

● INVITED REVIEW

Regeneration-associated macrophages: a novel approach to boost intrinsic regenerative capacity for axon regeneration

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Abstract

Axons in central nervous system (CNS) do not regenerate spontaneously after injuries such as stroke and traumatic spinal cord injury. Both intrinsic and extrinsic factors are responsible for the regeneration failure. Although intensive research efforts have been invested on extrinsic regeneration inhibitors, the extent to which glial inhibitors contribute to the regeneration failure *in vivo* still remains elusive. Recent experimental evidence has rekindled interests in intrinsic factors for the regulation of regeneration capacity in adult mammals. In this review, we propose that activating macrophages with pro-regenerative molecular signatures could be a novel approach for boosting intrinsic regenerative capacity of CNS neurons. Using a conditioning injury model in which regeneration of central branches of dorsal root ganglia sensory neurons is enhanced by a preceding injury to the peripheral branches, we have demonstrated that perineuronal macrophages surrounding dorsal root ganglia neurons are critically involved in the maintenance of enhanced regeneration capacity. Neuron-derived chemokine (C-C motif) ligand 2 (CCL2) seems to mediate neuron-macrophage interactions conveying injury signals to perineuronal macrophages taking on a solely pro-regenerative phenotype, which we designate as regeneration-associated macrophages (RAMs). Manipulation of the CCL2 signaling could boost regeneration potential mimicking the conditioning injury, suggesting that the chemokine-mediated RAM activation could be utilized as a regenerative therapeutic strategy for CNS injuries.

Key Words: axon regeneration; conditioning injury; neuron-macrophage interaction; regeneration-associated macrophage; cAMP; CCL2; M2 polarization; spinal cord injury

Introduction

A failure of spontaneous axon regeneration or limited extent of axonal plasticity following central nervous system (CNS) injury is largely responsible for poor functional recovery in patients with acute destructive lesions such as stroke, spinal cord trauma, and so forth. It has been traditionally thought that axon regeneration is hampered by both environmental factors surrounding injured axons and intrinsic cellular factors governing growth potentials. Recent several years have witnessed a plethora of exciting research data showing that manipulation of intrinsic regenerative capacity can attain axon regeneration in rodent models to the extent that has not been imaginable by previous approaches (Liu et al., 2011). It would be thrilling to await development of novel therapeutic measures translated from these lines of researches to boost intrinsic regenerative capacity by modulating specific signaling pathways. A potential concern, however, would be that the signaling pathways involved in intrinsic axon growth potential, phosphatase and tensin homolog (PTEN) or

B-RAF for example, are also relevant for tumor growth, raising likelihood of undesirable side effects when considering systemic delivery of drugs targeting the signaling molecules.

In this review, we propose that activating macrophages with pro-regenerative molecular signatures could be another approach for boosting intrinsic regenerative capacity of CNS neurons. The fact that macrophages can promote axon regeneration is not new. Benowitz and his colleagues reported that accidental lens injury during intravitreal injection stimulated regeneration of retinal ganglion neurons and infiltration of macrophages associated with lens injury was responsible for the regeneration-promoting effects (Yin et al., 2003, 2006). Another study showed that induction of chronic inflammation by zymosan together with degradation of inhibitory proteoglycans allowed functional regeneration of sensory axons through the dorsal root entry zone in the spinal cord (Steinmetz et al., 2005). After spinal cord injury, macrophages are activated into two divergent subsets: M1 (pro-inflammatory)

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or M2 (anti-inflammatory) types, and acquisition of either polarization is highly flexible and dynamic (Kigerl et al., 2009). *In vitro*, M2 macrophages were shown to be predominantly neuroprotective and promote axon growth whereas M1 macrophages were neurotoxic. Indeed, activation of macrophages by zymosan resulted in promotion of axon regeneration with concurrent neurotoxicity especially when zymosan was injected close to neuronal cell bodies (Gensel et al., 2009). To summarize, activation of macrophages is certainly associated with enhanced regeneration of axons, but the effects of macrophages on axon regeneration are complicated by their potential neurotoxic influence and dynamic changes between M1 and M2 polarization *in vivo* after injury.

Recent studies done by our lab demonstrated that macrophages are activated into a solely pro-regenerative phenotype in the dorsal root ganglia (DRG) following preconditioning peripheral nerve injury (Kwon et al., 2013). It has been well established that preceding peripheral nerve injury enhances axon regeneration of the central branches of the DRG neurons after subsequent injury to the spinal cord, which is so called “conditioning effects”. We first observed that perineuronal macrophages increased their numbers in the DRGs after preconditioning peripheral nerve injury, but not after an injury to the central branches, and the increase persisted for at least several months. Using minocycline, a macrophage deactivator, we provided evidence that macrophage activation is essential in the enhancement of axon regeneration by preconditioning peripheral nerve injury. Interestingly, when we injected cyclic adenosine monophosphate (cAMP), a reagent well known to increase intrinsic capacity of axon growth, into DRGs, we found that activation of macrophages occurred to the extent similar to that observed after preconditioning injury. Based on this observation, we set up an *in vitro* model in which pro-regenerative macrophage activation is achieved by cAMP-mediated neuron-macrophage interaction. In this model, cAMP treatment to the co-cultures consisting of DRG neurons and macrophages, not to either neuron or macrophage culture alone, resulted in accumulation of pro-regenerative factors in the culture medium, which greatly promoted neurite outgrowth of cultured DRG neurons. These findings led us to hypothesize that certain signals emanating from injury site at the peripheral branches or cAMP injection stimulate DRG neurons to produce factors that activate or recruit macrophages, which in turn secrete pro-regenerative factors directly affecting regenerative potential of DRG neurons (Figure 1). Further studies by our lab and others identified that a chemokine (C-C motif) ligand 2 (CCL2) is the neuron-derived factor activating macrophages (Kwon

et al., 2015). In the neuron-macrophage co-culture model, cAMP treatment failed to stimulate macrophages when CCL2 (–/–) neurons were co-cultured with wild type (WT) macrophages, but not when WT neurons were co-cultured with CCL2 (–/–) macrophages. Conversely, cAMP treatment effectively stimulated macrophages to produce pro-regenerative activities when chemokine (c-c motif) receptor 2 (CCR2) (–/–) neurons were co-cultured with WT macrophages, but the pro-regenerative activities were substantially attenuated when WT neurons were co-cultured with CCR2 (–/–) macrophages. These findings suggested that injury signals conveyed to neuronal cell bodies might produce CCL2 that in turn recruits macrophages around neuronal cell bodies and instructs them to acquire a pro-regenerative phenotype. We further demonstrated that CCL2 overexpression in DRG neurons were sufficient to enhance capacity of DRG neurons to regenerate axons in *in vivo* spinal cord injury model, confirming that CCL2 is necessary and sufficient for the enhancement of regenerative capacity in conditioning injury model.

One of the peculiar findings in our study was that macrophages activated in our model did not exhibit any neurotoxic effect *in vitro* or *in vivo*. The conditioned medium collected from the neuron-macrophages co-cultures treated with cAMP never resulted in a decrease in the number of cultured DRG neurons. Although intraganglionic CCL2 overexpression elicited marked activation of perineuronal macrophages, we did not observe any evidence of damages to the DRG neurons. In a previous study, macrophage activation resulted in degeneration of transplanted neurons and impaired axon growth when zymosan was injected close to the cell bodies (Gensel et al., 2009). Hence, the phenotype of activated macrophages following preconditioning peripheral nerve injury or CCL2 overexpression mimicking conditioning injury is solely pro-regenerative as opposed to that of chemically activated macrophages. We speculate that our *in vitro* neuron-macrophage co-culture model faithfully replicate the solely pro-regenerative macrophage phenotype observed following preconditioning peripheral nerve injury. It is well known that activated macrophages surrounding tumor tissue produce various growth-promoting molecules, thus contributing to further aggravation of tumor growth (Ostuni et al., 2015). These macrophages are called “Tumor-associated macrophages (TAMs).” Interesting analogies exist between TAMs and activated macrophages in our model. Activation of TAMs is initiated by molecules derived from tumor cells, and TAMs in turn provide growth-promoting factors to tumor cells. In our model, pro-regenerative macrophage activation is initiated by CCL2 derived from neurons with injury signals from the

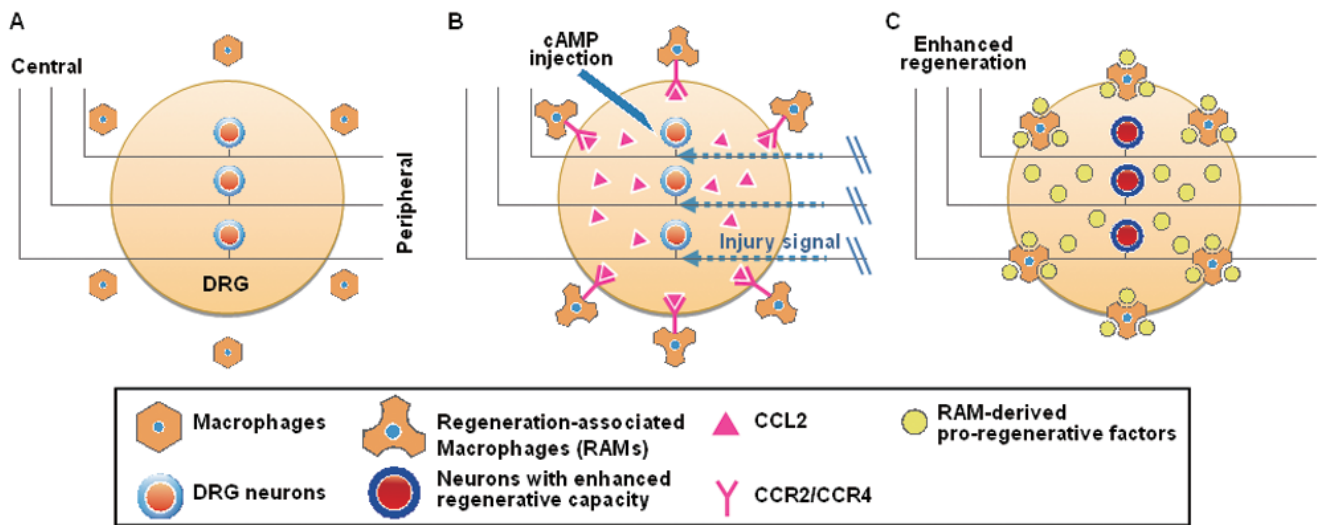


Figure 1 A schematic diagram illustrating our model for the neuron-macrophage interactions in conditioning injury-induced enhanced regenerative capacity of dorsal root ganglia (DRG) sensory neurons.

(A) A simplified illustration of the major players in a resting state. (B) Preconditioning peripheral nerve injury or cyclic adenosine monophosphate (cAMP) injection, either of which supports activation of regeneration-associated genes (RAGs), stimulates neurons to produce chemokine (C-C motif) ligand 2 (CCL2) to attract and activate regeneration-associated macrophages (RAMs). Not only chemokine (C-C motif) receptor 2 (CCR2) but also CCR4 on macrophages was found to bind to neuron-derived CCL2 (Kwon et al., 2015). (C) RAMs produce various molecular factors that enhance regenerative capacity of DRG neurons.

peripheral nerves or cAMP, and the activated macrophages in turn provide neurite outgrowth promoting factors to neurons surrounded by them. Therefore, we propose that the activated macrophages with a solely pro-regenerative phenotype is named as “regeneration-associated macrophages (RAMs)” to underscore the analogies with the TAMs.

What are functional implications of RAMs in a conditioning injury model? We addressed this question using two methods to suppress RAMs: pharmacological inhibition using minocycline and genetic deletion of CCL2 (Kwon et al., 2013, 2015). When minocycline was delivered intrathecally to DRGs *via* an osmotic minipump, conditioning effects on neurite outgrowth were almost completely abolished when DRG neurons were cultured 7 days after injury. Interestingly, minocycline injection did not attenuate neurite outgrowth activities observed just 1 day after conditioning injury. This finding suggested that activation of RAM might not be involved in the initial induction of conditioning effects. We obtained clearer pictures on this issue using CCL2 ($-/-$) mice. In CCL2 ($-/-$) mice, conditioning effects at 3 and 7 days after injury were completely abolished. However, the percentage of neurite-bearing DRG neurons at 1 day was similar between WT and CCL2 ($-/-$) mice. Moreover, expression of growth associated protein 43 (GAP-43), one of the most representative regeneration-associated genes (RAGs) reflecting growth potentials, was unambiguously observed at 1 day after injury in CCL2 ($-/-$) mice, where-

as the GAP-43 expression was abolished at 3 and 7 day. These findings verify that RAM activation is dispensable for the initial induction of RAGs after injury, but building up conditioning effects at later time points and their maintenance are dependent of CCL2-mediated RAM activation. To demonstrate the necessity of RAM activation in the maintenance of conditioning effects for a longer time period, intrathecal minocycline administration was delayed until 3 weeks after injury and lasted 7 days (Kwon et al., 2013). We observed almost complete abrogation of conditioning effects while delivery of control PBS with the same time schedule resulted in neurite outgrowth as robust as what is expected at 7-day time point. It was reported that increases in RAG expression and enhanced neurite outgrowth potentials persist for as long as several months (Ylera et al., 2009). The long-standing effects after conditioning injury may be necessary for the proper *in vivo* axon regeneration phenotype since the regenerating axons would require lasting and uninterrupted RAG expression to maintain sustained elongation through inhospitable molecular environment at the injury site. In this context, we argue that RAM activation is necessary to achieve the enhanced intrinsic capacity for axon regeneration in CNS by conditioning injury. The initial induction of conditioning effects, however, may be achieved by an entirely neural mechanism with retrograde injury signals. How the retrograde injury signals evolve generating molecules with chemokine activity (like CCL2) will be another interesting line of future researches.

Conclusion

How could the concept of “RAM” be utilized therapeutically to enhance axon regeneration after CNS injury? Our *in vitro* neuron-macrophage co-culture model would provide us a unique opportunity to characterize molecular signatures, especially in regard to transcriptional networks, of RAMs with an exclusively pro-regenerative phenotype without accompanying neurotoxicity. Then, it would be possible to create RAMs using cell-reprogramming technologies *ex vivo* or *in vivo*, and transplantation of reprogrammed RAMs or delivery of reprogramming factors designed to create RAMs could be envisioned as therapeutic interventions. Another therapeutic approach would be to activate signaling pathways leading to RAM activation after CNS injury. It is obvious that RAM activation does not occur following axotomy in CNS. Figuring out detailed upstream signaling requirements for proper RAM activation in CNS might enable us to develop a novel pharmacological strategy to enhance intrinsic regenerative capacity of injured CNS neurons. This approach would be relatively safe because potential signaling molecules for RAM activation are less likely to be coupled to oncogenic pathways.

Author contributions: *MJK and BGK designed the experiments and wrote the manuscript. MJK and HJY performed the researches and collected the data.*

Conflicts of interest: *None declared.*

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