**Immunomodulatory properties of muscle-derived stem cells associated with reduced NF-κB/p65 signaling**

**INTRODUCTION**

The nuclear factor kappa B (NF-κB) signal pathway has been implicated in both the normal and disease states of many different tissues. In skeletal muscle, for example, constitutive activation of inhibitor of kappa B kinase (IKKβ), a potent activator of NF-κB, leads to muscle wasting.[1] Inversely, muscle specific deletion of IKKβ in a murine model of muscular dystrophy improves dystrophic pathology and is accompanied by an increase in the number of cells fitting a muscle progenitor marker profile (CD34+/Sca1+), suggesting that NF-κB has a direct effect on muscle stem cells.[2] The NF-κB protein family includes five subunits, two of which, a p65-p50 heterodimer, are thought to play a role in blocking early myogenesis.[3] In this study, we examined the role of NF-κB signaling in the regenerative phenotype of muscle-derived stem cells (MDSCs) isolated from the gastrocnemius of p65 deficient mice (heterozygous, p65+/−) and wild type litters (p65+/+). We previously found that p65−/− MDSCs have enhanced cell proliferation, survival under oxidative stress, differentiation, and muscle regeneration capacity. Furthermore, we have found that p65−/− engraftments in wild type skeletal muscle are associated with reduced inflammation and fiber necrosis compared to p65+/+ MDSC engraftments. In vitro and in vivo experiments suggest that reduction of p65 signaling enhances the regenerative phenotype of MDSCs, suggesting this pathway as a candidate target to improve stem cell-based therapies for muscle disease and injury.

**MATERIALS AND METHOD**

**Cell Isolation:** MDSCs were isolated from five month old (n=3) p65+/− or p65+/+ mice via a preplate technique.[4] A population of slowly adhering cells was obtained and expanded in DMEM containing 10% fetal bovine serum (FBS), 10% horse serum, 1% penicillin-streptomycin, and 0.5% chick embryo extract. Cells were used between passages 15 and 30.

**In vivo regeneration assay:** Muscle injury was induced in C57Bl/6J mice by cardiotoxin injected into gastrocnemius. One day later, MDSCs were injected into the injured muscles. Six days following transplantation, mice were sacrificed and injected muscles were harvested and snap-frozen. Serial cryosections were prepared and immunohistochemistry was performed to assess inflammation (CD14) and necrosis (IgG). The number of CD14 (+) cells was counted to assess infiltration of macrophages/monocytes. Necrosis was determined by mouse IgG staining and quantified by assessing the percentage of positively stained area.

**In vitro Inflammation Model:** MDSCs were grown for 24 hours in proliferation medium, after which the medium was collected and sterile filtered. RAW264.7 cells, immortal murine macrophage-like cells, were activated by exposure to 100 ng/mL LPS in either p65+/+, p65−/−, or muscle-derived fibroblast conditioned medium for 24 hours.

**Gene Expression Analysis:** Cells were washed and RNA collected by Trizol extraction. Total RNA was reverse transcribed with Superscript III reverse transcriptase (Invitrogen) according to manufacturer’s protocols. The PCR reaction was carried out with Taq Polymerase (Promega), according to manufacturer’s protocols. PCR products were analyzed by electrophoresis on a 1.5% agarose gel.

**RESULTS**

Wild type mice were injected with either p65−/− (Fig 1A) or p65+/+ (Fig 1B) MDSCs in the gastrocnemius muscle and sacrificed six days later. Tissues were cryosectioned and immunostained with antibodies against CD14 to identify a monocyte/macrophage infiltrate, indicating inflammation, as well as with antibodies against mouse immunoglobulin G, a marker of necrosis. Injections of p65−/− cells were associated with a decrease in both necrosis and inflammation (Fig 1B-D), compared to p65+/+ injections (Fig 1A, C-D).

**DISCUSSION**

The data presented here provides evidence supporting that NF-κB inhibition stimulates MDSC-mediated muscle regeneration through multiple mechanisms, including through the expression of anti-inflammatory factors that attenuate inflammation and necrosis. These experiments identify the NF-κB signaling pathway as a potential therapeutic target to enhance muscle regeneration following injury or disease. Future directions for this project include investigating modulation of the IKK/NF-κB pathway as a means to rejuvenate the phenotype of aged muscle stem and progenitor cells. Clinical research should be conducted to test the efficacy of p65 inhibition therapy in patients suffering from muscle disorders.

**REFERENCES**