Molecular Therapy
Commentary

Carbon monoxide licensing of MSCs enhances their efficacy through autophagy-mediated miRNA mechanisms

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Sepsis is a complex condition leading to multiple organ failure including the development of acute respiratory distress syndrome (ARDS) secondary to infection. The mortality rate of sepsis is 40%–60%,[1] indicating an unmet need for the development of novel therapeutics for this condition. Mesenchymal stromal cells (MSCs) are known for their immunomodulatory and cytoprotective effects; however, their efficacy as a cellular therapy for sepsis has been disappointing, with less than ∼50% of patients responding to treatment. Thus, strategies to enhance MSC efficacy to increase the response rates in patients are eagerly awaited.

In this issue of *Molecular Therapy*, Hwang et al. demonstrate the ability of licensing human bone marrow MSCs with low levels of carbon monoxide (CO) to enhance their therapeutic efficacy in a preclinical model of sepsis, identifying a mechanism associated with autophagy-mediated miRNA expression in MSC-derived extracellular vesicles (EVs) (Figure 1).[2] Furthermore, this *ex vivo* conditioning of human MSCs (hMSCs) with CO led to increased bacterial clearance, neutrophil phagocytosis, and mouse survival after cecal ligation and puncture (CLP). Together, these novel findings demonstrate the importance of *ex vivo* MSC licensing prior to administration to improve MSC therapeutic success.

Although MSCs are one of the most extensively studied cell-based therapies, heterogeneity remains the primary concern leading to discrepancies in their clinical therapeutic efficacy. MSCs require activation to exert their full therapeutic potential, which can be carried out through licensing these cells *in vitro* with stimuli or *in vivo* in an inflammatory disease microenvironment. This can be achieved through various methods, such as exposure to pro-inflammatory stimuli, including injury, inflammation, and disease.[3,4] MSC licensing can lead to changes in the expression of genes, cell surface markers, and secreted molecules, subsequently influencing the behavior of these cells *in vivo*, optimizing their regenerative, immunomodulatory, or anti-inflammatory effects for a given application.

The application of MSCs for treatment of sepsis and septic shock has been well documented in phase 1 and 2 trials,[5,6] where MSCs isolated from different tissues had a positive effect on survival rates in early-stage sepsis (NCT02421484, NCT02328612, NCT01849237, NCT05283317, NCT04961658, NCT03369275, NCT05969275, and NCT05283317). However, further randomized controlled trials are required with important consideration given to the primary endpoints and overall trial design.

The strategy of licensing or pre-activated MSCs prior to their administration has demonstrated success in enhancing MSC therapeutic efficacy.[7] Preliminary results from a first-in-human trial for genetically modified MSCs (termed: GEM00220) in bacterial sepsis (AMETHYST – NCT04961658) were presented at ISCT 2024 with publication of the data eagerly awaited. There is currently an active phase 2 trial investigating the efficacy of inhaled CO for the treatment of ARDS (NCT03799874) investigating the potential direct cytoprotective effects of this compound. Interestingly CO licensing has been studied in other cell types, such as T cells, where CO increased mitochondrial biogenesis,[8] indicating the potential relevance of the novel work demonstrated by Hwang et al.[2] to other cell types and diseases.

Autophagy is required to maintain cellular homeostasis through the degradation of damaged proteins and organelles. Furthermore, the regulation of autophagy in MSCs can improve their therapeutic efficacy. Hwang et al. illustrate the benefits of licensing MSCs with CO *ex vivo*, to enhance autophagy and thus increase the expression of micro RNA (miRNA) in MSC-derived EVs. CO-licensed hMSCs demonstrated significant upregulation in the expression of autophagy-associated genes *LC3B*, *Beclin1*, *ATG5*, and *ATG1* compared to MSCs exposed to room air. Furthermore, silencing of beclin1 in MSCs or addition of autophagy inhibitor SP-1 or 3-MA impaired induction of autophagy in CO-licensed MSCs.

miRNA plays an important role in the pathogenesis of sepsis-induced ARDS, making miRNA modulation a potential therapeutic agent, where miRNA could be overexpressed with mimics or, conversely, blocked through the use of miRNA inhibitors. MSC-derived EVs or exosomes are known to contain specific miRNAs, highlighting their beneficial use in the context of sepsis.[9]

Hwang et al. hypothesize that neutrophils aid bacterial clearance through phagocytosis in their model of CLP-induced polymicrobial sepsis. Macrophages efferocytose these apoptotic neutrophils in an attempt to restore homeostasis and resolve inflammation. Mice that underwent CLP surgery and received 5×10^5 MSCs intravenously demonstrated increased bacterial clearance in the blood and peritoneum. Furthermore,

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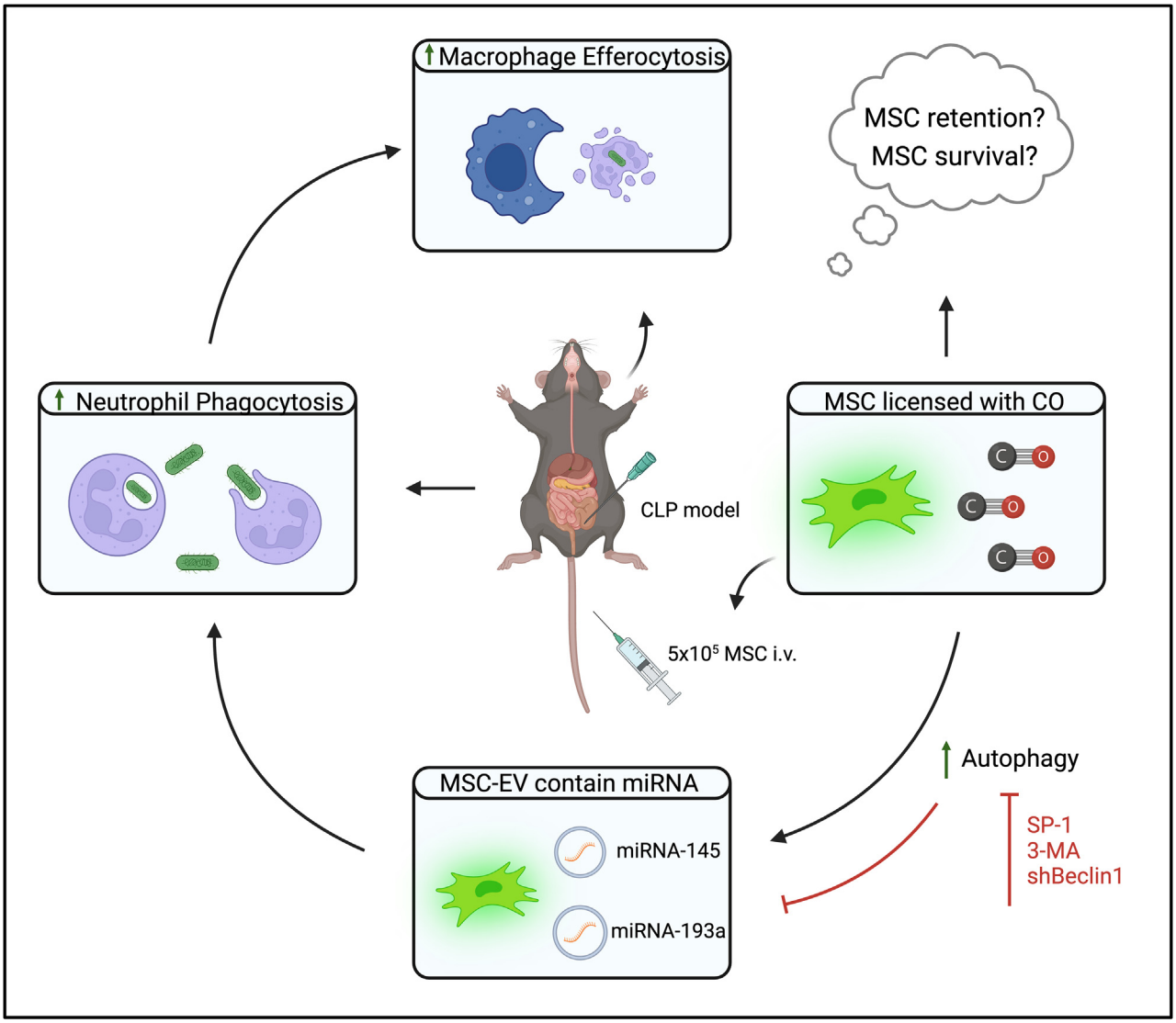

Figure 1. Carbon monoxide licensing enhances MSC efficacy in sepsis

Human bone marrow-derived MSCs licensed *ex vivo* with 250 ppm of carbon monoxide for 4 h have increased autophagy, which was abrogated after use of autophagy inhibitors SP-1 and 3-MA or beclin1 silencing. MSC autophagy leads to the production of extracellular vesicles (EVs) containing miR-145 and miR-193a. After systemic administration of 5×10^5 CO-licensed MSCs, MSC-derived EVs containing micro RNAs were responsible for MSCs therapeutic effects of increased neutrophil phagocytosis of GFP-labeled *E. coli,* increased macrophage efferocytosis of apoptotic neutrophils, and increased animal survival after cecal ligation and puncture (CLP). Possible future avenues would be to investigate MSC fitness and retention after CO licensing.

the study highlights the novel mechanistic role of miR-145-3p and miR-193a-3p in MSC therapeutic efficacy. In addition to the application of miRNA inhibitors, the use of mimic miRNA or antagomirs would be beneficial to further investigate the importance of miRNA activity in MSCs' ability to decrease neutrophil phagocytosis, macrophage efferocytosis, bacterial clearance, and animal survival.

The authors confirm that the therapeutic effects of CO-licensed MSCs are mediated through paracrine means, with miRNAs being present in isolated EVs. However, it is well documented that MSCs have multiple paracrine mechanisms of action, such as mitochondrial transfer or the secretion of anti-inflammatory cytokines, enzymes (cyclo-oxygenase or indoleamine 2,3-dioxygenase), or lipid mediators (PGE2). It would be interesting to further delve into these mechanisms and to investigate other possible avenues of MSC paracrine function in the context of sepsis, as it is unlikely that a single paracrine mediator is causing this pleiotropic effect on disease outcome.[9] To translate these findings to the clinic, it would be important for the authors to define a potency assay to accurately depict the MSC mechanism of action, as heterogeneity in

the results of MSC clinical trials is still an issue, impacting their therapeutic success.[10]

Modulation of macrophage polarization and function leading to a pro-resolving macrophage phenotype is an important mechanism mediating MSC therapeutic effects. As MSCs are known to become apoptotic shortly after systemic administration, it would be beneficial to elucidate the extent of MSC retention *in vivo* after administration to eliminate the hypothesis that MSCs are exerting their cytoprotective effects by means of being phagocytosed by macrophages, thus aiding bacterial clearance indirectly. Direct comparison between CO-licensed MSCs and the secretome/EVs from CO-licensed MSCs would provide definitive information on the efficacy of whole cells compared to the secretome/EVs. Lastly, *in vivo* imaging analysis would help to further characterize the fitness of MSCs after CO licensing, in terms of MSC survival or retention compared to MSCs exposed to room air.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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