Snapshot: Targeting Macrophages as a Candidate for Tissue Regeneration

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DOI: https://dx.doi.org/10.21775/cimb.029.037

Abstract
Macrophages are a specific mononuclear cell group abundant in almost every organ of higher animals. This group is a pivotal part of the immune system and is involved in immune responses against exogenous antigen invasion. Recently, accumulating evidence has demonstrated that macrophages participate in wound repair and tissue regeneration. In this review, we will first introduce the influences of regeneration after injury in various tissues and organs among macrophage-depleted animal models. Second, the possible relationship between macrophages and reparation capacities will be discussed. Finally, we provide a general idea about the roles of macrophages in the injury-regeneration process and then discuss the current challenges and prospects of their clinical application. The information compiled here may be useful for regenerative research and may promote macrophages as a therapeutic target in regenerative medicine.

Introduction
Macrophages, also known as "big eaters" due to their phagocytic capacity, were first annotated by Mechnikov in the late 18th century (Fraga et al., 2017; Tauber, 2003; Wan et al., 2017). They are a specific mononuclear cell group abundant in almost every organ of higher animals. In mammals, macrophages can derive from the yolk sac, fetal liver, and bone marrow (Geissmann et al., 2010; Wynn et al., 2013). Their differentiation is orchestrated by multiple growth factors (GFs) (Gordon, 2003; Sica et al., 2012). Tissue-resident macrophages, usually originating from the bone marrow hematopoietic progenitors, has the ability of self-renewing (Geissmann et al., 2010; Jenkins et al., 2011; Schulz et al., 2012; Davies et al., 2013). Generally, they are divided into two phenotypes, classically activated M1 macrophages and alternatively activated M2 macrophages, which refer to the state activated by Th1 and Th2 lymphocytes or with interferon-gamma (INF-γ) and interleukin 4 (IL-4), respectively (Goerdt et al., 1999; Gordon, 2003; Sica et al., 2012). This phenomenon is described as heterogeneity or polarization.

Tissue regeneration plays an important role in cardiovascular diseases (Sengupta et al., 2010; Wang et al., 2013), cerebrovascular diseases (Wang et al., 2013), and neoplasms (Jin et al., 2017), three major killers among human across the world (Jiang et al., 2017; Li et al., 2017; Liang et al., 2017; Lv et al., 2017; Ma et al., 2017; Wu et al., 2018; Xin et al., 2018; Zhang et al., 2018). The primary function of a macrophage is to serve as the effector of the innate immune system (Saini et al., 2016; Jackaman et al., 2017; Liang et al., 2017; Rahimifard et al., 2017; Ren et al., 2017). Accompanied by monocytes, macrophages occupy the front line of innate defense against exogenous antigens. They are highly flexible cells that exhibit dramatic changes in phenotypes in response to stimuli from neighboring tissues/cells and/or the external environment. They promptly infiltrate into

the pathogen invasion site of the organism, clear debris, and secrete chemokines and cytokines to initiate inflammation procedures, drive acquired immune responses and subsequently promote injury resolution from a morbid status (Gordon et al., 2005; Hume, 2006; Hume, 2008; Geissmann et al., 2010; Hume et al., 2012; Wynn et al., 2013). Additionally, macrophages fulfill indispensable functions in the patterning and formatting of tissues during developmental and mature stages. They are involved in erythropoiesis (Kawane et al., 2001), hematopoiesis (Gordy et al., 2011)(Gordy et al., 2011), angiogenesis (Rao et al., 2007; Machnik et al., 2009; Fantin et al., 2010; Stefater III et al., 2011), osteogenesis (Niida et al., 1999; Pollard, 2009), and neural homeostasis (Pollard, 2009; Erblich et al., 2011). Furthermore, macrophages maintain metabolic homeostasis in adult mammalian organs, such as adipose tissue, the liver, and the pancreas (Weisberg et al., 2003; Odegaard et al., 2007; Kang et al., 2008; Olefsky et al., 2010; Nguyen et al., 2011; Odegaard et al., 2013). However, chronically activated macrophages lead to a prolonged inflammatory reaction, which triggers substantial adjunctive tissue destruction. Indeed, these unregulated macrophages contribute to various chronic inflammatory and autoimmune diseases, including atherosclerosis, asthma, rheumatoid arthritis, and fibrosis (Kamada et al., 2008; Hansson et al., 2011; Libby et al., 2011; Murray et al., 2011). Apart from the disease-promoting effect, clinical and experimental evidence has demonstrated that macrophages participate in tumor initiation, progression, and metastasis (Marelli et al., 2017; Yao et al., 2017).

In this review, we aimed to mainly focus on the mechanism by which macrophages contribute to tissue/organ injury repair. We will first introduce the influences of regeneration after injury in various tissues and organs among macrophage-depleted animal models. Second, the possible relationship between macrophages and reparation capacities will be discussed. Finally, we provide a general idea about the roles of macrophages in the injury-regeneration process. Collectively, this review will serve as a comprehensive reference and hopefully aid in the design of future research and provide a potential therapeutic target in therapeutic methods.

Macrophage depletion and tissue regeneration

Blood vessel rupture and/or nerve regeneration

Physical damage of the brain is usually accompanied by the rupture of blood vessels. Scientists have discovered that macrophages are involved in the repair of brain vascular rupture and regeneration of peripheral nerves. Because vascular endothelial growth factor (VEGF) is one of the key factors in angiogenesis (Pollard, 2009; Fantin et al., 2010), Cattin and co-researchers (Cattin et al., 2015) used the *Cre* system and created transgenic mice in which the production of VEGFα in macrophages is inhibited. In the abovementioned work, they found that the loss of VEGF*α* did not inhibit macrophage recruitment during the early stage of nerve regeneration. However, a reduction in vascularization was observed in mutant animals, leading to the restraint of Schwann cells (SCs) at the stump site. Consequently, these cells failed to form tube-like structures, which are necessary to direct axons back to their original targets. Meanwhile, by studying transgenic zebrafish models (Liu et al., 2016), another group demonstrated that in some cases, following cerebrovascular rupture, macrophages were recruited by extracellular ATP and formed direct physical adhesions with both epithelial ends. With the help of mechanical forces, both the broken ends of cerebral vessels could be successfully reconnected, and a functional vessel was then restored (Figure 1, Table 1). Thus, macrophages promote the migration of SC and maintain the homeostasis of peripheral neural network by secreting cytokines, making physical ligation, and assisting neovessel formation.

Fracture healing

Macrophages have also been involved in bone healing. Schlundt et al. produced unilateral closed fractures in 8-week-old mice (Schlundt et al., 2015). They used a well-developed medical intravenous injection method to selectively deplete mouse macrophages. This fabulous work was performed by Dutch scientists VanRooijen and Van Nieuwmegen in 1984. The approach is to introduce an effector molecule, liposome-carried dichloromethylene diphosphonate, specifically called clodronate liposome, into the organism (Van Rooijen et al., 1984). This compound has been shown to have high affinity for calcium and, thus, adheres to the mononuclear phagocyte system (MPS). At the cellular and molecular levels, the accumulated intracellular effectors caused a "liposome-mediated macrophage suicide" effect, leading to the death of the macrophages (Figure 2). This novel method is reversible and less toxic, demonstrating effective elimination of macrophages in various organs, such as the spleen, lung, and testis (Van Rooijen et al., 1985; Thepen et al., 1989; van Rooijen et al., 1989; Delemarre et al., 1990; Van Rooijen et al., 1990;

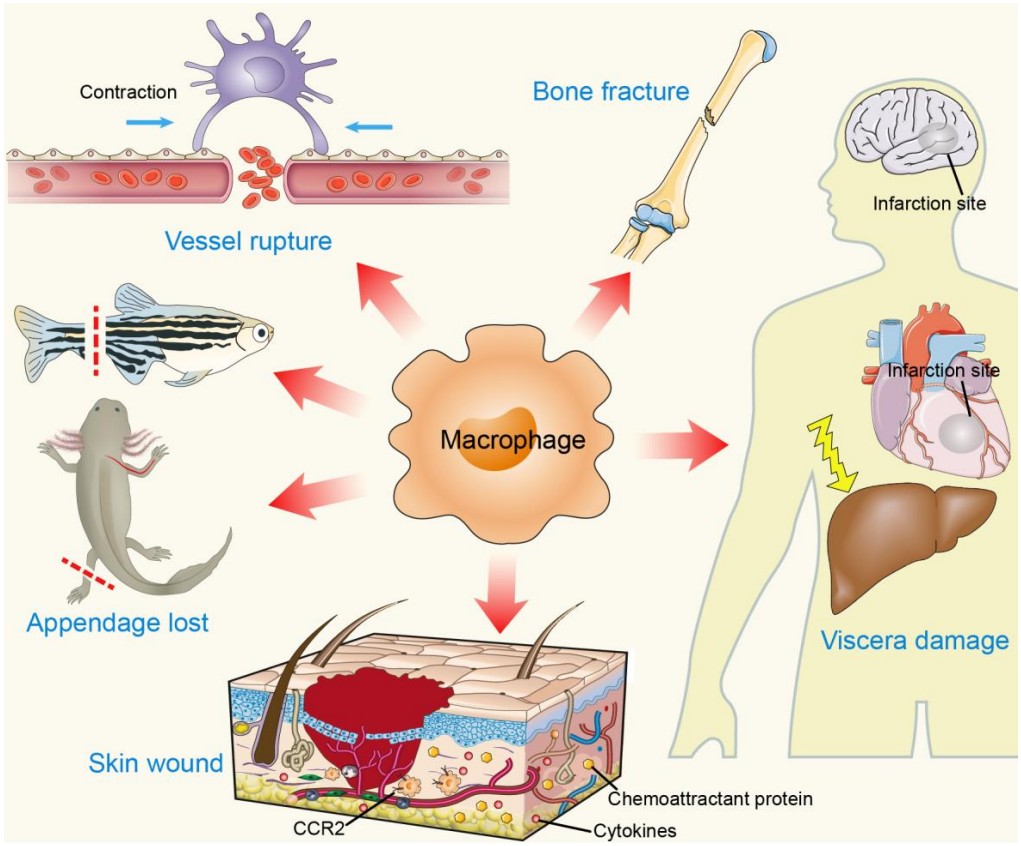

Figure 1. Roles of macrophage in regeneration. Accumulated studies have demonstrated that macrophage participated in regeneration in different organisms. In human, macrophage is able to promote regeneration of bone fracture, vessel rupture, and skin wound. Besides, it can also promote appendage growth of zebrafish and newt.

Table 1. Summary of macrophage involvement and function in different regeneration processes.

Organ/tissue/body parts	Model	Mechanism	Macrophage function	Reference
Blood vessel/nerve	Zebrafish	PI3K and Rac1 mediate mechanical traction of macrophages. The cords of Schwann cells, which interact directly with blood vessels, are used as scaffolds by axons to cross the rupture 'bridge'.	Facilitating the ligation of vessel endothelial rupture. Secreting VEGF-A to induce polarized blood vessels and drive angiogenesis.	(Cattin et al., 2015; Liu et al., 2016)
Appendage	Salamander and zebrafish	Appropriate TGF-β1 signaling activation and cytokine production in limb regeneration.	Orchestrating the early response to injury and activation of subsequent limb regeneration.	(Godwin et al., 2013; Petrie et al., 2014)
		Wnt/β-catenin signaling partially modulates cellular and biochemical events in tail regeneration.	Impacting the proliferative capacity of the blastema and extent of tail fin regeneration in a stage-dependent manner.	
Skin/muscle	Mouse	CX_3CR1 enhances phagocytosis and muscle trophic growth factor production.	Phagocytosing necrotic muscle fibers and neutrophils.	(Zhao et al., 2016)
Brain	Mouse	Recruitment of MOs/MPs via CCR2 and activating TGF-β1 signaling pathway.	Stabilizing the neurovascular unit in the subacute stage.	(Gliem et al., 2012)
Bone	Mouse	Changing of the macrophage phenotype from M1 to M2, thus enhancing the anti-inflammatory signaling cascade.	Improving callus mineralization and endochondral ossification.	(Raggatt et al., 2014; Schlundt et al., 2015)

Bergh et al., 1993; Biewenga et al., 1995; Van et al., 1996; Van Rooijen et al., 1996). Using this strategy, researchers have found that an enduring macrophage depletion resulted in non-union and impaired callus mineralization, appearing as a lower callus and bone volume. Both type II and type X collagens, which should graduate into mineralized tissue in the late healing phase, were still detectable at 21 days in animals from the macrophage-depletion group. This is a clear message that endochondral ossification was severely disturbed in mice without sufficient macrophages. A similar failure of bone reconstruction was observed by Raggatt's research group (Raggatt et al., 2014). They created transgenic mice, in which mature myeloid cells carried a Fas-based suicide gene. When these mice were treated with AP20187, the specific suicide gene-activating ligand, macrophages can be temporally depleted at any of the interested phases. Using this strategy to study femoral fracture mouse models, Raggatt found that soft callus formation in mice with ablated macrophages at the initial time of surgery was totally blocked because cartilage, and woven bone formations were absent. When AP20187 was delivered at the initiation of the early anabolic phase, some animals exhibited a small but morphologically normal callus, whereas others had minimal or no soft callus formation. Overall, macrophage depletion remarkably impacts endochondral callus formation and normal bone anabolism, thus influencing subsequent fracture healing. For more details on macrophage-depleted animal models, please refer to Figure 2.

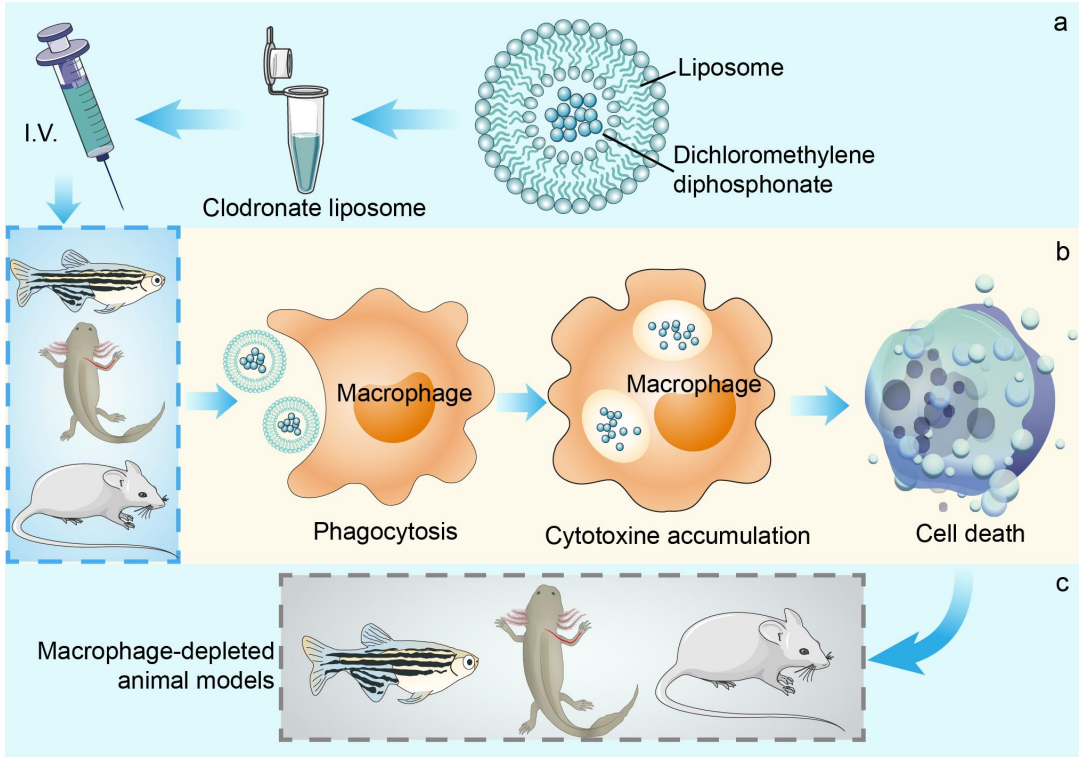

Figure 2. Acquisition of macrophage-depleted animal models. Clodronate (dichloromethylene diphosphonate) is artificially encapsulated in liposome, suspended in sterile phosphate buffered saline. After intravenous injection, clodronate liposome will be firstly ingested and digested by macrophage, and further contribute to the release of cytotoxic molecular clodronate. The intracellular release and accumulation of clodronate at a certain concentration may induce apoptosis of the macrophage. By this approach, temporally macrophage-depleted animal models are created, which offers opportunity for researching macrophages *in vivo*.

Viscera damage repair

Macrophages are also involved in the damage repair of internal organs of the body. Duffield et al. (Duffield et al., 2005) created a carbon tetrachloride (CCl_4) fibrosis model and observed the liver regeneration status in transgenic mice lacking macrophages. They found that macrophages acted differently during the injury and repair phases. In progressive inflammatory injury, macrophage depletion resulted in the amelioration of fibrosis. However, depleting macrophages during the recovery stage led to failure of resolution with the persistence of cellular and matrix components of the fibrotic response. Duffield et al. also discovered that two subgroups of macrophages: circulating monocytes and hepatic resident cells, plays pivotal roles in tissue injury and regeneration. It was also demonstrated that an insufficient number or dysfunction of macrophages is related to delayed clinical deterioration and hemorrhagic conversion of infarctions in mouse models of ischemic stroke. Frequent bleeding and dilated neovessels in the infarct border zone were also observed (Gliem et al., 2012). Another group, Vinuesa et al., first treated mice with clodronate liposome and then used the same ischemia/reperfusion surgery approach to study kidney damage in models lacking macrophages found that the absence of macrophages blocked the repair process, while re-injection of RAW 264.7 in macrophage recovered the regeneration ability of the organism, by augmenting cell proliferation and secreting anti-inflammatory cytokines in post-reperfusion period (Vinuesa et al., 2008).

Repair in skin wound and muscle injury

The skin is the first barrier of animals against external invaders; therefore, wound healing appears to be particularly important to organisms. A study shows that macrophages are inextricably involved in skin regeneration. Transgenic mice with depleted macrophages demonstrated delayed wound closure, reduced granulation tissue formation and collagen deposition, impaired angiogenesis and decreased cell proliferation during wound healing (Mirza et al., 2009). It has been demonstrated that the macrophage subtype was recruited soon at the lesion site after acute skeletal muscle injury via CCR2, a chemokine type 2 receptor expressed on the surface of monocytes/macrophages, and its ligand monocyte chemoattractant protein-1. Next, these macrophages switched into the $CX_3CR_1^{hi}$/Ly-$6C^{low}$ subtype to produce cytokines to accomplish muscle regeneration (Arnold et al., 2007; Contreras-Shannon et al., 2007; Sun et al., 2009; Lu et al., 2011; Lu et al., 2011). Inspired by this discovery, instead of using the macrophage-knock-out system, Zhao and colleagues (Zhao et al., 2016) generated *CX_3CR1* mutant transgenic mice as models, in which the function of macrophage was impaired. In barium chloride ($BaCl_2$)-injected acute muscle injury mouse models, both the number of necrotic fibers and percentage of the necrotic area at 7 d were significantly augmented compared with the WT controls, indicating unsuccessful injury repair. In general, macrophages seem to promote wound closure as well as dermal healing and are important to skeletal muscle injury repair by regulating tropic growth factor production and the cytokine microenvironment.

Appendage regrowth

In contrast to mammals, some vertebrate and amphibian species can regenerate scarless tissues (Lévesque et al., 2010) or even whole body parts, such as limbs, fins, the spinal cord, the tail, and sections of the heart and brain (Brockes et al., 2002; Ferretti et al., 2003; Brockes et al., 2005; Parish et al., 2007; Singh et al., 2010; Jiang et al., 2017; Li et al., 2017; Ma et al., 2017; Siegel et al., 2017; Di et al., 2018; Li et al., 2018). In salamanders, loss of an entire limb can be regenerated within weeks. Using the clodronate-liposome-depletion strategy again, Godwin and colleagues found that early macrophage engagement is crucial for limb regeneration (Godwin et al., 2013). They observed that the deletion of macrophages before and during early amputation days in axolotl exacerbated fibroplasia and collagen deposition, inhibiting blastema formation at the amputated limb site, thus repressing limb regrowth. On the other hand, macrophage interference during a later stage after blastema formation only resulted in delayed limb redevelopment associated with the reduction of vascularization, which appears less harmful to the axolotl itself.

Using the combination of cell tracking and genetic cell ablation approaches in zebrafish fin regeneration research, Petrie et al. (Petrie et al., 2014) found that macrophages responded quickly after the blastema formation stage in the tail fin regeneration process. In transgenic zebrafish lacking macrophages, the extent of new fin tissue growth was decreased compared with that of WT fish. Moreover, these fish demonstrated scattered distinct areas of abnormal tissue regeneration along the fin. Moreover, Petrie *et al.* identified stage-

dependent functional roles of macrophages in appendage outgrowth of adult zebrafish. They observed that the lack of macrophages throughout blastema formation during early amputation stage, led to the inhibition of regeneration. On the other hand, depleting macrophages during the later tissue outgrowth phase did not significantly affect the regeneration rate but resulted in an elevated aberrant growth phenotype (33% compared with 16% in the control group and 9% in the WT group). The depletion of macrophages during these two stages caused the failure of wound closure and aberrant fin patterning. Interestingly, no similar results were observed in mouse models so far (Dovi et al., 2003; Martin et al., 2009; Mirza et al., 2009; Evans et al., 2013).

Mechanism of macrophages involved in regeneration

Excreted factors

The primary role of macrophages associated with regeneration is their remarkable capacity for cytokine secretion. Macrophages have significant beneficial improvement in inducing angiogenic activity during blood vessel regeneration. One of the well-known angiogenesis molecules produced by macrophages is the multifunction-factor VEGF (Breen, 2007). During peripheral nerve rupture, macrophages could selectively sense the hypoxic environment between the proximal and distal stump and then secrete VEGFA to induce the polarized vasculature. Schwann cells can then use these newly generated blood vessels as "tracks" to cross the bridge, taking regrowing axons with them and assist the accomplishment of nerve regrowth (Cattin et al., 2015). In the course of wound healing, macrophage knockout mice exhibit disorganized cytokine expression at the wound bed, and the chaos resulted in disabled injury repair (Mirza et al., 2009). During fracture healing, either Fas- (Raggatt et al., 2014) or clodronate liposome injection-induced (Schlundt et al., 2015) macrophage depletion led to prolonged union and impaired mineralization. This finding is concurrent with the *in vitro* experiment indicating that the removal of macrophages diminished osteoblast mineralization (Chang et al., 2008). This decreased mineralization is probably associated with the lack of osteoblasts originated from macrophage-enhanced osteoblastic differentiation (Vi et al., 2015). Claudia et al. have proven that M2 macrophage enhancement driven by IL-4 and IL-13 improved bone remodeling (Schlundt et al., 2015). The M1 macrophage expresses high levels of pro-inflammatory cytokines and reactive nitrogen and oxygen intermediates, which exert microbicidal and tumoricidal activity. In contrast, M2 macrophage can secret scavenging molecules that participate in tissue remodeling and immuno-regulation (Mills et al., 2000; Sica et al., 2012; Mills et al., 2014; Zhou et al., 2014). Hence, changes in microenvironmental factors in the first few days of bone fracture led to the switch of M1 macrophage to M2. This transformation promotes the secretion of anti-inflammatory cytokine and bone regeneration.

Signaling pathways

Signaling pathways also participate in the process of regeneration. After myocardial infarction (MI), the Wnt pathway is activated in mice. Palevski et al. (Palevski et al., 2017) demonstrated that macrophages, recruited by MI, contributed to non-canonical activation because they highly expressed non-canonical Wnt ligands. Interestingly, the knockout of the Wnt ligand essential transporter *Wntless* (*Wls*) in macrophages, which inhibited ligand secretion, blocked the inflammatory autocrine loop and resulted in a shift toward the M2-like phenotype. These *Wls*-deficient M2-like macrophages exhibited anti-inflammatory characteristics and displayed augmented expression of inducible nitric oxide synthase (iNOS), transforming growth factor-β1 (TGFβ1), and insulin-like growth factor 1 (IGF1), which are positive factors to promote ischemic injury protection, infarct healing, and myocardial repair.

Another report proved that a single macrophage was sufficient to generate mechanical traction forces to pull the endothelial ends of brain vascular rupture and facilitating their ligation, thus mediating vessel regeneration (Liu et al., 2016). Transcriptional profile analyses identified the upregulation of phosphatidylinositol-3-kinase (PI3K) and Ras-related C3 botulinum toxin substrate 1 (Rac1) in macrophages at the lesion site. PI3K and Rac1 coordinated actin polymerization in microfilaments to accomplish the filopodia/lamellipodia-dependent migration. Notably, this capacity seems much more efficient during cerebrovascular regeneration than in other tissues. A previous study provided evidence that the existence of neutrophils retarded wound closure in mice (Dovi et al., 2003), possibly explaining the magnificent capacity of the macrophages mentioned above because neutrophils cannot pass the blood-brain barrier (BBB).

When studying the functions of distinct sub-populations of monocytes/macrophages (MOs/MPs) in the subacute stages of brain infarction, Gliem and

his colleagues detected co-localization of TGF-β1 and $CD68^+$ MOs/MPs in the infarct border zone. In addition, disruption of infarction repair, down-regulation of the TGF-β1 signaling activation indicator, and phosphorylation of Smad2 were observed in MO/MP-depleted mice. By injecting TGF-β1, the deficient mice displayed decreased secondary infarct bleeding and showed preferable neurovascular integrity maintenance, suggesting a critical role of TGF-β1 signaling in stabilizing the neurovascular unit and lesion remodeling (Gliem et al., 2012).

Similar TGF-β1 secretion in macrophages was found in the injury phase of CCl_4-induced liver fibrosis/cirrhosis models. It regulated the deposition of matrix and supported scar-forming cells, known as activated hepatic stellate cells (Duffield et al., 2005). Nevertheless, this TGF-β1 expression was attenuated during the repair phase, and macrophages assisted in the degradation and absorbency of redundant matrix components. This finding provides solid evidence that two phenotypes of macrophages were independently responsible for either the injury or repair phase.

Conclusion and perspective

Amputation and tissue repair have always been the hotspot of regenerative medicine. Considering well-differentiated human cells, human limb regeneration is often relatively difficult regarding certain tissues and organs, or the ability to regenerate may be lost. Unlike humans, lower animals such as zebrafish, salamanders, etc. have a strong ability to regenerate by repairing and remodeling the limbs and large areas of defective tissues. Therefore, scientists establish systems in model animals to explore the regeneration mechanism. A wealth of experimental evidence has demonstrated that macrophages have an irreplaceable role in the regeneration process. They not only infiltrate the damaged targets at the time of body injury and stop the continued invasion of pathogens but also coordinate in all post-tissue repair processes and promote the regeneration of the injured site by switching phenotypes to secrete growth factors and regulate signaling pathways. Relatively, functional and phenotypical switch of macrophage is controlled by crosstalk of signaling pathways, such as C-Jun N-terminal kinase (JNK), PI3K/proterin kinase B (PKB), Notch, Janus kinase (JAK)/signal transducer and activator of transcription (STAT), peroxisome proliferator-activated receptor (PPAR), Krüppel-like factor (KLF), interferon regulatory factor (IRF), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), and hypoxia-inducible factors (HIF) (Sica et al., 2012; Zhou et al., 2014). This balance of the M1/M2 transition is tightly associated with physiological and pathological activities. Emerging evidence has proved that M1/M2 macrophages exert different even opposite functions in a stage-dependent manner. Specifically, M1 type mainly initiates proinflammatory response while M2 type reverses this phenomenon (Gordon et al., 2010; Sica et al., 2012). However, studies have shown that macrophages have negative effects in tissue regeneration. Phenotypes lacking macrophage fractalkine receptor CX_3CR_1, exhibiting inefficient phagocytosis and abnormal cytokine secretion, has therapeutic actions in arthrosclerosis (Lesnik et al., 2003), acute spinal cord injury (Donnelly et al., 2011; Freria et al., 2017) and acute colitis (Kostadinova et al., 2010). However, Zhao et al. (Zhao et al., 2016) and Ishida et al. (Ishida et al., 2008) found that CX_3CR_1 deficiency mice showed delayed repair of acute skeletal muscle injury. These findings provide another vision to explore the dual function of macrophages in different tissue/organ/species during regeneration process. Thus far, the field of regeneration is still under study, and investigations to date have not been set forth for the identification of the precise mechanisms underlying the repair processes. The advance of genetic modification technique and transcriptome analysis provide new avenues for future research of macrophages. To sum up, the outstanding roles of macrophage in tissue regeneration may offer an elaborate picture of tissue injury and regeneration between robust regenerative organisms and less regenerative mammals. This might provide a new therapeutic target that can be manipulated for mammalian tissue/organ regeneration.

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