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.

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 optimize differentiation protocols.

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.

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
Runx1Cre/wt:Rosa26R26R-LacZ mice	Adult microglia derive from primitive myeloid progenitors that arise before embryonic day 8.	Ginhoux *et al.,* 2010
Tamoxifen-inducible *Flt3-Cre* mice	Adult HSCs contribute substantially to the pool of peritoneal macrophages.	Epelman *et al.,* 2014
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
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.	Gomez Perdiguero *et al*., 2015
Constitutive *S100a4-Cre* mice	FL monocytes contribute to many adult macrophage populations.	Hoeffel *et al*., 2015
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

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 depeletion 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 Gancyclovir 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.

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 capable to maintain themselves [17,18]. In contrast, Scott *et al.*, demonstrated that BM-derived KCs can stably engraft the liver after elimination of the resident KC using diphtheria toxin [35]. Moreover, in the lung, BM-derived macrophages could also still be detected at least 1 year after they had engrafted [45]. Therefore, further studies will be required to assess if and how BM monocytes can persist in some but not other tissues, and if this is affected by tissue inflammation.

LESSONS FROM DEVELOPMENT TO CREATE MICROGLIA FROM PSCs

To generate tissue-resident macrophages from pluripotent stem cells, it is not yet clear how important the origin of these cells is *in vitro*, but signals emanating by the tissue-specific macrophage niche appear to be an important factor. However, as pointed out in the preceding paragraphs much is still to be learned regarding what these factors are. In addition, this will also require that the exact phenotype of the 'to be generated'- cell type is well understood. As most studies that tried to recreate tissue-resident macrophages have focused on microglia, and because they are candidate targets to treat neurodegenerative diseases, we will here focus our review on these brain-specific macrophages.

The identity of microglia, the resident macrophages of the central nervous system, was poorly characterized until very recently. Former markers to identify microglia, including CD68, CD11b, MHCII and IBA1, were not very specific and were co-expressed by other macrophages [48-51]. Although Haynes *et al.,* described in 2006 the importance of *P2ry12* in mouse microglial function, identifying microglia-specific

markers remained a challenge [52]. The lack of tools to distinguish microglia from other monocytic cells limited their purification and *in vitro* manipulation, and precluded the derivation of microglia from PSCs. Hickman *et al.,* in 2013 [14] was the first to compare the transcriptome of CD11b positive microglia from mouse brain to whole brain tissue by direct RNAseq. These studies confirmed the expression of *P2ry12,* but also found *Trem2, Cd33, Cx3cr1, Hexb* and *Siglech,* amongst others, to be expressed in microglia. Butovsky *et al.*, in 2013, described a set of microglial genes (for example, *Fcrls*, *P2ry12*, *Tmem119*, *Olfml3*, *Hexb*, *Tgfbr1*, *Gpr34* and *Sall1*) uniquely expressed in mouse microglia but not other myeloid cells [15]. They also described a unique molecular signature of human microglia, represented by the highly or unique expression of *P2ry12*, *GPR34*, *MERTK*, *C1Qa*, *PROS1* and *GAS6* [15]. In addition, in 2016, Bennet *et al.,* identified the transmembrane protein 119 (*Tmem119*), coding for a cell-surface protein of unknown function, as a unique microglia-specific marker in both mouse and human, for which they generated monoclonal antibodies enabling the isolation of microglia [16]. Gosselin *et al.,* in 2017 confirmed these gene signatures in human microglia [4]. The ability to positively identify microglia from among other monocytes/macrophages has now made it possible to develop methods for the generation of microglia from PSCs, which is discussed in the following sections and schematically represented in Figure 1.

hPSC-derived microglia-like cells through a CD235a^{+} intermediate state

Generation of monocytes from PSCs and subsequent differentiation into macrophages *in vitro* through sequential exposure of hPSCs to specific cytokines was first described in 2008 [53-55]. In 2013 followed the first description of the generation of cells with microglia features from PSCs that appeared to co-develop with neuronal cells (Beutner *et al.,*2013), followed shortly thereafter by the description by Almeida *et al.,* that this may also be possible for human PSCs [56,57]. How the culture conditions induced microglia-like cells was not addressed. However, the derivation of microglia in the setting of neuronal development might mimic some factors present in the developing microglia niche and hence induce cells with a microglia fate.

Only recent, culture systems have been developed wherein primitive YS hematopoietic cells are generated from PSCs, characterized by the expression of *CD235a* [13,58,59], thought to be the lineage from which microglia are derived [44]. Based on this approach Muffat *et al.,* published in 2016 the generation of microglia-like cell (pMGLs)[60]. hPSCs were cultured as embryoid bodies (EBs) in a fully defined, serum-free neuroglial differentiation (NGD) medium, adjusted to match the components of human cerebrospinal fluid, and supplemented with IL-34 and colony-stimulating factor 1 (CSF1). Both cytokines signal via CSFR1 and are important for microglia differentiation and maintenance *in vivo*. In contrast to CSF1, which is also needed for the differentiation of bone marrow-derived progenitor cells into monocytes, IL-34 is a tissue-restricted ligand, mainly produced by neurons and keratinocytes, and uniquely supports microglia and Langerhans cell maintenance [61,62]. After 1 week of culture, neuralized spheroids and cystic structures (YS-EBs) formed. When subsequently plated on poly-d-lysine coated plates, these structures flattened into cell lawns reminiscent of endothelial cells. The cells stained positive for VE-cadherin, CD117, CD41 and

CD235a. Consecutive plating of these cells on modified polystyrene (Primaria) plates selectively allowed microglia to adhere and mature further over 30 days. After 8 weeks of differentiation the yield of pMGLs varied between 1×10^6 and $8x10^6$ starting from 2×10^6 PSCs. pMGLs could be maintained for several months in culture, even if they displayed limited proliferation potential. Mature pMGLs stained positive for TMEM119, P2RY12, IBA1 and CD45. The pMGLs transcription signature was highly similar to that of purified human fetal microglia maintained under the same culture conditions [60]. Functionally, pMGLs were highly phagocytic and acquired a ramified morphology, enhanced by co-culture with differentiated neurons and glia. Although similarty was shown with *in vitro* cultured human fetal microglia, it would also be of interest to further demonstrate the unique microglia signature of pMGLs in comparison to hPSC-derived monocytes and macrophages.

Table 2: Cytokines/small molecules used for microglia-like cell differentiation and yield of microglia-like cells from human PSCs

Reference protocol	**Duration of the protocol**	**Yield**	**Cytokines/ Small molecules**
Muffat *et al.,* 2016	±8 weeks	1-8x10^6 pMGLs from 2×10^6 hPSCs	CSF1, IL-34
Abud *et al.,* 2016	±5 weeks (38 days)	3-4x10^7 iMGLs from 1x10^6 hPSCs	FGF2, BMP4, Activin A, VEGF, TPO, SCF, IL3, IL6, CSF1, IL-34, TGFβ1, CX3CL1, CD200
Takata *et al.,* 2017	±4 weeks (25 days)	Start from 1x10^5 hPSC/well (yield not mentioned)	FGF2, BMP4, CHIR99021, VEGF, DKK1, SCF, IL3, IL6, CSF1
Haenseler *et al.,* 2017	± 4 weeks	1-4x10^7 pMacpre from 1x10^6 hPSCs	BMP4, VEGF, SCF, CSF1, IL3, GM-CSF, IL-34
Pandya *et al.,* 2017	4 weeks – 8 weeks	1-3x10^6 iPS-MG from 1x10^6 hPSCs	VEGF, BMP4, SCF, Activin A, Flt3, IL3, IL6, GM-CSF, CSF1
Amos *et al.,* 2017	±6 weeks (40 days)	15-30% ScMglia of EBs responsible for the majority of cells with microglia morphology	FGF2, BMP4, SB431542, GM-CSF, CSF1, IL-34, TGFβ1, Y-27632, BIO
Douvaras *et al.,* 2017	±6-8 weeks (45-60 days)	2-3x10^6 iPSC-MG from 1x10^6 hPSCs	BMP4, FGF2, SCF, VEGFA, IL-3, TPO, CSF1, Flt3, GM-CSF, IL-34

Also in 2016, Abud *et al.,* described the generation of human microglial-like cells (iMGLs)[63], generated in a two-step protocol. The initial step was adapted from Sturgeon *et al.*,[13] creating *CD43/CD235a/CD41* positive primitive hematopoietic progenitors, termed iHPCs. *CD43*$^+$ iHPCs were subsequently cultured in serum-free differentiation medium containing not only CSF1 and IL-34, but also transforming growth factor TGFβ1, described by Butovsky *et al.,* to be important for maintenance of microglia *in vivo* and *in vitro* [15]. On day 14, early iMGLs were CD117$^+$/CD45$^+$, even if CD45 and CD11b expression levels never reached those observed in macrophages, consistent with murine microglia development [64]. Exposure of iMGLs to the soluble CNS factors, CD200 and CX3CL1, further primed microglia to respond to neurodegenerative conditions [65,66]. By day 38, iMGLs resembled human microglia, but not monocytes or macrophages, demonstrated by their expression of many microglial-enriched proteins, including MERTK, ITGB5, CX3CR1,

TGFβR1, and PROS1. As iMGLs matured *in vitro,* they also became more ramified like microglia *in vivo*. The high similarity between primary microglia and iMGLs based on unbiased whole-transcriptome analysis further supported the notion that PSC-derived iMGLs approximate human microglia. iMGLs also secreted cytokines in response to inflammatory stimuli, migrated in response to calcium transients, and robustly phagocytosed CNS substrates. Furthermore, iMGLs transplanted into transgenic mice and human brain organoids resembled microglia *in vivo* [63]. Finally the authors assessed the effect of Amyloid β fibrils and brain-derived tau oligomers on AD-related gene expression in iMGLs and showed that iMGLs were able to internalize both the Amyloid β fibrils as well as the tau oligomers. The authors also stated that other cells are not present in PSC-iMGL progeny, which might be co-generated in the EB-derived pMGLs using the Muffat *et al.,* protocol [60]. The Abud *et al.,* approach is thus an efficient and rapid protocol that recreates microglia-like cells from a presumed YS-progenitor intermediate, with a thorough characterization of the gene expression profile and functional aspects. The inability of the $CD235^{+}$ cells generated in this protocol to generate T cells will however be required to fully demonstrate YS nature of this cell population.

Takata *et al.,* used Wnt modulation [13] to generate putative primitive hematopoietic progenitors, even though they did not demonstrate that the cells were *CD235a+*. Subsequent co-culture of these cells with iPSC-neurons yielded hiMacs, with some morphological and functional characteristics of microglia [44]. Buchrieser *et al.,* reported that during iPSC differentiation, most, if not all, monocytes/macrophages are produced in a *MYB*-independent fashion [67], consistent with what has been demonstrated in mouse [36]. They knocked out the transcription factor (TF) *MYB,* Runt Related TF 1 (*RUNX-1)*, or *Spi-1* Proto-Oncogene (*SPI1)*) in human PSCs. They demonstrated that monocytes and macrophages could be produced in the absence of *MYB*, while *RUNX-1* and *SPI1* were required, consistent with the notion that YS myelopoiesis is *MYB*-independent. Haenseler *et al.,* described a protocol wherein *MYB*-independent macrophages were co-cultured with iPSC-derived cortical neurons, yielding microglia-like cells after one month. These microglia-like cells expressed key microglia markers, developed highly dynamic ramifications, and were phagocytic [68]. Although this study demonstrated that cells with human microglia characteristics can be generated in a MYB-independent way, and hence are very likely derived from a cell with YS hematopoietic progenitor characteristics, they did not demonstrate that the progenitors were $CD235a^{+}$.

In conclusion, these studies suggest that microglia-like cells, transcriptionally and functionally resembling primary microglia, can be generated from a PSC-derived YS intermediary cell when cultured either with cytokines/growth factors (CSF1 and IL-34, ± TGFβ1 [15], CX3CL1, CD200 [63,65,66]) or with neurons.

hPSC-derived microglia-like cells through a monocyte/macrophage intermediate state

As described in the section "tissue-resident macrophages-niches", it might also be possible to derive microglia-like cells from definitive hematopoietic cells. Pandya *et al.,* were the first to report the generation of microglia by culturing hPSC on OP9 feeders (commonly used to model definitive hematopoiesis *in vitro*) or in a feeder-free protocol [69]. This did not generate $CD235a^{+}$ cells, but myeloid intermediate cells

positive for *CD34*, *CD43* and *CD45*. Following subsequent co-culture with human astrocytes, and a defined medium composed of serum and several different combinations of growth factors/cytokines, including SCF, VEGF, M-CSF, GM-CSF, FLT3, IL3, IL6, Activin A and BMP4, they isolated *CD39*$^{+}$ microglia (named iPS-MG)[69]. Although transcriptome studies demonstrated that the iPS-MG did not cluster very tightly together with commercially obtained human fetal microglia, high levels of expression of the six consensus human microglia genes, *P2RY12*, *GPR34*, *MERTK*, *C1QA*, *PROS1* and *GAS6* were found in iPS-MG but not in undifferentiated iPSC, macrophages, dendritic cells or the primary human fetal microglia. The hiPSC-MG displayed functional properties of microglia, such as phagocytosis of foreign particles, production of reactive oxygen species (ROS) and secretion of inflammatory cytokines. A similar protocol to differentiate microglia-like cells starting from stem cell-derived definitive myeloid progenitors was published by Amos *et al.,* [70]. An alternative protocol was described by Douvaras *et al.* [71]*,* who differentiated hPSC towards monocytes based on the Yanagimachi *et al.* publication [12]. Following isolation of *CD14*$^{+}$ and *CX3CR1*$^{+}$ "microglia progenitors", microglia-like cells were generated by culture with GM-CSF and IL-34 for 2 weeks [71]. The microglia-like cells were positive for *IBA1, CD11c, TMEM119, P2RY12, CD11b,* and *CX3CR1*. Transcriptome studies revealed that the microglia-like cells clustered together with primary human microglia and were distinct from all other macrophage subtypes. Microglia-like cells also functionally resembled microglia (cytokine profile and calcium release in response to ADP). They suggest that CD235a^{+} YS progenitors were formed, even though differentiations were done without Activin/Nodal modulation, commonly used to generate primitive hematopoietic cells [13]. We hypothesize that the microglia-like cells generated in the Pandya *et al.,*[69] Amos *et al.,*[70] and Douvaras *et al.,*[71] studies may represent transdifferentiation of monocytes to microglia reported upon microglia depletion [42,43].

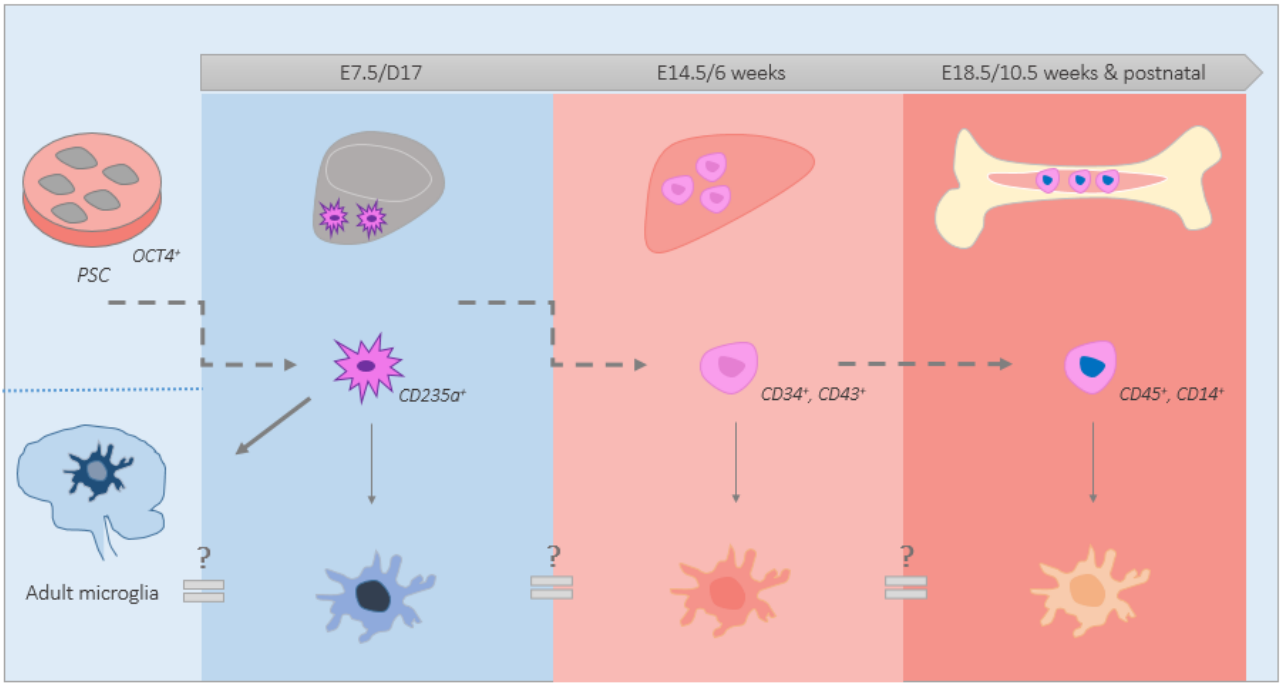

Figure 1: Different stages of embryonic hematopoiesis and the origin of pluripotent stem cell (PSC)-derived microglia-like cells: *In vivo, yolk sac (YS) progenitors colonize the brain and further mature to adult microglia up until*

birth. However, in vitro PSC (OCT4$^+$)-derived intermediates resembling either YS progenitors (CD235a$^+$), fetal liver (FL) precursors (CD34$^+$, CD43$^+$) or bone marrow (BM) monocytes (CD45$^+$, CD14$^+$) can also be further differentiated towards microglia-like cells in vitro. Although they resemble brain resident microglia to some extent, further optimization of in vitro differentiation conditions will still be required to fully recreate in vivo microglia. Different stages of the cells throughout development (murine/human) are indicated at the top of the figure.

Recreating the microglial niche to improve microglia differentiation from PSCs

As already indicated above, there is considerable evidence that the fate of postnatal tissue-resident macrophages is dictated by the niche wherein they reside. Although relatively little is known regarding brain-derived microglia, removal of the cells from the *in vivo* environment leads to very fast loss of the typical microglia gene signature [4,72]. For this reason, several of the published protocols co-cultured microglia-like progenitors/progeny with neurons, astrocytes, and / or oligodendrocytes to more closely mimic the *in vivo* environment wherein they were being fated [44,60,63,68,69]. Such co-cultures improved the microglia-like cell phenotype and functional features. A good example is the study by Haenseler *et al.,* demonstrating that co-culture of *MYB*-independent iPSC-derived macrophages with iPSC-derived cortical neurons decreased pathogen-response pathways, upregulated homeostatic function pathways, and promoted a more anti-inflammatory and pro-remodeling cytokine response than corresponding monocultures [68].

Aside from co-culture in 2D, a number of groups also addressed the question if creation of 3D cultures, encompassing neurons, astrocytes and/or oligodendrocytes, might favorably impact microglia differentiation and function. Performing 3D co-cultures might not only improve cellular interactions, but may also impact microglial function by better recreating the “soft” mechanophysical properties of brain tissue, and hence prevent activation of microglia [73]. For instance, Muffat *et al.,* created 3D co-cultures of pMGL, neurons and astrocytes. Live imaging showed rapid extension and retraction of filopodial arbor termini of pMGL branches, suggesting that pMGLs not only integrated into organotypic neural cultures but also matured into what are currently defined as resting, yet dynamically motile microglia. Of note, this magnitude of extension and retraction was not observed in 2D cultures on Primaria or glass coverslips, on which nonamoeboid pMGLs displayed only terminal ruffle movements on a much smaller scale [60]. Abud *et al.,* co-cultured hiPSC derived microglia (iMGLs) with hiPS-derived 3D brain organoids consisting of neurons, astrocytes, and oligodendrocytes [63]. They demonstrated that after 3 days of co-culture, most iMGLs had migrated into the complex organoids. By day 7, iMGLs also extended varying degrees of ramified processes within the 3D organoid environment. To determine if iMGLs could respond to neuronal injury, they pierced organoids with a 25G needle and found iMGLs that adopted a more amoeboid morphology, clustered near the injury site and, resembling ‘‘activated’’ microglia found in injured or diseased brains [74].

All these studies suggest that culture of iPSC microglia-like cells in a more complex multicellular brain environment, and this in 3D, recreating not only cell-cell interactions but also the physical properties of microglia niches, can improve maturation and prevent spontaneous activation of microglial progeny, while preserving their ability to react to injury. Obviously, once more is learned regarding the composition of the

microglial niche both during development and in the adult brain, further improvements in creating functional microglia from PSCs will be forthcoming.

FUTURE DIRECTIONS TO CREATE TISSUE-RESIDENT MACROPHAGES FROM PSCs

Although, as described in this review, efforts have been made to recreate microglia from stem cells, fewer studies have addressed generation of other tissue-resident macrophages. The fate of tissue-resident macrophages, as for any cell, is dictated by cell-extrinsic (cell-cell and cell-extracellular matrix interactions, and physicochemical properties of the cell "niche") factors as well as cell-intrinsic factors (transcriptional machinery and epigenetics). These factors are important for the generation of tissue-resident macrophages, both during development and postnatally, even if very little is currently known. Further insights in these factors will, however, be needed to generate more mature tissue-resident macrophages from stem cells.

Extrinsic Factor-driven efforts to create microglia – recreating the microglia niche

To enable creation of mature tissue-resident macrophages from hiPSCs, it will be necessary to develop culture conditions that allow maturation of progenitors *in vitro*, sustain this mature state and support their survival. This will require that culture medium is optimised and that engineering of 3D hydrogel environments where cell-cell and cell-ECM interactions, as well as mechanobiological characteristics of the developing/mature tissue of residence, are recreated *in vitro*. We hypothesize that correct supplementation of the culture medium will be obligatory. For instance, Bohlen *et al.,* showed that addition of CSF1, IL-34, TGFβ2 and cholesterol to the medium enhanced survival of highly ramified adult microglia *in vitro* [72]. Once more is learned about the microglia niche, additional chemical signals to be incorporated in the culture, will likely also be identified.

Furthermore, 3D (co-) cultures in synthetic hydrogels, e.g. based on poly(ethylene glycol) (PEG)[75], might allow enhanced survival and maturation by reconstructing the physical properties and extracellular matrix (ECM) composition of the fetal and adult brain *in vitro*. This highly tunable synthetic system will allow not only to modify mechanobiological properties of the hydrogel to resemble that of microglia niches by adjusting stiffness to 500-1000Pa, but will also allow decoration of the gels with ECM components [76-78] and signaling molecules that govern microglia fate and maintenance. Thus, synthetic hydrogels would not only support cell adhesion, but could serve as fully functionalized scaffolds to stimulate cell maturation and survival [79]. Last, single cell sequencing revealed the heterogeneity of microglia [80], therefore adjusting the environment to resemble the diseased brain might be needed to further study their altered functionality in neurodegenerative disorders such as AD [80].

Intrinsic Factor-driven: Transcriptome and epigenome guided differentiation

Acquisition of specific cell fates is driven by 'pioneer' TFs. Pioneer factors bind to regions of closed chromatin and transform them to accessible ($H3K4me1^{+}$) states [81], enabling subsequent binding of lineage-specific TFs. During blood formation, hematopoietic stem/progenitor cells differentiate into distinct lineages. Use of knockout mice and genome wide transcriptome analysis of cells of the immune system have identified candidate pioneer and lineage-specific TFs involved in the distinct lineage commitment [82]. For instance, the ETS-family TF, *PU.1* has been identified as a pioneer factor for macrophage (and B-lymphoid) development. PU.1 binds a number of chromatin remodelers (such as histone deacetylase-1 (HDAC1) and Methyl-CpG Binding Protein 2 (MeCP2)), DNA-methyl transferases (such as DNA-methyl transferase (DNMT)3a and 3b) as well as a number of TFs (such as GATA Binding Protein (GATA)-1, CCAAT-enhancer-binding protein (C/EBP) and RUNX-1 to execute both its chromatin remodeling and transcriptional activation functions. PU.1 induces a common myeloid progenitor (CMP) that further develops into megakaryocytic-erythroid progenitors (MEP) following binding of the lineage-specific TFs, GATA1 and GATA2, or to a granulocyte-macrophage progenitors (GMP) by binding of C/EBP-ε [82]. Another TF important in the regulation of lineage commitment and in myeloid cell maturation, including the decision for a CMP to differentiate into monocyte precursors, is interferon regulatory factor 8 (*IRF8*). IRF8 has been shown to co-operate with PU.1 to promote formation of promoter-distal enhancers that induce monocyte-related genes, including the critical downstream TF gene *Klf4* [83].

Genome wide transcriptome analysis has also identified TFs possibly responsible for fating of tissue-resident macrophages. Peritoneal macrophage identity is for example regulated by retinoic acid (RA) through the TF, GATA-6 [84]. For KC commitment, inhibitor of differentiation *Id3,* was shown to be important, as inactivation of *Id3* impaired development of liver macrophages and resulted in selective KC deficiency in adults [85]. In addition, induced expression of the TF *Spi-C* was observed following exposure to erythrocyte-associated heme, critical for the maintenance of the heme-metabolizing processes of splenic macrophages [86]. Langerhans cell development, on the other hand, requires transforming growth factor-β1 (TGFβ1)[87], and the TF BTB Domain and CNC Homolog 2 (*Bach2)* was reported to play a role in the function of alveolar macrophages [88]. Although less is known regarding microglia, distinct regulatory circuits have been described for early, pre-, and adult microglia, including the TFs Kruppel Like Factor 2 (*Klf2),* AT rich interaction domain 3A *(Arid3a),* Basic leucine zipper transcription factor, ATF-like (*Batf),* DNA (cytosine-5)-methyltransferase 1*(Dnmt1),* E2F transcription factor 6 *(E2f6),* Retinoid X Receptor Beta *(Rxrb),* Friend leukemia integration 1 TF *(Fli1),* Spalt Like transcription factor1 *(Sall1), Fos* Proto-Oncogene, AP-1 transcription factor Subunit *(Fos),* Early Growth Response 1 *(Egr1),* MAF BZIP transcription factor B *(Mafb),* Myocyte Enhancer Factor 2A *(Mef2a)* and *Jun* proto-oncogene, AP-1 TF (*Jun)* [89].

This knowledge combined with novel genome engineering approaches, especially the CRISPR technology [90], may now allow investigators to 'engineer' pluripotent stem cell lines to acquire tissue-specific

macrophage fates. Not only does CRISPR mediated homologous recombination (HR) in PSC allow creation of reporter cell lines to enrich for tissue-resident macrophages, it can also be exploited to drive lineage-specific differentiation. Indeed, when a dead Cas (dCas), unable to cause double strand breaks, is linked to a transactivator sequence (such as multiple herpes simplex virus Protein VP16 repeats (VP64)[91], or VP64 fused with p65, a subunit of the ubiquitous NF-kB TF complex (p65) and the Epstein-Barr virus R *transactivator (*Rta) to create the VPR [92]), activation of endogenous gene transcription is possible by a median of 150-fold [93]. When dCas is fused to a gene repressor such as Kruppel-associated box (KRAB) domain of *Kox1* [94] or four copies of the mSin3 domain (termed SID4X)[95], inhibition of gene transcription by up to 15-fold is possible [93]. By Incorporating an inducible CRSIPR-dCas-activator/repressor into a safe harbour locus such as the *AAVS1* locus [96], and/or using dCas9 orthologues from different bacteria (e.g. *S. thermophiles* (St1Cas9) and *N. meningitidis* (NmedCas9))[97] fused with a transactivator or a repressor sequence) combinatorial induction of TFs that are insufficiently expressed in PSC-derived tissue-resident macrophages, and inhibition of TFs that are incorrectly expressed (e.g. persistent pluripotency TFs, or TFs for lineages other than the desired lineage) now becomes possible. Alternatively, one could create hPSC encompassing necessary TFs under an inducible promotor, inserted in the safe harbour locus *AAVS1* [96], which might stimulate these cells to acquire a tissue-resident macrophage fate upon treatment with doxycycline and required cytokines.

With the advent of high throughput single cell RNAseq methods, it now also becomes possible to probe the expressed transcriptome at the single cell level. This does not only allow us to gain a better view on the heterogeneity of cell populations at any given time during development or in post-natal life, but also will aid in elucidating the developmental path taken, as this is important knowledge to create a specific cell type from PSCs. For instance, Amit *et al.,* recently performed PERTURB-seq, an integrated method for massively parallel single-cell RNA-seq and CRISPR-pooled screens, identifying important innate immune circuits in single cells. Such approaches will make it possible not only to elucidate TF involvement in developmental processes or specific cell fates, but also the exact function of specific TFs and their interacting partners in gene regulatory networks in a given cell at potentially all steps of development [98].

CONCLUDING REMARKS

Significant progress has been made in the creation of cells with microglia-like phenotypes from stem cells, mimicking both YS hematopoiesis or transdifferentiation from monocytes. Similar approaches could now also be used to create other tissue-resident macrophages from PSCs that closely resemble their *in vivo* counterparts. Moreover, combining novel genome engineering to recreate cell-specific transcriptional networks, and 3D-bioengineering to more faithfully recreate healthy and diseased tissue-resident macrophage niches, will further increase our ability to generate tissue-resident macrophages from any organ. Such cells would enable the study of mechanisms and genetics underlying the many diseases wherein tissue-resident macrophages are involved.

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