Immunomodulatory Strategies to Enhance Neuromuscular Regeneration After Composite Muscle-Nerve Injuries

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INTRODUCTION: Most musculoskeletal injuries involving volumetric muscle loss (VML) are composite, often involving damage to other tissues including bone and nerves [1]. Damage to skeletal muscle and its associated nerves can disrupt communication at the site of neuromuscular junctions (NMJs). Reduced nerve regeneration post-muscle trauma can inhibit functional skeletal muscle recovery outcomes [2]. Modulation of the inflammatory response after composite injuries is required to prevent excessive fibrosis, which has been shown to impede nerve regeneration [3]. Cell secretome including soluble proteins such as cytokines, chemokines, and growth factors, and extracellular vesicles (EVs) offer a promising targeted therapy. For example, mesenchymal stem cell (MSC)-derived EVs are being used as carriers for therapeutic cargo to develop potential treatments for immunomodulation and muscle and nerve regeneration [4]. Interleukin-4 (IL-4) is a pro-regenerative cytokine associated with M2 macrophages, and exogenous supplementation of IL-4 has been shown to aid in muscle function and myogenesis [5] and peripheral nerve regeneration [6]. Bioengineered MSC-EVs enriched with immunological factors will be encased in a biosponge scaffold and delivered to the sight of composite VML and nerve crush (NC) injuries in rats. Our hypothesis is that these specific immunological factors will support NMJ formation when encapsulated inside the MSC-EV-biosponge system and have sustained bioactivity. This work will unfold mechanisms underlying neuro-musculoskeletal repair, and to develop a potential therapeutic to aid functional recovery after composite muscle trauma.

METHODS: ***In vitro experiments*** **-** NSC-34 motor neuron cells were cultured in differentiation media supplemented with glial cell line-derived neurotrophic factor (GDNF; 100 ng/mL), IL-4 (50, 100 ng/mL), and interferon-gamma (IFN-γ; 50, 100 ng/mL) for 6 days. Brightfield images were taken, and neurite length was measured using ImageJ on days 2, 4, and 6. MSCs were cultured, and their EVs were collected. Their size and EV-specific markers were characterized using Nanoparticle Tracking Analysis and Exocheck^{TM} Exosome Antibody array membranes, respectively. T cell-EVs were transfected with green fluorescent protein (GFP) mRNA (Exo-Fect Exosome Transfection Kit). MSCs at a density of 10,000 cells/well were incubated with GFP-transfected EVs. After 24 and 40hrs, fluorescent images were taken and GFP+ cells per field of view were quantified. Biosponge scaffolds were fabricated by mixing type A gelatin, collagen type I, and laminin-111 with zero-length crosslinkers EDC and NHS. They were allowed to gel overnight and were flash frozen with superchilled methyl-2-butane and lyophilized. SEM images were taken to evaluate pore size. ***In vivo experiments*** **–** All animal procedures were approved by the Saint Louis University Institutional Animal Care and Use Committee (3172). A 6mm biopsy punch was used to create VML injury in the tibialis anterior muscle in 10-12 week old male Sprague Dawley rats, and hemostats were used to crush the peroneal branch of the sciatic nerve for 60sec to create the NC injury (n=6/group). Male rats were used due to financial constraints of the study. GFP-transfected EVs ($5x10^5$ EVs) were injected intramuscularly into the site of the VML defect, and on day 7 post-injury muscle was harvested for histological analysis. At 4 weeks post-injury, peak-isometric torque production was measured, and tissues were harvested. Statistical analysis was performed via one or two-way ANOVA tests.

RESULTS SECTION: NSC-34s cultured with GDNF (100 ng/mL) had a significantly higher percentage of 30-45 µm sized neurite extensions ($p<0.05$). NSC-34s cultured with IL-4 (100 ng/mL) extended neurites on days 4 and 6 comparable to those cultured with GDNF (100 ng/mL). When cultured with IFN-γ (50, 100 ng/mL), neurite extensions from NSC-34s were significantly shorter than controls ($p<0.05$). MSC-EVs had an average size range of 50-300 nm. MSC-EVs showed expression for characteristic EV markers, including CD63, ANXA5, TSG101, ICAM, and CD81. EVs transfected with GFP mRNA were uptaken by MSCs *in vitro* and cause production of GFP in a time-dependent manner. GFP-transfected EVs injected intramuscularly resulted in GFP+ cells throughout a VML injured muscle on day 7 post-injury. Biosponge scaffolds were fabricated with gelatin, collagen, laminin, EDC, and NHS, and have consistent small pores on average 8.607 µm. A novel injury model of VML+NC was developed, which resulted in peak isometric torque functional deficits at 4 weeks post-injury. Functional deficit of VML+NC compared to contralateral control was 87.9%, which was significantly higher than that of the VML at 64.6% ($p>0.05$).

DISCUSSION: Our results show that a neurotrophic factor, GDNF and anti-inflammatory factor, IL-4 have similar effects on neurite extension, while a pro-inflammatory factor, IFN- γ, inhibits neurite outgrowth. We successfully transfected EVs with GFP mRNA, demonstrating EVs as a promising gene delivery vehicle for potential therapeutics in composite muscle-nerve injuries. Peak isometric torque measurement showed successful development of a composite VML-NC injury in rats. Future work will involve encapsulation of bioengineered MSC-EVs into the biosponge scaffold to be delivered to the site of composite nerve-muscle injuries in this rat model.

SIGNIFICANCE/CLINICAL RELEVANCE: (1-2 sentences): Traumatic injuries to extremities are a major contributor to long-term disability, and an estimated 22% of patients with VML also suffer peripheral nerve injury. This work has translational relevance to clinical cases of severe composite musculoskeletal injuries like VML.

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Figure 1

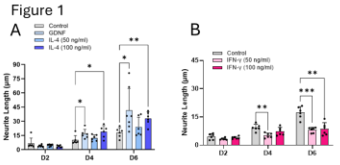

Figure 2

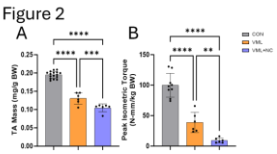

Figure 1: Quantified neurite extension lengths from NSC-34 motor neurons cultured with GDNF (A), IL-4 (A), and IFN-γ (B).
Figure 2: Mass (A) and peak isometric torque measurements (B) of untreated VML and VML+NC muscles at 4 weeks post-injury.