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Development and function of tissue resident macrophages in mice

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Abstract

Macrophages are important for tissue development, homeostasis as well as immune response upon injury or infection. For a long time they were only seen as one uniform group of phagocytes with a common origin and similar functions. However, this view has been challenged in the last decade and revealed a complex diversity of tissue resident macrophages. Here, we want to present the current view on macrophage development and tissue specification and we will discuss differences as well as common patterns between heterogeneous macrophage subpopulations.

Keywords

macrophage; erythro-myeloid progenitor; tissue specification; pulse labeling models; mononuclear phagocytes

Among the cells of the immune system, macrophages are professional phagocytes defined as mobile cells with the capacity to engulf and digest pathogens, particles and dead cells (1). Phagocytes were first described one century ago by Ellie Metchnikoff in the star fish larvae (2), and he described these cells as “the police” of the organism by fighting against invading pathogens and restricting injuries (3), but also as the “janitor” of the organism, since phagocytes ensure the “clean” removal of used epidermal pigmented cells in the star fish, thereby leading to renewal of the epithelium. Thus, phagocytes have dual functions, as immune sentinels but also as regulators of tissue homeostasis. In vertebrates, many professional phagocytes belong to innate immunity system, including neutrophils and

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mononuclear cells like dendritic cells, circulating monocytes and tissue macrophages (4). These mononuclear myeloid cells were hierarchically organized in developmental pathway named “the mononuclear phagocyte system” (MPS) in 1969, based on their similar morphology, putative common origin, overlapping immune functions and repopulation kinetics (5). According to the MPS, the most immature cells are the bone marrow promonocytes, which divide and give rise to circulating immature cells, or monocytes. Under favorable conditions and in anatomical locations where phagocytosis is needed, monocytes leave the blood stream and mature into tissue phagocytes or macrophages (5). The paradigm that circulating monocytes are the sole precursors of tissue macrophages (6–9) was challenged by results from bone marrow chimera, parabiotic mice and adoptive transfer experiments (7,10,11). Subsequent kinetic studies of the spleen macrophages led Van Furth to revise the MPS framework and he proposed a dual origin, with half of myeloid cells renewing from blood and the other half being produced locally within the spleen (12). This seminal work raised the question whether tissue resident macrophages could originate from another source than circulating monocytes and self-maintain in tissues.

Here, we will summarize the recent findings in the field of mammalian tissue resident macrophage biology and give an overview about our current understanding on the functions and the development of tissue resident macrophages, as well as their heterogeneity across various organs. The molecular mechanisms underlying macrophage self-renewal and tissue macrophage functions in response to infection and tissue injury will not be discussed, as reviewed elsewhere (13,14).

Functions of tissue resident macrophage in homeostasis

In mammals, tissue resident macrophages are found all over the body in all organs and serous membranes, which surround organs and the body cavities, like the well-studied macrophages of the peritoneal cavity (15,16). Long elongated processes or dendrites extend from their cell bodies and build up a 3D network-like structure throughout each organ (17–19), thereby allowing constant surveillance or scavenging of their tissue (20–22). In general, activation of macrophages leads to an immediate retraction of the elongated processes and a change in cell morphology (19,23,24). This can be accompanied by the migration of macrophages to the injury site or source of inflammation in their host tissue (25,26). Upon activation, macrophages actively phagocyte intruding pathogens or necrotic and apoptotic cells after tissue injury, release bio-active molecules like cytokines and chemokines and they can also serve as antigen presenting cells to activate the adaptive immune response (27).

Macrophages play homeostatic functions such as scavenging of macromolecules, debris, necrotic or apoptotic cells, and invading pathogens, clearance of senescent cells as well as the production of bioactive molecules, both concurring to tissue development. During mouse development, macrophages are involved in clearance of apoptotic and senescent cells during organogenesis, in the brain, limb and lung (28–30) and they scavenge and digest the nuclei released by maturing erythroblast (pyeroncyte) (31). Fetal macrophages are important during branching morphogenesis (such as in the mammary gland, pancreas, lung testis and kidney) (32–37) and critical regulators of blood and lymphatic vessel morphogenesis and maturation during fetal and postnatal development (32,38–40).

While macrophages have similar properties across different tissues in regards to their phagocytic abilities and trophic functions (11), they also have highly specific functions depending on their anatomical location. Tissue of residence accounts for the first source of heterogeneity in phenotype and function(s) among different tissue macrophages populations during steady-state For example bone osteoclasts, brain microglia, liver Kupffer cells or lung alveolar macrophages share common functions, however, they are also highly adapted to their organ-specific purpose: osteoclasts in the bone are important for the continuous resorption and restructuring of the bone mass (41,42), whereas microglia in the CNS are highly adapted to support the neuronal network and neuronal circuit development under steady-state conditions (28,43,44). In the liver, Kupffer cells are important for the uptake of blood particles and dying red blood cells from the circulation and iron recycling while lung alveolar macrophages are critical for the uptake of surfactant and removal of particles form the alveoli (45–47) (Table 2). This functional tissue specialization is reflected at the gene expression and epigenetic (enhancer landscapes) of different tissue resident macrophages (48) (49).

A second source of heterogeneity is found among different macrophages populations within the same organ or tissue. Beside the differences due to their anatomical location, distinct macrophage subsets within the same organ can coexist next to each other, and display different phenotypes and functions, as seen in the lung or spleen. Within the spleen, macrophages are highly diverse in phenotype and specialized function. They are found in the three regions of the spleen: the red and white pulp, as well as the marginal zone at the interface between both. Red pulp macrophages are efficient in phagocytizing aged erythrocytes that arrive with the circulating blood and they play a major role in iron metabolism (50). Marginal zone macrophages scavenge antigens and play an important role in retaining B cells in the marginal zone (51), while marginal metallophilic macrophages are known to scavenge viral antigens and release type I interferon (52). Finally, white pulp tingible body macrophages are efficient phagocytes for apoptotic B cells that are left over from germinal center reactions in the spleen (14,53).

Origin of tissue resident macrophages

Tissue macrophages are present in the developing embryo prior to the detection of circulating monocytes (54) and before the emergence of the first hematopoietic stem cells (HSCs) in the aorta-gonado-mesonephro (AGM) region (55). Since this early observation, three hematopoietic waves that can produce macrophages have been described: a first wave of “primitive” progenitors and second wave of “definitive” progenitors are generated in the extra-embryonic yolk sac, and a third “definitive” wave of macrophages from fetal and adult HSCs (56,57). While Palis and colleagues characterized two waves of YS progenitors with macrophage potential *in vitro* (58), Bertrand and colleagues identified two progenitor types that coexist and can give rise to macrophages in the mouse YS *in vivo*: monopotent macrophage-restricted progenitors and multipotent myeloid and erythroid progenitors (59). However, the contribution of the different hematopoietic waves to tissue macrophages in adulthood remained unclear.

The yolk sac (YS) origin of macrophages was first proposed by Alliot *et al* (60). In this pioneering study it was shown that microglial progenitors are found in the brain rudiment of the developing embryo around 8.0 dpc (days post coitum), shortly after the detection of first hematopoietic progenitors with myeloid potential around 7.5 dpc in the YS. These YS progenitors are capable of giving rise to microglial cells on astrocytic feeder monolayers, whereas hematopoietic progenitors isolated from the embryo proper around 9.0 dpc, failed to differentiate into microglia (60). Nevertheless, there was no direct formal proof that YS progenitors give rise to microglial cells or to other tissue macrophages in the developing embryo and adult.

To understand hematopoietic waves *in vivo*, a non-invasive pulse labelling system based on the Cre/loxP system was utilized, which used a tamoxifen-inducible MER Cre-MER recombinase gene (Cre recombinase fused to two mouse estrogen receptor sequences) under control of the *Runx1* promoter (61). This study labeled for the first time hematopoietic progenitors in the early embryo and YS, and followed these cells during development until adulthood. Tamoxifen injection at 7.25-7.5 dpc in the *Runx1*$^{MER\text{-}Cre\text{-}MER}$ strain showed that microglia derive from YS progenitors (62). It was proposed that YS macrophages invade the embryo proper and colonize the central nervous system. However, labeling was also detected in *bona fide* fetal and adult HSCs, albeit to a lesser extent, hampering interpretation of the data. Another caveat of the study is that the knock-in Runx1-MER-Cre-MER animals are heterozygous for *Runx1, and Runx1* gene-dosage is critical for definitive hematopoiesis. *Runx1* haploinsufficiency results in a dramatic change in the temporal and spatial distribution of HSCs, leading to their early appearance in the AGM region and also their ectopic emergence in the YS (63).

The respective contribution of HSCs and YS progenitors to tissue macrophages remained unclear, especially outside the central nervous system. During embryonic development, two distinct subsets of myeloid cells can be characterized in $CX_3CR1^{GFP/+}$ reporter mice (64), based on kinetics of emergence, phenotype and genome-wide gene expression profile (Figure 1). Both populations are *bona fide* myeloid populations as revealed by analysis of embryos lacking *Pu.1* (65), a master regulator of the macrophage lineage (66). CD45$^+$ CX^{3}CR1^{high} CD11b$^+$ F4/80^{bright} macrophages were detected in all embryonic tissues starting from 9.5 dpc while a second myeloid cell population, described as a CX^{3}CR1$^+$ CD45$^+$ CD11b^{high} F4/80^{low}, appeared in most tissues (except the brain) around 12.5 dpc (65), when the definitive HSC-derived hematopoiesis in the fetal liver starts and the first HSCs begin to differentiate into hematopoietic lineages. These two myeloid populations can be genetically distinguished by their dependency on the transcription factor *Myb*. *Myb* is required for HSC maintenance and self-renewal (67,68), and *Myb*-deficient embryos have a complete loss of hematopoietic progenitors in the embryo proper but not in the YS (67,69). F4/80^{low} myeloid cells in the different embryonic tissues are generated in a *Myb*-dependent manner from HSCs. In contrast, fetal F4/80^{high} macrophages developed independently of *Myb* and in the absence of fetal HSCs (Table 1) (65). The presence of “phenotypic” fetal monocytes in the fetal liver suggests the existence of definitive progenitors with myeloid potential in *Myb*-deficient embryos (70). Pulse-labeling with tamoxifen in *Csf1r*$^{MER\text{-}iCre\text{-}MER}$ animals at 8.5 dpc showed that *Csf1r*-expressing cells present in the YS generate fetal F4/80^{bright} macrophages and adult tissue resident macrophages (65). These

YS-derived F4/80^{bright} tissue macrophages are found in most adult organs and are maintained within their tissue of residency without further input from bone marrow HSC and progenitors (Figure 1) as evidenced in non-irradiation chimeras and BM progenitor fate mapping animals (Flt3-Cre) (65). Subsequent studies using parabiotic animals, genotoxic depletion of macrophages or CX^{3}CR1-based fate mapping systems confirmed the self-maintenance of tissue macrophages with no contribution of monocytic or CCR2$^+$ bone marrow progenitors (71,72) and extended them to a subset of cardiac macrophages, at least in young animals (73,74). Fate mapping models (S100A4-Cre) with high labeling efficiency in hematopoietic stem and progenitor cells in the bone marrow and circulating monocytes, but a low labeling in tissue resident macrophages from lung, spleen and brain further exclude a contribution of circulating monocytes and HSC-derived hematopoiesis as a source for tissue resident macrophages (71).

These approaches do not allow yet to conclude on the YS progenitor type (primitive or definitive) that gives rise to fetal HSC-independent macrophages and it remains to be elucidated whether all YS-derived tissue macrophages originate from the same precursor/progenitor. The YS Csf1r-expressing cells labeled with a tamoxifen pulse at 8.5 dpc in *Csf1r*$^{MER\text{-}iCre\text{-}MER}$ animals are Csf1r$^+$ AA4.1$^+$ ckit$^+$ CD45^{low} progenitors. Functionally defined as erythro-myeloid progenitors (EMPs), they first appear in the YS at the 16-18 somite pairs stage and are a common progenitor for resident tissue macrophages (70) (Figure 1). EMPs and HSCs have distinct differentiation and repopulation potentials and can be identified by distinct cell surface phenotypes (70,75). EMPs are identified as kit^{high}CD41$^+$CD16/32$^+$ cell population around 9.5 dpc (Table 1) and they develop within the YS vasculature (blood islands and remodeled vascular plexus) in a *Runx*-dependent manner (76) and not in the embryo proper, the placenta or vitelline and umbilical vessels. YS-EMPs are generated from 8.5 until 11.5 dpc and they seed the fetal liver where they expand and differentiate into macrophages, erythrocytes, monocytes, granulocytes, and mast cells (70,75). YSEMP-derived Kit+ progenitor cells and short-lived YS-EMP-derived monocytes and granulocytes are found until at least 16.5 dpc in the fetal liver, in accordance with reports that EMP-derived hematopoiesis is not only necessary but also sufficient to support survival of embryos lacking HSCs until the time of birth (77). EMPs and HSCs differentiate from distinct populations of hemogenic endothelial cells (77), and the developmental lineages of HSC-derived myeloid cells and YS-EMP-derived myeloid cells are disconnected from each other (70) (Table 1). YS-EMPs and tissue resident macrophages were only labeled in *Tie2*$^{Mer\text{-}iCre\text{-}Mer}$ mice when tamoxifen was applied before 9.5 dpc, and did not equilibrate with HSC labeling efficiency (Figure 1).

Immature macrophages present in the YS at 8.5 dpc could also be labeled in*Csf1r*$^{MER\text{-}iCre\text{-}MER}$ embryos, and as such, this pulse-labeling strategy does not allow to rule out a contribution of “primitive” macrophage progenitors to tissue resident macrophages. Brain microglia is labeled more efficiently than other tissue resident macrophages when tamoxifen is applied at E7.5 in *Runx1*$^{MER\text{-}iCre\text{-}MER}$, *Tie2*$^{MER\text{-}iCre\text{-}MER}$ and *Kit*$^{MER\text{-}iCre\text{-}MER}$ (73,80,92). These findings, together with the comparison of the labeling efficiency over time of microglia and macrophages in other tissues after a single tamoxifen pulse (62,65,70), has led some authors to hypothesize that they could originate from different YS progenitors. Therefore, more investigations are required to demonstrate

that the differences observed between microglia and other tissue resident macrophages correspond to different YS progenitor populations. Collectively, these findings suggest that (i) “primitive” progenitors can contribute to and only microglia pools or that (ii) colonization of the brain is performed only in a very limited time window from YS-EMPs, and these two hypotheses are not mutually exclusive. In favor of the latter, pulse-labeling at E8.5 only allows to label YS-EMPs in a narrow time window following the pulse, while YS-EMPs continue to emerge from the YS until at least 11.5 dpc (70,75,78) and expand in the fetal liver after colonization (70). In the central nervous system, it was shown that microglia enter the neuroectoderm between 9.0 dpc and 9.5 dpc (60,62,79), while the colonization of other tissues by macrophages is not yet fully defined.

From EMP to tissue resident macrophages

Further investigations tracking the differentiation and migration of YS-EMPs *in vivo* might be necessary to completely identify the differentiation pathway of the progenitors. It is currently not known what are the differentiation steps followed by YS-EMPs during their differentiation into mature tissue macrophages. Myeloid progenitors/precursors derived from YS-EMPs were described in *ex vivo* culturing systems (79). These intermediate progenitors seemed to have different myeloid differentiation levels characterized by gradual upregulation of mature macrophage/myeloid markers like CX_3CR1 and down regulation of immature macrophage markers like CD31 (59,79).

In regards to the migration of YS-EMPs into the embryo, most available data supports the importance of blood circulation. This hypothesis is supported by the analysis of blood-circulation-deficient animals like the cardiac Na^+-Ca^{2+} exchanger (*Ncx1)* knockout mice. *Ncx1*-deficient animals die *in utero* at 9.5 dpc due to heart failure and lack of blood circulation (80,81) and they show an accumulation of macrophages in the YS, but a decrease of microglial cells in the developing neuroectoderm (62). Similarly, YS-EMPs are not detected in the fetal liver in *Ncx1*-deficient embryos, but increase massively in the YS (75). Thus, an established blood circulation is needed for the migration of macrophage progenitors to the embryo proper. However, once in the embryo proper, the progenitors or their daughter cells do not necessarily need any established vascularization to colonize tissues, as microglia are found in the brain before the neuroectoderm is vascularized and further support angiogenesis or new vessel formation in the brain parenchyma (32).

Further work is also necessary to establish whether tissue seeding is performed by EMPs, EMP-derived myeloid precursors or by their mature progeny, the macrophages. In favor of the latter, before or prior to their entrance into the CNS, myeloid cells express mature microglial/macrophage markers like F4/80 and CX_3CR1 (60,62,79), thereby suggesting that colonization in the brain is performed by macrophages. It will also be interesting to identify the cues and factors triggering the entrance of tissue macrophages into tissues. In the case of brain colonization, Csf1 and CXCL12 are secreted from neural progenitors in the developing brain parenchyma and play a role in the recruitment of macrophages to the developing cortex in mice (32,82), while loss of vascular endothelial growth factor (*Vegf*) and classical chemokine receptor signaling does not impact brain colonization (32,79). It was recently proposed that senescent cells in the limb could be responsible for macrophage recruitment at

12.5 dpc (30), however, CX_3CR1^{gfp} $F4/80^{bright}$ macrophages are found in the limb as early as 10.5 dpc (65).

Albeit the identification of the YS-EMP as a major source for $F4/80^{bright}$ tissue macrophages, there are still many open questions concerning the development of tissue macrophages. It is not clear yet how the YS-EMP migrate from the YS to the embryo proper and how this cell differentiates into different “varieties” of tissue macrophages, each of them highly adapted to their specific function. Furthermore, the signals recruiting resident macrophages to the tissue remain unclear.

Extrinsic cues involved in maintenance of EMP-derived macrophages

It was proposed that YS-derived macrophages would be replaced late during gestation by HSC-derived fetal monocytes in the lung, skin and gastro-intestinal tract. While available data does not yet fully support this model for the lung alveolar macrophages and epidermal Langerhans cells from young adults (83,84), it has now been clearly characterized for the lamina propria macrophages in the small intestine and colon (85,86). Within the intestine, YS-derived macrophage numbers decrease progressively until weaning, after which all lamina propria macrophages are continuously replenished by infiltrating $CCR2^{+}$ $Ly6C^{high}$ monocytes (85).

A hallmark of embryonic tissue macrophages after entrance into the tissue is that they disseminate and proliferate, resulting in a massive increase in their cell number in the developing tissue, as shown for Kupffer cells and microglia (60,87). This “burst of proliferation” is also well described for Langerhans cells after entrance in the epidermis at 18 dpc (18). The proliferation rate of Langerhans cells is stable at 5% throughout adulthood, further indicating that Langerhans cells maintain themselves in the tissue by local proliferation rather than recruitment of new progenitors, under physiological conditions of the skin (18,88). The decline of proliferation is seen for all adult tissue macrophages, and their proliferation never stops completely during adulthood. Kupffer cells labeled by injection of latex beads still show the same labeling efficiency with beads 3 months after injection and have a low mitotic index under physiological conditions (89). Microglial cells have also been shown to be a stable cell population over life time with no contribution from circulating progenitors (10,90,91) and can repopulate by local proliferation after genetic depletion (92). In the lung and heart, EMP-derived macrophages appear to be replaced during aging (70,73,74). In specific settings where tissue macrophages are depleted, such as lethal irradiation or Listeria infection, monocytes can replace tissue macrophages (92,93). However, the functional consequences of macrophage replacement for tissue homeostasis require further investigation. All these results point towards a new paradigm for tissue macrophage homeostasis, with long-lived tissue resident macrophages with a low proliferation rate and nearly no contribution of circulating progenitors. A long life span demands the maintenance of resident macrophages in the tissues and in the aging organism. Therefore, the host tissues have to provide “cues and factors” which support and help to maintain the “resident” tissue macrophage population.

One of the key factors important for the maintenance and also differentiation of myeloid progenitors is Colony stimulating factor 1 (*Csf1*). Discovered in the 1970s in the Jackson Laboratories, *Csf1*$^{op/op}$ mice harbor a spontaneous point mutation in the locus of the *Csf1* gene (94). *Csf1*$^{op/op}$ mice have severe osteopetrosis and a compromised hematopoietic compartment, especially the monocytic/macrophage lineage, where differentiated cells are severely decreased (95). The loss of differentiated myeloid cells cannot be rescued by bone marrow transplantation in *Csf1*$^{op/op}$ mice, indicating a defect in the microenvironment of the hematopoietic compartment rather than a cell-intrinsic defect of myeloid cells (96). Csf1 binds to a 165kDa surface glycoprotein which is encoded by the *c-fms* protooncogene and is expressed on nearly all murine macrophages, the Csf1 receptor (Csf1r) (97). Detailed characterization of macrophage populations in *Csf1*$^{op/op}$ demonstrated that not all tissue resident macrophages are affected by the loss of *Csf1* (98). Epidermal Langerhans cells and brain microglia remained mostly unaffected by the loss of Csf1, whereas osteoclasts are massively reduced in these animals (99,100) (Table 2). *Csf1r*-deficient (*Csf1r*$^{-/-}$) animals phenocopy the osteopetrotic pathology, as well as the reduced fertility and life span found in *Csf1*$^{op/op}$ mice, but have a severe reduction of microglia and Langerhans cells (62,101). *Csf1r* is widely expressed in the hematopoietic system, in embryonic and adult tissue macrophages, as well as bone marrow derived myeloid cells (102). Csf1 was considered to be the only ligand of the receptor, but the discrepancy between the phenotype of *Csf1*$^{op/op}$ and *Csf1r* $^{-/-}$ suggested the existence of another ligand. In a systematic functional screen, interleukin-34 (Il-34) improved monocyte viability, whereas other cell types remained unaffected (103). Il-34 binds to Csf1r and triggers downstream signaling upon binding in a similar manner as known for Csf1. *Il-34*-deficient mice have reduced numbers of microglia and Langerhans cells while other tissue macrophage populations are unaffected (104,105) (Table 2). Il-34 is released by “stromal” cells, such as neurons in the CNS and keratinocytes in the epidermis. Both ligands, Csf1 and IL-34 share the same receptor and it is not yet clear how and if they can trigger different activation cascades upon Csf1r binding. A recent study revealed that Il-34 and Csf1 are structurally similar but do not share a common sequence (106). Both can support the survival and maintenance of myeloid cell lines *in vitro*, but they trigger different activation responses in regards to cytokine release. IL-34 induces a stringer activation of the signaling cascade downstream of Csf1r, but this activation lasts much shorter compared to Csf1 binding (107). Further investigations are needed to elucidate if Csf1 and IL-34 play completely independent functions on tissue resident macrophages or if their effects are complementary. Yet, it remains unclear why tissue resident macrophages are dependent on one of these two Csf1r ligands depending on their tissue.

Depending on the tissue of residence, a complexity of secreted factors from the surrounding cells maintains the macrophage pool. Transforming growth factor beta (*TGFβ*) was long known to differentiate hematopoietic stem cells and bone marrow myeloid progenitors into cells with a Langerhans cell-like phenotype *in vitro* (108). Mice deficient for TGFβ have an epidermis completely devoid of Langerhans cells even before the autoimmune phenotype is established in these animals (109). Deletion of the TGF beta receptor (*Tgfbr*) in epidermal Langerhans cells led to a reduced number of Langerhans cells during the first week of life, albeit the initial seeding of the epidermis at birth was normal. It was proposed that Langerhans cells leave the epidermis and adopt a migratory phenotype without TGFβ signal.

These studies indicate that TGFβ is not important for the colonization of the epidermis with tissue resident macrophages, but rather important to maintain the macrophages in a resting state within the epidermis (110) (Table 2).

Among environmental factors shaping tissue macrophages at steady-state, products of intestinal commensals could contribute to tissue macrophage maintenance and function in the mouse brain. Housing of mice under complete sterile conditions (also termed 'germ-free') leads to a pronounced immature phenotype of microglia, including upregulation of several surface proteins such as F4/80 and increased numbers in different brain regions, in line with altered expression of genes regulating the cell cycle (111). Notably, such macrophage alterations were found to be extremely plastic in adult mice, as eradication of intestinal bacteria by antibiotic treatment drives microglia to acquire an immature status. Vice versa, recolonization of mice harboring a reduced flora with complex microbiota leads to microglia 'maturation', indicating a great plasticity of the gut-microglia connection (112).

Transcription factors in tissue macrophage development and specialization

Various transcription factors have been identified in myeloid cell development and as discussed above, most efforts were focused during the last 20 years in describing the detailed development of myeloid cells in the bone marrow (113–117). While the detailed differentiation steps of tissue macrophages from their YS progenitor are not yet fully understood, transcription factors important for tissue specific macrophage differentiation and diversification are starting to emerge (48).

The most studied transcription factor in macrophage differentiation is Pu.1 (*Spi1, Sfpi1*). Pu. 1 belongs to the *ets* transcription factor family, which is characterized by the ETS DNA binding domain (118). Expression of Pu.1 is restricted to hematopoietic cells (mostly B cells and myeloid cells). Animals deficient for the transcription factor Pu.1 show multiple defects in hematopoiesis and die either prenatally or a few days after birth (66,119). Further analysis of the hematopoietic defects by McKercher *et al* revealed a loss of B cells and mature myeloid cells, but normal erythrocytes and megakaryocytes, which revealed a key role of Pu. 1 in myeloid differentiation and the development of myeloid progenitors. *Pu.1*-deficient HSCs have a homing and maintenance defect in the fetal liver and *Pu.1*-deficient embryos can be rescued by wild-type HSC transplantation *in utero*. *Pu.1*-deficient mice completely lack tissue resident macrophages from bone marrow and YS origin (65) but an earlier report found *Csf1r*-expressing cells but no F4/80$^+$ cells in the absence of *Pu.1* (120). However, the activity of Pu.1 is not an "on-or-off" signal. High concentrations of Pu.1 trigger development of macrophages, whereas low levels favor B cell development (121). Gradients of Pu.1 are also involved in the lineage decision of myeloid progenitors, where low levels of Pu.1 lead to the development of granulocytic progenitors and high levels of Pu.1 trigger macrophage differentiation (122). Pu.1 also plays a role beyond myeloid differentiation, in mature macrophage functions. *In vitro* knockdown of Pu.1 in bone marrow derived macrophages leads to a reduction in proliferation, which is associated with downregulation of *Csf1r* on the cell surface (123). Pu.1 is not only able to regulate macrophage-specific genes like *Csf1r*.

Chromatin immunoprecipitation (ChIP) for Pu.1 reveals its association with binding sites all over the genome in adult macrophages (124). Pu.1 binding to the enhancer motifs or promoter regions in macrophages is most often associated with monomethylation of lysine 4 in histone 3, which leads to an opening of the chromatin structure at these positions and allows further recruitment of a secondary set of transcription factors, for example liver X receptors (LXRs) or Nfκb. Therefore, Pu.1 is also important in mature macrophages to modulate the chromatin landscape and orchestrate other transcription factors to their binding sides, which might be induced by external stimuli and are important for the functional properties of macrophages (125).

Whereas Pu.1 activity is essential in all tissue macrophages for their development and for their function, other transcription factors are found to play a role in specific subsets of tissue macrophages. Another member of the Ets transcription factor family is Spi-C (*Spic*). Spi-C was initially found in a yeast-two hybrid-screen with a cDNA library of LPS-stimulated B cells and high expression was described in mature B cells, whereas macrophages only expressed lower levels of Spi-C (126). However, Spi-C is highly expressed in one specific subset of tissue resident macrophages, the red pulp macrophages (RPMs) of the spleen. This specialized subset of macrophages is important for the phagocytosis of old erythrocytes from the blood stream and to maintain iron homeostasis. *Spic*-deficient mice showed a selective loss of RPMs in the spleen, whereas B cells and other myeloid cells like monocytes and dendritic cells were not affected. Furthermore, these mice showed a disturbance in iron homeostasis with an accumulation of iron specifically in the red pulp of the spleen, indicating the importance of RPMs for maintaining iron homeostasis in this organ. Additional studies confirmed the tissue-specific expression and significance of Spi-C for homeostasis (48,127). Spi-C is a transcription factor crucial for the development of one single subset of tissue macrophages and further indicates that myeloid cells adapt specified transcriptional programs after they reach their organ of residency. This indicates that tissue macrophage subsets acquire a specified transcriptional program after reaching the target tissue to develop specific properties required in these organs.

Peritoneal macrophages are one of the best-studied tissue macrophages over the last decades. Two functionally distinct macrophage subsets are found in the peritoneal cavity: large peritoneal macrophages (LPMs) and small peritoneal macrophages (SPMs), with LPMs largely outnumbering SPMs under physiological conditions (16). Both subsets express the macrophage markers CD11b and F4/80 and phagocytize bacteria injected *in vivo*. However, LPMs are more efficient than SPMs in apoptotic cell clearance (128) and both subsets show a differential expression of other surface markers like MHC class II and respond differently to various stimuli *in vitro* (16). Several studies suggest that LPMs are derived from an embryonic source and are not maintained by monocytic turn over (72,129). Beside functional differences, C/EBPβ (*Cebpb*) was found to be highly expressed in LPMs and at a lower level in SPMs. *Cebpb*-deficient mice show a massive decrease in LPM generation, but show also in lung alveolar macrophages (128). Transplanted *Cebpb*-sufficient SPMs into *Cebpb*-deficient mice can differentiate into LPMs in the peritoneal cavity, but this differentiation was not observed after transplantation of SPMs into wildtype animals, which suggests another differentiation pathway for LPMs under steady state conditions. While C/EBPβ is important in general for monocyte and macrophage activation

upon different stimuli. C/EBPβ is also a transcription factor that controls the differentiation and function of these two specific subsets of tissue macrophages.

Several recent studies further characterized the macrophage compartment of the peritoneal cavity. Two independent studies identified GATA-6 as a major transcription factor inducing peritoneal macrophage specific gene expression in LPMs (130,131). GATA-6 is a zinc finger transcription factor involved in different lineages from mesoderm during embryonic development like precardiac mesoderm or the primitive gut (132). GATA-6 is highly and specifically expressed in LPMs, whereas SPMs and other tissue macrophages do not express it (130,131). *Gata6*-deficiency in myeloid cells leads to reduced LPMs numbers, altered gene expression and loss of proliferation capacities in these cells. *Gata-6*-deficient LPMs failed to regulate peritoneal B1 cells (130), which are the main producers of IgM antibodies and continuously migrate to the lamina propria of the gut to give rise to IgA producing B cells (133–135). These two studies concluded that Gata-6 links peritoneal-specific identity (gene expression profile), function (regulation of peritoneal B1 cells) and renewal (proliferation) of LPMs.

Tissue-specific adaption of macrophages seems to occur in nearly each tissue, at the phenotypic, genetic and epigenetic levels (Table 2). To understand how the tissue environment and the different genetic programs of macrophages could lead to unique macrophage identities and specific functions in each tissue, two studies analyzed different subsets of tissue macrophage populations in different sets of high-throughput sequencing data, like RNA sequencing (RNA-seq, transcriptome) or chromatin immunoprecipitation sequencing (ChIP-seq, chromatin structure and modifications). Tissue resident macrophages revealed distinct gene expression patterns depending on their tissue of residence and they have different enhancer landscapes, especially active enhancers (48,49). As expected from their different developmental and cellular origin, resident tissue macrophages have a distinct chromatin landscape signature from bone marrow-derived myeloid cells like monocytes and neutrophils (48). Among tissue macrophages, there was a striking variability in active enhancers depending on the tissue of residency. Bone-marrow-derived macrophages, which can repopulate various tissues after tissue macrophage depletion by irradiation and bone marrow transplantation, can adopt a tissue-specific chromatin landscape similar to host macrophages. When transplanted into the alveolar cavity, peritoneal macrophages “adopt” a lung-like phenotype and gene expression profile. Based on these, tissue environment was proposed to be responsible for the differences in gene expression and cell identity between macrophages in different tissues. Nevertheless, tissue microenvironment alone cannot account for the diversity of macrophage identities within each tissue. Within the peritoneal cavity, two subsets of macrophages co-exist, the large peritoneal macrophages and small peritoneal macrophages. These two macrophage populations are thought to share the same origin and are located in the same anatomical structure, however their chromatin structure and the enhancers they use are quite different (49).

Collectively, both the developmental pathway and the tissue microenvironment contribute to create a unique chromatin structure in each adult macrophage subset, which is highly adaptable depending on the physiological status and the organ. It will be most interesting to investigate whether adult tissue macrophages bear in the chromatin landscape marks from

their common origin and whether they carry a “basic” genetic program that persists until adulthood.

Summary and Outlook: What are the missing links in the macrophage puzzle?

In the last years, many studies using new genetic mouse models and high-throughput gene expression analyses have provided insights into how macrophages develop and that their heterogeneity can be partially driven by their tissue environment, respectively. Albeit many questions are still open, it is now demonstrated that most tissue resident macrophages are generated from yolk sac progenitors but do not develop or renew from blood monocytes and hematopoietic stem cells. Many challenges lie in the identification of the missing steps in their developmental pathway(s) and its transcriptional control, as further differentiation steps as well as migratory pathways of macrophage precursors in the developing embryo remain undefined. Our knowledge on macrophage specialization to their host tissues has dramatically increased, especially in terms of their intrinsic and extrinsic (epi)genetic programs playing a role in this process in different tissues. However, our understanding of how all these different layers are integrated to give rise to the newly defined heterogeneity of resident macrophage is still limited, in particular when addressing macrophage functions. Because macrophages were most often considered to have activation properties and functions specific for the stimulus but independent of their location, macrophage functions in homeostasis and disease were transposed from one tissue to another. It is thus an exiting scientific time to reinvestigate macrophage functions more carefully, in regards to their developmental origin and their cell-intrinsic tissue specialization, to identify the environmental factors supporting or driving tissue macrophage specialization and maintenance, and to characterize their contribution to tissue homeostasis and function, but also to disease pathogenesis and tissue repair, distinguishing them from infiltrating monocyte-derived macrophages in inflamed tissues.

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Highlights

-Macrophage subsets are heterogeneous in their developmental hematopoietic origin

-Erythromyeloid progenitors give rise to tissue macrophages persisting until adulthood

-Tissue macrophages have homeostatic functions depending on their anatomic location

-Tissue adaption of macrophages occurs on phenotypic, epigenetic and genetic levels

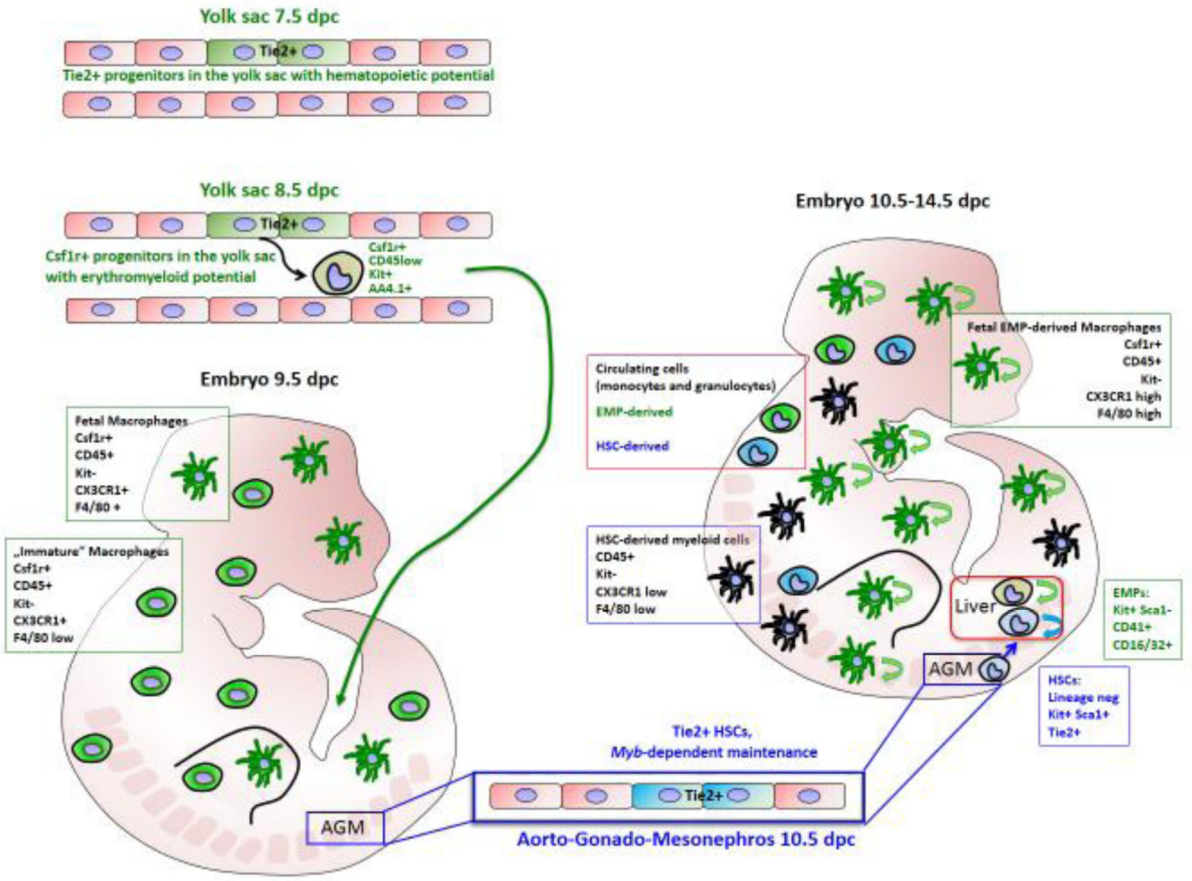

Figure 1. Embryonic development of resident tissue macrophages from erythromyeloid-progenitors in the yolk sac

The first progenitors of tissue resident macrophages arise from Tie2^{+} hemogenic/endothelial progenitors in the blood islands of the yolk sac (YS) as early as 7.5 days post coitum (dpc). These Tie2^{+} progenitors give rise to Myb-independent erythromyeloid progenitors (EMP), which are first detectable around 8.5 dpc in the YS and characterized by the expression of Csf1r. The EMP migrates to the embryo proper and gives rise to resident tissue macrophages, which are seeding embryonic tissues from 9.5 dpc onwards. These EMP-derived tissue macrophages actively proliferate within their developing tissues. Beside the EMP-derived tissue macrophages, there is another wave of myeloid cells developing after Myb-dependent hematopoietic stem cells (HSCs) emerge in the embryo starting at 10.5 dpc in the AGM (aorta-gonado-mesonephro) region, and their myeloid progeny can be detected between 10.5 and 14.5 dpc. In contrast to the proliferating self-renewing tissue macrophages, these cells are renewed by differentiation from proliferating HSCs in the fetal liver.

Table 1

Comparison of YS-EMP-derived tissue macrophages and HSC-derived myeloid cells/macrophages

	Myb-independent tissue macrophages	**HSC-derived myeloid cells/macrophages**
Progenitor	erythromyeloid progenitor (EMP)	hematopoietic stem cells (HSC)
Progenitor surface phenotype	$CD45^{low}$ Kit^{+} $AA4.1^{+}$ $CD16/32^{+}$ $CD41^{+}$ $Sca1^{neg}$	LSK-SLAM (Lin^{neg} $Sca1^{+}$ $Kit^{+}CD150^{+}CD48^{-}$)
Hematopoietic niche	Yolk sac/fetal liver	AGM region and large embryonic arteries/ fetal liver; Bone marrow
Macrophage Surface markers	$F4/80^{high}$ $CD11b^{low}$	$F4/80^{low}$ $CD11b^{high}$
Common markers (gene expression pattern)	Csf1r, CX_3CR1	CCR2, Flt3
Maintenance in the adult	Local self-renewal, proliferation, depending on Csf1r-signaling (Il-34 or Csf1)	Replacement by circulating HSC-derived progenitors
Transcription factors for differentiation	Pu.1, Runx1	Pu.1, Runx1, c-Myb, Gata-2 (?)
Transcription factor for tissue adaption	Different factors depending on tissue (see table 2)	Not described yet
Physiological function within tissues	Tissue homeostasis, removal of apoptotic cells and secretion of growth factors, stem cell survival	Not described yet
Function under challenge within tissues	Tissue inflammation, phagocytosis of dead cells and pathogens, antigen presentation Tissue repair, phagocytosis	Tissue inflammation, secretion of pro-inflammatory cytokines, antigen presentation

Table 2

Diversity of EMP-derived tissue macrophages in adulthood

	Brain	Epidermis	Liver	Lung	Spleen	Peritoneum	Heart
Surface markers	F4/80^{+} CD45^{low} CD11b^{+} CX$_3$CR1^{+} Siglec-H^{+} P2YR12^{+}	F4/80^{bright} CD45^{+} CD11b^{+} Epcam^{+} MHCII^{+}	F4/80^{bright} CD45^{+} CD11b^{+} Tim4^{+} MHCII^{+}	F4/80^{bright} CD45^{+} CD11b^{low} Siglec-F^{+} CD64^{+} CD11c^{+} CX$_3$CR1^{gfp}	F4/80^{bright} CD45^{+} CD11b^{low} CD68^{+} CD64^{+} MHCII^{+} CX$_3$CR1^{+}	F4/80^{bright} CD45^{+} CD11b^{+} Tim4^{+} MerTK^{+}	F4/80^{bright} CD45^{+} CD11b^{+} CD14^{+} MerTK^{+} CX3CR1^{+} MHCII^{+}
GF requirements	CSF1, IL-34, TGFβ	CSF1, IL-34, TGFβ	CSF1	GM-CSF, CSF1	CSF1	CSF1	CSF1
Transcription factor for tissue adaption	Mef2c Smad Irf8	Id2 Runx3	Lxr-α Rxr-α	Jun PPARγ C/EBPβ Bach2	Spi-C C/EBPβ	Gata6 RAR-α/β C/EBPβ	Not known
Physiological function within tissues	removal of apoptotic cells						
	Synaptic pruning; Immune quiescence	Tolerance induction	RBC recycling; Hemoglobin clearance and heme degradation	Surfactant removal; Clearing of inhaled particles	RBC recycling; T reg differentiation	Maintenance of B1 cell pool in the gut	Angiogenesis Fibrosis Immune quiescence Maintaining the cardiac stem cell niche
Function under challenge within tissues	phagocytosis of dead cells and pathogens						
	Cytokine release to recruit further immune cells and blood-brain barrier break down;	Antigen presentation in draining lymph nodes	Cytokine release to initiate of acute phase proteins	Immune suppression in asthma	Type I interferon production against parasites	Clearing Nematode infection; NO production	Angiogenesis; Fibrosis