in the complete absence of hematopoiesis apart from primitive erythroid cells. These KOs are useful when coupled with targeted approaches. For example, the *Cbf*β KO model can be used to deplete EMP or HSC by

TABLE 1 | Mouse model for studying the contribution of different hematopoietic waves to hematopoietic system.

Knockout models	Function	Affected wave	Major impact on hematopoietic waves	References
Csf1r (Dai et al., 2002)	Early/Late-EMP differentiation	1, 2	Lack of early EMP and reduced late-EMP contribution to TRM	Ginhoux et al., 2010; Hoeffel et al., 2012
Csf1 (null mutation) (Marks and Lane, 1976)	Early EMP differentiation	1	Reduced early EMP contribution to TRM	Cecchini et al., 1994; Kondo and Duncan, 2009; Easley-Neal et al., 2019
Myb (Mucenski et al., 1991)	Late-EMP and HSC differentiation	2, 3	Lack of late-EMP and HSC contribution to TRM	Schulz et al., 2012; Hoeffel et al., 2015
Nur77 (Lee et al., 1995)	BM monocytes differentiation		Reduced BM monocytes/macrophages contribution to TRM	Hanna et al., 2011
Plvap (Rantakari et al., 2015)	FL macrophage migration		Reduced FL monocytes/macrophages contribution to TRM	Rantakari et al., 2016
Ccr2 (Hanna et al., 2011)	BM monocytes/macrophages migration		Reduced BM monocytes/macrophages contribution to TRM	Boring et al., 1997
KitL (Ding et al., 2012)	Late-EMP and HSC maintenance	2, 3	Reduced late-EMP and HSC contribution to TRM	Ajami et al., 2007; Azzoni et al., 2018
Cx3cr1 (Jung et al., 2000)	Mediates monocyte retention in the BM		None	Imai et al., 1997; Jacquelin et al., 2013
Pu.1 (Scott et al., 1994; McKercher et al., 1996)	YS myeloid differentiation and HSC maintenance	1, 2, 3	Lack of EMP and HSC contribution to TRM	Scott et al., 1994; Olson et al., 1995; McKercher et al., 1996; Kim et al., 2004; Iwasaki et al., 2005; Kierdorf et al., 2013
Runx1 (Okuda et al., 1996; Wang et al., 1996a; North et al., 1999)	Master regulator of hematopoiesis, expressed from HE onwards	1, 2, 3	Lack of EMP and HSC	Okuda et al., 1996; Wang et al., 1996a; North et al., 1999
Cbfβ (Sasaki et al., 1996; Wang et al., 1996b; Niki et al., 1997)	Essential RUNX1 co-factor	1, 2, 3	Lack of EMP and HSC	Sasaki et al., 1996; Wang et al., 1996b; Niki et al., 1997
Cbfβ (*Tie2-Cbfβ*) (Miller et al., 2002)	Essential RUNX1 co-factor	1, 2, 3	Lack of HSC	Chen et al., 2011
Cbfβ (*Ly6a-Cbfβ*) (Chen et al., 2011)			Lack of EMP	Chen et al., 2011

(Continued)

TABLE 1 | Continued

Constitutive fate mapping models	Relevance	Labeling efficiency (assay time point)				References
		LMP (Wave 2)	**pMP/Early EMP (Wave 1)**	**Late-EMP (Wave 2)**	**HSC (Wave 3)**	
S100a4 (Bhowmick et al., 2004) (Transgene)	Active in FL monocytes and not in FL macrophages	Not done	∼20% (5wo)	64.5 ± 6.7% (5wo)	Not done	Hoeffel et al., 2015
		Not done	∼20% (Adult)	∼60% (Adult)	∼100% (Adult)	Hashimoto et al., 2013
Flt3 (Benz et al., 2008) (KI)	Active in HSC progeny	Not done	Not done	Not done	∼80% (NB)	Hoeffel et al., 2015
		Not done	Not done	∼20% (Adult)	∼80% (Adult)	Hashimoto et al., 2013
		Not done	<2% (P8 onwards)	10–30% (P8 onwards)	∼80% (P8 onwards)	Gomez Perdiguero et al., 2015
		Not done	Not done	10–20% (4wo)	∼80% (4wo)	Schulz et al., 2012
Ms4a3 (Liu et al., 2019) (KI)	Active in GMP	Not done	None (NB onwards)	Trace (NB onwards)	BM GMP: 68.7 ± 1.58% (Adult)	Liu et al., 2019
Tnfrs11a (Maeda et al., 2012) (KI)	Essential for osteoclast development. Tracks YS progenitors.	Not done	∼80% (E14.5)	∼90% (6wo)	∼10% (E14.5 onwards)	Mass et al., 2016
Tnfrs11a (Percin et al., 2018) (KI)		Not done	Not done	∼90% (Adult)	None (Adult)	Percin et al., 2018
Rag1 (McCormack et al., 2003) (KI)	Lymphoid-specific activity	∼100% (E14.5)	Trace (E14.5)	<5% (E14.5)	Not done	Boiers et al., 2013

(Continued)

TABLE 1 | Continued

Inducible fate mapping models	Relevance	Pulse timepoint	Labeling efficiency (assay time point)			References
			pMP/Early EMP (Wave 1)	Late-EMP (Wave 2)	HSC (Wave 3)	
Csf1r (Qian et al., 2011) (Transgene)	Mainly a myeloid marker	E8.5	63.2 ± 5.6% (E13.5)	Trace (E13.5)	Not done	Hoeffel et al., 2015
			∼60% (E11.5)	Marked (E12.5)	Not done	Gomez Perdiguero et al., 2015
Cx3cr1 (Yona et al., 2013) (KI)	Mainly a myeloid marker	E9.0	∼40% (E16.0); ∼30% (NB onwards)	Trace (6wo onwards)	Trace (6wo)	Hagemeyer et al., 2016
Runx1 (Samokhvalov et al., 2007) (KI; driven by P2/Runx1b promoter)	Master regulator of hematopoiesis, expressed from HE onwards	E7.25-E7.5	∼30% (E10.5)	<5% (8wo)	<3% (8wo)	Ginhoux et al., 2010
			Not done	Not done	none (9–12mo)	Samokhvalov et al., 2007
			∼30% (E10.5); ∼20% (E13.5)	Not done	Not done	Hoeffel et al., 2012
		E7.5	22.2 ± 0.9% (E13.5)	< 5% (E13.5); <10% (E16.5)	Trace (E13.5)	Hoeffel et al., 2015
			Not done	∼10% (8wo)	∼10% (8wo)	Ginhoux et al., 2010
			Not done	Not done	1–10% (9–12mo)	Samokhvalov et al., 2007
			Not done	∼12.5% (8wo)	∼7.5% (8wo)	Hoeffel et al., 2012
		E8.5	∼15% (E13.5); <5% (E16.5)	∼25% (E13.5); ∼30% (E16.5)	1–3% (E11.5); <5% (E13.5)	Hoeffel et al., 2015
			Not done	∼30% (8wo)	∼30% (8wo)	Ginhoux et al., 2010
			Not done	Not done	1–50% (9–12mo)	Samokhvalov et al., 2007
			Not done	∼30% (8wo)	∼30% (8wo)	Hoeffel et al., 2012
		E9.5	<5% (E13.5)	∼15% (E13.5)	∼30% (E13.5)	Hoeffel et al., 2015
			Not done	∼20% (8wo)	∼30% (8wo)	Ginhoux et al., 2010
			Not done	Not done	50–100% (9–12mo)	Samokhvalov et al., 2007
			Not done	∼25% (8wo)	∼30% (8wo)	Hoeffel et al., 2012
Tie2 (Gomez Perdiguero et al., 2015) (KI)	Endothelial marker (including HE) also expressed in subset of HSC and myeloid cells	E7.5	∼60% (E12.5)	∼40% (6–8wo)	∼40% (E12.5)	Gomez Perdiguero et al., 2015
		E8.5	∼30% (E12.5)	∼20% (6–8wo)	∼75% (E12.5)	
		E9.5	trace (E12.5)	∼10% (6–8wo)	∼80% (E12.5)	
		E10.5	none (E12.5)	∼5% (6–8wo)	∼40% (E12.5)	
Kit (Sheng et al., 2015) (KI)	Express in early HSPC and YS HE cells	E7.5	∼70% (E13.5)	Trace (E13.5)	Trace (6wo)	Sheng et al., 2015
		E8.5	∼70% (E13.5)	∼40% (E13.5)	∼60% (6wo)	
		E9.5	∼50% (6wo)	∼50% (6wo)	∼40% (6wo)	
Cdh5 (Sorensen et al., 2009) (Transgene)	Endothelial marker (including HE)	E7.5	∼80% (E10.5); ∼90% (E14.5)	∼80% (E10.5)	<10% (E14.5)	Gentek et al., 2018a
		E10.5	Trace (E14.5)	Not done	∼80% (E14.5)	

EMP, erythroid–myeloid progenitors; HSC, hematopoietic stem cells; TRM, tissue-resident macrophages; FL, fetal liver; BM, bone marrow; pMP, primitive myeloid precursors; HSPC, hematopoietic stem and progenitors; YS, yolk sac; LMP, lymphomyeloid progenitors; GMP, granulocyte-macrophage progenitors; HE, hemogenic endothelium; KI, knock-in; NB, new born; wo, week old; mo, month old.

combination with respectively *Ly6a-Cbf*β or *Tie2-Cbf*β rescue alleles (Chen et al., 2011).

Constitutive lineage tracing models rely on lineage-specific promoter activity to drive *Cre* recombinase expression, which in turn irreversibly activates or deletes a target gene (Hoess and Abremski, 1984; Sauer and Henderson, 1988). Such models have been established to trace long term lineage contribution of HSC- and YS-derived hematopoietic cells. *Flt3*-Cre, *Ms4a3*-Cre, *S100a4*-Cre predominantly track HSC progeny, albeit with several restrictions. *Flt3*-Cre (Schulz et al., 2012; Hashimoto et al., 2013; Gomez Perdiguero et al., 2015; Hoeffel et al., 2015) and *S100a4*-Cre (Hashimoto et al., 2013; Hoeffel et al., 2015) mark the majority of HSC-derived cells (>80%). However, both also mark some YS-derived hematopoietic cells (**Table 1**). In contrast, *Ms4a3*-Cre does not mark any YS cells from the first or second wave and only marks HSC-derived GMPs (∼70%) (Liu et al., 2019). The *Tnfrs11a*-Cre model is currently best suited to track YS hematopoiesis with little (Maeda et al., 2012; Mass et al., 2016) or no (Percin et al., 2018) HSC labeling. However, this model cannot distinguish the two YS waves of hematopoiesis. Currently, the only option to track LMP progeny is the *Rag1*-Cre fate mapping model (Boiers et al., 2013) which marks all FL B and T cells alongside a small number of myeloid cells.

Inducible tracing models [tamoxifen-inducible Cre-mediated recombination (Metzger et al., 1995; Feil et al., 1997)] add an extra layer of specificity that can overcome certain limitations of the constitutive models. This approach allows not only for reporter activation or gene deletion in specific cell types but also during a defined developmental time window. The latter has allowed for the specific marking of the first hematopoietic wave in the YS (pMP/early EMP) using multiple models (**Table 1**). In this context, a caveat of the *Csf1r*-Mer-iCre-Mer and *Cx3cr1*-CreER based systems is that they only label myeloid progeny (Gomez Perdiguero et al., 2015; Hoeffel et al., 2015; Hagemeyer et al., 2016). In contrast, *Tie2*-Mer-iCre-Mer, *Kit*-Mer-Cre-Mer, *Runx1*-Mer-Cre-Mer, and *Cdh5*-CreERT2 provide less restricted marking. Distinguishing progeny from late-EMP (wave 2) and HSC (wave 3) is still challenging, as illustrated in **Table 1** (Samokhvalov et al., 2007; Ginhoux et al., 2010; Hoeffel et al., 2012, 2015; Gentek et al., 2018a).

HSC-INDEPENDENT HEMATOPOIETIC CELLS CONTRIBUTE TO THE ADULT HEMATOPOIETIC SYSTEM

In the adult hematopoietic system, several hematopoietic populations have been shown to consist of cells with an HSC-independent embryonic origin (**Figure 1B**). This has been best studied for tissue-resident macrophages (TRM), which were traditionally thought to be continuously replenished by BM HSC-derived monocytes. However, this view was challenged by the discovery of radiation-resistant and self-repopulating Langerhans cells (Merad et al., 2002), microglia (Ajami et al., 2007; Ginhoux et al., 2010), and alveolar macrophages (Guilliams et al., 2013; Hashimoto et al., 2013; Jakubzick et al., 2013) in BM transplantation and parabiosis studies. Subsequent lineage tracing studies have convincingly demonstrated that microglia are the progeny of myeloid cells produced during the first wave of hematopoiesis. *Csf1r*-Mer-iCre-Mer, *Cx3cr1*-CreER, *Runx1*-Mer-Cre-Mer, and *Kit*-Mer-Cre-Mer lineage tracing models all support the pMP/early EMP origin of microglia (Gomez Perdiguero et al., 2015; Hoeffel et al., 2015; Sheng et al., 2015; Hagemeyer et al., 2016). This has been further strengthened by the analysis of *KitL* and *c-Myb* KO models in which the number of late-EMP and HSC (waves 2 and 3) is drastically decreased, while the microglia population remains unaffected (Ding et al., 2012; Schulz et al., 2012; Azzoni et al., 2018).

It is now widely accepted that TRM populations do not have a unified common origin. Some tissues retain and maintain YS-derived cells while in others they are replaced or co-exist with BM-HSC-derived cells (for review Ginhoux and Guilliams, 2016; Mass, 2018; Wittamer and Bertrand, 2020). Below, we highlight recent findings concerning the persistence of YS-derived embryonic hematopoietic cells in adults.

HSC-Independent Origin of Adult TRM

Alveolar macrophages (AM) and interstitial macrophages (IM) are two major subsets of lung TRM (Lehnert et al., 1985; Liegeois et al., 2018). Around E10.5, YS pMPs/early EMPs (Tan and Krasnow, 2016) seed in the primordial lung buds. Parabiosis, adoptive transfer, and lineage tracing experiments have shown that a subset of pMP/early EMP-derived IM (Hoeffel et al., 2012, 2015; Guilliams et al., 2013; Gomez Perdiguero et al., 2015; Tan and Krasnow, 2016; Liu et al., 2019) and AM (van de Laar et al., 2016; Li et al., 2020) persist into adulthood. The functional significance of these sub-populations, if any, is currently unknown. However, it has been shown that FL monocyte-derived TRM possess enhanced mitochondrial respiratory and glycolytic capacity (Li et al., 2020) versus their HSC-independent counterparts.

Adipose tissue macrophages (ATM), are the most abundant immune cells in adipose tissues and play a prominent role in regulating inflammation and insulin sensitivity (Russo and Lumeng, 2018). ATM, which can proliferate and self-renew, are partially derived from embryonic HSC-independent cells (E9.0 pulsed *Cx3Cr1*-CreER) (Hassnain Waqas et al., 2017; Waqas et al., 2017). Further studies using the *Ms4a3*-Cre fate-mapping model, which traces BM-HSC monocyte-derived cells (Jaitin et al., 2019; Liu et al., 2019), demonstrated that half of the white adipose tissue ATM is Ms4a3 positive under normal physiological conditions. Interestingly, a high-fat diet increases the proportion of HSC-derived monocytes within ATM population (Jaitin et al., 2019).

The *Ms4a3*-Cre fate-mapping model has also revealed that adult renal macrophages (RM) are of mixed origin (Schulz et al., 2012; Epelman et al., 2014; Gomez Perdiguero et al., 2015; Hagemeyer et al., 2016; Mass et al., 2016; Liu et al., 2019; Munro et al., 2019). Furthermore, YS-derived RM (E9.5 pulsed *Cx3cr1*-CreER) are more proliferative than their HSC-derived counterpart and their contribution to the kidney TRM population expands with age (Ide et al., 2020).

Osteoclasts are multinucleated myeloid cells that resorb bone tissue and are critical for the development, repair, and remodeling

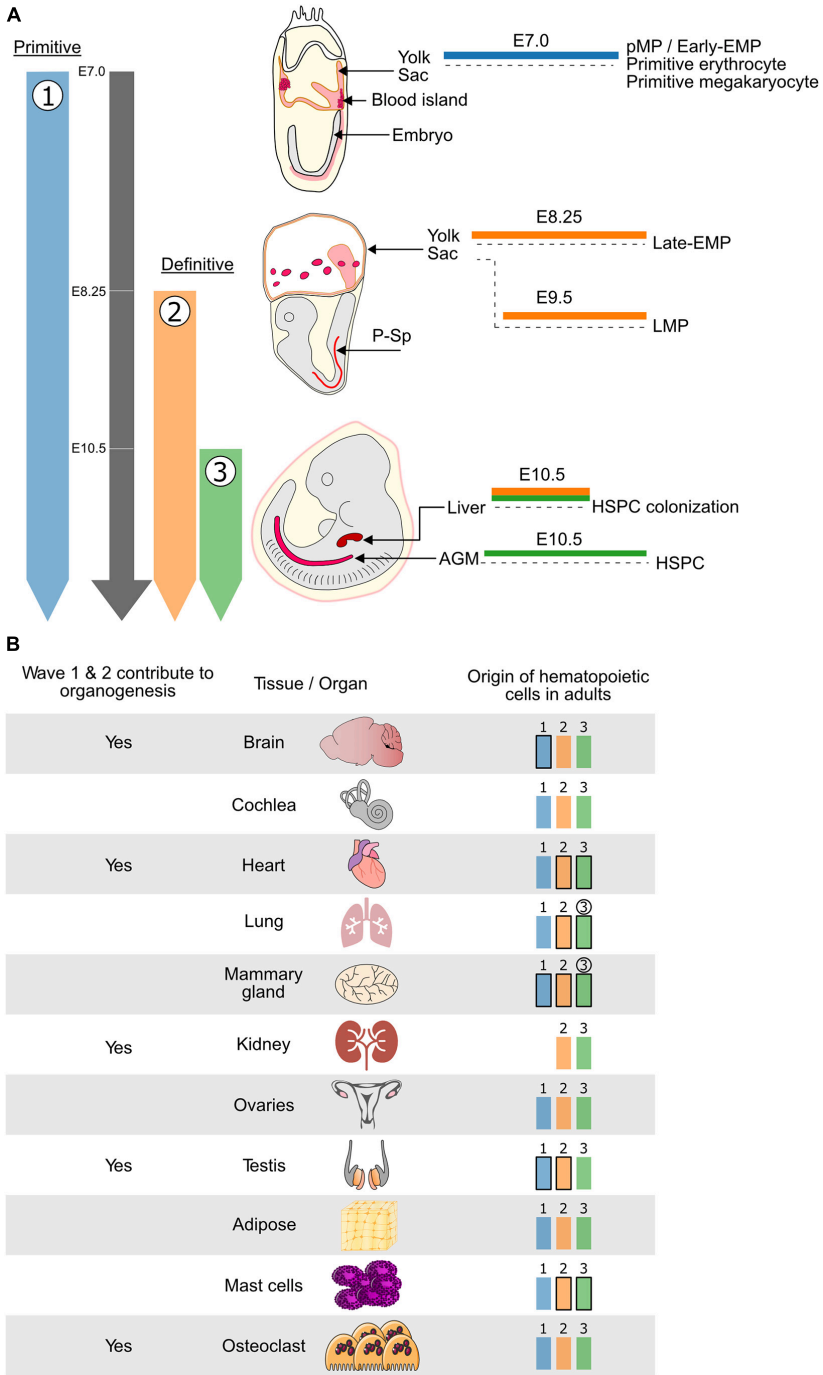

FIGURE 1 | The hematopoietic system is established in developmental waves that differentially contribute to embryonic organogenesis and the adult hematopoietic system. **(A)** Schematic representing the timing and embryonic sites of hematopoietic activity during mouse development. The three waves of hematopoiesis are represented in Blue (wave 1: primitive erythrocytes, megakaryocytes, macrophages and Early EMP), Orange (wave 2: Late EMP and LMP) and Green (wave 3: HSPC). Wave 1 is known as the primitive wave while waves 2 and 3 constitute the definitive waves of hematopoiesis. Waves 1 and 2 which do not generate HSCs originate in the Yolk Sac while wave 3, which generates the HSPCs, is initiated in the AGM region of the embryo proper. Cells from both waves 2 and 3 can colonize the fetal liver where they can mature and expand before moving to their final destination. **(B)** (Left) HSC-independent macrophages which originate from the first two hematopoietic waves have been shown to play important roles during embryonic development of several tissues and organs. (Right) In addition to the wave 3 (Green) HSC-derived hematopoietic cells, the HSC-independent hematopoietic cells generated during wave 1 (Blue) and 2 (Orange) persist, to varying degrees, in adult tissues and organs. Waves that contribute most of the hematopoietic cells are depicted in bold and waves for which the contribution increases overtime are circled. Blue: hematopoietic wave 1 (primitive), Orange: hematopoietic wave 2 (Late-EMP/LMP), Green: hematopoietic wave 3 (HSC). E, embryonic day; Mφ, macrophage; HSC, hematopoietic stem cell; EMP, erythroid–myeloid progenitor; LMP, lymphoid–myeloid progenitor; HSPC, hematopoietic stem and progenitor cell; P-Sp, para-aortic splanchnopleura; AGM, aorta-gonad-mesonephros.

of the skeleton (Udagawa et al., 1990; Takahashi et al., 1994). The HSC-independent origin of osteoclast was first suggested by *ex vivo* co-culture experiments (Thesingh, 1986) and has been recently confirmed *in vivo* using *Csf1r*-Mer-iCre-Mer and *Cx3cr1*-CreER models (Jacome-Galarza et al., 2019; Yahara et al., 2020). Early/late EMP-derived osteoclasts not only persist but also expand in the adult BM (Yahara et al., 2020). Furthermore, a subpopulation of early/late EMP-derived osteoclasts was found to reside in the spleen. Interestingly, this spleen population can fuse with BM-derived monocytes creating long-lived osteoclast syncytia with a mixed origin (Jacome-Galarza et al., 2019; Yahara et al., 2020).

Based on CD206 and MHCII expression, three testis subpopulations of TRM can be distinguished. These populations possess different levels of phagocytic capacity (Lokka et al., 2020). Tracing (E8.5 pulsed *Csf1r*-Mer-iCre-Mer, E13.5 pulsed *Cx3cr1*-CreER) and KO (*Ccr2*, *Nur77*, *Plvap*) studies have shown that all three waves of hematopoiesis contribute to adult testis TRM. Strikingly, antibody-based macrophage depletion experiments have demonstrated that adult BM-derived cells play no part in testis TRM maintenance (Lokka et al., 2020).

Finally characterization of *Csf1* KOs, the *Csf1r*-EGFP constitutive tracing model, and E8.5 pulse-labeled *Csf1r*-Mer-iCre-Mer mice point to an HSC-independent origin for part of the TRM in adult cochlea (Kishimoto et al., 2019), ovaries (Jokela et al., 2020), and mammary glands (Gouon-Evans et al., 2000; Jappinen et al., 2019; Stewart et al., 2019).

Beyond Tissue Resident-Macrophages: Mast- and Lymphoid-Cells

Mast cells (MC) can be classified into two groups. Connective tissue MC (CTMC) populate the skin, tongue, trachea, esophagus, adipose tissues, and peritoneal- and pleural cavities while mucosal MC (MMC) are found in the gut and respiratory mucosa. The exclusive BM HSC-derived (van Furth and Cohn, 1968) origin of MC was first challenged by transplantation assays that showed BM only minimally contributes to MC repopulation in MC-depleted hosts (Kitamura et al., 1977, 1978). Subsequent fate-mapping studies using *Csf1r*-Mer-iCre-Mer and *Runx1*-Mer-Cre-Mer suggested that the majority of MMC are derived from HSC, whereas CTMC are largely derived from the HSC-independent EMP (Li Z. et al., 2018). Interestingly, EMP-derived and HSC-derived MC have distinct transcriptional profiles suggesting distinct biological functions (Gentek et al., 2018a; Li Z. et al., 2018). If the CTMCs derived from HSC-independent cells can persist in significant numbers in the adult is unclear. *Csf1r*-Mer-iCre-Mer and *Runx1*-Mer-Cre-Mer fate-mapping studies suggest that they can, while *Cdh5*-CreERT2 based lineage tracing suggests a mostly fetal HSC origin of adult CTMC (Gentek et al., 2018a). These contradictory findings highlight that data from fate-mapping models should be interpreted with caution and that currently, no model can definitively distinguish the progeny of late-EMP from fetal HSC.

Finally, YS-derived lymphoid cells have also been found to persist into adulthood. Early B and T-cells [B1a (Yoshimoto et al., 2011; Kobayashi et al., 2014) and γδ T (Boiers et al., 2013; Gentek et al., 2018b) cells], a primary source of innate immunity in early embryo development (Yoshimoto et al., 2012), persist into adulthood and remain functionally distinct from their HSC-derived counterparts. The ontogeny and contribution of these YS-derived lymphocytes has been reviewed previously (Yamane, 2018; Ghosn et al., 2019). Finally, the existence of EMP-derived NK cells, possessing a potent degranulation response, has been reported recently (Dege et al., 2020). This is particularly striking as NK cells are considered to be of lymphoid origin. However, it is unclear whether these EMP-derived NK cells are part of the myeloid lineage or if these findings have revealed lymphoid potential in EMP. Similarly, it is not clear to what extent EMP-derived NK cells persist into adulthood (Sun et al., 2011; Wu et al., 2014; Corat et al., 2017; Schlums et al., 2017).

HSC-INDEPENDENT MACROPHAGES PARTICIPATE IN EMBRYONIC ORGANOGENESIS

The role of macrophages in tissue remodeling is an exciting field of ongoing research (Hoeffel and Ginhoux, 2018; Wittamer and Bertrand, 2020). The discovery of adult YS-derived TRM populations with an M2-like non-inflammatory phenotype, associated with wound healing and tissue repair, hints at potential roles in embryonic organogenesis (Takahashi et al., 1998; Rae et al., 2007; Pucci et al., 2009; Fantin et al., 2010; DeFalco et al., 2014; Italiani and Boraschi, 2014; Munro et al., 2019; Shigeta et al., 2019). However, identifying unique and specific roles of HSC-independent cells is complicated by the fact that embryonic organ development spans across all hematopoietic waves. Below we highlight the instances where specific roles for HSC-independent macrophages have been identified (**Figure 1B**).

HSC-Independent Embryonic Macrophages Guide Vascular Network Organization in Developing Organs

Vascular networks are established by tip- and stalk- endothelial cells. Endothelial tip-cells, guided by vascular endothelial growth factor (VEGF) gradients, drive the direction of the vessel while the endothelial stalk-cells follow and establish the vessel lumen (Herbert and Stainier, 2011). During embryonic organogenesis, macrophages have been shown to play an essential role in organizing endothelial networks. A role for HSC-independent macrophages in blood vessel anastomosis was first described in detail in the mouse embryonic hindbrain where it is entirely dependent on pMP/early EMP-derived macrophages (Fantin et al., 2010; Rymo et al., 2011). These macrophages invade the brain in a CSF1-dependent manner. Subsequently, upon brain vascularization, the macrophages closely associate with tip-endothelial cells. Macrophage depletion in the brain [*Pu.1* (Scott et al., 1994; McKercher et al., 1996) and *Csf1* KO (Wiktor-Jedrzejczak et al., 1990; Cecchini et al., 1994)], but not specific depletion of FL-derived macrophages (*Lysm*-Cre-mediated diphtheria toxin) (Clausen et al., 1999), significantly reduces the number of vessel intersections and thereby limits the overall complexity of the brain vascular network (Fantin et al., 2010; Rymo et al., 2011).

Hematopoietic stem cells-independent macrophages also play a role in kidney and testis vascular network formation. In mouse embryos, the mesonephros (a temporary kidney structure) and the gonads (which will give rise to the testis in males) are established near the extending nephric ducts around E9. Further extension of the nephric duct results in the generation of uretic buds and the metanephros (precursor to the adult kidneys) around E10-E11.5 (Takasato and Little, 2015). Proliferating primitive pMP/early EMP-derived macrophages (E7.5 pulsed *Csf1r*-Mer-iCre-Mer) are first detected in the gonadal region around E10.5 (DeFalco et al., 2014). By E11.5-E13.5, these macrophages closely associate with and engulf EC of the mesonephros vascular plexus and testis vasculature. Depletion of fetal macrophages (*Cx3cr1*-Cre-mediated diphtheria toxin) results in an enlarged mesonephros vascular plexus, reduced migration of EC into the testis, and impaired development of the coelomic artery. Blood vessels start entering the metanephros between E11.5-E12, and YS-derived macrophages are consistently found perivascular at developing vascular fronts (Rae et al., 2007; Hoeffel et al., 2015; Munro et al., 2019). Analysis of macrophage depleted E12.5 kidney explants (anti-CSF1R depletion) showed increased numbers of unconnected endothelial structures and a reduction in vascular network size, consistent with a role for macrophages in vessel anastomosis.

The developing heart harbors macrophages derived from both the HSC-independent and HSC-dependent hematopoietic waves (*Ccr2*-GFP, *Cx3cr1*-GFP) (Leid et al., 2016). HSC-independent macrophages (E7.5 pulsed *Rosa26-tdCsf1r*-MerCre) appear in the heart around E12.5 and predominantly populate the myocardium where they accumulate near perfused coronary vessels. Genetic depletion of macrophages ($Csf1^{op/op}$) results in retarded primitive coronary plexus development. However, specific depletion of HSC-derived macrophages (*Ccr2* KO) does not affect primitive coronary plexus development, indicating that HSC-independent macrophages are responsible for the modulation of the myocardial vascular network.

Finally, recent RNA-seq of a human Hofbauer cells (Zulu et al., 2019), a fetus-derived macrophage population found in the placenta, suggests that they may play a role in angiogenesis and remodeling (Thomas et al., 2021). Although Hofbauer cells have also been identified in mice (Takahashi et al., 1991), their role has not yet been investigated *in vivo*.

It is tempting to postulate a generalized role for HSC-independent macrophages in the establishment of vascular networks during embryonic organogenesis. In support of this, HSC-independent macrophages have a similar role in organizing vascular networks in zebrafish, independently of specific organs (Fantin et al., 2010).

HSC-Independent Macrophages Directly Support Organogenesis

Hematopoietic stem cells-independent macrophages have also been directly implicated in embryonic organ development. The central nervous system is arguably one of the best-studied systems in this context, with YS-derived microglia playing a role in multiple perinatal brain developmental events (Li and Barres, 2018; Low and Ginhoux, 2018). Around E14.5, microglia accumulate near developing axonal tracts and their genetic (*Pu.1* KO)/antibody-based depletion (anti-CSF1R) or their inappropriate activation (E13.5 lipopolysaccharide maternal immune activation), affects the development of interneuronal connections and dopaminergic axon outgrowth (Squarzoni et al., 2014). Similarly, in the peripheral nervous system, macrophages/microglia are found in close contact with developing sensory neurons in dorsal root ganglia from E11. Genetic (*Pu.1* KO) or antibody-based depletion (anti-CSF1R) of these macrophages alters the developmental trajectory of the sensory neurons (Angelim et al., 2018).

During gonad development, macrophages associate with and engulf mislocated germ (E10.5–E11.5) and Sertoli (E12.5) cells, and their absence (*Cx3cr1*-Cre/diphtheria toxin) results in irregularly branched and shortened testis cords (DeFalco et al., 2014). Furthermore, depletion of macrophages during embryogenesis ($Csf1^{op/op}$, anti-CSF1R depletion) but not postnatally (*Ccr2* KO, anti-CSF1 depletion at birth) results in impaired spermatogenesis after birth (Lokka et al., 2020). Similar observations have been made in kidney development where the clearance of rostral nephrogenic cells and uretic bud formation are delayed in the absence of YS-macrophages (*Cx3cr1*-Cre/diphtheria toxin) (Munro et al., 2019). These results suggest that embryonic macrophages are participating in gonad/testis and kidney development.

Yolk sac-derived osteoclasts are essential for normal skeletal development in the embryo and their absence (*Csf1r* KO model and *Csf1*-Cre-mediated *Tnfrsf11a* KO) disrupts tooth eruption, skull formation, long bone formation, and their timely hematopoietic colonization (Yoshida et al., 1990; Dougall et al., 1999; Dai et al., 2002; Jacome-Galarza et al., 2019). This phenotype is not observed when HSC-derived macrophages are deleted (*Flt3*-Cre-mediated and *Csf1r* KO) (Jacome-Galarza et al., 2019).

Finally, heart development also depends on HSC-independent macrophages which, interestingly, originate locally from HE cells populating the endocardium (Nakano et al., 2013; Yzaguirre and Speck, 2016; Shigeta et al., 2019). An important phase in heart development is the establishment and remodeling of the heart valves which starts around E9.5 and concludes after birth. Specific depletion of endocardial macrophages (*Nfatc*-Cre-mediated *Csf1r* KO) demonstrated that they are essential for heart valve development and that macrophages of other sources cannot compensate for their loss (Shigeta et al., 2019).

HSC-Independent Macrophages Support HSC Formation

Arguably the most striking function of HSC-independent macrophages is that they can affect HSC ontogeny. This has been studied in detail in zebrafish. HSCs generated in the dorsal aorta of zebrafish enter the circulation via the postcardinal vein (PCV) (Bertrand et al., 2010; Kissa and Herbomel, 2010; Lam et al., 2010). This requires newly formed HSC to traverse the mesenchyme separating the two vessels. Primitive macrophages accumulate in this subaortic mesenchyme and, via metalloproteinases mediated

extracellular matrix degradation, create tracks for the HSC to enter the subaortic mesenchyme. These primitive macrophages then join the PCV from where they migrate to the zebrafish FL equivalent, known as the caudal hematopoietic tissue (CHT; Travnickova et al., 2015). Once the HSC reaches the CHT, a specific set of primitive VCAM+ macrophages (usher macrophages) interact with and "capture" passing HSPC and guide them into the CHT (Li D. et al., 2018).

In mice, HSC-independent macrophages also play an important role in HSC ontogeny. At E10.5, HSC-independent macrophages are found in close association with EC and IAHC in the AGM, where they possibly participate in moving KIT+ IAHC cells towards the aortic lumen (Mariani et al., 2019). The CXCL3 chemokine (expressed amongst others by HE and IAHC) is important for the macrophage accumulation in the AGM (Mariani et al., 2019). The deletion of its receptor, *Cxcr3*, results in increased numbers of macrophages in the YS and reduced numbers in the AGM. Reduction of the number of macrophages in the AGM, either by genetic (*Cxcr3* KO) or chemical (clodronate and CSF1R inhibitor BLZ945) depletion, negatively affects HSC generation in the AGM (Mariani et al., 2019). Furthermore, both direct and indirect (transwell) co-culture experiments of AGM-derived aortic endothelial cells (including HE) with aortic macrophages result in an increase of the hematopoietic colony-forming capacity of the endothelial cells (Mariani et al., 2019). These results indicate an essential role for macrophage secreted factors in AGM EHT. RNA-seq of the aortic macrophages revealed that despite having an immune phenotype associated with anti-inflammatory or M2 type phenotype, they have a distinct pro-inflammatory transcriptome (Mariani et al., 2019). Currently, it is unclear if these macrophages promote emergence of all, or only subsets, of HSC.

CONCLUDING REMARKS

In the last decade, it has become clear that HSC-independent hematopoietic cells have previously unanticipated roles in both embryos and adults. They have been found to participate in organogenesis and persist in adults as distinct hematopoietic populations. There are however still many open questions about their exact role, origin, and contributions. In this context, the development of more precise and efficient genetic tracing models would be beneficial. Specifically, models that can efficiently differentiate wave 2 (late-EMP and LMP) from wave 3 (HSC) are needed.

It is also essential to acquire more detailed knowledge of the different hematopoietic waves, both mechanistically and in terms of their exact sites of origin. Indeed, the observation that heart-specific HE can give rise to a specialized population of macrophages, raises the question whether other specialized hematopoietic cells are produced in a site or organ-specific way. Both in the AGM and the YS, multiple sites of hematopoietic emergence have been described (Muller et al., 1994; Medvinsky and Dzierzak, 1996; de Bruijn et al., 2000; Li et al., 2012; Frame et al., 2016; Kasaai et al., 2017). Closer investigation of these known sites as well as the identification of new sites could reveal the existence of new, functionally unique, hematopoietic populations. Furthermore, understanding if and how the distinct hematopoietic cells generated by the different waves interact to optimize blood production is equally fascinating. Altogether such knowledge could provide cues to develop better strategies for *in vitro* generation of HSCs and/or specific blood lineages from embryonic- and induced pluripotent stem cells (ES and IPSC). IPSC generated from different cellular sources may be inherently primed towards specific hematopoietic lineages. Additionally, it may be beneficial to incorporate mature hematopoietic cells into *in vitro* blood production protocols. Along these lines, it has been recently reported that macrophages can support the *in vitro* production of mature enucleated erythroid cells (Lopez-Yrigoyen et al., 2019).

Finally, findings in animal model systems are starting to be confirmed in humans. Macrophages have been found to accumulate in the human AGM at the time of HSC formation (Travnickova et al., 2015), and single-cell sequencing indicates that human microglia are also derived from HSC-independent hematopoietic waves (Bian et al., 2020). In conclusion, it is now well established that HSC-independent hematopoiesis is essential for embryonic organogenesis and its progeny can, and does, persist after birth. This has opened up a new and fascinating field of hematopoietic research.

AUTHOR CONTRIBUTIONS

WHN and ML wrote the manuscript. MZHF produced the figure. GL revised and edited the manuscript. All authors approved the final manuscript.

FUNDING

The work in our laboratory is supported by Cancer Research UK C5759/A20971 (GL) and Blood Cancer UK 19014 (GL).

ACKNOWLEDGMENTS

We thank Renaud Mevel for critical reading of the manuscript. Due to space limitations we were, regrettably, unable to cite all manuscripts that have contributed to the discussed research topic.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.