Developmental Biology xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/locate/developmentalbiology

The phenotypic and functional properties of mouse yolk-sac-derived embryonic macrophages

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ARTICLE INFO

Keywords:
Yolk sac
Macrophages
Microglia
Angiogenesis
Endothelial Cells
Neural Stem/Progenitor Cells

ABSTRACT

Macrophages are well characterized as immune cells. However, in recent years, a multitude of non-immune functions have emerged many of which play essential roles in a variety of developmental processes (Wynn et al., 2013; DeFalco et al., 2014). In adult animals, macrophages are derived from circulating monocytes originating in the bone marrow, but much of the tissue-resident population arise from erythro-myeloid progenitors (EMPs) in the extra-embryonic yolk sac, appearing around the same time as primitive erythroblasts (Schulz et al., 2012; Kierdorf et al., 2013; McGrath et al., 2015; Gomez Perdiguero et al., 2015; Mass et al., 2016). Of particular interest to our group, macrophages have been shown to act as pro-angiogenic regulators during development (Wynn et al., 2013; DeFalco et al., 2014; Hsu et al., 2015), but there is still much to learn about these early cells.

The goal of the present study was to isolate and expand progenitors of yolk-sac-derived Embryonic Macrophages (EMs) *in vitro* to generate a new platform for mechanistic studies of EM differentiation. To accomplish this goal, we isolated pure (> 98%) $EGFP^{+}$ populations by flow cytometry from embryonic day 9.5 (E9.5) *Csf1r-EGFP*$^{+/tg}$ mice, then evaluated the angiogenic potential of EMs relative to Bone Marrow-Derived Macrophages (BMDMs). We found that EMs expressed more pro-angiogenic and less pro-inflammatory macrophage markers than BMDMs. EMs also promoted more endothelial cell (EC) cord formation *in vitro,* as compared to BMDMs in a manner that required direct cell-to-cell contact. Importantly, EMs preferentially matured into microglia when co-cultured with mouse Neural Stem/Progenitor Cells (NSPCs). In conclusion, we have established a protocol to isolate and propagate EMs *in vitro*, have further defined specialized properties of yolk-sac-derived macrophages, and have identified EM-EC and EM-NSPC interactions as key inducers of EC tube formation and microglial cell maturation, respectively.

1. Introduction

Beside their key role in immune response, macrophages have been shown to mediate the development and maintenance of normal structure and function of several tissues including the vascular system. For instance, macrophages were previously shown to regulate vascular complexity during retinal development by secreting VEGF-C, which activates VEGFR3 on vascular endothelial cells and reinforces Notch expression (Tammela et al., 2011). Additionally, previous studies have suggested that macrophages function as cellular chaperones to enhance vascular anastomosis in the developing hindbrain and retina by mediating the fusion of endothelial tip cells (Fantin et al., 2010; Rymo et al., 2011). Others have found that macrophages function in vascular remodeling and regression such as in the case of the transient hyaloid vasculature of the eye. Here, resident macrophages mediate vascular endothelial cell apoptosis *via* the cooperation between the angiopoietin-2 and *Wnt7β* pathways (Rao et al., 2007). In addition, recent work has indicated that macrophages promote pupillary membrane capillary regression by engulfing endothelial cell membrane particles emanating from the pupillary membrane vasculature (Poché et al., 2015). Together, these studies suggest that macrophages play various roles during the development and remodeling of the vascular

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https://doi.org/10.1016/j.ydbio.2018.07.009
Received 4 July 2018; Accepted 11 July 2018

system, but the exact mechanisms underlying this functional diversity are not fully understood.

Beyond development, macrophages have also been implicated in pathological angiogenesis of several diseases such as cancer. For instance, tumor associated macrophages (TAMs) play an important role in tumor angiogenesis as they regulate the angiogenic switch leading to increased tumor vascularization, which is required for the transition to the malignant state (Owen and Mohamadzadeh, 2013; Lin et al., 2006). Additionally, studies have shown that TAMs can secrete many pro-angiogenic factors and proteases. TAMs are also characterized by the expression of the mannose receptor 1 (*Mrc1*), which is also highly expressed on embryonic macrophages during development (Takahashi et al., 1998; Lin and Pollard, 2007; Mazzieri et al., 2011). These data are in line with the previous finding that TAMs and EMs share a common gene signature suggesting overlapping functions possibly at the level of angiogenic potential (Pucci et al., 2009). While these studies investigated the function of adult macrophages in a tumor environment, our project aimed at elucidating the functional properties of yolk sac-derived embryonic macrophages (EMs) isolated from the embryo proper.

The many described roles for macrophages point to tremendous functional heterogeneity, but also plasticity. Many groups have described the ability of macrophages to respond to altered cellular environments by dramatically changing their functional role. For example, in the broadest sense, the presence of specific signaling molecules are well known to instruct macrophages to adopt either the so-called M1 (pro-inflammatory) identity *versus* the M2 (anti-inflammatory) identity (Jetten et al., 2014). Importantly, EMs have been shown to express M2 markers such as *Mrc1* and Arginase 1, suggesting they are more similar to the M2, *versus* the M1, classification scheme (Takahashi et al., 1998; Rőszer, 2015). Additionally, M2 macrophages have been reported to exhibit pro-angiogenic activities. However, it is important to recognize that within these two very general classifications of macrophages, other levels of functional variation likely exist along a continuum (Ovchinnikov, 2008; Pucci et al., 2009; Mazzieri et al., 2011; Jetten et al., 2014).

In addition to environmental cues, macrophages may also be influenced by developmental origin and the corresponding cell intrinsic transcriptional identity (Matcovitch-Natan et al., 2016). Macrophages derived from various progenitor populations (yolk sac, fetal liver, and bone marrow) appear at different stages of development, coincident with different sites of hematopoiesis. The earliest macrophages in the mouse embryo are found at embryonic day (E) 7.5, but are actually a transient population of maternally derived macrophages that are cleared by E9.0 (Bertrand et al., 2005; Kierdorf et al., 2013). Embryonic macrophages derive from the extra-embryonic yolk sac and arise from both primitive hematopoietic progenitors, which are evident as early as E7.5, as well as from erythromyeloid progenitors, which are detected by E8.5 (Bertrand et al., 2005; Schulz et al., 2012; Kierdorf et al., 2013; McGrath et al., 2015; Gomez Perdiguero et al., 2015; Mass et al., 2016). By E10.5, definitive hematopoietic stem cells (HSCs) are present within the aorto-gonado-mesonephros (AGM) region and generate a second population of embryonic macrophages. Subsequently, AGM HSCs cells migrate to the fetal liver where they expand and differentiate starting at E12.5 (Bertrand et al., 2005; Orkin and Zon, 2008; Schulz et al., 2012; Gomez Perdiguero et al., 2015). At this point, the fetal liver is a transient source of definitive hematopoiesis from which circulating monocytes are derived. During neonatal stages, fetal liver hematopoiesis declines and is replaced by bone marrow hematopoiesis (Lichanska et al., 1999; Lichanska and Hume, 2000; Geissmann et al., 2010; Wynn et al., 2013).

Yolk-sac-derived macrophages form the first wave of tissue resident macrophages. Cell lineage tracing studies have shown that mouse yolk sac macrophages colonize the developing central nervous system (CNS) and persist to adulthood as tissue resident microglia (Ginhoux et al., 2010). These cells self-renew within the CNS throughout adulthood without significant contribution from the bone marrow and circulating blood monocytes and were shown to play important roles in the sprouting, migration, anastomosis and refinement of the CNS vascular system (Herbomel et al., 2001; Ajami et al., 2007; Ginhoux et al., 2010; Fantin et al., 2010; Hashimoto et al., 2013; Arnold and Betsholtz, 2013). For example, the microglia-deficient mice (Pu.1 knockouts and Csf1 op/op mutants) have reduced numbers of vascular branch points in the hindbrain (Fantin et al., 2010). Similarly, previous studies indicates that microglia play roles in shaping the vascular plexus in the retina (Rymo et al., 2011; Arnold and Betsholtz, 2013). However, microglia share a number of molecular characteristics with other macrophage classes, making it difficult to distinguish microglia from other macrophages using molecular marker analysis. Therefore, the detailed characterization of morphological features has been widely used as an alternative to quantitatively differentiate between microglia and other myeloid cells in culture (Fujita et al., 1996; Eder et al., 1999; Szabo and Gulya, 2013; Sheets et al., 2013).

In this study, we first investigated the possibility that EMs are endowed with greater pro-angiogenic potential than BMDMs. Despite several studies indicating that EMs express pro-angiogenic factors and gene expression signatures distinct from fetal liver and adult macrophages (Bertrand et al., 2005; Ovchinnikov, 2008; Pucci et al., 2009; Schmieder et al., 2012), very little is known about the phenotypic properties and functional capabilities of EMs before the production of the definitive HSCs. Furthermore, to date, most research investigating the pro-angiogenic potential of macrophages has been performed using adult macrophages. Therefore, isolation and characterization of EMs is necessary for determining the full potential of macrophages in driving vascular development as well as other roles served by microglia.

Here, we report successful isolation, *in vitro* expansion and characterization of EMs derived from E9.5 *Csf1r-EGFP*$^{+/tg}$ transgenic mice. We confirmed the macrophage identity in our isolated/cultured EMs by their expression of the specific macrophage markers *Csf1r* and *F4/80*. We also determined that EMs possess pro-angiogenic and pro-inflammatory properties distinct from adult macrophages. Most importantly, we demonstrated that EMs have a greater capability to differentiate into microglia as compared to adult macrophages when co-cultured with NSPCs, consistent with data showing that microglia derive from EMs (Ginhoux et al., 2010). The purified EM population is thus suitable to investigate a wide range of molecular properties and functional capabilities of macrophages *ex vivo*.

2. Material and methods

2.1. Mice

Flk1-myr::mCherry$^{tg/tg}$ mice (Larina et al., 2009) were crossed to *Csf1r-EGFP*$^{tg/tg}$ mice (Sasmono et al., 2003) to generate *Csf1r-EGFP*$^{+/tg}$; *Flk1-myr::mCherry*$^{+/tg}$ mice. *Csf1r-EGF* $^{+/tg}$; *Flk1-myr::mCherry*$^{+/tg}$ mice were intercrossed to generate *Csf1r-EGFP*$^{tg/tg}$; *Flk1-myr::mCherry*$^{tg/tg}$ mice. *Csf1r-EGFP*$^{tg/tg}$ males were mated to CD1 females to generate *Csf1r-EGFP*$^{+/tg}$ mice. CD1 females were purchased from the Charles River Laboratories. All animal procedures were reviewed and approved by Baylor College of Medicine Institutional Animal Care and Use Committee in compliance with the National Research Council Guide for the Care and Use of Laboratory Animals.

2.2. In vivo confocal imaging macrophages in the embryo

CD1 females were mated to *Csf1r-EGFP*$^{tg/tg}$; *Flk1-myr::mCherry*$^{tg/tg}$ males to generate *Csf1r-EGFP*$^{+/tg}$; *Flk1-myr::mCherry*$^{+/tg}$ transgenic embryos. The morning of a vaginal plug formation was counted as embryonic day 0.5 (E0.5). Embryos were isolated at E9.5 and dissected under a stereomicroscope at 37 °C. Embryos were kept in a pre-warmed dissection media (1XDMEM-F12 contains 10% Fetal Bovine Serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin Life technologies). The