Title: Specialized functions of resident macrophages in brain and heart

Summary sentence: Ontogeny and tissue specialized functions of microglia and cardiac macrophages and their role in heart and brain homeostasis and inflammation

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Abbreviations

Cardiac tissue macrophages (cTM)

Milk fat globule-EGF factor 8 protein (MFGE8)

Central Nervous System (CNS)

Erythro Myeloid Progenitors (EMP)

Subventricular Zone (SVZ)

Rostral migratory stream (RMS)

Phosphatidylserine (PS)

Myocardium infarct (MI)

Experimental autoimmune encephalitis (EAE)

Neural stem/progenitor cells (NSPC)

Alzheimer's disease (AD)

Amyotrophic Lateral Sclerosis (ALS)

Disease-associated Microglia (DAM)

Atrioventricular Node (AVN)

Abstract

The functions of macrophages in healthy tissues extend beyond their well-established roles as immune sentinels and effectors. Among tissues, cells of the brain and heart possess unique excitatory properties that likely demand special support. Accordingly, existing evidence demonstrates that microglia in the brain has an active role in synaptic organization, control of neuronal excitability, phagocytic removal of debris and trophic support during brain development. In the heart, recent studies suggest that cardiac macrophages are involved in the regulation of heart homeostasis by phagocytosis, production of trophic and immune-related factors, and by forming direct contacts with cardiomyocytes to regulate electrical conduction. In this review, we discuss mechanisms associated with the high degree of specialization of resident macrophages in both tissues, their origin and heterogeneity, and their contributions in regulating homeostasis under steady-state and pathological conditions.

Introduction

Most mammalian systems and tissues contain a complex network of cells endowed with specialized roles that work together during the organism's lifetime to ensure its correct functioning. In order to optimise their performance during this long-lasting relationship, some tissues such as the hematopoietic compartment in the bone marrow and the intestinal epithelium continuously self-renew through the action of dedicated stem cell lineages. Other tissues, such as the liver, are normally quiescent in the adult but can robustly activate after injury to replenish lost cells. A third group of tissues that includes the heart and the brain, do not regenerate well after injury leading to scaring rather than true tissue regeneration. In addition, these two tissues contain highly differentiated excitatory cells (cardiomyocytes and neurons) that do not re-enter the cell cycle in adult animals [1]. Both neurons and cardiomyocytes form excitatory networks with important anatomical constrains: in the case of the heart, organization of cardiomyocyte into packed functional networks ensures rhythmic contraction and the adequate propagation of electrical impulses along conduction pathways to pump blood. In the brain, axonal projections from different neuronal subtypes propagate electrical impulses to specific anatomical locations, which allows transmission and integration of signals received from the environment. It is therefore likely that neurons and cardiomyocytes require special support from other tissue-resident cells. Among these resident cells, heterogeneous populations of macrophages have emerged as a versatile toolkit that accomplishes multiple tasks in virtually every tissue [2], and these macrophages have been shown to be necessary for normal tissue function.

Tissue-resident macrophages colonize the brain and the heart early in development and accumulating evidence indicates that their activity extends beyond their well-established roles as immune sentinels and effector cells. For example, microglia has an active role in synaptic organization [3, 4], control of neuronal excitability [5], phagocytic debris removal [6], and trophic support for brain protection and repair [7]. In the case of the heart, functional data examining the role of cardiac tissue macrophages (cTM) in adult heart homeostasis is still limited; however, gene expression profiling and recent functional experiments reveal surprising roles for cTM in the healthy heart. These processes include clearance of tissue debris [8], electrical conduction [9], angiogenesis [10], fibrosis [11, 12] and maintenance of immune quiescence [12, 13]. We review here the functions of microglia and cTM during CNS and heart homeostasis, and focus on relevant properties of these cells: their origin, phagocytic and trophic functions, and other highly specialized roles in each tissue.

Ontogeny of Microglia and Cardiac Macrophages

The last decade has revolutionized our understanding of macrophage development, primarily by challenging the notion that tissue macrophages are monocyte-derived. Fate-mapping studies demonstrated that a significant number of macrophages are derived from primitive precursors that arise in the yolk sac [14, 15]. These macrophages or their progenitors seed tissues before birth and proliferate to populate the growing tissues, with negligible or only minimal dependence on circulating monocytes [14, 16]. Microglia has a very low rate of replacement in steady-state in adult animals as evidenced by transplantation, parabiosis and lineage-tracing studies, and it is considered as a prototypical example of a yolk sac-derived macrophage with minimal dependence on adult hematopoiesis [14, 15, 17, 18] (**Figure 1**). Similarly, most macrophages in the spleen, peritoneum, liver, and lung appear to be maintained by embryonic precursors that take residence in tissues before birth, independently of adult hematopoiesis [14, 15, 19]. cTM have been proposed to also belong to this type of macrophages [20], although there is some controversy as discussed below. Other adult tissues, including regions of the skin and intestine, continue to be colonized by monocytes through adulthood, such that these tissue-resident macrophage populations are ultimately derived from adult hematopoiesis [21, 22]. We discuss here the ontogeny and specification of microglia and cTM.

Microglia: origin and environment

Different populations of myeloid cells have been described to inhabit the CNS. These populations include non-parenchymal macrophages such as perivascular, subdural meningeal and choroid plexus macrophages, and by parenchymal myeloid cells mainly formed by microglia [23]. Microglia respond to various brain insults, adopt activated phenotypes and secrete a range of cytokines and neurotrophic factors to modify disease progression [24]. Microglia, like other tissue-resident macrophages, originate from Erythro Myeloid Progenitors (EMPs) that emerge in blood islands in the yolk sac early during development, and gain their microglia-specific transcriptional profile only upon migration into the CNS [16-18, 25]. In humans, microglial cells can be identified in the extracerebral mesenchyme as early as 4.5 gestational weeks and invade the parenchyma at around the fifth weeks [26, 27]. In rodents, microglia precursors defined by expression of CSF1R and CD45 but not cKit ($CSF1R^{+}CD45^{+}c\text{-}Kit^{-}$) start seeding the neuroectoderm as early as embryonic day 9.5 [18]. Following CNS colonization and establishment of its cellular identity, microglial cells self-renew stochastically in the

healthy brain and expand clonally during pathology [28] (**Figure 1**). An elegant demonstration of the embryonic origin of microglia and its potential implications in human neurodegenerative disease was the observation that mosaic expression of the BrafV600E allele in the resident macrophage lineage during early embryonic development (E8.5) gives rise to ERK-activated microglia that causes neuronal loss and late-onset neurodegeneration [29].

Although microglial cells share expression of several important genes with other mononuclear phagocytes, they have a unique signature defined by genes such as *P2ry12* and *P2ry13*, *Tmem119*, *Gpr34*, *Siglech* or *Trem2* [30]. In addition to intrinsic signals, the local environment is a key influence in shaping the microglial phenotype Transcriptional and epigenetic studies have shown that microglia gene signature is not as dependent on individual factors such as age, sex or disease status as it is on the correct environment with its identity-defining factors PU.1, IRF or AP-1. These findings suggest a model by which microglia establishes its identity and function through intersecting developmental and environmental networks [31].

The CNS microenvironment displays important variations in neuronal subtypes, neurotransmitter profiles, hemodynamics and type of metabolism that likely influences the local microglial phenotype. In this regard, a genome-wide analysis of microglia from discrete brain regions found that microglial cells have distinct regional identities [32]. Specifically, expression profiles in the cerebellum and hippocampus are relatively distant from those of the cerebral cortex and striatum, which share a closer relationship. Interestingly, while the cerebellar immunophenotype become more differentiated with age, differences in the hippocampal phenotypes dissipate [32]. Microglia residing in the Subventricular Zone (SVZ) and the adjacent rostral migratory stream (RMS) also comprise a morphologically and antigenically distinct subset. For example, SVZ/RMS microglia is clearly distinguishable by low expression of purinoceptors and lack of ATP-elicitable chemotaxis [33].

Regional variations in microglia density have also been reported. Highly populated areas include the hippocampus, olfactory telencephalon, basal ganglia and substantia nigra [34]. Moreover, *in vivo* fate mapping to visualize the proliferative dynamics identified high proliferation frequencies in the olfactory bulb, hippocampus and cerebellum, a finding that has challenged the prevailing concept of microglial longevity, which considers microglia as long-lived cells with an overall low turnover rate [28].

It is likely that the “sensome”, the set of genes used by microglia to sense their environment, could be responsible for the transduction of signals that mediate this phenotypic and functional variability. The sensome comprises around 100 genes that allow microglia to sense chemokines and cytokines, purinergic molecules, inorganic substances, changes in pH and amino acids [30]. Moreover, microglia express numerous types of neurotransmitter receptors [35], a finding that suggests that interneuronal communication can also have indirect effects on the neighbouring microglia that surround the active synapse.

Ontogeny of cardiac macrophages

The heart is composed of multiple cell types including cardiomyocytes, cardiac fibroblasts, endothelial cells, smooth muscle cells, and immune cells, which constitute around 10% of non-myocytic cells in the heart [36]. GFP expression in CX3CR1$^{GFP/+}$ reporter mice allowed visualization of cTM in the normal myocardium, where they account for a large fraction of total leukocytes [13]. To date, cTM heterogeneity has been proposed based on the expression of different markers and on their ontogeny. Although the extent of cTM heterogeneity remains incomplete, most of these cells express *bona fide* tissue macrophage markers such as F4/80, CX3CR1, CD68 and CD64 [13, 20, 37]. cTM are interspersed between cardiomyocytes, in perivascular areas [13], and in the epicardium [38]. The initial seeding of fetal yolk macrophages to the developing heart is through the mesothelium, a structure within and just below the epicardium. Accordingly, epicardial ablation results in defective macrophage settlement in the heart [39].

The ontogeny of cTM has been addressed by genetic fate mapping and parabiosis [20, 40, 41]. Lineage-tracing analysis revealed that primitive macrophages originating from yolk sac progenitors entered the heart via FLT3-independent pathways as early as E9.5–E10.5. Genetic labelling at E8.5 using *Runx1*^{CreER} or *Csf1r*^{CreER} models showed that significant numbers of these cells persisted well into adult life [20]. A second wave of macrophages originating from fetal liver hematopoietic progenitors appears in the heart through a mix of FLT3-dependent and independent mechanisms at late developmental stages [20]. Similar results were obtained using CX3CR1-induced labelling [41]. These studies showed that early yolk-sac-derived macrophages persist longer in the heart than in other tissues, suggesting a largely embryonic origin for these cells in the adult. After birth, cTM expand and are maintained by local proliferation [20, 38, 40]. This is consistent with studies showing that the contribution of blood monocytes to the resident macrophage pool is minimal even after 4-8 weeks in parabiosis [20, 40, 41]. The interpretation of these results varies between groups: some support the idea of the

cardiac pool as a closed, embryonically derived system with low contribution of adult hematopoiesis [20, 40], while others interpret it as a progressive substitution of embryonic derived macrophages by monocyte-derived macrophages with age [41]. Supporting the latter, both circulating monocytes as well as residual cTM contribute to repopulate the heart after severe inflammation [41], angiotensin II infusion, sub-lethal irradiation or depletion of cTM [20]. Interestingly, self-renewal of the embryo-derived cTM pool declines with age, and may allow progressive replenishment by circulating monocytes, even in the absence of injury [41]. Additional studies will be needed to sway the weight of evidence one way or the other (**Figure 1**).

Like in the brain, various populations of macrophages reside in the myocardium. Using a combination of flow cytometry, tissue immunostaining, and genetic lineage tracing, Leid et al. showed that the developing heart contains at least two macrophage subsets that can be subdivided on the basis of expression of the chemokine receptor CCR2 [10], which are similar to populations found in the adult [20]. In the embryo, CCR2-negative ($CCR2^-$) macrophages overexpress transcripts previously associated with yolk sac–derived and resident macrophage subsets (e.g., *Cx3cr1*, *Lyve1*, *Emr1*, *Cd207*, and *Ccl12),* while the CCR2-possitive ($CCR2^+$) subset shows increased expression of monocyte-derived macrophages transcripts, including *Ly6c*, *Cxcr2*, *Sell*, *Irf5* and *Nr4a1* [10]. During development, $CCR2^+$ and $CCR2^-$ cTM occupy different locations, with $CCR2^-$ cells residing predominantly in the myocardial wall and $CCR2^+$ mostly found in trabecular projections of the endocardium [10]. Interestingly, these subsets appear to be functionally specialized; $CCR2^-$ macrophages are recruited to coronary vessels at the onset of perfusion and participate in remodelling of the coronary plexus through selective expansion of the perfused vasculature, and are therefore important for normal cardiac development. In contrast, $CCR2^+$ macrophages are dispensable for this process [10].

Together, these data argue that cTM exist independently from blood monocytes in the steady-state and self-renew through *in situ* proliferation with low monocyte input, with an estimated turnover of about one month [40]. When cTM are depleted, they preferentially replenish through both local expansion and monocyte replacement to differing degrees. This shift in the source of cardiac macrophages through development, disease and aging is biologically intriguing and discussed below.

Phagocytosis by microglia and cardiac macrophages in tissue homeostasis

The word “macrophage” derives from the Greek *“*makros*”* (large) and *“*phagein*”* (eater). True to their name, macrophages are highly phagocytic, as first discovered by Elie Metchnikoff in 1882. While macrophage ontogeny and activity are highly heterogeneous [42], all macrophages participate in the clearance of tissue debris as well as the detection of pathogens and tissue damage. Many of the homeostatic roles of macrophages rely on their ability to phagocytose. Specifically, microglia-mediated phagocytosis plays an important role in brain development, synaptic pruning and in the maintenance of the neurogenic niche. In the case of cTM, their phagocytic activity has been associated with debris clearance and immune defense. Specific mechanisms of phagocytosis will not be discussed here unless relevant, and the reader is referred to recent reviews on this topic [43].

Microglial phagocytosis

Synaptic pruning: Microglia are the brain’s professional phagocytes. They can remove dead and dying neurons as well as synapses and cellular processes of live neurons. Two-photon imaging of GFP-labelled microglia showed that the termini of microglial extensions are highly dynamic in the intact mouse cortex [44] Multiple components of the neuronal synapses are a major target of microglia surveillance, and interactions of microglia and synapses have been described in several settings [3, 45, 46]. Using models with fluorescent-labelled neurons and microglia, Wake and colleagues demonstrated that cellular extensions from resting microglial cells make brief contacts (approximately 5 min) with neuronal synapses at a frequency of about once per hour, which are prolonged following cerebral ischemia [46]. In the visual cortex, physical contacts between microglia and dendritic spines were demonstrated to change morphology, display phagocytic structures and to envelope synapse-associated elements during visual experience, suggesting that microglia actively contribute to experience-dependent modification or elimination of a specific subset of synapses in the healthy brain [45]. Super-resolution imaging demonstrated that post-synaptic density protein-95 (PSD95, now known as DLG4) was present within microglial processes in the mouse hippocampus during normal development, suggesting that microglia can uptake pre- or postsynaptic material [3]. Consistent with a role of microglia in this process, mice lacking the chemokine receptor CX3CR1, which exhibit a transient reduction of microglia during the early postnatal period, presented a deficit in synaptic pruning [3]. This defect was later associated with weak synaptic transmission, decreased functional brain connectivity, deficits in social interaction and increased repetitive-behavior phenotypes [47], establishing a link between microglia and neurodevelopmental disorders.

Complement proteins C1q and C3 mediate the specific interactions of microglia with neuronal synapses [4]. Complement proteins are localized in developing CNS synapses during periods of active synapse elimination and are required for normal brain wiring [48]. Seminal studies on the formation of synaptic connections in the retinothalamic system during early postnatal development identified the complement protein C1q or the downstream complement protein C3 as important mediators of synapse elimination [49]. Phagocytosis of the developing synapses tagged with complement proteins was mediated through the complement receptor 3 (CR3, also known as Mac-1), and consequently disruption of microglia-specific CR3/C3 interactions resulted in sustained deficits in synaptic connectivity [4]. Retinal transforming growth factor (TGF)-β has been proposed as a key regulator of neuronal C1q expression because mice lacking TGF-β receptor II (TGFβRII) in retinal neurons present reduced C1q expression in retinal ganglion cells together with a reduced synaptic localization of complement proteins, which phenocopy the synaptic defects observed in complement-deficient mice [50].

In addition to C3, CR3 also recognizes various types of neuroinflammatory stimuli such as lipopolysaccharide (LPS), β amyloid, high-mobility group box 1 (HMGB1) or α-synuclein, all of which can cause neurotoxicity. Interestingly, activation of microglial CR3 by LPS and hypoxia triggers long-term synaptic depression in the hippocampus, an effect that may contribute to memory impairments and synaptic disruptions in neuroinflammation-related brain disorders [51]. In addition, inappropriate activation of the complement-dependent pathway has been proposed to mediate synapse loss in experimental models of Alzheimer's disease (AD) [52]. These evidences suggest that immune activation of microglia during neuroinflammation impairs brain function, and highlights the importance of these cells in sculpting the neuronal networks. Accordingly, complement proteins are strongly upregulated in neurodegenerative disorders such as AD, glaucoma, and amyotrophic lateral sclerosis [53-55] (**Figure 2**).

Neurogenesis: In addition to synaptic pruning, microglia-mediated phagocytosis of neurons is an important process to shape neural circuitries. During development, programmed cell death occurs among neuronal subsets [56], including both postmitotic neurons and proliferating neuroblasts. Tracking of dying cells by microglia is thought to be mediated by exposure of "eat me" signal at the surface of neurons. One of the best studied "eat me" signals is phosphatidylserine (PS), a plasma membrane lipid that is 'flipped-out' and exposed on the surface of apoptotic cells [57]. Scavenger receptors (e.g., CD36 or SRA-1), integrin αvβ3, Mer, TIM-1 and 4, BAI1, and Stabilin1 and 2 have all been shown to act as PS receptors [58]. Studies of neuron engulfment by microglia

in embryonic zebrafish brains indicate that microglia phagocytose dying neurons by extending cellular branches that form phagosomes at their tip in a BAI1 and TIM-4 dependent fashion [59]. In mice, microglial-mediated phagocytosis in the adult neurogenic niche relies on PS recognition by the TAM receptors Mer and Axl, and adult mice lacking these receptors exhibit a marked accumulation of apoptotic cells in neurogenic regions of the CNS [60]. Similar findings were made in mice lacking Gas6 and ProS, two bridging proteins that mediate PS recognition by TAM (Tyro3/Axl/Mer) family receptors [60]. This phagocytic mechanism mediated by Mer is analogous to the one employed by retinal pigment epithelial cells to phagocyte photoreceptor outer segments [61] or by astrocytes that engulf CNS synapses [62], indicating that TAM-mediated phagocytosis is a general supportive function that reshapes neuronal circuits by different CNS cell types.

In the neurogenic niches of the adult mouse brain, rapidly dividing transit-amplifying cells are constantly producing new neurons of which only a small proportion integrates into the neural circuitry of the hippocampus or in the granule and glomerular cell layers of the olfactory bulb [63]. In the adult hippocampus, newborn neuroblasts are rapidly cleared out through phagocytosis by unchallenged microglia present in the adult subgranular zone [64]. This capacity of microglia to phagocytose neuronal progenitors seems to be required for normal neurogenesis, because disruption of engulfment *in vivo* leads to an accumulation of apoptotic nuclei in the neurogenic niches and generally impairs neurogenesis [65]. The importance of microglia in maintaining homeostasis of the neurogenic cascade could be of mayor significance in processes of memory, depression, and neurodevelopmental or neurodegenerative disorders such as Alzheimer's and Parkinson's disease. In this regard, microglial expression of *Axl* is prominently upregulated in the inflammatory environment that develops in a mouse model of Parkinson's disease [60], and augmentation or repopulation of brain phagocytes, or improvement of their phagocytic activity, has been shown to arrest pathology in a mouse model of Rett Syndrome [66].

Phagocytosis and pathology: As described above, unchallenged microglia phagocytose apoptotic cells during development and in adult neurogenic niches. However, microglial phagocytosis has been traditionally studied under inflammatory conditions leading to the assumption that microglia needs to be activated in order to become phagocytic. Under inflammatory conditions, specific PS-binding opsonins and their receptors are strongly upregulated, enabling potent detection and phagocytosis of PS-exposing cells [67]. As an example, Mer and Milk fat globule EGF-like factor 8 (MFGE8) are transiently up-regulated following focal brain ischemia and deficiency in either receptor completely

prevents long-term functional motor deficits and strongly reduces brain atrophy as a result of inhibiting phagocytosis of neurons [68]. This results challenge the traditional notion that phagocytosis of dead or dying neurons from the infarcted brain may be beneficial by clearing harmful cellular components and decreases inflammation. In this regard, it appears that inflammatory activation of microglia impairs their ability to discriminate between apoptotic and viable neurons for phagocytosis, resulting in *phagoptosis* during inflammation [67].

Beyond phagocytosis of neurons, microglia activation during inflammatory conditions has an important role in phagocytosing infiltrating leukocytes to the inflamed tissue. Time lapse imaging on organotypic brain slices showed that microglia rapidly engulf apoptotic and also viable motile neutrophils, thus mediating neuroprotection against the neurotoxic effects of these leukocytes [69]. Clearance of neutrophils was shown to be important for neuroprotection after stroke [70, 71]. In addition, microglia exhibits a high capacity to uptake apoptotic autologous thymocytes, as well as apoptotic autoreactive T cells [72], myelin debris [73] or amyloid beta [74]. These observations raise important issues on the therapeutic manipulation of microglial phagocytosis, because detrimental effects could be achieved. On one hand, enhanced phagocytosis of synapses and viable neurons by microglia impairs CNS homeostasis [51-53, 68], but on the other phagocytosis can ameliorate CNS-related pathologies [66, 70, 72-74] and it is necessary for the correct functioning of the neuronal circuitry [3, 4, 32, 45, 46, 64, 65]. Further work will be needed to further establish how microglia-mediated phagocytosis is shaped by environmental signals or pathological conditions. In this regard, recent studies on AD using transcriptomic on single-cells identified a novel microglia type associated with neurodegenerative diseases (disease-associated microglia or DAM) with a unique potential to restrict neurodegeneration [74]. In a physiological context, microglial heterogeneity may be confined to distinct brain regions at sites where they interact with specific neurovascular structures. Whether the emergence of the DAM phenotype is restricted to a specific microglia population or if it is transiently acquired during neurodegeneration, as well as the signals that induce this phenotype, remain open questions (**Figure 2**).

Further studies have investigated the differential role of resident microglia and infiltrating monocytes to the inflamed CNS in the progression of neurodegeneration [75-78]. Disruption of the blood-brain barrier during inflamation has a high impact on the heterogeneity of the CNS phagocyte subsets due to infiltration of HSC-derived monocytes into the CNS parenchyma. Infiltrating monocytes are thougth to play different functions compared with microglia during the acute inflammatory response. In the onset of experimental autoinmune encephalitis (EAE), the phagocytic activity of monocytes has

been associated with the initiation of demyelinization, whereas microglia appeared to clear debris [77]. Differences have been also reported in the context of stroke, where changes in the expression profiles between microglia and macrophages 72 hours post-ischemia indicated that proinflammatory cytokines were preferentially upregulated in the infiltrating myeloid population [78].

Despite their vigorous accumulation during neuroinflammation, bone marrow-derived cells do not contribute to the pool of microglia as they are thought to disappear during resolution [79]. However, long-term engraftment of bone marrow-derived macrophages can take place in the brain when microglia are impaired in their ability to repopulate the niche. In these conditions, brain-engrafted macrophages do not alter behaviour but display a different transcriptional signature when compared to microglia [80].

Phagocytosis by cardiac macrophages

Incorporation of cardiomyocyte-derived material: Contrary to microglial cells, little is known about the phagocytic role of cTM in the steady state heart. Recent studies have explored the transfer of cardiomyocyte-derived material into cTM using αMHC-reporter mice to fluorescently label cardiomyocytes. Results from these experiments indicate that cTM can incorporate large amounts of cardiomyocyte-derived material *in vivo*, even in the unperturbed hearts [8]. In this settings, a cTM subset expressing low levels of the major histocompatibility complex II ($MHCII^{lo}$) was phagocytically more active than other subsets both in vitro and *in vivo* [8, 20]. This result is striking given the low renewal rate of adult cardiomyocytes [81], and the lack of cardiomyocyte precursors. It will be interesting to assess if this transfer of material from the cardiomyocyte is caused by a "trimming" process similar to synaptic pruning mediated by microglia in the brain [3], and to define the possible mediators of this type of phagocytosis in the heart. More important, the phagocytic content and the physiological consequences of cTM phagocytosis for heart homeostasis are currently unknown and deserve further studies (**Figure 3**).

While phagocytosis in the healthy myocardium has received little attention, studies in injured hearts reveals extensive functions of macrophages in clearance of damaged material and myocardial remodelling. In the context of myocardial infarction (MI), cTM disappear in the first 24 hours, and are replaced by monocyte-derived macrophages [40]. Although monocyte-derived macrophages evoke tissue inflammation early after MI, they also have phagocytic activity against infiltrating inflammatory cells (i.e. neutrophils) that die *in situ* [82], cardiomyocyte-derived debris and against other apoptotic cells [83]. Given the anti-inflammatory properties of phagocytosis, disruption of this function in the infarcted myocardium can result in failed myocardial remodelling [84]. Conversely, attempts have been made to stimulate this beneficial property elicited by phagocytosis

using PS-expressing liposomes that mimic apoptotic cells [85]. In the post-infarcted heart, this treatment reduced the expression of the pro-inflammatory genes TNFα and CD86, and increased expression of CD206, TGF-β and IL-10, all of which have anti-inflammatory and pro-healing properties. As a result, liposome-treated hearts displayed increased angiogenesis, reduced infarct area, and improved cardiac function [85]. In the same direction, studies analysing the effect of macrophage depletion with clodronate in the context of MI, showed a net beneficial role for macrophages in scar formation after MI, given that macrophage-depleted mice display poor infarct healing and myocardial rupture [86].

Macrophages are endowed with several molecules/mechanism to detect apoptotic cells that can work synergistically or independently [87]. Mutants in both Mer and Mfge8 molecules have larger infarcts, increased accumulation of apoptotic cells, enhanced fibrotic area, reduced angiogenesis and worse outcomes than either single mutant mice [88]. In contrast, macrophage-borne Mer appears to be necessary and sufficient for efferocytosis of cardiomyocytes *ex vivo* [89] (**Figure 3**). In infarcted human hearts, the expression of this receptor increases in necrotic areas, mimicking what is found in experimental ischemic mouse hearts. Supporting the role of Mer in the phagocytosis of necrotic cardiomyocytes, Mer-deficient macrophages contain reduced myocyte debris after MI [90]. Surprisingly, *ex vivo* co-culture of primary macrophages and adult cardiomyocyte apoptotic bodies revealed that phagocytosis of cardiomyocyte-derived material was inefficient. This was associated with myocyte-induced inactivation of Mer, while genetic elimination of a proteolytic site that enhances functional expression of Mer partially rescued this defect [90]. Further supporting this idea, an inactive form of Mer (solMER) was identified in the infarcted myocardium, implicating a natural mechanism of Mer inactivation after MI [8, 89]. In contrast, a cleavage resistant form of Mer improves MI outcome by enhancing efferocytosis [8]. Because uptake of cell debris by macrophages after MI has been shown to induce IRF3 and type I IFN production, which are detrimental for post-MI recovery, limiting phagocytosis in the infarcted myocardium may be a way to control macrophage activation and to protect heart function [91].

Immune defence and immune quiescence: In the adult heart, cTM are one of the first lines of defense against infections by direct ingestion of bacteria [40]. Exposure to the type-2 pathogens *Schistosoma mansoni* or *Heligmosomoides polygyrus* results in increased macrophage density in the heart, adoption of a stellate morphology, and increased expression of Ym1, RELMα and CD206, which is indicative of M2-like polarization. This was dependent on the recruitment of $Ly6C^{high}$ $CCR2^{+}$ monocytes,

suggesting that changes in cardiac macrophage pool could occur through *de novo* recruitment of monocyte-derived macrophages with different phenotypes and immune functions [92].

cTM-mediated phagocytosis also participates in immune quiescence by performing non-phlogistic elimination of cellular debris and dead cells, which could otherwise cause inflammation by the release of intracellular contents (reviewed in [12]). In the steady state, cTM produce factors such as C1q and galectins [13] that directly bind apoptotic cells and antibody-bound targets thus eliciting phagocytosis [93], while simultaneously dampening inflammation [94]. cTM also produce lipoxin A4, resolvin E1 and protectin D1 [13], which promote non-phlogistic phagocytosis by directly stimulating macrophages to further phagocytosis and by inhibiting the production of inflammatory cytokines [95, 96]. These findings suggest that cTM may inhibit aberrant inflammatory reactions in the heart to maintain tissue homeostasis [13]. In agreement with this possibility, macrophages in the healthy heart have been shown to express molecules associated with an immunomodulatory phenotype, including cytoprotective factors such as IGF-1 and immune repressors such as IL-10 [13] (**Figure 3**).

Trophic functions: influence on tissue homeostasis

The secretory microglia

Microglia has the ability to react rapidly to modifications of their environment by secreting soluble factors. Its close association with neurons and astrocytes and other cell types suggests an involvement of microglia-released products in several homeostatic processes in the CNS. Indeed, a wealth of reports have demonstrated that microglia-derived soluble factors influence development, inflammation, synaptic activity, oligodendrogenesis, astrocyte differentiation or sensitivity to pain (**Figure 2**).

Regulators of neuronal cell death: Neurons require trophic support during the formation of neural circuits. Factors produced by the microglia can deliver these signals as they constitutively release different cytokines. This release is highly sensitive to environmental conditions and can augment by several orders of magnitude in response to certain stimuli. Early in *vitro* studies demonstrated that co-culture of neurons from various CNS regions with microglia or with microglia-conditioned medium promoted neuronal survival and neuritic extension [97-99]. In the mouse brain, Ueno and colleagues described that layer V cortical neurons require support from microglia for survival during postnatal development. Microglia near the subcerebral and callosal projection axons in the postnatal brain secretes insulin growth factor 1 (IGF1), which

inhibits caspase-3-mediated apoptosis of neurons [100]. In line with these findings, studies during embryonic development found that microglia localizes at decision points along specific axonal tracts. For instance, microglia transiently associates with the extremities of tyrosine-hydroxylase (TH)-positive midbrain dopaminergic axons, and perturbation of microglial activity affects the outgrowth of dopaminergic axons in the forebrain and the laminar positioning of subsets of neocortical interneurons [101]. In contrast, microglia can induce retinal ganglion cell death in the developing chick retina by releasing nerve growth factor (NGF) [102]. Thus, microglia-derived factors can differentially modulate neuronal survival during brain development.

Following inflammation, microglia activation is normally accompanied by partial retraction of processes to the cell body, proliferation, and expression and release of pro-inflammatory cytokines, including TNFα, IL-1β, IL-6 and IFN-γ. All these molecules have been shown to amplify the inflammatory response and to drive neuronal cell death in different pathological conditions [103]. Microglial secretion of Il-1α, TNFα and C1q also induces a subtype of reactive astrocytes termed A1, which contribute to the death of neurons and oligodendrocytes in neurodegenerative disorders [104]. A1 astrocytes lose their ability to promote neuronal survival, outgrowth, synaptogenesis and phagocytosis, and secrete a yet-to-be-identified toxin that kills neurons and oligodendrocyte [104]. Further studies have suggested a role for the microglial-astrocytic-neuronal axis in neurodegenerative diseases and cognitive dysfunctions. In this context, Habbas and colleagues demonstrated that TNFα triggers an astrocyte-neuron signalling cascade resulting in persistent functional modification of synapses that mediates hippocampal synaptic alteration and contextual learning-memory impairment during EAE [105]. TNFα secretion by microglia was suggested to be the prime event for this alteration as this cytokine is mainly secreted by activated microglia during EAE [106]. In addition to cytokine release, recent reports in the context of AD have shown that inflammation triggers microglia release of the inflammasome adaptor protein ASC (Apoptosis-associated speck-like protein containing a CARD), which binds rapidly to amyloid-β and increases the formation of amyloid-β oligomers and aggregates spreading pathology [107]. Contrasting with these pathogenic functions, microglial activation during inflammation has also been proposed to promote neuroprotection by releasing anti-inflammatory molecules and growth factors including NGF, brain-derived neurotrophic factor (BDNF) or neurotrophin-3 (NT-3) [108]. All these evidences point to the microglial secretome as a versatile modulator of neuronal survival during development and inflammation.

Synaptic partners: Microglia also maintain synaptic plasticity by releasing various soluble molecules. Among them, BDNF has emerged as a mediator of microglia-induced disinhibition of neuronal excitability by disrupting Cl^- homeostasis [109]. This effect is crucial in the pathogenesis of pain hypersensitivity after injury to peripheral nerves. Coull and colleagues demonstrated that ATP stimulation of P2X4 receptors in microglia initiates a core pain signalling pathway mediated by the release of BDNF, which causes collapse of the transmembrane anion gradient in spinal cord neurons [5]. These evidences indicate that neuropathic pain critically depends on microglia-to-neuron signals, which alter GABA/glycine-mediated inhibition.

Further studies have implicated microglial cells in the modulation of synaptic transmission; microglia-astrocyte crosstalk modulates excitatory neurotransmission in hippocampal slices [110]. Activation of microglia induces a rapid increase of spontaneous excitatory postsynaptic currents through release of small amounts of ATP, which targets astrocytes and leads to and increase postsynaptic current frequency in hippocampal neurons [110]. Similarly, microglial release of ATP and glutamate has also been described in zebrafish in the context of acute injury [111]. Increased production of glycine and serine by microglia has been also suggested to enhance long-term potentiation responses in hippocampal neurons *in vitro* [112]. Moreover, microglia have been suggested to play a role in synaptic scaling, a type of homeostatic plasticity that entails uniform adjustments in the strength of all synapses of a neuron in response to a prolonged stimulus. In this setting, TNFα has been proposed to mediate synaptic scaling in response to prolonged blockade of activity [113]. Because both astrocytes and microglia can produce TNFα, the relative contribution of these two cell types to this phenomenon remains unclear.

These studies show that microglia is a modulator of glutamatergic or GABAergic synaptic transmission, either directly (neurons) or indirectly (astrocytes), through the release of soluble mediators. It is important to note that several other molecules produced by microglia, such as cytokines (IL-1β, TNFα), gaseous molecules (nitric oxide) or lipidic mediators (prostaglandins) can also modulate synaptic transmission and further studies are needed to determine their role during microglia-to-neuron communication. Since pathological activation of microglia and alteration of neurotransmission are two early symptoms of brain disorders, it is likely that dysregulated microglia secretion underlies early disease [105, 111].

Microglia and CNS cell differentiation: Neural stem/progenitor cells (NSPC) proliferate and differentiate depending on their intrinsic properties and local environment and microglial cells are closely associated with NSPC in the neurogenic niche. Although the precise cellular and molecular interplay between microglia and NSPC *in vivo* are still poorly understood, available data indicate that the effect on NSPC is mainly mediated by soluble factors rather than by cell-to-cell contact. Among these factors, pro-inflammatory cytokines (e.g. IL-1β, TNFα and IL-6) impair neurogenesis, whereas anti-inflammatory cytokines (IL-4 and IL-15) and trophic factors (IGF-1, BDNF) are proneurogenic [114]. Thus, the activation state of microglia affects NSPC differentiation in different ways: IL-6 and leukemia inhibitory factor (LIF) released by activated microglia promote astrocytic differentiation of NSPC [115], whereas resting microglia inhibits the differentiation of human NSPC towards neurons [116].

In vitro studies also suggest that differentiation of oligodendrocyte precursors can be influenced by microglia [117]. CSF-1R mutant mice display a prominent reduction in the number of Nogo1+ oligodendrocytes in the cerebral cortex and hippocampus [118], an observation that correlates with highly activated amoeboid microglia in myelinating regions of the murine brain during early postnatal stages, and with the finding that inhibition of CSF-1R reduces the number of oligodendrocyte precursors in the corpus callosum [119]. However, the specific microglia-derived molecules that induce oligodendrocyte progenitor survival and differentiation to oligodendrocytes during brain development have not been identified. In the context of lysolecithin-induced injury, microglia promotion of oligodendrocyte differentiation is proposed to involve arginase 1-expressing microglia that secrete activin A to trigger regeneration [117].

cTM: a secretory cell with potential homeostatic functions

The interspersed localization of cTM between cardiomyocytes places them in a privileged position to exchange signals with these cells. Like microglia, cTM release products that have been proposed to regulate cardiac physiology. Conditioned media from $CCR2^{-}$ macrophages induces neonatal rat cardiomyocyte proliferation *in vitro* [120]. This effect may be critical under hypoxic conditions because an increase in $CCR2^{-}$ resident macrophages induces cardiomyocyte proliferation in neonate human and animal models during hypoxia [121], suggesting that cTM regulate cardiomyocyte growth and division. One interesting candidate to mediate these effects is IGF-1, which is highly expressed by cTM when compared with other resident macrophage populations of the brain or the spleen [13]. IGF-1 is know to have an effect in cardiomyocyte proliferation since its overexpression under the control of alpha-myosin heavy chain (αMHC)

promoter, a cardiomyocyte-specific gene, led to increased numbers of myocytes and cardiomegaly [122], an effect that was latter associated with the Hippo signaling pathway in the context of heart development [123]. However, a direct link between cTM-mediated IGF-1 production and cardiomyocyte proliferation has not been formally demonstrated (**Figure 3**).

cTM also contribute to cardiac fibrosis, a hallmark of cardiac senescence that increases with age [38]. cTM secrete a number of pro-fibrotic factors such as *Retnla*, *Ccl24*, Osteopontin and *Tgf1b* that may induce fibroblast proliferation and collagen deposition by both TGFβ-dependent and -independent pathways [11, 38]. In addition, expression of these genes by cTM increases with age, particularly at the epicardium, suggests that cTM-mediated ECM remodelling influences cardiac ageing [38]. Accordingly, cTM have been found to affect diastolic function of the heart by controlling extracellular matrix deposition by fibroblasts [11]. These evidences, as with microglia, suggest trophic roles for cTM in heart homeostasis and highlight a potential role for these resident cells in many more cardiac processes than previously anticipated.

Cell-to-cell contact: CNS vascular networks and electrical conduction in the heart

Histologically, macrophages form grids within tissues and organize in regular networks with long cytoplasmic extensions (filopodia) that project around specialized tissue cells such as epithelial cells, neurons, endothelial cells or cardiomyocytes. Their unique spatial localization within the brain and cardiac tissue allows them to establish narrow and physical interactions with other constituents of the tissular architecture. Two different mechanisms associated with cell-to-cell contact of macrophages with parenchymal cells in those organs have been reported. One is regulation of angiogenesis [124] by serving as a bridge to promote endothelial cell tip fusion in the brain; the second is to favour electrical conduction by cTM that form direct connections with cardiomyocytes [9].

Angiogenesis and tip cell fusion

Macrophages have been proposed to play an important role during developmental angiogenesis. Fentin and collaborators combined the analysis of mouse mutants defective in macrophage development or VEGF signaling with live imaging in zebrafish to show that macrophages in the developing brain promote endothelial tip cell fusion [124], an effect that was necessary for vascular network formation. Indeed, PU.1- or CSF1-mutant mice that lack microglia displayed reduced complexity of the brain vascular

network early in development [124]. This has been additionally shown to be dependent on hypoxia-induced factor 1 (HIF1α) signalling during vessel injury because HIFα-deficient macrophages are not able to assist repair of blood vessels [125]. An elegant study of vascular injury in zebrafish visualized a process by which macrophages mediated repair of cerebrovascular rupture through direct physical interaction with endothelial cells. Brain macrophages were proposed to generate mechanical traction forces that brought together endothelial ends and facilitated their ligation, because depolymerization of microfilaments and inhibition of phosphatidylinositide 3-kinase or Rac1 activity in macrophages impaired cerebrovascular repair [126].

The role of macrophages in cerebrovascular remodeling can be also extended to other tissues, especially during vascular development, as these intimate interactions between macrophages and endothelium occurs all across the developing embryo [124]. In the heart, cTM are also in intimate contact with the dense cardiac vascular bed and express Nrp1, a positive regulator of angiogenesis [13]. Interestingly, CCR2$^-$ cTM are also important for the normal development of the coronary vasculature [10] and essential for cardiac regeneration following injury in the neonatal heart, presumably by promoting angiogenesis [127]. Altogether, these results argue in favour of a proangiogenic role for cTM and microglia during development and after injury. These roles rely on direct cell-to-cell contact but also on the release of proangiogenic factors that assist or promote stabilization of endothelial tip cell fusion [128]. These proangiogenic functions could be exploited to stimulate tissue vascularization after cardiac infarct or stroke (**Figure 2 and 3**).

Modulation of the electrical activity of the heart

A unique property of the cardiac tissue is its autonomous pacemaker activity that controls its beating function. The cardiac conduction system is composed of a specialized group of cardiomyocytes responsible for initiating and transmitting electrical activity. The sinoatrial node located in the right atrium is the master pacemaker of the heart, which sends electrical signals to the atrioventricular node (AVN), a structure that coordinates contraction between the upper (atria) and lower (ventricles) chambers. The regulation of this unique function has been recently associated with a cluster of cTM localized in the AVN region, in the proximity of cardiomyocytes of the conduction system [9]. These macrophages form direct gap junctions with cardiomyocytes via connexin 43 (Cx43), and depolarize in synchrony with the cardiomyocytes to which they connect. By using patch clamp techniques, Hulsmans and collaborators showed that these cells have positive effects on the excitability and a shortening of the refractory period of the AVN conduction

structure (**Figure 3**). Elegant optogenetic approaches directed to cTM showed that these cells modulated the conducting properties of the AVN. Accordingly, macrophage-restricted deficiency (using the Cx3cr1^{CreER} driver) in Cx43 or general myeloid cell depletion, using the CD11b-DTR model, had similar alterations in electrical conduction of the heart, and caused arrhythmias [9]. These surprising findings extend the repertoire of support activities that macrophages can exert in a tissue, and reveal a high degree of specialization within each organ.

Open questions and concluding remarks

It is now clear that tissue-resident macrophages are extremely specialized cells whose functions extend beyond their roles as immune sentinels. In the brain and heart, they sense and respond promptly to environmental changes. Phagocytosis and secretion of cytokines, trophic factors and other molecules have a direct impact on both neurons and cardiomyocytes. Microglia participate in synaptic pruning, maintenance of the neurogenic niche and in cerebral inflammation through their phagocytic activity. Secretion of soluble mediators regulate neuronal differentiation, synaptic activity and immunity. In turn, phagocytosis of cardiomyocyte-derived material, cellular debris, immune cells and pathogens by cTM maintain homeostasis and modulate immune responses in the heart. Other sophisticated tasks, such as the regulation of the electrical activity of the heart, reveal that, like microglia, cTM are uniquely specialized to support the demands of their host organ.

The many, well-defined mechanisms by which microglia support CNS function, however, still contrast with the scarce roles ascribed to cTM in the heart. Nevertheless, we draw functional parallelisms between macrophages in these tissues, including their capacity to modulate excitatory currents, to phagocytose cellular content or to secrete essential factors. Whether these similitudes extend to macrophages in other tissues is unclear, but we emphasize that the rigid anatomical constrains and low regenerative potential shared by the brain and the heart likely demand special support by dedicated cells in both tissues.

The impact of microglia and cTM alterations on tissue function during disease or aging is a major focus of current research. In this regard, ontogenic differences between resident microglia and infiltrating monocytes to the inflamed CNS have been linked to a differential contribution of both myeloid subsets in the progression of neurodegeneration. In the heart, the effect of cTM replacement by monocyte-derived macrophages is still unclear. In this regard, recent studies suggest that macrophages at day 30 post-MI and basal cTM display similar, but not overlapping, transcriptional programs [129].

Myeloid cell replacement (embryonic vs. adult) and inflammation or aging-mediated myeloid cell infiltration could therefore have an impact on the heterogeneity and functioning of cTM and microglia and, by extension, on tissue homeostasis. The role of cTM on modulating electrical heart conduction [9] and the association of monocyte-derived macrophages with fibrotic deposition and diastolic dysfunction [11] prompt redefinition of the contribution of immune cells to idiopathic cardiomyopathies, and might direct generation of novel therapeutics tailored to this organ. Along the same line, phenotypic changes in microglia during age and disease, as well as the contribution of brain-engrafted macrophages to CNS homeostasis could unveil novel therapeutic approaches to fight neurodegeneration. Phenotypic modulation and genetic manipulation of brain and heart phagocytes, transplantation of specific myeloid progenitors and depletion of deleterious subsets may therefore provide efficient means to interfere with disease progression.

Disclosures

The authors declare no conflict of interest

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Figures and Figure Legends

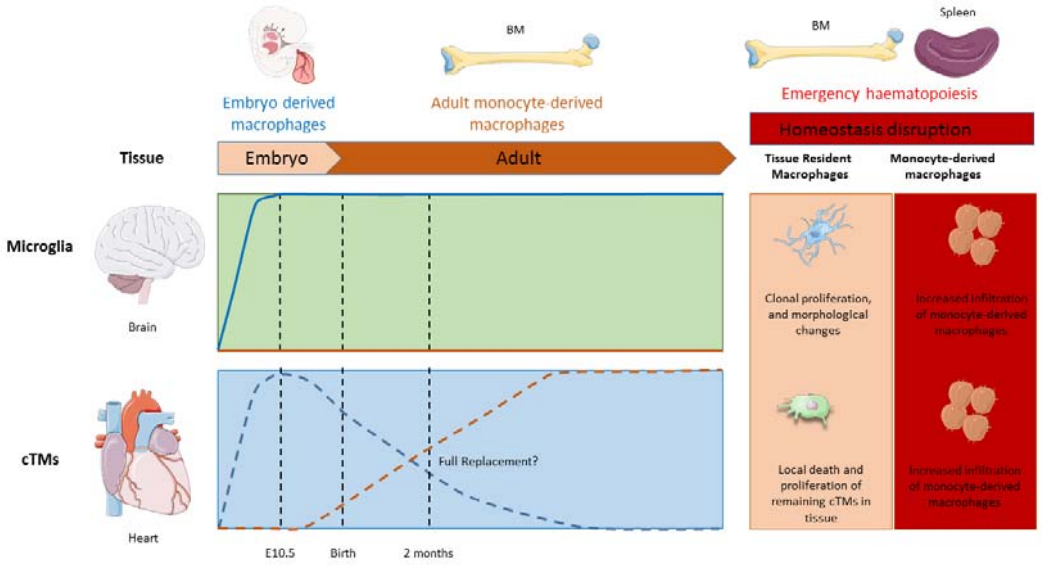

Figure 1 Origin of microglia and cardiac macrophages. Microglia emerges from yolk sac-derived EMPs that colonize the brain early during development. Contribution of HSC-derived monocytes under homeostatic conditions is minimal as shown by different experimental settings, including parabiosis and fate-mapping. Following inflammation microglia proliferates clonally and activates. Disruption of the blood brain barrier leads to the infiltration of blood leukocytes to the brain parenchyma. cTM arise from fetal precursors that colonize the heart early in development. After birth, a progressive substitution of fetal macrophages by blood monocytes has been proposed. In the inflamed or aged heart, cTM death and recruitment of blood monocytes have been shown to contribute to the heterogeneity of the cardiac phagocytic pool.

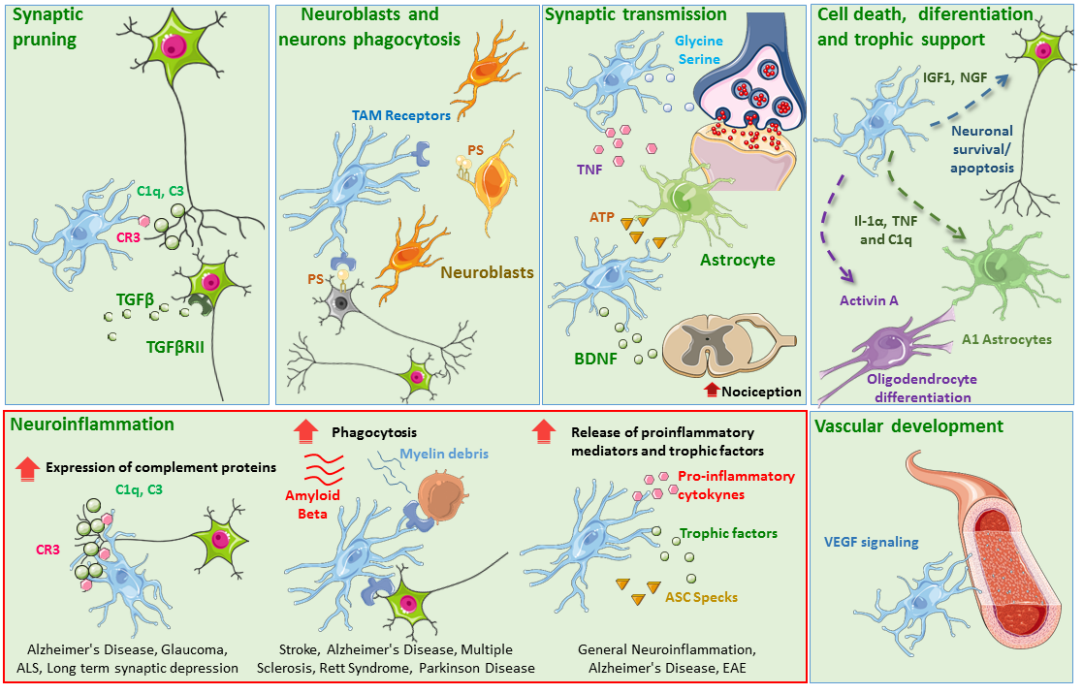

Figure 2 Microglia functions under homeostasis and inflammation. Complement tagged synaptic spines are phagocytosed by microglia leading to synaptic pruning and configuration of neuronal networks, especially during development. TGFβ has been proposed as a regulator of neuronal complement expression and C3 expression by microglia recognizes tagged synapses. Phagocytosis of neuroblasts and healthy neurons by microglia take place through phosphatidylserine (PS) recognition by microglial TAM receptors. Microglia released byproducts modulate synaptic activity, either directly by releasing BDNF, glycine or serine, or through astrocytes by ATP or TNFα release. The secretory activity of microglia also controls neuronal differentiation and survival, astrocyte activation and oligodendrogenesis. Repair of damaged blood vessels and proangiogenic properties form part of microglia functions. During inflammatory conditions, upregulation of complement proteins and excessive pruning have been proposed to play a role in different CNS pathologies. Upregulation of microglia-mediated phagocytosis during inflammation could play a deleterious role during stroke. In contrast, improved phagocytic performance of microglia has beneficial effects in pathology by phagocytosing amyloid beta, myelin debris and infiltrating leukocytes. An increased release of proinflammatory molecules and ASC specks by microglia has been related with neuroinflammation and amyloid-β oligomerization. Production of trophic factors by microglia is suggested to ameliorate neuroinflammatory diseases.

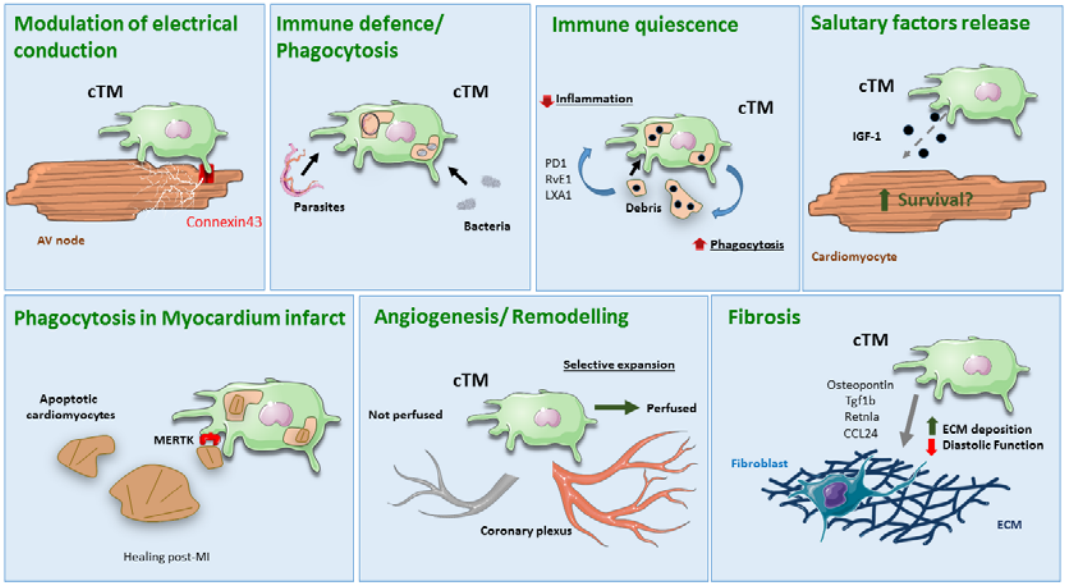

Figure 3 Functions of cTM during homeostasis and myocardial injury. cTM couple to cardiomyocytes through Connexin43 in the AV node, thus modulating their electrical properties. cTM participate in immune defense by directly phagocytosing pathogens, thus acting as the first line of defense. cTM also promote immune quiescence by performing non-phogistic phagocytosis: cTM release C1q, Galectins and other factors that promote removal of debris and apoptotic cells. Release of lipoxin A4, resolvin E1 and protectin D1 inhibit the production of inflammatory cytokines. cTM produce high levels of IGF-1, likely acting on neighboring cardiomyocytes and regulating their metabolic activity and survival. After myocardium infarct, cTM phagocytose necrotic and apoptotic cells through Mer receptor and contribute to healing. Vascular remodeling and selective expansion of perfused vascular beds in the coronary plexus are also mediated by cTMs, especially during embryonic development. cTM have been shown to release profibrotic factors that activate extracellular matrix (ECM) deposition by fibroblasts, thereby affecting diastolic function of the heart.