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Research paper

Generating tissue-resident macrophages from pluripotent stem cells: Lessons learned from microglia

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ABSTRACT

Over the past decades, the importance of the immune system in a broad scope of pathologies, has drawn attention towards tissue-resident macrophages, such as microglia in the brain. To enable the study of for instance microglia, it is crucial to recreate *in vitro* (and *in vivo*) assays. However, very fast loss of tissue-specific features of primary tissue resident macrophages, including microglia, upon *in vitro* culture has complicated such studies. Moreover, limited knowledge of macrophage developmental pathways and the role of local 'niche factors', has hampered the generation of tissue-resident macrophages from pluripotent stem cells (PSC). Recent data on the ontogeny of tissue-resident macrophages, combined with bulk and single cell RNAseq studies have identified the distinct origins and gene profile of microglia compared to other myeloid cells. As a result, over the past years, protocols have been published to create hPSC-derived microglia-'like' cells, as these cells are considered potential new therapeutic targets for therapies to treat neurodegenerative diseases. In this review we will provide an overview of different approaches taken to generate human microglia *in vitro,* taking into account their origin, and resemblance to their *in vivo* counterpart. Finally, we will discuss cell-extrinsic (culture conditions) and intrinsic factors (transcriptional machinery and epigenetics) that we believe can improve future differentiation protocols of tissue-resident macrophages from stem cells.

1. Introduction

Extensive genome wide sequencing (GWAS) over the past years revealed that the tissue-resident macrophages of the brain, known as microglia, are more than just the constant gardeners of the central nervous system (CNS). Not only do they play a key role in tissue homeostasis and inflammation, but it has become clear that they are important contributors to many neurodegenerative diseases that were not immediately recognized to comprise an inflammatory component. For instance, Alzheimer's disease (AD) was only described as an inflammatory disease in 1994, even if the first description of an AD patient dates already from 1906 [1,2]. Multiple immune system related genes have been shown to be risk factors for sporadic Alzheimer's disease (AD), e.g. SNPs in Clusterin (*CLU*), Complement receptor 1 (*CR1*), EPH receptor A1 (*EPHA1*), ATP binding cassette subfamily A member 7 (*ABCA7*), Triggering receptor expressed on myeloid cells 2 (*TREM2*), Siglec-3 (*CD33*) [3], all expressed by microglia. Thus, microglia-mediated inflammation might influence AD pathology, and microglia could serve as a novel therapeutic target.

Therefore, it has become crucial to create *in vitro* (and *in vivo*) assays encompassing microglia. However, limited access of human tissue and difficulties to isolate microglia complicated these studies. In addition, microglia very quickly lose tissue-specific functions and characteristics upon *in vitro* culture, turning them into "generic" macrophages [4].

An alternative to harvesting microglia or other tissue-resident macrophages would be the creation of these cells from a readily expandable stem cell source, pluripotent stem cells (PSCs) [5,6]. Although hematopoietic cells can be generated from PSCs [7–13], differentiation to microglia was until recently not possible. However, recent bulk- and single-cell-RNAseq has identified the expressed gene profile of different tissue-resident macrophages in comparison with other myeloid cells [4,14–16]. Moreover, the yolk-sac ontogeny of microglia has been elucidated, at least in mice [17,18]. This novel information can now be exploited for the creation of microglia, provided that culture systems can be adapted to allow their functional and phenotypic generation and maintenance *in vitro*. In this review, we will discuss the ontogeny of microglia compared to other tissue-resident macrophages and we will provide an overview of the differentiation of microglia from human pluripotent stem cells, how good they resemble their *in vivo* counterparts, and highlight remaining questions and future directions to

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optimize differentiation protocols.

2. Tissue-resident macrophages – ontogeny

For many years, it was believed that all tissue-resident macrophages were continuously repopulated by circulating monocytes. However, more recent insights in the ontogeny of monocytes, dendritic cells, and macrophages [19–23], suggest that monocytes can only repopulate tissue macrophage compartments under very specific conditions, and that, depending on the target organ, differences may exist [18]. To gain insight in the possible different origins of tissue-resident macrophages, it is important to understand that several waves of hematopoiesis during embryogenesis are responsible for the creation of the adult hematopoietic system [17,18]. The first wave, or "primitive hematopoiesis", originates from mesodermal blood islands in the yolk sac (YS), which itself is derived from the posterior plate mesoderm at ± E7.0 in mouse. These cells give rise to the primitive erythroblasts, megakaryocytes, and macrophages [24,25]. A "transient definitive wave", also termed erythro-myeloid precursors (EMPs), that generate erythroid and myeloid cells, but not lymphocytes, arises from the YS hemogenic endothelium between E8.0 and E8.5 [26]. As these EMPs cannot create long-term repopulating hematopoietic stem cells (HSCs) that form the basis of the adult hematopoietic system, they are termed "transient". Nevertheless, late EMPs generated on E8.25 migrate from E9.5 into the fetal liver generating myeloid progenitors including fetal liver monocytes [27]. The "definitive hematopoiesis" wave is derived from hemangioblasts originating in the para-aortic splanchnopleura region that give rise to fetal HSCs in the aorta/gonad/mesonephros (AGM) region at E10.5 [28,29], and subsequently establish long-term, definitive hematopoiesis.

A number of fate mapping models have been used to trace the contribution of different hematopoietic progenitors to adult tissue-resident macrophage populations (Table 1). Early EMPs, derived from primitive myeloid progenitors arising before E8, form the first population of tissue-resident macrophages, and this without a monocytic intermediate [17,30]. By contrast, monocytes from late EMPs and definitive HSCs in the fetal liver colonize many tissues at later developmental times and outcompete early EMP-derived YS macrophages [18]. Furthermore, fetal macrophages can also gradually be replaced by adult BM-derived cells following birth [31], although this is higly organ-specific. An exception to this is the brain, which is shielded by the blood-brain-barrier. Thus, formation of the BBB very early during fetal life [32], might prevent late EMPs and, later on, adult monocytes to enter the brain. To conclude, it is believed that the majority of brain-resident macrophages, microglia, are derived from early EMPs.

3. Tissue-resident macrophages – niches

How local environments influence the phenotype and properties of tissue-resident macrophages is not yet well understood. Lavin et al., demonstrated that following macrophage depletion in the lung, BM-derived macrophages that replaced them were transcriptionaly ± 90% identical to the embryonic macrophages that were eliminated [33]. In contrast, BM-derived macrophages that repopulated the liver Kupffer cell (KC) pool were only 50% transcriptionally identical to embryonic KCs and Bruttger et al., demonstrated even less congruence between the gene expression pattern of embryonic and BM-derived microglia [34]. These studies therefore suggested a role for tissue-specific signals in modifying BM-derived macrophage fate. One drawback of these studies was that replacement of embryonic by adult macrophages was the result of irradiation-mediated macrophage depletion, leaving the question whether irradiation also affected the niche function unanswered. That this might, at least in part, be responsible for differences between the resident and BM-derived repopulating macrophages was demonstrated by Scott et al. [35]. In this study, KCs were conditionally depleted using a diphtheria toxin approach. The gene expression profile of BM-derived KCs repopulating the liver following elimination of the resident KCs, without damage to the other liver cells by irradiation, was highly similar to the initial KCs [35]. This demonstrates that irradiation-mediated damage of the niche might occur, and thus the niche is crucial to instruct the macrophage, in this case, the KC phenotype. Less data is available for microglia: It is generally accepted that microglia are derived from $CD235a^{+}$ yolk-sac primitive progenitors and are not replaced by monocytes [17,36–39]. Nevertheless, following for instance irradiation, peripheral monocytes enter the brain. However, their gene expression pattern differs significantly from that of embryonic microglia [34] and they do not become tissue resident long-term [39–41]. It is possible that this is the result of irradiation-mediated damage of the microglial niche, as has been described for KCs [35]. Conform this idea is the fact that when the endogenous microglial niche is completely vacant, such as in the Purine-rich box1 (*Pu.1)* KO mouse [42], or microglia are experimentally depleted using Ganciclovir in mice expressing thymidine kinase under the control of the *Cd11b* promoter [43], adult BM cells appear to differentiate into microglia. However, Takata et al., found that there are distinct differences in for instance morphological changes of primitive and definitive (bone-marrow) hematopoiesis-derived macrophages to neurons *in vitro*, and they suggested that although transdifferentiation of monocytes to microglia appears possible, transdifferentiated microglia only partially resemble true microglia [44]. More studies will be needed to understand the exact signals emanating from tissue-resident macrophage niches that could possibly govern the fate switch from BM-derived monocytes into tissue-resident macrophages.

4. Tissue-resident macrophages – selfrenewal

Some studies have suggested that the capacity of embryonic and fetal macrophages to self-renew is significantly greater than that of BM-derived monocytes [27,45]. Consistent with this notion is the fact that monocytes recruited to specific organs often fail to stably engraft once inflammation resolves [38,46,47]. For instance, intestinal, dermal, and cardiac macrophages derived from BM precursors appear to not be

Table 1
Summary of the most important mouse fate mapping models used to trace the contribution of embryonic progenitors to adult-tissue macrophage population.

Mice	Most important findings	Reference
Tamoxifen-inducible Runx1-Mer-Cre-Mer mice	RUNX1 expression is necessary for the sequential emergence of EMPs and HSCs from the hemogenic endothelium	Samokhvalov et al. (2007) [98]
Runx1Cre/wt:Rosa26R26R-LacZ mice	Adult microglia derive from primitive myeloid progenitors that arise before embryonic day 8	Ginhoux et al. (2010) [17]
Tamoxifen-inducible Flt3-Cre mice	Adult HSCs contribute substantially to the pool of peritoneal macrophages	Epelman et al. (2014) [99]
Tamoxifen-inducible Tek-Mer-Cre-Mer mice (Tie2-Cre)	Fetal and adult macrophages originate predominantly from a progenitor cell type that expresses Tie2 at E7.5 but not after E9.5, and the authors propose this progenitor to be YS EMPs	Hoeffel et al. (2015) [27]
Tamoxifen-inducible Csf1r-Mer-Cre-Mer mice	Early EMPs differentiate locally, predominantly generating YS macrophages before the onset of blood circulation, and late EMPs can spread through the blood circulation	Perdiguero et al. (2015) [100]
Constitutive S100a4-Cre mice	FL monocytes contribute to many adult macrophage populations	Hoeffel et al. (2015) [27]
Tamoxifen-inducible Kit-Mer-Cre-Mer mice	Adult macrophages, with the exception of microglia and, partially, epidermal Langerhans cells, arise from definitive fetal HSCs.	Sheng et al. (2015) [101]