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Origins and functions of phagocytes in the embryo

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Objective. **To review the data on the origins, phenotype, and function of embryonic phagocytes that has accumulated over past decade.**

Data Sources. **Most of the relevant articles were selected based on the PubMed database entries. In additional, the Interactive Fly database (*http://sdb.bio.purdue.edu/fly/aimain/1aahome.htm*), FlyBase (*http://flybase.bio.indiana.edu:82/*), and TBase (*http://tbase.jax.org/*) were used to search for relevant information and articles.**

Data Synthesis. **Phagocytes in a vertebrate embryo develop in two sites (yolk sac and liver) and contribute to organogenesis in part through their ability to recognize and clear apoptotic cells. Yolk sac-derived phagocytes differ in differentiation pathway and marker gene expression from macrophages produced via classic hematopoietic progenitors in the liver.**

Conclusion. **We argue that yolk sac-derived phagocytes constitute a separate cell lineage. This conclusion raises the question of whether primitive phagocytes persist into the adulthood.** **© 2000 International Society for Experimental Hematology. Published by Elsevier Science Inc.**

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Macrophages in the developing embryo

During embryonic development of multicellular organisms, there is a continuous cycle of cell proliferation, differentiation, and death that permits tissue remodeling and organogenesis. As cells die, they are recognized and engulfed by specialized phagocytes. The process of cell death and engulfment is controlled by many genetic loci; perhaps the best characterized are of *Caenorhabditis elegans*, the so-called cell death (CeD) genes. In *C. elegans*, dying cells are engulfed by their neighbors, but in organisms ranging from *Drosophila melanogaster* to higher vertebrates, the cells that remove apoptotic bodies are specialized mobile phagocytes. These cells are commonly referred to as macrophages, but their origin and functional relationship to myeloid hematopoietic cells that arise later in development are not clear.

Hematopoiesis in vertebrate embryos is first detected in the yolk sac. The yolk sac hematopoietic cells develop in the splanchnic mesoderm of the yolk sac from the inner cells of the angiogenic clusters (hemangioblasts, also called blood islands) [1–3]. Although the hematopoietic progenitors in the yolk sac apparently can differentiate into all hematopoietic lineages when stimulated with appropriate growth factors in vitro, studies with chick/quail chimeras [3,4] indicate that the avian yolk sac blood cells are transitory. Definite blood cells in the chick embryo proper arise not from the yolk sac-derived cells but from the intraembryonic cells originating in the aortic mesoderm. Similar observations were made in mammalian embryos. The intraembryonic angiogenic clusters were identified in the paraortic splachnopleura and in the aorta, gonads, and mesonephros (AGM) region [5,6]. Hence, the model of embryonic hematopoiesis (reviewed in [7,8]) in mice suggests the existence of two waves of progenitor cell migration into the liver, one from the yolk sac at 9.5 to 10.0 dpc and the second from the AGM at 10.0 to 10.5 dpc forming a mixed population of progenitors in the liver. Subsequently, only the AGM-derived cells contribute to definite blood cells [9–11]. The difference in potential is manifested in the inability of the yolk sac cells to reconstitute an irradiated animal in contrast to those of the fetal liver or bone marrow cells [6,9,11–13].

The transition from yolk sac (primitive) to hepatic (definitive) hematopoiesis is marked by a clear transition in the phenotype of the erythroid cells, with a change from fetal to adult hemoglobin types and from nucleated to enucleated red cells. By contrast, the development of the myeloid lineage is less well characterized. There is considerable evi-

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dence that macrophage-like cells in the embryo differ phenotypically from those of the adult [14]. In mammals at least, this difference probably reflects in part the absence of exposure to immunologic challenges that contribute to macrophage development postpartum. Less attention has been paid to a possible transition in phenotype between phagocytes produced by the yolk sac and those arising from classic hematopoietic pathways in the liver.

A consideration of this issue leads one to consider the definition of a macrophage. The term itself was invented by Metchnikoff [15] to describe large mononuclear phagocytic cells able to take up microorganisms. Subsequently, all phagocytic cells were classified together as the reticuloendothelial system (RES) [16], an approach that persisted until the 1970s. The main problem with the RES concept was that it grouped together cells based on their function rather than their origins. van Furth and Cohn [17] proposed a classification of cells. Mononuclear phagocytes were defined as a family including committed hematopoietic precursors in bone marrow, their immediate progeny blood monocytes, and the cells in tissues that derive from transendothelial migration and maturation of monocytes to become professional phagocytes (macrophages). More recent information casts some doubt on whether tissue macrophages are continuously replenished from circulating monocytes [18]. There is certainly evidence for local proliferation of macrophages, which will not be reviewed here. However, the basic concept can accommodate inclusion of the progeny of cells that are seeded into the tissue from hematopoietic precursors arising at any time during development, including monocytes produced by the fetal liver. A more fundamental problem arises if yolk sac-derived phagocytes arise from an independent pathway. As noted earlier, facultative phagocytes arise in *C. elegans*, and professional phagocytes (referred to as macrophages) exist in insects that lack an obvious equivalent of the hematopoietic pathway. Most studies of "phagocytes" in the embryo are unable to make any assessment of the differentiation pathway, and the cells are identified based on their involvement in phagocytosis, and enzymatic, surface marker or mRNA expression that is shared with macrophages in adults [19–23].

In this article, we will review the origins, phenotype, and function of embryonic phagocytes. The term macrophage will be used advisedly, without any implication that such cells are "mononuclear phagocytes" as defined by van Furth and Cohn [17]. In fact, the available evidence indicates that a separate population of phagocytes exists in the embryo. If these cells are retained into adulthood, the concept of a "mononuclear phagocyte system" may require revision.

Embryonic origins of phagocytes

Yolk sac is the first tissue of embryonic origin containing cells described as macrophages in vertebrates. Their presence at early stages of development has been detected based on morphologic and histochemical criteria (light and electron microscopy), or surface markers (Mac-1 integrin, the receptor for macrophage colony-stimulating factor [CSF-1], *c-fms*, and mannose receptor) in chick, mouse, rat, and human [21–27]. Despite their appearance and markers, the earliest macrophage-like cells are functionally immature compared with classic adult macrophages. They do not appear actively phagocytic in tissue sections (A.M. Lichanska and D.A. Hume, unpublished observations) and lack markers such as F4/80 antigen (in mouse) and RM1 (in rat) and the secretory product lysozyme M [19,23,28–31]. Isolated pig yolk sac phagocytes were unable to ingest zymosan particles in vitro [32]. By contrast, human yolk sac cells were shown to avidly ingest dying erythrocytes and contain lysosomal enzymes [24], but these studies were performed at a later stage of development than in pig and mouse.

The question of whether yolk sac phagocytes might arise in the blood islands and differentiate as they migrate away is unresolved in the mouse, but studies in zebrafish suggest an alternative pathway. Because of the rapid progress of the process and accessibility of single labeling techniques, it is possible to watch individual cell migration and behavior during the early stages of yolk sac formation in the fish. Herbomel et al. [33] used a combination of video microscopy and in situ localization of hematopoietic marker genes to show that phagocytes appear in the yolk sac before red cells. Most importantly, the phagocyte precursors arise from a quite separate location than red blood cell progenitors.

After their infiltration of the yolk sac, progenitor cells in the zebrafish differentiate into mature phagocytes and invade the head mesenchyme in large numbers. Interestingly, *Drosophila* hemocytes (insect blood cells) also develop in the anterior head mesoderm [34]. Similarly, numerous phagocytes expressing macrophage markers are identifiable in chick and rodent head mesenchyme well before any circulation is established in embryo [25,35,36]. Studies with chimeras indicate that at least some of the early avian phagocytic cells come from the yolk sac [36,37], but the migration route has not been established and the additional presence of local stem cells has not been ruled out. The local phagocytes in the head mesenchyme and yolk sac remain extravascular and are able to proliferate [24,35,36,38–40].

Whether or not the yolk sac phagocytes originate from the blood island precursors, there is no apparent intermediate stage that resembles a blood monocyte (Table 1). This conclusion initially was based on the absence of characteristic morphologic features (such as lack of phagosomes and lysosomes) and histochemical markers (such as peroxidase). Using electron microscopy, Naito et al. [41] found no evidence of expression of peroxidase-positive granules on the nuclear envelope and the rough endoplasmic reticulum, a feature of immature macrophages in liver and bone marrow. More recently, we identified a definitive marker for the transition from yolk sac to liver myelopoiesis. The S100 proteins S100A8 and S100A9, otherwise known as MRP-8

and MRP-14, are expressed transiently during the process of macrophage differentiation from bone marrow precursors in vitro [42] and are definitive markers for the onset of myelopoiesis in the liver [23,43], but neither is expressed at all in the yolk sac.

Macrophages in the liver

As noted earlier, the initial appearance of macrophage-like cells in the mouse embryo proper occurs before the development of a blood circulation and the onset of liver hematopoiesis. Once the liver becomes a major source of myeloid cells, it becomes difficult to distinguish cells of yolk sac and liver origin in the absence of definitive markers. The former may decline, or they may continue to proliferate locally in particular tissues; there is no firm evidence either way. Nevertheless, there have been many studies of the cellular phenotype (Table 2) and location of macrophage-like cells in later embryonic development in a wide range of species. These studies suggest that although the myeloid cells become more like those found in an adult after the liver becomes a major source of myeloid cells, their phenotype remains quite distinct from cells in an adult. In the mouse, in particular, we see the appearance of cells expressing the widely studied surface marker F4/80, the macrophage scavenger receptor (MSR) and lysozyme, and the macrophage-specific transcription factor, PU.1 [19,23], but the level of lysozyme is low [31,44] and there is evidence that these phagocytes are unable to participate in wound healing [20].

The first detectable macrophages in the liver are clearly associated with hematopoietic islands, and adopt a stellate appearance resembling the macrophages of hematopoietic islands in the adult bone marrow [19]. They are physically associated with proliferating erythroblasts and may be engaged in erythropoiesis in several ways, as the major source of the red cell growth factor erythropoietin [57] and ingesting the nuclei expelled by maturing erythrocytes [58]. The first phagocytes infiltrating the liver are probably of yolk sac origin, raising the possible role of these cells, in combination with the environment of the liver, in initiating the establishment of definitive hematopoiesis.

With the onset of hematopoiesis in the liver, the number of phagocytes continues to increase to a point where they are one of the most abundant cell types in the embryo, constituting as much as 10% to 15% of the total cells in many organs. Various markers have been used to identify embryonic macrophages both in the liver and in other tissues (Table 2). Cells positive for macrophage markers are present in all developing organs and tissues: spleen, thymus, brain, lung, kidney, heart, muscles, branchial arches, epidermis, limbs, and eye [19,20,25,35,50,55,56,59–62]. In the brain, phagocytes form an almost continuous lining of the ventricular surfaces of the brain from 12.5 dpc and are abundant in the choroid plexus (A.M. Lichanska and D.A. Hume, unpublished observations). The actively phagocytic cells in the brain by this stage also express the adult macrophage markers MSR and lysozyme [23]. At later embryonic stages (16 dpc), the serial sections have shown that F4/80 and Mac-1 positive macrophages associate with regions of developing cortical white matter, corpus callosum, meninges, and choroid plexus [63,64]. Numerous actively phagocytic macrophages are found not only in the brain but also in other parts

Table 1. Characteristics of yolk sac, liver, and adult phagocytes

Characteristics	Yolk sac		Fetal liver	Adult
	9.0 dpc*	10.0 dpc		
Origin	Yolk sac mesoderm	Yolk sac mesoderm	Hematopoietic precursors in liver	Hematopoietic precursors in bone marrow
Intracellular organization	Large nucleus, microvilli	Large nucleus, microvilli	**?**	**?**
Peroxidase activity	−	−	Initially −ve; +ve later in gestation	+ or −
Localization of peroxidase activity	−	−	nuclear envelope	Nuclear envelope
Presence of lysosomes	−	+	+	+
Presence of lysosomal granules	−	+	+	+
Ability to phagocytose latex	+	+	+	+
Ability to phagocytose zymosan	−	**?**	+	+
Lysozyme M	−	−	+	+
c-fms (CSF-1 receptor)	+	+	+	+
F4/80 antigen	−	+	+	+
Macrophage mannose receptor	+	+	+	+
CD11b	+	+	+	+
Macrophage scavenger receptor	−	±	+	+
PU.1	−	−	+	+
mitf	+	+	+	**?**

*The cells can be induced to become phagocytic in in vitro culture [41].

Table 2. Macrophage markers used to describe embryonic phagocytes in various species

	Marker	Drosophila	Fish	Xenopus	Birds	Rodents	Human	References
Receptors	RMI					+		45
	F4/80					+		19
	Mac-1					+		21
	Mannose receptor					+		22,23
	c-fms					+		25
	Scavenger receptor	+						23
	crq receptor	+						46
	SR-CI					+		47
	ABC transporter					+		48
	DEP-1					+	+	49
	Lectin binding						+	35,50
	CD68							50
Enzymes	Lysozyme				+	+	+	23,31,51
	PU.1					+		23
Transcription factors	Mitf					+		23
	glide/gcm	+						52
	L-plastin		+					33
Other	Protein-X	+						53
	MDP-1	+						54
	WLC 15		+					55
	XL-1			+				56

of central nervous system (CNS), such as the cervical spinal cord [65]. They also are associated with sensory organs (e.g., eye and neural retina in developing eye) [66,67].

As the distribution of the cells expressing macrophage markers expands, they are initially most numerous in areas of active tissue remodeling such as the dorsal midline and branchial arches [20,23,53]. Cells in these regions are actively involved in phagocytosis of dying cells. This function has been most obviously demonstrated at a later stage of development in the interdigital zone in the developing footpad [20,25] and in the developing retina [66]. In nonmammals, well-documented examples of this association include metamorphosis in small intestine in *Xenopus*, suboptic necrotic center development in chick, and loss of the tail by tadpoles [68–70]. Apoptosis is an integral part of embryonic development, and tissue remodeling is used by all organisms from the most primitive multicellular ones. The association of specialized phagocytes with dying cells is a function conserved from *Drosophila.* There is some reported evidence that macrophages themselves, or rather their secretory products, cause cell death [71,72], but studies on *Drosophila* argue against such a role [34,73]. Studies of the retina demonstrated that there is a clear temporal distinction between the appearance of apoptotic bodies and subsequent migration of phagocytes toward the site of cell death [66], suggesting that dying cells elicit extravasation and chemotaxis of cells to clear the cell bodies.

The exact way in which phagocytes detect the potential targets and migrate toward them is not known. Dying cells express surface molecules that allow macrophages to recognize and engulf the apoptotic bodies (reviewed in [74]; see later), a signal that may cause concomitant maturation of the potential phagocytes. Direct evidence for a link between phagocyte maturation and cell death has been observed in insects. Terminal differentiation of insect hemocytes into fully functional phagocytes is very closely associated with apoptosis, but their absolute numbers are not increased in embryos with higher cell death [75–77]. Instead, qualitative rather than quantitative change is observed in *fork head*, *knirps*, and *stardust Drosophila* mutants with nearly 100% of hemocytes developing into fully mature macrophages. The converse phenomenon is observed in mutants with lower than normal levels of cell death. There is a significant reduction in numbers of fully mature macrophages detected by measuring the deposits of macrophage-derived proteoglycan-1 (MDP-1) [54]. In mouse models with natural or introduced mutations, the relationship between the level of apoptosis and numbers of macrophages in embryo has not been studied in detail, making any sort of comparison impossible. This area clearly requires further investigation.

Given the ability of macrophages to secrete a wide range of mediators [78] that can regulate the function of other cells, it seems very unlikely that their sole function is to ingest cells that have undergone autonomous programmed cell death. As noted earlier, erythropoietin from macrophages is known to be one of the factors secreted to allow primitive erythropoiesis [57]. Other factors presumably produced by embryonic macrophages are interferon γ (INF-γ), tumor growth factor β (TGF-β), and trombospondin [79–81]. Macrophages could contribute to vascularization of embryonic tissues by secreting appropriate cytokines [33], a proposal supported by their close association with the developing vasuclature. In the adult, macrophages produce several factors affecting endothelia, such as vascular endo-

thelial growth factor (VEGF), vascular permeability factor (VPF), basic fibroblast growth factor (bFGF), angiotropin, and substance P [82,83]. Embryonic macrophages have been implicated in depositing components of basement membrane such as proteoglycans, laminin, tiggrin, type IV collagen, and glutactin [54,84]. Studies of the optic nerve suggest that macrophages also might produce factors that are necessary for axon growth and guidance [85,86]. The identity of such factors is unknown except for macrophage-derived factor, an uncharacterized activity referred to as PIF (P388D1 cell line-derived inducing factor) able to induce anterior neural and mesodermal tissue in *Xenopus* [87].

Although a mammalian embryo is largely protected from pathogen challenge, embryonic macrophages can contribute to protecting the embryos against infection. The most detailed studies on immune abilities of embryonic phagocytes in vertebrates come from zebrafish. Both gram-negative and gram-positive bacteria can be cleared from the embryo by phagocytes migrating to the site of infection [33]. An infection in zebrafish also appears to induce a systemic response similar to macrophage activation in mammals.

Phagocytosis by embryonic macrophages

It is not the purpose of this review to deal in detail with mechanisms of phagocytosis, as the topic has been recently reviewed [74,88]. There are clear parallels in recognition of apoptotic cells by macrophages between mammals, *C. elegans*, and *D. melanogaster*. For example, the murine homologue of the Ced-7 gene in *C. elegans*, which encodes a member of the ABC transporter family (ABC1), is expressed specifically in embryonic phagocytes, and antibodies against the protein product block engulfment of apoptotic thymocytes by mature macrophages but have no impact on yeast particle uptake [48].

By far the biggest and the most diverse group of receptors involved in the uptake of dying cells are the scavenger receptors. There are three groups of scavenger receptors (SRs). The first includes all collagenous trimeric receptors and consists of the type I and type II receptors generated by alternative splicing, and MARCO SR encoded by a separate gene [89]. The second group of scavenger receptors contains CD36-related proteins, such as *croquemfort* (*crq*, also called class B), which are structurally unrelated to the first class but can take up oxidized low-density lipoproteins (LDLs) and apoptotic cells [89,90]. A third class, class C scavenger receptor dSR-CI, has been described in *Drosophila* [47]. Uptake of apoptotic cells is not the only function of SRs; they have high affinity for LDLs, lipopolysaccharide (LPS), fucoidan, lipotechoic acid, and nonopsonized particulate matter. They also can mediate adhesion to other cells [91,92]. The presence of multiple scavenger receptors could explain why a single targeted disruption of the scavenger receptor A type I or II in mice has no effect on the embryonic development and clearance of apoptotic cells [93]. The mutant mice do have impaired immunologic functions and resistance to atherosclerosis, suggesting that the main role of this receptor might be clearance of LDLs and bacteria. In *Drosophila*, the *crq* receptor is expressed exclusively on the macrophages engulfing apoptotic cells and is not required for taking up of bacteria, indicating that the two processes use separate pathways in species [90].

Mouse models of macrophage development

We mentioned in context the impact of molecular genetics approaches in *C. elegans* and *D. melanogaster* in identifying the processes that control the recognition and engulfment of dying cells and the maturation of embryonic phagocytes. A number of *Drosophila* mutants with various impacts on phagocytes have been described (Table 3). They all have mammalian homologues, but only some (NFκB, IL-1R, and TNF-R) are involved in the myeloid lineage in mammals; other genes are much more widely expressed. Several natural mutations or introduced disruptions in mice have possible impacts on embryonic myelopoiesis (Table 4), but so far, there are no described mutations that are defective in yolk sac-derived macrophages. Given the roles that have been ascribed to early embryonic phagocytes, it is difficult to see mouse development proceeding much beyond 10.5 dpc in their complete absence. The critical questions remaining to be asked include the nature of the growth signals that control early phagocyte development and proliferation and whether one can define markers and processes that differentiate yolk sac- and liver-derived cells. In pursuit of such a marker, we performed a targeted disruption of the S100A8 gene, which is first expressed in the liver (see earlier). However, the gene also is expressed transiently in early migrating trophoblasts, and in its absence the mother resorbs the developing embryos before liver hematopoiesis starts [43]. At the least, this finding indicates that S100A8 is not functionally redundant, and it may be possible to devise ways to overcome the maternal rejection phenotype to permit study of the onset of myelopoiesis in the livers of S100A8 (−/−) embryos.

Growth factors

There are several potential growth factors that could control embryonic macrophage proliferation and differentiation. CSF-1 is the only essential macrophage differentiation and survival factor known [135]. CSF-1 mRNA and protein are expressed in a developmentally regulated manner in the mouse embryo [136]. There is a natural mutation in the CSF-1 gene resulting in the absence of a measurable protein [120,121,137]. Animals carrying this mutation (*op/op* mice) are osteopetrotic, which means that they lack bone marrow due to the lack of osteoclasts and bone resorption. At birth, they also have reduced numbers of some macrophage populations (e.g., in kidney, liver, bone marrow, blood monocytes, spleen, and intestine), but some populations are unaf-

Table 3. Genes affecting *Drosophila* phagocytes and their mammalian homologues

Gene	Phenotype	Effect on macrophages	Mammalian homologue	Effects of KO in mice	References
glial cell missing/gcm	Lack of glial cells	Reduced hemocyte numbers	*Gcm1, Gcm2*	No knockout	52,94
bicaudal D	Head, thorax, and anterior abdomen missing	No detectable hemocytes	*Bicd1, BicD1*	No knockout	34,95
serpent	No mid-gut, no endodermal differentiation	No mature hemocytes	*GATA4*	Embryonic lethal at 9.5 dpc	96–100
reaper	Excess mid-line cells due to defect in apoptosis	None	*Fas*	Defective cell death	101–103
cactus	Melanocytic capsules, enlarged lymph glands	Overproliferation of macrophages	*IkappaBR, IkappaBT*	Increase of monocytes/mϕs numbers in spleen	104–106
toll	Defects in motorneuron number and muscle patterning	Lower density of hemocytes in hemolymph	*HTollR1-5*	Defective pathogen recognition	104,107–110
pelle, tube		Lower density of macrophages	*IRAK (pelle homolog)*	Attenuated response to IL-1	104,111,112
snail and *twist*	No mesodermal tissues	No macrophages	*Sna, Mtwist, Htwist*	Deficits in mesenchyme	34,113–117
domino	Melanized lymph glands	No circulating hemocytes	*None described*	No knockout	73,118
single minded	Lack of ventral midline, reduced brain size	No mid-line migration of hemocytes	*Sim1, Sim2*	No knockout	103,119

fected (e.g., bone marrow monocytes, lymph nodes, and thymus) [138]. Also unaffected in *op/op* mice are dendritic cells and Langerhans cells. In addition to macrophage and osteoclast deficiencies, the animals have developmental defects of the nervous and reproductive systems, which may reflect roles of embryonic macrophages [29,139–142]. The interpretation of the *op/op* phenotype is difficult because of transplacental trafficking of CSF-1, but recent evidence indicates that some embryonic macrophage populations can develop normally even when the mother is also *op/op* [143].

In principle, CSF-1 might be partly substituted by granulocyte-macrophage colony-stimulating factor (GM-CSF), another factor able to elicit macrophage proliferation in vitro and in vivo. However, mice with targeted disruption of the GM-CSF gene were shown to develop normally with no major abnormalities in hematopoiesis at birth [144]. They do develop abnormal lungs with a progressive infiltration by lymphocytes, the presence of numerous large intraalveolar phagocytic macrophages and an accumulation of surfactant in the lungs, and with the development of lung infec-

Table 4. Natural and introduced mutations in genes important for macrophage differentiation and/or function in adult animals

Gene	Phenotype	Effect on embryonic macrophages	References
CSF-1	Osteopetrotic mice, lack of bone marrow, macrophage deficient	None described	120–122
PU.1	Embryonic or neonatal lethal	Lack of mature macrophages, but embryonic phagocytes present	23,123,124
Scavenger receptor class A types I and II	Impaired response to *Listeria* and *Herpes* virus infections	None described	93
Core binding factors (CBF)	Embryonic lethal due to extensive hemmorrhaging	Lack of mature myeloid cells	125,126
CCAAT/enhancer binding proteins (C/EBP)	α: Neonatal lethal, lack of hepatic glycogen stores, no mature neutrophils, normal levels of monocytes β: Distorted immune regulation with defective macrophage activation	α: White blood cells appear immature	127–129
c-myb	Embryonic lethal due to severe anaemia	Not investigated	130
c-fos	Osteopetrotic	None described	131
Microphthalmia (mi)	Unpigmented, osteopetrotic, mast cell deficient, reduced NK activity	Not affected	132–134

tions (bacterial and fungal). Such pathology would be consistent with the decrease or absence of dendritic cells, which also respond to GM-CSF, but the development of the dendritic cells is not affected [145]. The lung pathology is even worse in mice with both GM-CSF (−/−) and *op/op* phenotype, suggesting that both factors contribute to alveolar macrophage function [146].

Development with age of osteoclasts and macrophages in *op/op* mice suggests that there is at least one additional growth factor that can act on the macrophage/osteoclast lineage. Niida et al. [147] presented strong evidence that VEGF is the factor involved. This finding is not totally unexpected, given that blood cells and endothelial cells develop from the common precursor during embryogenesis [2,33,148]. Macrophages in zebrafish recently were shown to originate in mesoderm, as do endothelial cells [33]. Moreover, evidence from *Drosophila* mutant bicaudal D also shows that lack of mesoderm results in lack of phagocytes [34]. It would be interesting to look at the effect of a VEGF null mutation on early phagocyte differentiation, especially because VEGF is highly expressed in embryonic tissues, but embryonic lethality and impacts of endothelial dysfunction [149] may preclude interpretation.

Transcription factors

The transition between yolk sac and liver hematopoiesis is likely to involve expression of key transcriptional regulators that, in turn, control expression of other genes. In the macrophage lineage, the most obvious candidate is PU.1, a novel member of the Ets transcription factor family. PU.1 is expressed at high levels in a macrophage restricted manner. Numerous macrophage promoters have functionally essential PU.1 sites (e.g., *c-fms*, tartrate-resistant acid phosphatase (TRAP), lysozyme M, macrophage mannose receptor, interleukin 1 (IL-1), F_c receptors (F_cRI and F_c RIIIA), MSR, and CD11b) [150–159]. In fact, macrophage-specific promoters have an archetypal structure in which purine-rich motifs recognized by PU.1 substitute for conventional TATA box and GC-rich elements found in classic mammalian promoters [158,159]. Two groups have made targeted disruptions of the PU.1 gene. Null mice created by Scott et al. [123] die in utero at 16.5 dpc, whereas the PU.1 (−/−) mice generated by McKercher et al. [124] die within 24 hours after birth. Independent of the time of death, many of the mature macrophage markers listed are absent in PU.1 (−/−) mice, and no mature macrophages were detected in tissues [160,161]. However, studies on the PU.1 null mice at early stages of development indicated that *c-fms* transcript was still detectable [124,160]. We showed that PU.1 is actually not expressed at detectable levels in early yolk phagocytes and appears first at 10.5 to 11.0 dpc when liver hematopoieis has commenced. Moreover, yolk sac-derived phagocytes detected by localization of the *c-fms* gene were unaffected by the PU.1 null mutation [23]. Although later stages of differentiation of liver-derived macrophages clearly are affected by the PU.1 mutation, the disruption here is not absolute. Henkel et al. [162] provided evidence for partial differentiation of immature phagocytes.

The CBF family of transcription factors is another group of regulators that bind to macrophage promoters [163]. These factors bind DNA as heterodimers of α and β subunits [125]. Three core binding factors have been mutated. The targeted disruption of CBFα1 has been shown to affect ossification of the skeleton attributed to lack of osteoblasts [164]. In contrast, the targeted disruption of both the CBFα2 (AML-1) and the β subunit led to an embryonic lethal phenotype due to extensive hemorrhaging [125,126]. Cbfa2 is expressed in both hematopoietic and endothelial lineage in the yolk sac from 8.5 dpc [165]. Analysis of fetal livers at 12.5 dpc revealed the presence of mainly yolk sac-derived erythrocytes and lack of definite myeloid cells. These findings could indicate that this family also has a distinctive role in the transition from yolk sac to definitive myelopoiesis. It would be of great interest to determine whether development of yolk sac phagocytes is effected by the CBF mutations.

Future directions

It is a common observation that ontogeny recapitulates phylogeny. In primitive organisms without an acquired immune system, macrophages constitute the primary mechanism of host defense. In species where the embryos are exposed to the elements, the host defense function of embryonic phagocytes may be required for survival even during development [33]. The functions of primitive phagocytes in mammalian embryos, and indeed the markers they express, seem to recapitulate the functions of hemocytes in *Drosophila*. Taking an evolutionary perspective, we suggest that there is a fundamental transition associated with the onset of liver hematopoiesis that correlates with the production of a new class of macrophage, coevolved with the acquired immune system to provide regulators of T- and B-cell function and to act as effector cells in host defense. Primitive yolk sac-derived phagocytes may be related to adult macrophages only in as much as they are both large and phagocytic. There is no compelling evidence that they develop from definitive hematopoietic precursors. Conversely, there is clear evidence that they do not progress via a monocyte intermediate and they do not require the transcription factor PU.1, which controls expression of so many mature macrophage genes, for their development.

The most interesting question that arises from this hypothesis is whether primitive phagocytes persist into the adult and constitute an independent arm of the host defense system, or alternatively an independent regulatory cell type that contributes to homeostasis. This question may be addressed by identification of definitive markers that distinguish “primitive phagocytes” from classic mononuclear phagocytes.

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