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The significance of M1 macrophage should be highlighted in peripheral nerve regeneration

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Summary. Macrophage influences peripheral nerve regeneration. According to the classical M1/M2 paradigm, the M1 macrophage is an inhibitor of regeneration, while the M2 macrophage is a promoter. However, several studies have shown that M1 macrophages are indispensable for peripheral nerve repair and facilitate many critical processes in axonal regeneration. In this review, we summarized the information on macrophage polarization and focused on the activities of M1 macrophages in regeneration. We also provided some examples where the macrophage phenotypes were regulated to help regeneration. We argued that the coordination of both macrophage phenotypes might be effective in peripheral nerve repair, and a more comprehensive view of macrophages might contribute to macrophage-based immunomodulatory therapies.

Key words: Macrophage, Macrophage polarization, Peripheral nerve injury, Peripheral nerve regeneration, Regenerative medicine, Immunomodulatory therapy

Introduction

Peripheral nerve injury (PNI) is a common traumatic disease. Although research on repair surgeries has advanced considerably, the outcomes might still be unsatisfactory, resulting in morbidities, such as the loss of sensory or motor functions and a decrease in the quality of life (Jones et al., 2016; Modrak et al., 2020). The treatment choices for PNI include advanced microsurgeries, such as end-to-end repair with tensionless epineurial sutures, autologous nerve grafting, and nerve transfer (Campbell, 2008; Modrak et al., 2020; Liu et al., 2022). However, these surgeries have some limitations, such as neuroma formation, donor site morbidity, and limited autologous nerve graft supply (Carvalho et al., 2020; Vijayavenkataraman, 2020). Although nerve autografting is the gold standard for the treatment of PNI, achieving satisfactory recovery via this method is difficult (Faroni et al., 2015; Kubiak et al., 2020). Thus, detailed studies on the mechanism of regeneration of nerve injury and the development of better therapeutic means are necessary.

The mechanisms of peripheral nerve injury and regeneration involve complex interactions among many kinds of cells and molecules (Mahar and Cavalli, 2018; Ydens et al., 2020), and macrophages play a key role (Zigmond and Echevarria, 2019; Li et al., 2022b). After PNI, macrophages are one of the earliest responders (Mueller et al., 2001; Li et al., 2022b). They directly or indirectly participate in several programs, such as angiogenesis, the migration of Schwann cells (SCs), clearance of myelin and axonal debris, and outgrowth of regenerative neurites (Gaudet et al., 2011; Cattin et al., 2015; Li et al., 2022b). Macrophages are often classified into two main categories, the M1 and M2 macrophages. The M2 macrophages can be further divided into M2a, M2b, M2c, and M2d subtypes (Zigmond and Echevarria, 2019; Dervan et al., 2021). Several studies have reported the role of M2 macrophages in peripheral nerve regeneration (Huang et al., 2020; Yu et al., 2022), but

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Abbreviations. Mo/Mac, Monocyte/macrophage; PNI, Peripheral nerve injury; MPS, mononuclear phagocyte system; TRMs, tissue-resident macrophages; HSCs, hematopoietic stem cells; IFN-γ, Interferon-γ; LPS, Lipopolysaccharide; Th, helper T cell; IL, interleukin; TNF-α, Tumor Necrosis Factor-α; NOX2, NADPH oxidase 2; TGF-β, Transforming growth factor-β; PTEN, phosphatase and tensin homologue delet2ed on chromosome ten; PI3K, phosphatidylinositol-3-kinase; Akt, protein kinase B; PBS, phosphate balanced solution; Nos, nitric oxide synthase; MCS-F, Macrophage colony-stimulating factor; BMDMs, bone marrow-derived macrophages; SCs, Schwann cells; TLR, Toll-like receptor; PNS, Peripheral Nervous System; CNS, Central Nervous System; WT, wild type; CM, conditioned medium; DRG, dorsal root ganglion; PDGF, Platelet-derived growth factor; GDNF, Glial Derived Neurotrophic Factor; VEGF, Vascular endothelial growth factor; Ly6C, lymphocyte antigen 6C

studies on the effects of M1 macrophages are sporadic. This is partly because pro-inflammatory M1 macrophages were traditionally considered to have inhibitory effects on regeneration, while pro-healing M2 macrophages were considered to promote regeneration (Wynn and Vannella, 2016; Zigmond and Echevarria, 2019). Many studies have also shown that decreasing the ratio of M1/M2 can improve regeneration (Jia et al., 2019; Huang et al., 2020). However, recent studies have stated that designating M1 macrophages as inhibitory to regeneration and M2 macrophages as promoting regeneration is arbitrary. Both macrophages play key roles in different phases of the regenerative process (Tomlinson et al., 2018; Zigmond and Echevarria, 2019), and successful recovery requires cooperation between them.

Due to technological advancements, researchers can characterize macrophages at a higher resolution. Describing macrophage polarization using a multidimensional model might be more accurate than the simple M1/M2 bipolar paradigm (Murray, 2017; Hu et al., 2021). This model extends the classical M1/M2 polarization phenotypes to a dynamic, complex, and continuous spectrum of macrophage activation states (Xue et al., 2014; Ginhoux et al., 2016; Murray, 2017; Orecchioni et al., 2019; Zigmond and Echevarria, 2019; Mesquida-Veny et al., 2021), where the classical characteristics of M1 and M2 macrophages can even be expressed simultaneously in an individual macrophage under specific conditions, as shown in some studies (Bazzan et al., 2017; Kalinski et al., 2020). Therefore, these phenotypes should be considered together (Qiao et al., 2021). They play different but complementary roles in driving the regeneration process.

We argue that strategies that coordinate activities of M1 and M2 macrophages might have better regenerative outcomes after PNI. As most studies have investigated the M2 phenotype, but only a few studies have determined the significance of the M1 phenotype in peripheral nerve regeneration, we summarized some important roles of M1 macrophages in PNI to draw the attention of researchers to M1 macrophages. We highlighted some studies that have discussed the coordination between M1 and M2 phenotypes for facilitating regeneration. We speculated that these studies might provide new ideas for developing ways to manipulate different macrophages and achieve better peripheral nerve regeneration.

Overview of peripheral nerve injury and repair

Classification of peripheral nerve injury

The peripheral nervous system (PNS) includes peripheral, cranial, and spinal nerves, as well as, neuromuscular junctions (Manoukian et al., 2020). The basic element of the PNS is nerve, which is composed of nerve tissue, connective tissue, and blood vessels. The outermost surface of a nerve is a fibrous connective tissue called the epineurium. Inside the epineurium, nerve fibers are bundled into fascicles surrounded by the perineurium. Finally, individual fibers are sheathed by the endoneurium (Manoukian et al., 2020). The nerve fibers can be further classified as myelinated or unmyelinated fibers based on the presence of the insulating myelin sheath surrounding the axon.

Based on the extent of damage to the connective tissues and nerve axons, PNI can be classified by Seddon's or Sunderland's classification (Manoukian et al., 2020; Ye et al., 2022). In Seddon's scheme, PNI is classified into three categories, neurapraxia, axonotmesis, and neurotmesis. Neurapraxia is the mildest form of injury and is characterized by local demyelination without lesions in the axon or connective tissue. In axonotmesis, the axons are damaged or destroyed, and the perineurium and endoneurium might be disrupted, but the epineurium remains intact (Yi et al., 2020). Neurotmesis indicates the most severe peripheral nerve injury, where the entire nerve stump, including the axon, endoneurium, perineurium, and epineurium, is completely severed with the loss of continuity (Vijayavenkataraman, 2020; Yi et al., 2020).

Sunderland further expanded Seddon's classification to five degrees. Sunderland's Grade I is equal to Seddon's neurapraxia. Sunderland's Grades II, III, and IV correspond to Seddon's axonotmesis. In Grade II, the axon is disrupted, but the endoneurium, perineurium, and epineurium remain intact. In Grade III, the damage spreads to the endoneurium. In Grade IV, only the epineurium is intact-the continuities of the axon, endoneurium, and perineurium are impaired. Sunderland's Grade V corresponds to neurotmesis in Seddon's classification, which represents the highest degree of nerve injury (Yi et al., 2020). When the degree of injury reaches the level of axonotmesis (Sunderland's Grade II) or above, Wallerian degeneration occurs.

Wallerian degeneration

Wallerian degeneration (WD), characterized by Dr. Augustus Waller, is a well-regulated, self-destructive process, where the injured axons undergo fragmentation and disintegration and become debris, which is cleared by the glia and immune cells (Thomas, 1964, 1972; Thomas and King, 1974; Zhang et al., 2021). Several studies have identified key molecular components of WD, and a core regulating pathway has been established.

Nicotinamide mononucleotide adenylyltransferase 2 (NMNAT2) is an axon survival factor (Ding and Hammarlund, 2019). It catalyzes the formation of nicotinamide adenine dinucleotide (NAD^+), which is a crucial coenzyme and metabolite for maintaining axon homeostasis (Zhang et al., 2021). The level of NMNAT2 in axons is regulated by the balance between continuous anterograde transport and degradation in the axon. The E3 ubiquitin ligase Phr1 and MAPK signaling regulate the turnover of NMNAT2. Injuries cause the anterograde transport to shut down, thereby the NMNAT2 is rapidly

degraded within several hours, leading to the rapid loss of NAD^+ synthesis and accumulation of its substrate NMN (Ding and Hammarlund, 2019; Zhang et al., 2021).

Sterile alpha and Toll/interleukin receptor motif-containing protein 1 (SARM1) is the intrinsic trigger of axon self-destruction. It is a multidomain protein containing an N-terminal armadillo repeat motif (ARM), two tandem sterile alpha motif (SAM) domains, and a C-terminal TIR domain (Osterloh et al., 2012). The SAM domain mediates protein-protein interaction between SARM1 molecules, and the TIR domain possesses NAD^+ glycohydrolase (NADase) activity and can hydrolyze NAD^+ to adenosine diphosphate ribose (ADPR), cyclic ADPR (cADPR), and nicotinamide (Nam) (Gerdts et al., 2015; Essuman et al., 2017; Horsefield et al., 2019). The intrinsic NADase activity of the TIR monomer is low but can be boosted substantially when dimerized or multimerized by the SAM domain (Wan et al., 2019). The ARM domain self-inhibits the prodegenerative activity of SAM-TIR so that SARM1 is maintained in the inactive conformation in axons under normal conditions (Gerdts et al., 2015; Zhang et al., 2021).

NAD^+ acts as the inhibitor of the NADase activity of SARM1 (Jiang et al., 2020; Sporny et al., 2020), while NMN acts as an activator (Zhao et al., 2019; Bratkowski et al., 2020). The MAPK pathway may also facilitate the activation of SARM1, as c-Jun N-terminal kinase (JNK), a protein downstream of the MAPK pathway can phosphorylate the SAM domain, which increases the NADase activity of SARM1 (Murata et al., 2018; Zhang et al., 2021). Upon activation, SARM1 consumes NAD^+ rapidly, leading to a positive feedback-related enhancement of the SARM1 activity and further decreasing NAD^+. NAD^+ is an important coenzyme and metabolite required for the maintenance of axonal homeostasis and integrity. NAD^+ depletion leads to mitochondrial dysfunction and energy failure in injured axons, which leads to the production of ROS, Ca^{2+} imbalance, and protease activation (Sheng, 2017; Kiryu-Seo and Kiyama, 2019; Zhang et al., 2021). Activated proteases (e.g., calpain) mediate the disintegration of the axonal cytoskeleton, which leads to the fragmentation of the axons (Ma et al., 2013; Conforti et al., 2014). The resultant debris of the injured axons is eliminated by macrophages and Schwann cells. These cells produce chemokines to further recruit hematogenous macrophages to facilitate the removal of debris, and they also release cytokines and neurotrophic factors to drive the following axonal regeneration (Ye et al., 2022).

Axonal regeneration

Axonal regeneration mainly consists of three processes. First, regenerative programs are activated in injured neurons. After injury, the neurons change their gene expression patterns and epigenetic modifications (Curcio and Bradke, 2018; Renthal et al., 2020). For example, the DRG sensory neurons upregulate regeneration-associated genes (RAGs) and downregulate genes that define the neuronal identity (Renthal et al., 2020). Axotomy of the peripheral branch of sensory neurons induces acetylated histone H3 and nuclear PCAF, which leads to the expression of different RAGs (Puttagunta et al., 2014; Curcio and Bradke, 2018). Besides neurons, external regulation by other types of cells is also needed to boost the activation of regenerative programs. The most important regulators are Schwann cells and macrophages. Following peripheral nerve injury, Schwann cells reprogram to acquire reparative phenotypes (Jessen and Arthur-Farraj, 2019), which transmit survival and regrowth signals to the injured neurons by producing several neurotrophic factors (Arthur-Farraj et al., 2012; Fontana et al., 2012; Rigoni and Negro, 2020). Macrophages also release important mediators to drive the expression of RAGs by neurons (Hervera et al., 2018). Under the action of regenerative programs, thousands of neurites emanate from the proximal part of the severed nerve and regrow toward their targets.

The second process involves the directional regrowth of neurites. The cells that guide the regrowing neurites are Schwann cells (Parrinello et al., 2010; Min et al., 2021). After acquiring the reparative phenotypes, SCs form chains and migrate toward the center of the nerve bridge. They extend long parallel processes and align in tracts called bands of Büngner to provide a pathway for regenerating axons to navigate across the nerve bridge (Nocera and Jacob, 2020; Min et al., 2021). Macrophages help SCs to migrate. Following peripheral nerve transection, a hypoxic nerve bridge is formed between the distal and proximal nerve stumps. This hypoxia is sensed by macrophages in the bridge, which leads to the activation of the transcription factor HIF-1α. Activated HIF-1α induces VEGF-A expression in macrophages. VEGF-A, in turn, induces the formation of polarized vasculatures in the bridge, which serve as substrates for the migration of Schwann cells (Cattin et al., 2015).

The third process involves the reinnervation of the target. The Schwann cells strongly influence this process. They produce motor-specific or sensory-specific neurotrophic factors that guide the motor or sensory neurites to regrow into their corresponding branches. Additionally, at the endplate region of the neuromuscular junctions, the non-myelinating perisynaptic SCs (PSCs) extend processes and guide the axon sprouts to reinnervate the denervated endplates of neighboring muscle fibers. Details regarding this process are reviewed elsewhere (Gordon, 2020).

Overview of macrophage polarization

The term macrophage was coined by Metchnikoff. Macrophages are defined based on their nature of phagocytosis and usually belong to the mononuclear phagocyte system (MPS) (Radzun, 2015). They are

found in most tissues of the body and have critical roles in most physiological and pathophysiological processes (Shapouri-Moghaddam et al., 2018; Locati et al., 2020). Therefore, an imbalance in their homeostasis because of some problem leads to the onset of a disease.

Due to the close association between macrophages and health, macrophages have been studied for a long time and have been classified in different ways to understand their features systematically (Murray et al., 2014). The most frequently used classification is the M1/M2 system (Zigmond and Echevarria, 2019; Mohapatra et al., 2021). This nomenclature was introduced by Mills et al. (2000) to describe an intrinsic propensity of macrophages from different mouse strains to express specific phenotypes when stimulated (Zigmond and Echevarria, 2019). For example, *in vitro* LPS-treated macrophages from C57Bl/6 mice display TH1-like cytokine responses, whereas LPS-treated macrophages from BALB/c mice produce TH2-biased responses. Thus, the researchers proposed that macrophages induce an "M-1" or "M-2" response that is analogous to the TH1 or TH2 response (Ginhoux et al., 2016). Before this definition based on M1/M2 responses, macrophages were often classified into classically or alternatively activated macrophages in response to different activities from Th1 or Th2 cells, respectively (Murray et al., 2014; Zigmond and Echevarria, 2019). Later, the two nomenclatures were used concurrently, where M1 referred to classically activated macrophages while M2 referred to alternatively activated macrophages. Although some researchers might disagree (Orecchioni et al., 2019), for better narration, we considered the M1/M2 nomenclature equivalent to classically/alternatively activated macrophages in this review.

Inflammatory stimuli such as lipopolysaccharide (LPS) and interferon-γ (IFN-γ) induce the M1 phenotype, while anti-inflammatory stimuli, such as IL-4, IL-13, or IL-10, induce the M2 phenotype. M1 macrophages support inflammatory responses by producing pro-inflammatory factors, such as IL-6, IL-12, and tumor necrosis factor (TNF). In contrast, M2 macrophages suppress inflammation and promote tissue repair by secreting anti-inflammatory cytokines and growth factors. (Jia et al., 2019; Yunna et al., 2020). M2 macrophages show subtle phenotypic variations in response to different micro-environmental signals and thus can be further subdivided into four subtypes: M2a, M2b, M2c, and M2d (Ginhoux et al., 2016; Dervan et al., 2021). Different subtypes have specific roles. For example, M2a macrophages can remove dead cells and secrete anti-inflammatory cytokines and growth factors (IL-10 and TGF-β). M2b macrophages promote cell growth and the synthesis of the extracellular matrix (ECM), releasing IL-10 and VEGF-A. M2c macrophages help in enhancing tissue repair and remodeling the ECM. M2d macrophages induce polarized angiogenesis to restore blood flow (Dervan et al., 2021).

For better characterizing the M1 and M2 phenotypes, a marker system was established. In humans, M1 macrophages express several markers, including CD86, CD64, nitric oxide synthase 2 (NOS2), IL-6, IL-12, IL-1α, and TNF-α. M2 macrophages express CD206, CD163, transforming growth factor-beta (TGF-β), peroxisome proliferator-activated receptor gamma (PPARγ), C-C motif chemokine ligands 14 and 22 (CCL14 and CCL22), and arginase-1 (ARG-1) (Boutilier and Elsawa, 2021). Most macrophage markers in mice are similar to those in humans. However, some exceptions exist, for example, murine M1 macrophages do not express surface markers CD86 or CD64 (Boutilier and Elsawa, 2021). Details on M1/M2 markers are presented in Tables 1 and 2.

Table 1. Markers of M1 macrophages.

Species	Tissue	Marker
Human	Brain	TSPO
Human	Lung	CD68, HLA-DR
Human	Lung	CXCL10
Human	Undefined	ROS
Mouse	Adipose tissue	Ccl2, Ccl5, Il18, IL-6, Nos2
Mouse	Adipose tissue	CD274, TNF-alpha
Mouse	Adipose tissue	Il1b, Nos2
Mouse	Artery	CD16, CD32
Mouse	Artery	IL-6, iNOS
Mouse	Brain	CD40
Mouse	Lung	CD197, CD40, inducible nitric oxide synthase(iNOS)
Mouse	Lung	IL-12, iNOS, IRF5, TNF-alpha
Mouse	Serum	CCR7
Mouse	White adipose tissue	CD11c
Human	Undefined	CD16, CD32, CD369, CD64, CD68, CD80, CD86, IRF5, MerTK, MHC class II, STAT1
Mouse	Undefined	CD14, CD16, CD204, CD32, CD369, CD64, CD68, CD80, CD86, IRF5, Ly6C, MerTK, MHC class II, NOS2, STAT1
Human	Peripheral blood	CCR7, CD127, CD16, CD32, CD62, CD64, CD80, CD86, CXCL10, IL-15R, IL-17R, IL-1R-1, IL-2R, TLR2, TLR4

Data were acquired from CellMarker database (http://xteam.xbio.top/CellMarker/).

Although these markers are used to identify macrophages, this strategy might cause some problems because some macrophages can express both M1 and M2 markers (Vogel et al., 2013; Bazzan et al., 2017; Zigmond and Echevarria, 2019; Kalinski et al., 2020), especially *in vivo* (Tomlinson et al., 2018; Kalinski et al., 2020). On the other hand, macrophages in some homeostatic or pathological situations (e.g., embryonic macrophages, resolution-phase macrophages, and macrophages from some cancers) did not show a clear M1 or M2 phenotype (Ginhoux et al., 2016). Therefore, many researchers agree that the dichotomous M1/M2 model is insufficient to describe macrophage activation (Ginhoux et al., 2016), and further studies should be conducted to describe macrophage polarization more accurately (Zigmond and Echevarria, 2019). To address the relative ambiguity of the M1/M2 paradigm, the multidimensional model of polarization was proposed (Ginhoux et al., 2016).

The main characteristics of the multidimensional model of macrophage polarization are multi-dimensionality, dynamicity, continuity, and spectrum. This model integrates multiple signals that influence the polarization of macrophages, including ontogeny-related signals, tissue-specific signals, and stress signals (Ginhoux et al., 2016; Murray, 2017). Although this model can be used to define macrophages accurately, further research on multiple aspects is still required before it can be applied. First, the marker system that can characterize the multidimensional state of macrophages has not been established, making it difficult to identify different macrophages. Second, mapping different macrophage populations to their function is challenging (Ginhoux et al., 2016). This is not only because functional studies on the cells are lacking but also because dynamic macrophages always change their activity based on the changing environment. Finally, the excessive heterogeneity of macrophages in this model prevents researchers from determining the commonalities of the cells, which makes the classification lose its significance. To summarize, the multidimensional polarization model of macrophage is promising but still in its infancy. From a practicality perspective, it is still reasonable to use the M1/M2 paradigm, especially in the field of tissue engineering, as demonstrated by recent studies (Freedman et al., 2022; Jiang et al., 2022; Li et al., 2022a, 2023).

The roles of M1 macrophages in peripheral nerve injury

Activation of neural regenerative potential

As mentioned above, the activation of the neural regeneration programs requires external signals (Tedeschi and Bradke, 2017; Rigoni and Negro, 2020).

Table 2. Markers of M2 macrophages.

Species	Tissue	Cell Marker
Human	Lung	CCL18
Mouse	Artery	CD206
Human	Bone marrow	ARG, CCL2, CD163, CD206, FIZZ1, IL-10, MRC1
Human	Brain	CD163
Human	Lung	CD163, CD68
Human	Peripheral blood	CD14, CD163
Human	Serum	CD163
Human	Undefined	HLA-DR
Human	Undefined	IL-10
Mouse	Adipose tissue	Chil3
Mouse	Adipose tissue	Clec10a, Il4, Mrc1
Mouse	Artery	Arg-1, CD206, TGFbeta1
Mouse	Artery	Arginase-1, CD206, Ym-1
Mouse	Bone marrow	Arg-1, CCL22, CD11b, CD206, F4/80, MRC1
Mouse	Bone marrow	Arg-1, CD163, IL-10
Mouse	Bone marrow	IL-13R-alpha1
Mouse	Lung	Arg-1, CD163, CD206
Mouse	Lung	Arg-1, IL-10
Mouse	Lung	Arg-1
Mouse	Lung	CD206, IL-10
Mouse	Serum	CD16, CD200, CD206, CD32
Mouse	Serum	CD206
Mouse	Undefined	IL-10
Mouse	White adipose tissue	CD206
Human	Undefined	CD115, CD163, CD204, CD206, CD209, Fc-epsilon RI-alpha, IRF4, STAT6, VSIG4
Mouse	Undefined	CD115, CD14, CD163, CD204, CD206, CD209, CSF1R, Fc-epsilon RI-alpha, IRF4, Ly6C, RELMalpha
Human	Peripheral blood	CD163, CD206, CD23, CD36, IL-1, C-type lectin-like receptor dectin-1, CD209, DCIR, CLACSF13, FIZZ1, M60, CD184, TRAIL

Data were acquired from CellMarker database (http://xteam.xbio.top/CellMarker/).

Macrophages are the key players in this process (Niemi et al., 2013, 2016). For example, after axotomy, macrophages accumulate around the cell bodies of injured neurons and stimulate the outgrowth of neurites under the conditioning lesion (Niemi et al., 2013). The overexpression of CCL2 is sufficient to induce macrophage accumulation in uninjured DRGs and increase the regenerative capacity of DRG neurons via a STAT3-dependent mechanism (Niemi et al., 2016). Hervera et al. found that macrophages are recruited to the injured site following axotomy and release exosomes containing functional NADPH oxidase 2 complex (NOX2 complex). These exosomes are incorporated into injured axons via endocytosis. In axonal endosomes, the NOX2 complexes are retrogradely transported to the cell body, where they oxidize and inactivate PTEN, thus stimulating PI3K-phosphorylated (p-)Akt signaling and driving the expression of regeneration-associated genes (RAGs) (Hervera et al., 2018). In that study, the researchers found that exosomes can be generated by macrophages treated with LPS, which is a classical M1 macrophage inducer (Hervera et al., 2018). Their findings suggested that the M1 macrophages might activate the regenerative potential of the axons of injured neurons (Fig. 1).

Clearance of Wallerian degenerative debris

The Wallerian degenerative debris must be eliminated as it inhibits the regeneration of severed injured nerves (Kang and Lichtman, 2013; Vaquié et al., 2019). Schwann cells (Gomez-Sanchez et al., 2015) and macrophages (Forese et al., 2020; Wofford et al., 2022) are responsible for removing degenerative debris. The M2 macrophages can perform phagocytosis to scavenge debris and apoptotic cells (Shapouri-Moghaddam et al., 2018). However, some studies have shown that M1 macrophages also possess this property (Fig. 1). For example, Vereyken et al. showed that after incubation with LPS and IFN-γ (both are M1 inducers), macrophages exhibited a higher capacity to engulf myelin and neuronal fragments compared to macrophages treated with IL-4 (an M2 inducer) (Vereyken et al., 2011). Wang et al. reported that bone marrow-derived macrophages (BMDMs) treated with recombinant MCS-F (M2 inducer), followed by myelin debris, reduced the expression of M2 markers and increased the expression of M1 markers, as determined by Western blot assays, which suggested a phenotypic transition of macrophages from M2 to M1 after the treatment of myelin debris (Wang et al., 2015). Although Kroner et al. (2014) found that macrophages treated with LPS downregulated M1 markers and upregulated M2 markers upon myelin phagocytosis, these conflicting results probably because of differences in experimental protocols, specifically, depending on whether exposure of macrophages to myelin occurs before or after polarization, as reviewed by Zigmond et al. (Zigmond and Echevarria, 2019) and Kopper et al. (Kopper and Gensel, 2018).

The findings of some *in vivo* studies also indirectly suggested that M1 macrophages might help in eliminating myelin debris. Boivin et al. reported that the genetic ablation of the LPS receptor TLR4 led to impaired Wallerian degeneration compared to WT mice after sciatic nerve transection (Boivin et al., 2007).

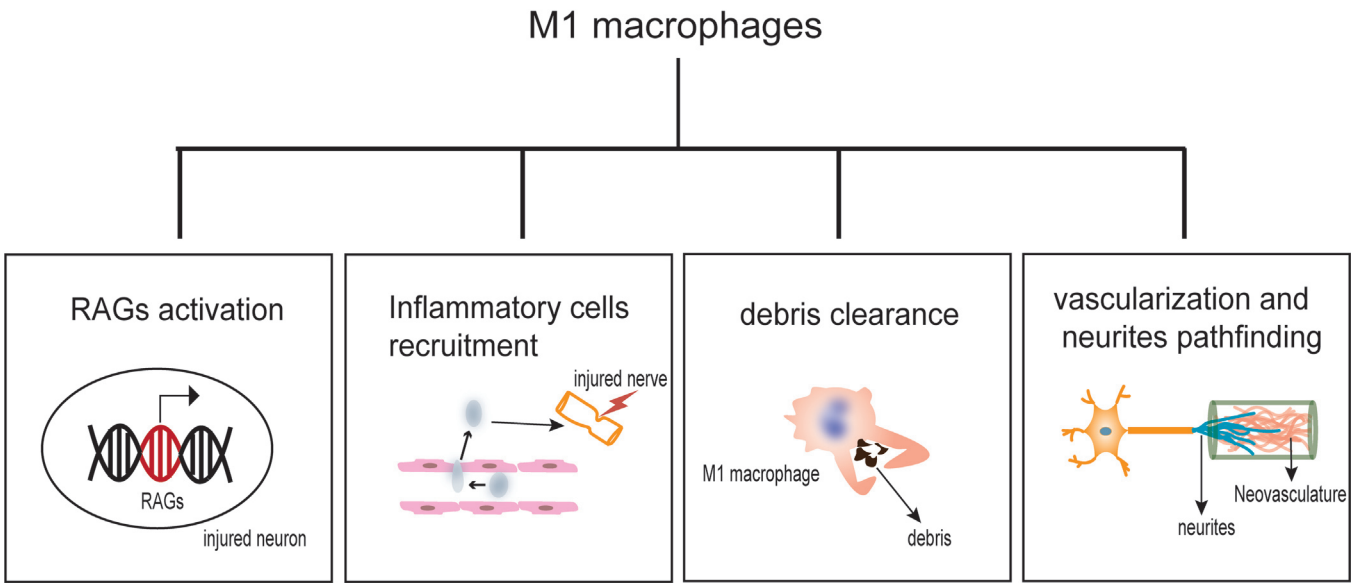

Fig. 1. The role of M1 macrophages in peripheral nerve regeneration. The M1 macrophages contribute to the peripheral nerve regeneration in many aspects, which include activation of regeneration-associated genes (RAGs) in the injured neuron, recruitment of inflammatory cells, clearance of Wallerian degenerative debris, vascularization of injured nerves and pathfinding of neurites.

Conversely, intraneural injection of LPS into the transected sciatic nerve of rats enhanced the recruitment of macrophages in the distal nerve and accelerated myelin phagocytosis compared to the injection of PBS (Zigmond and Echevarria, 2019). Mice deficient in secreted factors commonly associated with M1 macrophages, such as NOS2, IL-1β, or TNF-α, showed a similar phenotype to TLR4-deficient mice, i.e., impaired macrophage recruitment and delayed Wallerian degeneration (Zigmond and Echevarria, 2019). These studies suggested that M1 macrophages are required for Wallerian degeneration; however, further studies are needed to differentiate between the roles of M1 and M2 macrophages in this process.

Production of neurotrophic and growth factors

Macrophages can release different types of neurotrophic and growth factors (Elkabes et al., 1996; Tomlinson et al., 2018). These neurotrophic factors promote nerve regeneration (Anderson et al., 2018; Nocera and Jacob, 2020). Some of the mechanisms underlying the effects of neurotrophic factors have been elucidated (Chen et al., 2007; Sarhane et al., 2019). M2 macrophages are considered to be the main pro-regenerative factors producing cells (Takeda et al., 2011; Zajac et al., 2013). However, Cavel et al. found that LPS stimulation leads to an increase in the expression of GDNF by macrophages (Cavel et al., 2012). Similarly, Tomlinson et al. reported that pro-inflammatory stimulation increased the expression of PDGF-b (Tomlinson et al., 2018). In that study, the researchers found that the expression of the genes of neurotrophic and growth factors of M1 (IFN-γ + LPS) and M2 (IL-4) was not limited to the M2 (IL-4) phenotype (Tomlinson et al., 2018). Among the nerve repair-associated growth factors examined, approximately half were upregulated by IL4-conditioned BMDMs, and half were upregulated by IFN-γ + LPS-conditioned BMDMs (Tomlinson et al., 2018). Thus, the M1 macrophages are also important contributor to the production of the neurotrophic or growth factors (Fig. 1).

Pro-inflammatory macrophages respond early to nerve injury

As discussed above, M1-associated activities are required for recruiting blood-borne macrophages after injury (Boivin et al., 2007; Zigmond and Echevarria, 2019). M1 macrophages can produce several pro-inflammatory cytokines, such as interleukin-1β (IL-1β), IL-6, and TNF-α, to promote the infiltration of more immunocytes (e.g., monocytes and macrophages) into the injured site (Chen et al., 2015) (Fig. 1). Therefore, M1 macrophages are required to enrich the pool of macrophages within the injured nerve during the early phase of inflammation. The pro-regenerative M2 macrophages come from the initially formed pool. In a study, a parabiosis model was used in which wild-type mice and td-Tomato reporter mice shared blood circulation, which allowed researchers to track the origin of immune cells in the injured nerve. In this study, a cytometric analysis showed that most macrophages in the injured site were blood-borne cells, confirmed by the high proportion of td-Tom+ macrophages in the injured nerve (Kalinski et al., 2020); Further analysis showed that the blood-borne monocytes entered into the injured nerve and produced different macrophage sub-populations (Kalinski et al., 2020). An analysis of the expression of lymphocyte antigen 6C (Ly6C, which is expressed at high levels on pro-inflammatory, circulating monocytes) showed that one day after post sciatic nerve crush injury (SNC), the number of macrophages increased sharply in the injured nerve, and the Ly6C expression profile of macrophages consisted of 50% classically activated $Ly6C^{hi}$ cells (cells with high expression of Ly6C), 41% $Ly6C^{int}$ cells (cells with intermediate expression of Ly6C), and 9% $Ly6C^{-}$ cells (cells with negative expression of Ly6C) compared to the expression profile of naive nerve tissue. At day 3, the proportion of the $Ly6C^{hi}$, $Ly6C^{int}$, and $Ly6C^{-}$ cells were 28%, 47%, and 25%, respectively. At day 7, most macrophages were non-classical $Ly6C^{-}$ (65%) and intermediate $Ly6C^{int}$ (25%), with few $Ly6C^{hi}$ cells (10%). These results indicated that the MPS in the injured nerve was initially pro-inflammatory and then transitioned to a pro-resolving state (Kalinski et al., 2020; Dervan et al., 2021). Tomlinson et al. found a similar result, where an early pro-inflammatory response declined over time after sciatic nerve transection, and M2 markers (Retnla/Fizz1) increased throughout the following time course (Tomlinson et al., 2018).

Macrophages can switch from the M1 to the M2 phenotype (Spiller et al., 2015). Thus, the transition of macrophages from a pro-inflammatory to a pro-resolving state is at least partly due to the conversion of the predominant macrophage population from M1 to M2-like in the injured nerve. These results also indicated that M1 and M2 macrophages play different roles in different phases of peripheral nerve injury. M1 macrophages are active in the early phase, whereas M2 macrophages are active in the later phase. Emphasizing one phenotype while neglecting the other will yield a sub-optimal repair strategy (Dervan et al., 2021).

Indirectly guiding the regrowth and reinnervation of regenerative axons

Nerve regeneration mainly occurs for the proper reinnervation of target organs. Successful reinnervation largely relies on the migration and guidance of Schwann cells (Gordon, 2020; Min et al., 2021), which in turn require the newly formed blood vessels induced by macrophages to serve as substrates for migration (Cattin et al., 2015). Therefore, macrophages indirectly guide axon regeneration.

However, the phenotype of the macrophages in this new blood vessel formation process is not resolved.

Most researchers support that angiogenesis is mainly regulated by the M2 macrophages (Jia et al., 2019; Yang et al., 2021). However, recent studies have shown that M1 macrophages are also necessary for this process (Spiller et al., 2014; Tomlinson et al., 2018). The results of RNA-sequencing showed that VEGF-A expression was higher in M1 (IFN-γ + LPS) than in M2 (IL-4) (Tomlinson et al., 2018). Cattin et al. showed that the vasculature within the nerve bridge was formed before day 3 after transection (Cattin et al., 2015), which is a period when macrophages are pro-inflammatory, as discussed above (Lee et al., 2018; Kalinski et al., 2020). The findings of these studies suggested that the M1 macrophages can indirectly guide the regrowth and reinnervation of axons through pro-vascularization effects within the nerve bridge (Fig. 1).

Regulation of the phenotype of macrophages in regenerative medicine

Enhancing the M2 phenotype in peripheral nerve repair

The M2 macrophages can resolve inflammation and angiogenesis, thus promoting tissue repair. Based on these findings, researchers designed various nerve-guidance conduits (NGCs) to promote the M2 phenotype for improving the treatment of peripheral nerve injury. The methods to endow the NGCs with pro-M2 activity include 1) changing the physical properties of NGCs; 2) loading NGCs with bioactive factors. For example, Jia et al. prepared an aligned poly (l-lactic acid-co-ε-caprolactone) (P(LLA-CL)) nanofiber scaffold using the electrospinning technique. They found that the macrophages that seeded on aligned nanofiber scaffolds tended to show the M2 phenotype compared to those macrophages that seeded on random nanofibers. The results of *in vivo* experiments also showed that aligned and random nanofibers simulated macrophage polarization toward the M2 and M1 phenotypes, respectively. Immunolabeling studies showed that the aligned nanofiber NGC group exhibited significantly better results than the random NGC group regarding SC infiltration and axonal regrowth, as determined by the S100 and NF160 immunofluorescence results (Jia et al., 2019). Similarly, Dong et al. also found that the oriented nanofibers induced macrophage polarization toward the M2 phenotype both *in vitro* and *in vivo* and improved nerve regeneration outcomes regarding electrophysiological performance, histological evaluations, and behavioral assessments (Dong et al., 2022). Besides the alignment of the nanofibers, the porosity of NGCs also influences the polarization of the M2 macrophages. Yu et al. found that after being implanted for five months, the zein conduit with high porosity (PZC-high) achieved a better recovery of a 15-mm sciatic nerve defect in rats than the zein conduit with low porosity (PZC-low). Additionally, the number of M2 macrophages in PZC-high was higher than that in PZC-low, which suggested that the porosity of the zein conduit promoted M2 macrophage polarization and accelerated nerve regeneration (Yu et al., 2022). Some bioactive factors loaded on NGCs are also used to induce the M2 phenotype. For example, Lv et al. filled polycaprolactone (PCL) electrospun conduits with self-assembling peptide-collagen VI hydrogel to fabricate NGCs capable of gradually releasing collagen VI. The results of an *in vivo* study showed that the release of collagen VI could considerably shift macrophage polarization toward the M2 phenotype and improve nerve regeneration and functional recovery (Lv et al., 2017). Yadav et al. found that autologous platelet-rich growth factor (PRGF) inhibited the expression of M1-associated markers and increased the expression of the M2 marker CD206 during the induction of M1 macrophages. The immunohistochemistry (IHC) results showed that PRGF administration could promote axonal regrowth and remyelination and hence, improve the reinnervation of the targeted muscles (Yadav et al., 2022).

Although the above-mentioned studies showed the therapeutic potential of various strategies in enhancing the M2 phenotype in peripheral nerve repair, some issues still need to be addressed. First, most studies have characterized the phenotypes of macrophages within a time frame of weeks to months after the repair surgery, i.e., the period when macrophages naturally and intrinsically upregulate the M2 phenotype and downregulate the M1 phenotype (Dervan et al., 2021). Therefore, the information is insufficient to determine whether the benefits are derived from the transition from M2 to M1. M1 macrophages also actively participate in the early phase of peripheral nerve injury (approximately within seven days after injury), but the studies characterizing macrophages during this period are rare; thus, the potential actions of M1 macrophages might have been overlooked. Second, macrophage characterization lacks dynamicity in these studies, and thus, a lot of information is missing, considering that the phenotypes of macrophages change rapidly. Finally, the above-mentioned studies only showed the events where the M2 phenotypes were enhanced, but they did not investigate the causality between phenotype and regeneration. Therefore, further investigation is required to understand the role of the M2 phenotype in peripheral nerve repair and the underlying mechanisms.

Coordinating M1 and M2 phenotypes to improve regeneration

Strategies that enhance the M2 phenotype to promote peripheral nerve regeneration are common. No study has investigated the coordination between M1 and M2 macrophages to improve peripheral nerve repair. However, in studies on bone regeneration, researchers have coordinated M1 and M2 macrophages to repair bone defects. Because the mechanism of regeneration is common among different tissues, particularly concerning vascularization, we argue that studies on bone repair might provide new ideas to design more advanced scaffolds for improving peripheral nerve regeneration.

Spiller et al. designed a scaffold based on the

modifications of decellularized bone for a short-term release of IFN-γ to promote the M1 phenotype, followed by a more sustained release of IL4 to promote the M2 phenotype (Spiller et al., 2015). In that study, IFN-γ was absorbed in the scaffolds, while IL4 was attached via biotin-streptavidin binding. The results of the *in vitro* study confirmed that this scaffold partially achieved the sequential release profile of IFN-γ and IL-4, and at least at the level of gene expression, the M1 and M2 macrophages could be activated in different phases, although there were overlapping releasing phases (Spiller et al., 2015). The murine subcutaneous implantation model showed higher vascularization in this scaffold compared to that in the negative control. The scaffold absorbing IFN-γ alone showed the strongest effect in promoting the $CD31^+$ vascular density. This finding suggested that the M1 macrophage strongly influences angiogenesis (Spiller et al., 2015).

In another study, researchers designed a tissue-engineered bone (IFN-γ@ CaSiO3-β-TCP) using 3D printing technology (Li et al., 2018). The biomaterial contained silicate and was loaded with IFN-γ by physical absorption (Li et al., 2018); the former polarized M2 macrophages, and the latter stimulated and recruited M1 macrophages. The results showed that IFN-γ@CaSiO3-β-TCP scaffolds released IFN-γ in the early stage (1-3 days), followed by the sustained release of Si, while the scaffolds degraded (Li et al., 2018). The releasing profile contributed to the sequential polarization of macrophages, where the M1 macrophages were activated at an early stage, and the M2 macrophages were activated at a later stage. An *in vitro* tube formation assay and an *in vivo* subcutaneous implantation experiment showed that IFN-γ@CaSiO3-β-TCP significantly promoted angiogenesis compared to other groups (CaSiO3-β-TCP, IFN@β-TCP, β-TCP) (Li et al., 2018). These results also indicated that strategies that only use M2 macrophages ($CaSiO_3$-β-TCP) could not better facilitate the revascularization of the material.

As reviewed by Martin et al. M1 and M2 macrophages contribute to different stages of the revascularization of biomaterials; M1 macrophages promote the early stages of vascularization, such as endothelial cell sprouting, while M2 macrophages influence the later stages of vascularization and vessel maturation (Martin and Garcia, 2021). M1 macrophages can promote angiogenesis over time by inhibiting vessel growth and stimulating vessel regression; vessel regression is necessary for healthy angiogenesis (Korn and Augustin, 2015; Graney et al., 2020; Wang et al., 2021). Therefore, the coordinated efforts of M1 and M2 macrophages are required for angiogenesis (Wang et al., 2021), which is a prerequisite for the regeneration of any tissue, including peripheral nerves.

Conclusions and future perspectives

In this review, we proposed that the M1 macrophage strongly influences peripheral nerve regeneration, and strategies coordinating M1 and M2 macrophages should be developed to assist nerve repair.

First, the M1 macrophages contribute to several processes associated with peripheral nerve regeneration, including the activation of neural regenerative potential, clearance of Wallerian degenerative debris, production of neurotrophic and growth factors, and guidance of the regrowth of axons. Although these processes that occur in M1 macrophages are necessary for nerve regeneration, their application in current treatment strategies is insufficient and superficial.

Second, the inadequate evidence provided by most studies where researchers increased the activity of M2 macrophages to aid regeneration does not exclude the possibility of the involvement of M1 macrophages. Further studies are needed to determine the roles of M1 and M2 macrophages in tissue engineering strategies, for example, by setting more time points for the dynamic characterization of the phenotype or identifying macrophages at the early stage of injury rather than only performing post-repair characterization.

Third, the multidimensional model might better describe macrophage activation. However, the marker system and functional data of this model need further development. The approaches to determine cellular heterogeneity are usually expensive (e.g., single-cell transcriptome analysis, cytometry by time-of-flight mass spectrometry (Ginhoux et al., 2016)), which might discourage researchers. Therefore, the M1/M2 paradigm is still widely accepted and used. We believe that the multidimensional polarization represents the future direction to define the state of macrophage. The best macrophage-based immunomodulatory strategy might be to manipulate any macrophage population when required.

Finally, we propose two questions to be addressed in the future. 1) Can the dynamic phenotypic changes of macrophages and their association with peripheral nerve regeneration be determined precisely? 2) Can macrophages be modulated more precisely to obtain the desired phenotypes? We speculate that resolving these issues can promote the repair of PNI.

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