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Modeling spinal muscular atrophy with patient derived iPSCs to characterize the role of
SMN in cardiomyocytes and microglia

by

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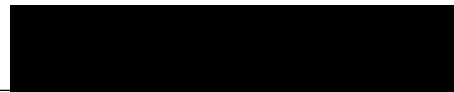
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ABSTRACT

Characterizing the role of SMN in Cardiomyocytes and Immune cells in Spinal Muscular Atrophy:

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Spinal muscular atrophy (SMA) is a neurodegenerative disease characterized by loss of alpha motor neurons and skeletal muscle atrophy. The disease is caused by mutations of the *SMN1* gene that result in reduced functional expression of survival motor neuron (SMN) protein. Global restoration of SMN in a SMA model mouse suggests that non-neuronal cells may contribute to the disease progression. In severe SMA, reports of cardiovascular and glia dysfunction are observed prior to the onset of motor neuron degeneration. In this dissertation, we utilize a patient-derived iPSC model to assess the role of SMN in cardiomyocytes and microglia function.

The first study focuses on the role of SMN in cardiomyocyte function. Markers of heart failure, including brain natriuretic peptide (BNP), fatty acid-binding protein 3 (FABP3) and creatine kinase (CK) are elevated in Type I SMA patients. We found impaired cardiovascular function in severe SMA mice and in cardiomyocytes differentiated from iPSC(s) derived from SMA patient(s). Reducing SMN levels in cardiomyocytes from control patient iPSCs slowed relaxation kinetics and calcium

reuptake similar to SMA patient derived cardiac cells. Importantly, restoring SMN in iPSC derived cardiomyocytes increased calcium reuptake. Transcriptome data of whole hearts taken from juvenile SMA mice indicated a reduced level of SERCA, the key ATPase pump responsible for calcium reuptake and muscle relaxation. Taken together, these results indicate that SMN deficiency impairs cardiomyocyte function at least partially through SERCA mediated intracellular Ca^{2+} cycling dysregulation.

The second study focuses on the role of SMN in microglia. Increased microglia reactivity and small lymphoid organs have been reported in the SMA mice. How SMN deficiency affects immune cell function in SMA remains unresolved. In this study, we sought to characterize the immune cells in the CNS of SMA model mice and characterize the reactive profile of CNS resident microglia. Our studies revealed that lymphocytes play a minor role in disease progression. However, microglia from spinal cords of SMA model mice exhibit a reactive morphology and unique transcriptome profile. Next, we generated microglia from control and SMA patient iPSCs. Microglia were validated by flow cytometry, immunocytochemistry and transcriptome profiling. SMN-deficient microglia displayed an aberrant migration pattern and increased phagocytosis. Moreover, conditioned media released from SMA microglia altered electrophysiological properties and cell morphology of control and SMN deficient human iPSC differentiated motor neurons.

Current available treatments for SMA are not effective in all patients, suggesting other disease modifying therapeutics are critically needed. Here we show that SMN-deficiency alters cardiomyocyte and microglia function, representing potential modifiers

of SMA pathology. Developing therapeutics that target these tissue-specific pathways could bring us closer to a cure.

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CHAPTER 1: Introduction

SPINAL MUSCULAR ATROPHY

Spinal muscular atrophy (SMA) is a progressive autosomal recessive disorder, with a carrier frequency of 1 in 50 (202). SMA is one of the leading causes of infant mortality and is characterized by a homozygous deletion or mutation in the *SMN1* gene, resulting in insufficient production of the survival motor neuron (SMN) protein (193). The first clinical descriptions of SMA were published in the 1890s by Guido Werdnig and Johan Hoffman, attributing muscle weakness to the loss of alpha motor neurons in the anterior horn of the spinal cord (116). It was not until 1995 that SMA was linked to *SMN1* gene mutations (147). There is no known cure for SMA but current therapeutic interventions aim to increase levels of SMN protein (80, 184, 235, 283). Effectiveness and toxicity vary among patients, suggesting additional avenues for therapy are needed. In humans, SMN is encoded by two *survival motor neuron* genes: *SMN1* and *SMN2* (193). *SMN1* and its paralog gene *SMN2* lie in a unique location within a complex duplication region (307). Due to this position, unusual gene copy variations occur for both *SMN1* and *SMN2* in different populations (307). The major difference between the two genes is a C to T transition in exon 7 of *SMN2*, resulting in alternative splicing (276). *SMN1* makes primarily full-length SMN, and the *SMN2* gene primarily produces an unstable SMN protein lacking exon 7 that is rapidly degraded (193).

Due to deletion or mutations of the *SMN1* gene, SMA patients rely exclusively on the *SMN2* gene products, resulting in insufficient levels of the SMN protein. For reasons unknown, motor neurons in the ventral horn of the spinal cord are most susceptible to reduced levels of SMN protein. While numerous studies characterizing the role of SMN in

motor neuron vulnerability continue to be reported, emerging literature strongly suggests that peripheral tissues also play a role in disease progression (42, 49, 51, 64, 65, 174). Moreover, co-administering therapies selectively targeting peripheral tissues with SMN-boosting therapies, may eventually provide a cure to the disease.

Epidemiology

Spinal muscular atrophy is a debilitating disease with an incidence of 1 in 11,000 (288) suggesting a carrier frequency in the general population of roughly 1 in 50 (202). While this incidence rate is relatively high, particularly in Europe, the rate seen in Sub-Saharan Africa is lower with a carrier frequency of only 1 in 209. The reason for this variability is due to the fact that people residing in sub-Saharan Africa have several copies (≥ 3) of the *SMN1* gene, (177, 215) reducing the chances of observing homozygous deletion of the gene (254, 307).

Clinically, SMA is characterized by the inability of patients to achieve motor milestones and the disease severity correlates with the number of *SMN2* copies. Due to the range of *SMN2* copy number, the clinical and phenotypic manifestations of SMA fall on a continuum, ranging from severe (inability to sit independently) to mild (ambulatory). A version of the original Hammersmith Functional Motor Scale is typically used to assess the motor deficits seen in SMA patients and monitor therapeutic efficacy (238). Motor function performance is determined using 36 unique motor performance skills (238). Items ranging from sitting to standing, to rolling to the side, and ability to bring one's hands to their head. Additionally, there are two timed tests (238). The final assessment can be divided into five groups: very severe (type 0), severe (type I), intermediate (type II), mild (type III), and very mild (type III and type IV) (193). Type 0 has an onset in

utero, where movements are absent or reduced. Polyhydramnios was observed in these patients, requiring ventilation assistance for severe respiratory distress at birth (6). These patients live to only six months of age (6). Type I, also known as Werdnig-Hoffmann disease, accounts for roughly 50-60% of all SMA cases (21, 236). These patients have symptomatic onset at 6 months of age and typically do not live past the age of two years. They lack the ability to hold their heads up, support themselves, or walk. Type II, also known as Dubowitz disease, has an onset of muscle weakness seen at the age of 6-18 months (87). Type III, also known as Kugelberg Weller disease, has symptom onset at 18 months of age (143). The mild Type IV patients have symptoms onset occurring in adulthood (193, 316, 139). Despite the variety in phenotype groups, 95% have a homozygous deletion or mutation of SMN1 gene on chromosome region 5q13 (193).

Pathology

The common pathology seen in all SMA patients is symmetrical progressive muscle weakness due to skeletal muscle atrophy brought on by the loss of alpha motor neurons and denervation of muscle fibers (104, 269). Motor neurons appear especially sensitive to low levels of the ubiquitously expressed SMN protein. Patients typically display proximal muscle weakness compared to distal muscles and those of the lower extremities (48, 104). Weakening of these proximal muscles results in the inability to support the head and the trunk while sitting, as well as an increase in gait instability leading to lateral gait lean and compromised abductor muscles (104). Due to progressive muscle weakness, fasciculations can occur in the limbs, as well as in the thoracic wall. Furthermore, fasciculations of the tongue may develop as the disease progresses (104). Although weakness, muscle fatigue and deterioration are predominant phenotypes in

SMA, children with Type I and Type II ultimately die from respiratory failure (140). The intercostal muscles that support normal breathing are weakened in these patients (104). Children with severe SMA must rely solely on their diaphragm to support breathing and coughing. As a result, children with SMA type I or II often have a collapsed chest wall. Additionally, the ability to properly clear the lower respiratory track is impaired. Patients with severe SMA require ventilation assistance for respiratory failure, particularly during sleep (14).

Although the majority of SMA research is focused on motor neurons, recent evidence suggests that SMA is a multi-system disorder (64, 155, 269, 275). A retrospective study utilizing SMA patient insurance claims indicate symptoms associated with skeletal muscle, heart, autonomic nervous system, vasculature, gastrointestinal, bones and connective tissue dysfunction (155). Additionally, SMA patient autopsies include reports of impaired kidney function, as well as abnormalities in the spleen and pancreas, supporting a multi-organ pathology.

Cardiac dysfunction is one of the more frequent symptoms reported by patients prior to muscular degeneration (155). Accounting for a majority of the SMA cases, the severe Type I patients have been shown to have electrocardiogram (ECG) abnormalities, bradycardias, hypertrophy, as well as structural cardiac defects including atrial and ventral septal defects (11, 71, 180, 191, 201, 244, 293, 303, 311, 315). Children with SMA are predisposed to dyslipidemia, with elevated blood levels of cholesterol and triglycerides. Serum levels of fatty acids and lipids may serve as potential biomarkers of SMA (66, 152). Accumulation of fatty substances results in predisposition for fatty liver disease as well as cardiac dysfunction in SMA patients (66). Cardiomyopathies can lead

to improper circulation, depleting the distal limbs of the necessary nutrients (126). For example, digital necrosis was observed in patients with Type I SMA (12). This phenomenon was also noted in severe SMA model mice where vascular necrosis of the extremities was often observed (110).

Low SMN levels in pancreas, kidney, bone, and gastrointestinal tract have been previously reported in severe SMA Type I patients (14, 32, 66, 77, 98, 206, 269, 270, 275). Deletion or mutation in *SMN1* may affect pathways involved in bone-resorption (270). SMN protein is thought to interact with osteoclast stimulating protein to promote production of osteoclasts (270). Patients with SMA often suffer extreme osteopenia and may have repeated injuries linked to minor trauma (133). Bone mineral density is also shown to decrease with age (133). Thinning of the rib cage has been observed in severe patients, adding to the respiratory distress (77). Decrease in bone mineral and density has been commonly observed in patients with gastrointestinal diseases due to malabsorption (306). SMA patients have also reported gastroesophageal reflux, as well as constipation (98). As 37% of the enteric nervous system that innervates the gastrointestinal (GI) tract is comprised of motor neurons, it is not surprising that GI immobility and thus delayed gastric emptying is observed (98). A reduction of smooth muscle, as well as disruption in the gastrointestinal system has been recapitulated in SMA model mice (99).

SMA Type I patients often exhibit glucose intolerance, similar to the fasting hyperglycemia and glucose resistance demonstrated in the severe mouse models (32). This was correlated to high levels of circulating glucose observed in patient urine samples (206). Moreover, abnormal levels of cystatin C, sodium, calcium, and creatinine further implicates kidney dysfunction in SMA patients (206). SMN deficiency affects

many tissues and organs. The body protects itself through a functioning immune response. Unfortunately, reduced SMN levels also target the spleen and thymus, resulting in immune system abnormalities (64, 275). The spleen size has been shown to be reduced in three different mouse models of SMA (64, 197). Additionally, an elevated number of lymphocytes were observed in these conditions (64). How these immune cells influence the outcome of disease pathogenicity is still not well understood.

Although SMA is primarily a motor neuron disease, emerging clinical observations have uncovered additional features that could be treated to further improve the disease outcome. In most cases, SMA is diagnosed only after muscle weakness is apparent, which is too late in the disease progression to reverse motor neuron loss. Therapies that increase SMN levels in motor neurons slow motor neuron loss. However, physiological deficits that cannot be reversed by restoring SMN could provide additional therapeutic opportunities to improve the efficacy of current treatments.

Physiological role of SMN

The SMN protein is ubiquitously expressed (193). Loss of SMN protein in mice is embryonic lethal, highlighting its importance in development and cell survival (246). The human 38kD SMN protein is made up of 294 amino acids, comprised of several functionally important domains (90, 137, 169, 255, 256, 282, 318).

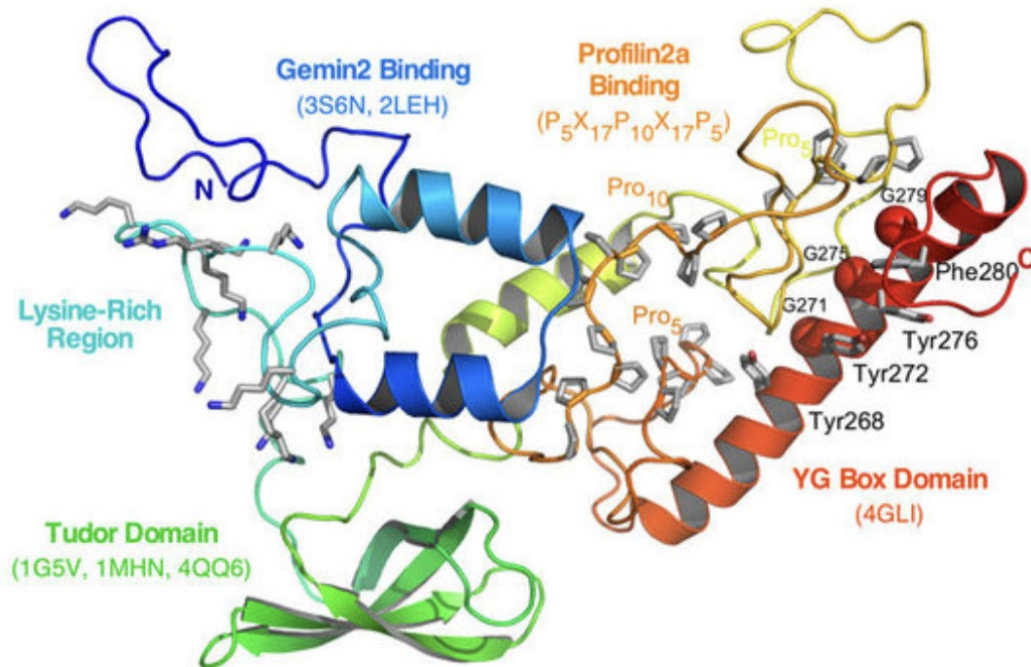


Figure 1. Structure of the SMN protein with functionally important domains including Gemin2-binding domain, a lysine-rich region, a Tudor domain, a Profilin2a binding domain and a YG box. Figure adapted from Singh et al. 2017 (158).

SMN protein and Gemin 2-8 form the core SMN complex. This complex is assembled in the cytoplasm and migrates to the nucleus where it is concentrated into nuclear gem structures, crucial sites in RNA processing (211). These nucleolar gem-like structures, or coiled bodies, were found to be heavily concentrated with small nuclear ribonucleoproteins (snRNPs) spliceosome subunits (19). Within these coiled bodies, SMN has been associated with fibrillarin, an RNA binding protein (43).

Findings indicate SMN protein has a role in the assembly of small nuclear ribonucleoproteins (snRNPs). snRNPs are essential for pre-mRNA splicing and have the potential to alter gene transcription (18, 276, 320). Therefore, changes in SMN levels can cause alterations in snRNPs biogenesis, impacting protein homeostasis (188). The splicing machinery is not unique to neurons and motor neurons, and is carried out in all cells (92).

Thus, the reduction in SMN protein dramatically impacts protein production in all tissues. Timely and efficient protein expression is required during critical periods of development, as well as for maintaining cellular homeostasis (43, 197). In fact, a proteomic analysis in SMN deficient mice showed a widespread alteration in protein expression during embryonic development (19, 119, 312). Irregularities in protein expression could explain the systemic pathologies previously mentioned.

SMN protein may play a cell specific function. Within the cell, SMN has been shown to not only localize in the nucleus and cytoplasm, but in dendrites, synapses, and along the axon (23, 197). A transcriptome profile conducted in SMA model mouse brains highlighted changes in gene clusters linked to cytoskeletal dynamics, axon guidance, somatodendritic compartmentalization, regulation of cytokine production, and vesicular transport compared to controls (28).

The cytoskeleton dynamics within the cell, particularly those of the actin filaments and microtubules, are the driving force responsible for maintaining many basic cellular properties (26, 28). Remodeling of the cytoskeleton can be attributed to the polarized cellular shape, required for endocytosis and migratory capabilities of glial cells (128). Additionally, the cytoskeleton facilitates in the transport of mRNA, proteins, and organelles along the cell axon (233, 234). One of the proteins mediating these actin and microtubule dynamics throughout the cell body is profilin (233). In mammals, five profilin isoforms have been described. These include profilin I, IIa, IIb, III, and IV (144). Of these, profilin IIa is the most abundant in the CNS tissue (271). Although profilin IIa has the most affinity to SMN by binding to its proline-rich region, knockdown of either isoform profilin I or profilin II results in inhibition of neurite outgrowth and aggregation of SMN in the

cytoplasm (29, 31). Furthermore, the alterations in profilin II seen due to SMN deficiency lead to an upregulation in the RhoA-ROCK signaling pathway in the spinal cord and skeletal muscle from SMA model mice (197). In SMA model mice, actin cytoskeleton signaling was disrupted in the skeletal muscle, spinal cord, heart, and liver (29). Pharmacological targeting of ROCK pathways increases myofiber and neuromuscular junction endplate area size, and improves the survival of SMA model mice (200, 257). In other neurodegenerative conditions, ROCK inhibitors have also been used to reduce inflammation, as well as phagocytosis in microglia, and improve survival of neurons (170, 290, 309).

Muscle weakness is attributed to denervation and compromised synaptic transmission of motor neurons in SMA pathophysiology (290). Severe SMA patients displayed an age dependent decline in motor unit number estimation (MUNE), as well as a decline in maximum compound motor action potential amplitude (CMAP) (134). CMAP and MUNE are electrophysiological approaches for tracking the operating state of a motor unit pool in real time. Following stimulation of a peripheral nerve, the CMAP response reflects the electrophysiological contribution from a muscle or group of muscles. MUNE reflects the approximate number of motor neurons or axons supplying the muscle or a group of muscles under investigation (321). Autopsy results of SMA patients indicate anterior horn cell loss occurred, while the remaining neurons were decreased in size (120, 175, 247). A motor unit is comprised of the lower motor neuron, its axon and the muscle fiber it innervates. Reduced levels of SMN seem to modulate the axonal outgrowth, resulting in a truncated length in both motor neurons as well as sensory neurons (121, 247). Moreover, both sensory and motor neurons present with shorter neurites and smaller

growth cones, as well as reduced expression of beta-actin mRNA and protein (141). In a study looking at severe SMA model mice, there was actually very minimal denervation and Wallerian degeneration of motor neurons occurring throughout the disease progression (141, 170, 301, 309). SMN deficiency results in pre-synaptic as well as post-synaptic abnormalities at the neuromuscular junction (141). While the packaging of the neurotransmitter remained consistent between groups, reduced synaptic vesicle release as well as reduced quantal content was indicated in a severe SMA model mouse (141). Additionally, decreased acetylcholine receptors (AChR) clusters, and tiny myofibers reflect an immature myosin heavy chain sequence (141, 301). Together, maturation of motor nerve terminals may be compromised in SMA (2).

Next, we look at the muscle fibers of the SMA motor unit. As the name suggests, there is significant atrophy of the muscle, particularly that of the limbs and trunk (111). Muscle biopsies of case reports indicate increased interfascicular fibrosis as well as muscle fiber atrophy (61). Interestingly, reducing SMN to SMA levels in muscle alone using a Myf5-Cre driver did not result in a neuromuscular phenotype in an animal model (118). Moreover, restoring SMN in muscle of SMA animals did not improve survival (118). Interestingly, boosting SMN protein concentrations via an ASO treatment in a severe SMA model mouse was able to stop motor unit loss, but there was a delay in restoring the full function of the motor unit, suggesting that there are other cells that contribute to its repair (94, 113).

Although SMN has a widespread effect on cellular and tissue composition, there is still much we do not know. For example, what the threshold of SMN protein level that is required for optimal processing of cellular activities is yet to be determined. Additionally,

the SMN levels each cell type and tissue require may be unique to its function. The key to an ideal SMA therapeutic is to target the aberrant mechanisms that occur prior to the onset of motor neuron degeneration and muscle decline.

IMMUNE CELLS IN NEURODEGENERATIVE CONDITIONS

Immune cells have played as the body's major defense in countless neurodegenerative diseases (9, 232, 292). Although many cells can contribute to inflammation, when considering neuroimmunology, the focus is mainly on microglia, the resident immune cell in the CNS (47, 105, 210). In healthy conditions, cell-surface and secreted microglial inhibitors are constitutively expressed by neurons (105). In neurodegenerative conditions, it is likely that the death or damage of neurons removes this inhibition, resulting in an activated microglial state. Additionally, cytokines released by the lymphatic system can influence microglia phenotype (47). In fact, microglial and lymphocyte reaction to motor neuron damage has been an inherent immune response in ALS disease progression (292). Activated microglia were observed in spinal cord sections of severe SMA model mice (69). Moreover, there is a significant increase in the lymphocyte population in SMA mice compared to controls, potentially contributing to neuroinflammation. Both lymphocytes and microglia can be pro-inflammatory or anti-inflammatory (281). It is still not fully understood whether these immune cell populations are beneficial or harmful to the SMA disease progression.

Neuroinflammation in SMA

The central nervous system (CNS) is composed of two major types of cells; neurons and glia. Depending on the species, glia' cells can make up to 33-66% of the total CNS population, (122) comprised of three distinct cells types: astrocytes, oligodendrocytes, and

microglia (67). Oligodendrocytes and astrocytes both originate from the neural progenitor cells within the neuroectoderm (16, 67). Microglia on the other hand are unique in that they arise from hematopoietic stem cells in the yolk sac during early embryogenesis, and migrate to the CNS for further development (96). As supporting cells, glia ensure homeostasis within the CNS, and aid in neuronal survival through pH buffering, ion exchange, glutamate recycling, synapse structure stabilization and debris removal (16, 294).

Oligodendrocytes

Studies outlining the contribution of oligodendrocyte to SMA pathology are conflicting (212, 215). Oligodendrocytes are critical for myelin formation to facilitate the propagation of action potentials in the CNS (37). All aspects of oligodendrocyte development and CNS myelination were normal in a severe mouse model of SMA (215). Despite that, decreased expression of myelin basic protein and NG2 were observed in SMA mice spinal cords (212). NG2 glia are capable of generating not only oligodendrocytes, but also astrocytes and neurons, so aberrant NG2 expression may reflect dysfunction in these subsequent cell populations (212). As oligodendrocytes are shown to be preserved throughout the time course of a severe model of SMA, further studies clarifying the role of oligodendrocytes in SMA are warranted (313).

Astrocytes

Astrocytes play a contributing role to SMA pathology (35, 167, 172, 213, 229, 242). Glial fibrillary acidic protein (GFAP), a marker for neurological injury, was increased in regions near the sites of degenerating alpha motor neurons in human post mortem spinal cords (242, 319). There was no change in GFAP staining found in white matter (242).

Similar findings were observed in SMA model mice, as well as an altered astrocyte morphology and cytokine expression profile (19, 213). Gene expression analysis showed significant elevation of IL-1 β , IL-6 and TNF α in SMA mice, potentially driving neuroinflammation (242). Elevated levels of calcium can induce astrocytes to produce pro-inflammatory cytokines (108). Intriguingly, increased basal levels of calcium were observed in SMA astrocytes compared to controls in studies using patient-derived iPSCs differentiated astrocytes (172). Administration of ATP, a known trigger of intercellular calcium signaling, only caused a minimal calcium response (172). A deficit in internal calcium signaling could indicate a compromised cellular interaction, as well as reduction in secreted glial cell line-derived neurotrophic factor (GDNF) levels by SMA astrocytes (172, 229). Like GDNF, monocyte chemoattractant protein 1 (MCP1) is secreted by astrocytes to promote neuronal survival. Importantly, reduced MCP1 secretion was described previously in SMA (167). Restoring SMN to astrocytes alone partially attenuated the pro-inflammatory profile, improved neuromuscular circuitry, and extended the life span of SMA mice, suggesting that immune cells may contribute to SMA disease progression (35, 50, 167, 172, 212, 213, 229, 242, 259).

Microglia

Microglia play a vital role in mediating innate immunity in the CNS (96, 151, 260). Not only do they protect against neuronal damage, microglia play a significant role throughout development (218). The main function of microglia is to maintain homeostasis and clear cellular debris (97, 210). In the healthy mature brain, tissue microglia have elongated cell bodies, with extensive branches and are referred to as “ramified microglia”. These “ramified”, or resting microglia, are responsible for immune surveillance and show

a suppressed immune phenotype adapted to the specific microenvironment of the CNS. In neurodegenerative conditions, microglia become rapidly activated in response to pathological conditions or immunological stimuli (232). In SMA, ionized calcium binding adaptor molecule 1 (Iba1), a marker for microglia, is upregulated prior to the onset of severe motor neuron degeneration (295). Microglia were found to co-localize with degenerating alpha motor neurons in spinal cords of SMA model mice (295). Like astrocytes, murine studies indicate SMN-deficient microglia adopt an inflammatory profile including increased reactive oxygen species, increased TNF α production, and increased NFkappaB activity (10). Additionally, CD68, a marker of phagocytic activity, was dramatically increased in SMA microglia. This trend in phagocytosis due to reduced SMN levels in microglia was corroborated by several labs (10, 308). Although ablation of microglia did not spare motor neuron degeneration, motor function was improved (308). However, all studies on microglia described thus far have been done in animal models (10, 295). In Alzheimer's research, microglia differentiated from mouse vs human cells indicated over 700 differentially expressed genes in humans versus mice, confirming the importance of validating current SMA microglia findings in humans (106).

Lymphocytes

Activation of lymphocytes, whether T cells or B cells, induces an adaptive immune response (50, 259). Lymphocytes originate from bone marrow progenitor cells. After which, T cells travel to the thymus to undergo further maturation and specialization (64, 166, 297). Once reaching maturity, lymphocytes express unique receptors for the identification of antigen patterns that determine highly specialized cell-mediated responses. Additionally, these immune cells can transfer to the spleen and other lymph

nodes (129). Conditions where development is impacted in the spleen or thymus can thus impact the production of lymphocytes (50, 129). T cells can be subcategorized into two main classes, the T helper cells and cytotoxic T cells (228). Lymphocytes interact with antigen presenting cells, such as microglia, which cause them to differentiate into disease relevant phenotypes. For example, T helper cells, or CD4 T cells, can further differentiate into Th1, Th2, Th9, Th22, follicular helper T cells (Tfh), or regulatory T cells (Treg) (1). Each of these differentiated cells are specific in their cytokine secretion and instigation of cellular cascades. Activated cytotoxic T cells, or CD8 T cells, on the other hand, once activated are capable of directly destroying cells that are malignant or infected with a virus via apoptosis (1). Moreover, these specialized T cells can further activate microglia through release of cytokines in a positive feedback loop (9, 47, 292). Similar to T cells, B cells also can further differentiate into different classes upon antigen peptide encounter (1). How these lymphocytes impact the SMA disease progression is still not well understood.

Restoring SMN levels in motor neurons alone is not sufficient to rescue the disease in SMA mice (275). However, preserving basal levels of SMN in the rest of the animal while reducing SMN solely in motor neurons resulted in a very mild SMA phenotype with close to normal life span (219). In these mice, motor function was impaired early in postnatal development but normal after post-natal day 13 when compared to controls (220). Together these data strongly suggest that supporting immune cells play a critical role in motor neuron degeneration in SMA. Moreover, these studies support the growing body of evidence that SMA is a multi-system disorder with contributions from several different cells and tissues (64, 168, 213, 242, 243, 275, 308).

Animal models in SMA

Reduced expression of SMN protein has been generated in animals to study SMA, including zebrafish, flies, worms, rats, mice, and pigs (190). Only primates have multiple *SMN1* gene copies, whereas *SMN2* is found only in humans (44). SMN gene deletion is embryonic lethal requiring most models to include an SMN2 transgene (265). Similar to humans, the SMA phenotype observed in these genetically modified mice was inversely proportional to the copies of SMN2 gene introduced (44). For example, two copies was akin to a severe model, whereas eight copies of SMN2 was able to fully rescue the mouse (44). Moreover, these mice were able to recapitulate phenotypes observed in the SMA patients, including the loss of alpha motor neurons (18, 261, 279).

While SMA model mice are routinely used to study the disease, worms, *Drosophila*, and zebrafish allow for high-throughput screening and analysis of discrete cellular pathways in the nervous system (225, 261). The yeast *Schizosaccharomyces pombe* was used in early studies looking at SMN protein cellular localization (261). Like SMN in humans, the *S. pombe*'s ortholog ySMN protein is critical for its survival. Using this simple model, researchers were able to show that SMN localized in not only the nucleus, but also the cytoplasm (225). The idea that SMN interacted with Gemin 2 within the complex, as well as its involvement in spliceosome machinery, was discovered using *C. elegans* (261). Additionally, SMN's robust expression in the nervous system and its role in synaptic vesicles was first described in *C. elegans* (261). Subsequent studies demonstrating the importance of SMN in both muscle and the nervous system were performed in a *Drosophila* model of SMA (49, 187, 237).

In the past 30 years, zebrafish have also emerged as an excellent model to study neurodegenerative diseases (5, 41, 252, 261). The neuroanatomical and neurochemical

pathways of the cortex of zebrafish have a similarity to the human brain (252). Moreover, the visual clarity of the embryos makes neurological imaging possible in real-time (41). In SMN deficient zebrafish models, altered morphology of motor neurons due to SMN deficiency was readily observed. Motor neurons in zebrafish that received morpholinos to knock down SMN showed defects in axonal branching bifurcations compared to controls (190). Although these findings are consistent with other animal models, early stages of development show that motor neurons still innervate muscles, suggesting that lower organisms may not fully replicate the disease phenotype observed in humans (175). Nevertheless, studies using zebrafish show synaptic pruning deficits in motor neurons due to SMN deficiency, which is consistent with findings from SMA patient autopsies (261).

Mice are excellent mammalian models for enabling researchers to study not only physiological mechanisms but to interrogate gene and protein function (280). Furthermore, they provide an invaluable tool for preclinical testing of possible therapeutics (280). Many SMA mouse models have been generated, ranging from severe models that live only a few days to mild models with an almost normal lifespan. Because Type I SMA patients account for the largest proportion of SMA, the severe SMN Δ 7 mouse (*Smn*^{-/-}; *SMN2*^{+/+}; *SMN Δ 7*^{+/+}) is the most widely used (279). Like the motor deficits in SMA patients, the SMN Δ 7 mouse has the inability to right itself, a test used to assess motor function. Other symptoms include heart defects as early as post-natal day 2, impaired neurotransmission, NMJ denervation, and muscle tone deficiency starting at post-natal day 5 (280). This model faithfully recapitulates the progressive nature of the disease with mice having an average life span of 12-16 days (280).

With such a short life span, the severe mouse model may not be ideal to truly tease out the mechanisms that underlie SMN deficiency. The SMN^{2B/-} mouse model was generated to study a milder SMA phenotype. The SMN^{2B/-} mouse has a SMN transgene that has a three nucleotide substitution within the exon 7 splicing enhancer (72). Unlike the SMN Δ 7 mouse, this model has a slower disease progression and an average lifespan of 28 days (54, 182). Altogether, these animal models are crucial to advancing our understanding of SMA pathophysiology and are valuable tools in preclinical assessment of SMA therapeutics.

Induced Pluripotent Stem Cells

Animal models of SMA have so far facilitated the development of three groundbreaking treatment options for SMA. However, the translational capacity of current cell and animal models is limited by the degree of conservation of genes and pathways between these models and human patients. Specifically, how do our findings using immortalized cell lines, and systemic studies in mouse models reflect the conditions of patients? Animal models have been used extensively to study the etiology of neurodegenerative diseases (5, 41, 72, 225, 252, 261, 280). Although they may mirror key features of disease progression and functional outcomes, they do not completely replicate the complexity of the condition seen in humans (36, 62). Patient-derived induced pluripotent stem cells (iPSC) have emerged as powerful tools to refine understanding of pathogenic mechanisms underlying complex human diseases (79, 223). iPSCs have now been used to study a wide range of neurodegenerative diseases including Alzheimer's, amyotrophic lateral sclerosis (ALS), spinocerebellar atrophy, Parkinson's disease, Huntington's disease, and multiple sclerosis (20, 55, 57, 88, 198, 227). Stem cells offer the

advantage of a human genomic background and the ability to be differentiated into disease relevant cells. iPSCs are thus invaluable tools to study specific cell types in various injury and disease models, while retaining the patient's genetic background. In fact, Shinya Yamanaka's discovery of iPSCs in 2006 is considered one of the major breakthroughs in the field of stem cells (69, 111, 139, 193). iPSCs have now been successfully differentiated into astrocytes and motor neurons to study SMA pathophysiology (109, 167, 203).

Studying human cardiomyocytes, motor neurons and microglia is challenging due to the difficulty in isolating these cell types directly from human tissue [30]. Due to the time constraints for optimum yield, extracting these human cells postmortem could be unsuccessful (69). Thus, postmortem isolated cells do not afford the ability to study the disease progression (52). Alternatively, patient derived iPSCs have been differentiated into a variety of cells types and used to model disease pathogenesis and progression (20, 57, 109, 198, 203, 204, 227, 278). Reprogrammable tissue, such as fibroblasts, from an individual can be isolated through the life span of the patient (286). In this study, we sought to differentiate SMA and control patient iPSCs into cardiomyocytes, motor neurons and microglia. Prior to these findings, there has been no exploration for how SMN deficiency affects the function of human microglia and cardiomyocytes.

Current Treatments for SMA

Until 2016, there were no treatments available for SMA (262, 167). Current FDA approved therapeutics including Risdiplam (Evrysdi™), Nusinersen (Spinraza®), and Onasemnogene ebeparvovec (Zolgensma®) aim to increase levels of SMN protein (81, 100, 114, 183, 185, 193, 222, 240, 262, 272, 284). They do so by introducing exogenous levels of full length SMN, or targeting the SMN2 gene to boost inclusion of exon 7,

thereby increasing full length SMN protein. Additionally, many SMN independent drugs, designed to complement the current therapies, are currently in various stages of preclinical or clinical assessment (30).

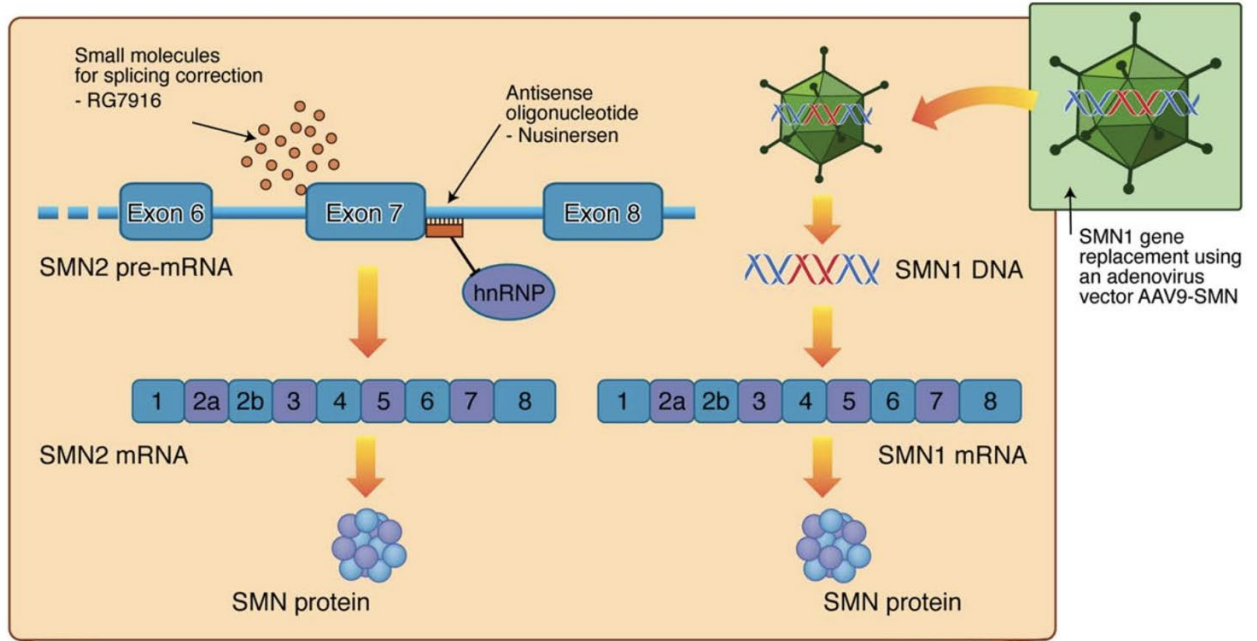


Figure 2. Therapeutic targets available for SMA that promote the production of fully functional SMN protein. Risdiplam (RG7916) is an orally administered splicing modifier. Zolgensma is an SMN1 gene replacement therapy utilizing a self-complementary adeno-associated viral vector (AAV9-SMN). Nusinersen is an intrathecally administered antisense oligonucleotide that works by displacing heterogeneous nuclear ribonucleoprotein (hnRNP) proteins from the intronic splicing silencer site on the SMN2 pre-mRNA. Figure adapted from Farrar et al. 2017 (323).

Nusinersen, an antisense-oligonucleotide (ASO) was the first approved drug for treatment of SMA (4, 224). ASOs are single-stranded nucleic acid sequences intended to target pre-mRNA and to modulate gene expression (130). With high target sensitivity, they allow for reduced toxicity (130). The effects of ASO are reversible, requiring chronic treatment (130). Nusinersen works by modifying the splicing of the *SMN2* gene. By suppressing the binding of splice-factors to the intronic splice-silencing site in intron

7 of *SMN2*, Nusinersen promotes exon 7 inclusion, increasing the production of the full length SMN protein. Unfortunately, Nusinersen does not cross the blood-brain-barrier, relying on intrathecal administration three times a year. Treated SMA patients show dramatic improvement in reaching motor milestones as indicated by the Hammersmith Functional Motor Scale-Expanded analysis (239). Nusinersen has also extended life expectancy with most treated patients still alive after 5 years compared to the two year life expectancy of untreated patients (224).

Approved in May of 2019, Onasemnogene ebeparvovec (Zolgensma®), or AVXS-101, was the next FDA approved drug for treating SMA (60, 284). A copy of the complementary human SMN gene is packaged in an adeno-associated viral serotype 9 (scAAV9) and is administered to patients in a one-time intravenous dose (60). To date, it has been approved for children under the age of two suffering from severe SMA (284). The adeno-associated virus is engineered from and belongs to the parvovirus family (205). While there are benefits to this therapeutic, after an initial exposure to the vector, an immune response is generated and memory T-cells are formed (189). Re-administration of a similar vector in the future will thus cause a potentiated response. Long term adverse effects are not yet known as it remains unclear what sustained high expression of SMN protein does in all tissues (179).

Risdiplam (Evrysdi™) is the most recent FDA approved drug for SMA therapy. Approved on August 7th of 2020, Risdiplam is an orally administrated drug that modifies *SMN2* splicing to enhance the production of full-length SMN by stabilizing the U1 snRNP binding to the 5' splice site of exon 7 (179). SMN levels in the CNS and peripheral tissues were increased with Risdiplam administration (235). Due to the ease of

drug administration, compliance is more effective with reduced side effects due to the noninvasive route of drug administration (54). Four open-label trials are currently ongoing for Risdiplam including FIREFISH (Identifier: NCT02913482), SUNFISH (Identifier: NCT02908685), JEWELFISH (Identifier: NCT03032172) and RAINBOWFISH (Identifier: NCT03779334) (15, 52, 179, 182). The drug is currently approved for treatment of ages two and above, although other trials are ongoing to test the efficacy in different SMA patient types (179) (163).

While these drugs have changed the SMA disease landscape, none of the currently available compounds cure the disease, and many patients remain nonrespondent or severely debilitated. Access to treatment is hampered by the high cost of these treatments. The average cost per year for patients suffering from severe SMA is approximately \$104,197 (146). Initial diagnosis and inpatient costs can exceed \$11,000 in the US (22). A lifetime treatment of Nusinersen will cost approximately \$3.9 million (179). Currently the annual cost of Risdiplam is capped at \$340,000 (214). Zolgensma, as of January 2021, is considered the most expensive drug in the world, at \$2.1 million for a single dose (214). Not only is this cost of care for SMA patients expensive, but it is also taxing for the caregivers. While patient motor function is improved, they are not fully ambulatory. The treatment efficacy depends on not only the age of the patient, but also the severity of the disease prior to treatment (125, 184). For example, Nusinersen administered to adult patients with Type 2 SMA does not reach the same efficacy as patients who received early intervention (179). An important limitation of the current therapies is that they are all designed to increase full-length SMN and there are no therapeutic options to reverse the disease course in tissues where the developmental

damage is not reversed by SMN induction (138). Greater understanding of where and when SMN is needed in specific tissues will be immensely helpful in developing these adjuvant therapies.

PURPOSE OF RESEARCH

Rationale and Studies

While SMA is primarily a motor neuron disease, studies indicate system wide dysfunctions exists across all severity types of SMA patients. It is still unknown how reduced SMN expression causes cardiomyopathies in SMA. SMA patients present with structural cardiac defects, arrhythmias, bradycardias, and hypertrophy (268). Severe SMA model mice have smaller hearts compared to their unaffected littermates. Cardiomyopathies can lead to inadequate circulation to the extremities and cutaneous necrosis in very severe SMA patients (310).

How deficiency in SMN affects immune responses is an active area of research in SMA. In a healthy individual, the interaction between glia and neurons maintains CNS homeostasis (210, 232). Previous work by others, as well as our preliminary findings, show retracted microglial processes and enlarged soma, characteristic of a reactive state, in the later stages of SMA disease (295, 308). However, it remains unclear if intrinsic factors, extrinsic factors, or a combination of both are causing activation of microglia in the SMA mice. SMN deficiency could potentially alter gene expression profiles in microglia, triggering adoption of a reactive state. Alternatively, SMA motor neurons may be releasing factors inducing reactivity in microglia. Microglia respond to various inflammatory factors due to constitutive and inducible surface receptors that amplify the innate immune response. Targeting any one of these receptors may induce microglia

activation. Whether the microglia is reactive early and maintain reactivity throughout the disease is unknown.

Studying human cardiomyocytes and microglia is challenging due to the difficulty in isolating these cell types from human tissue [30]. Mouse models have been used extensively to study neurodegenerative disease etiology. Although they may mirror disease progression and functional outcomes, they do not completely replicate the complexity of disease seen in humans. Additionally, cells isolated from mice CNS lose their unique identity when cultured alone (199). Patient derived iPSCs have previously been differentiated into target cells that retain their identity and are used to model disease pathophysiology (55, 57, 79, 88, 163, 203, 223, 227, 323). They offer advantages in that they retain the patient's genetic background. In this study, we proposed to differentiate SMA patient iPSCs into cardiomyocytes and microglia. Currently, no SMA study has utilized iPSCs to model microglia or cardiomyocytes. Characterizing these cells of interest in SMA disease progression may provide a better understanding of the disease etiology.

Hypothesis and Specific Aims

SMA patients do not become fully ambulatory with current FDA approved treatments. Our long-term objective is to establish novel pathways for treating SMA patients in order to find a cure. We hypothesize that non-neuronal cells play a critical role in the SMA disease phenotype. To test this hypothesis, we propose the following two aims:

Aim 1: To determine the role of SMN in cardiovascular dysfunction using patient derived iPSC differentiated cardiomyocytes.

Aim 2: To characterize microglia in SMA mice, as well as in patient derived iPSC differentiated microglia.

CHAPTER 2: SMN-deficiency disrupts SERCA2 expression and intracellular Ca²⁺ signaling in cardiomyocytes from SMA mice and patient-derived iPSCs.

ABSTRACT

Spinal muscular atrophy (SMA) is a neurodegenerative disease characterized by loss of alpha motor neurons and skeletal muscle atrophy. The disease is caused by mutations of the *SMN1* gene that result in reduced functional expression of survival motor neuron (SMN) protein. SMN is ubiquitously expressed, and there have been reports of cardiovascular dysfunction in the most severe SMA patients and animal models of the disease. In this study, we directly assessed the function of cardiomyocytes isolated from a severe SMA model mouse and cardiomyocytes generated from patient-derived iPSCs. Consistent with impaired cardiovascular function at the very early disease stages in mice, heart failure markers such as brain natriuretic peptide were significantly elevated. Functionally, cardiomyocyte relaxation kinetics were markedly slowed and the T_{50} for Ca²⁺ sequestration increased to 146 ± 4 ms in SMN-deficient cardiomyocytes from 126 ± 4 ms in wild type cells. Reducing SMN levels in cardiomyocytes from control patient iPSCs slowed calcium reuptake similar to SMA patient-derived cardiac cells. Importantly, restoring SMN increased calcium reuptake rate. Taken together, these results indicate that SMN deficiency impairs cardiomyocyte function at least partially through intracellular Ca²⁺ cycling dysregulation.

BACKGROUND

Spinal muscle atrophy (SMA) is one of the leading genetic causes of infant mortality. SMA is caused by survival motor neuron (SMN) protein deficiency due to deletions or mutations of the *SMN1* gene. Individuals also carry two or more copies of a paralog gene, *SMN2*, which produces suboptimal levels of the SMN protein. The amount

of full-length protein produced from *SMN2* is an important determinant of disease severity (78, 148). SMA patients with more than two copies of *SMN2*, as a result of duplication, tend to have less severe forms of the disease. Recently, therapeutic breakthroughs, gene-replacement therapy using viral delivery (178) and altering *SMN2* splicing using antisense oligonucleotides (80), have remarkably improved motor function and survival of SMA patients, but patients remain prone to cardiopulmonary complications leading to opportunistic upper respiratory tract infections (7).

Motor neuron loss and consequent muscle atrophy are the defining features of the SMA phenotype. SMN is ubiquitously expressed in all tissues, where it is known to play a key role in snRNP biogenesis and spliceosome assembly (176, 177, 226). It is unclear why reduced levels of the ubiquitously expressed SMN protein selectively target anterior horn cells of the spinal cord. Since spliceosome assembly occurs in all cells, it is possible that reduced SMN function in other tissues may contribute to the clinical manifestation of the disease.

Indeed, recent observations suggest a role of SMN in tissues outside of the motor neuron with possible contribution to the pathology of SMA (33, 38, 63, 112, 129, 300). Notably, in a study designed to develop an SMA plasma protein biomarker panel to monitor disease progression and therapeutic efficacy, 84 putative markers regressing to motor function were found in patients ($n = 108$ total; type I = 17; type II = 49; type III = 42) enrolled in the BforSMA study, including several markers of congestive heart failure and cardiovascular dysfunction (135). Indeed, marked elevation of heart failure markers such as brain natriuretic peptide (BNP), fatty acid-binding protein 3 (FABP3), and

creatine kinase (CK) in SMA patients suggests that normal cardiac function may require sufficient functional expression of SMN. Importantly, in vivo assessment of cardiovascular function indicates that SMA mice have impaired cardiac function (25). Sympathetic denervation of the heart in SMA model mice (25, 73, 107, 268, 311) has been suggested as a cause of cardiac pathophysiology, but the mechanisms fully responsible for these cardiac deficits are unknown. Importantly, there have been no direct measurements of cardiomyocyte function in any SMA model so it has been difficult to conclude with certainty whether SMN directly impairs cardiac function.

We sought to determine if SMN deficiency compromises contractile function in ventricular cardiomyocytes isolated from the SMN Δ 7 mouse (*SMN Δ 7;SMN2; Smn^{-/-}*) that recapitulates a severe form of SMA with an average life span of 14 days and muscle weakness onset at post-natal day 5 (P5) (157). In this model, we observe significant elevation of heart failure marker expression as early as P5 and marked impairment of contractile function in isolated cardiomyocytes. Moreover, we observe marked reduction in SERCA2 expression and consequent slowed Ca²⁺ transient kinetics in cardiomyocytes isolated from SMA mice and generated from SMA patient-derived iPSCs. These results show that SMN deficiency also causes cardiac deficits directly in cardiac myocytes, potentially by altering SERCA2 expression and calcium cycling kinetics, in addition to any autonomic dysfunction.

METHODS

Generation of transgenic mice

The generation and phenotypic characterization of the SMN-deficient transgenic SMN Δ 7 mouse line (*SMN Δ 7; SMN2; Smn^{-/-}*) has been described previously (145).

Briefly, the SMN Δ 7 mouse line is a triple mutant model with a double copy of SMA cDNA lacking exon 7 and two copies of the human *SMN2* on an *Smn*^{-/-} background. SMN Δ 7 mouse transgenic and unaffected littermate mice (post-natal day 5 (P5) and 10 (P10)) were used in the present study. All procedures complied with the standards for the care and use of animal subjects as stated in the *Guide or the Care and Use of Laboratory Animals* (NIH publication no. 85-23, revised 1996), and all protocols were approved by the IACUC at the Uniformed Services University of the Health Sciences.

RNA extraction and quantification

Tissue (50–100 mg) was homogenized in 1 ml TRIzol reagent (Thermo Fisher, cat 15596018), and total RNA was isolated and converted to cDNA as previously described (127). Gene-specific Taqman primer and probe sets were purchased from Applied Biosystems: atrial natriuretic peptide (ANP; *Nppa*, *Mm01255748_g1*), brain natriuretic peptide (BNP, *Nppb*, *Mm01255770_g1*), skeletal muscle actin (*Acta1*, *Mm00808218_g1*), SERCA2 (*Atp2a2*, *Mm01201431_m1*), hypoxanthine guanine phosphoribosyl transferase (*Hprt*, *Mm01318743_m1*), and glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*, *Mm01180221_g1*). qRT-PCR reactions were performed in triplicate using the StepOnePlus™ Real-Time PCR System (Applied Biosystems). The following primers were purchased from Integrated DNA Technologies: *SERCA2*, 5'-TGAGACGCTCAAGTTTGTGG-3'; *SERCA2a*, 5'-ATGCAGAGGGCTGGTAGATG-3'; *SERCA2b*, 5'-ACAAACGGCCAGGAAATG-3'; and *GAPDH*, 5'-GCATGGCCTTCCGTGTTC-3', 5'-ATGTCATCATACTTGGCAGGTTTC-3'. The level of each transcript was quantified by the threshold cycle (Ct) method using *Gapdh* and *Hprt* as endogenous controls.

Values were normalized to the mean of the unaffected group for each gene, which was assigned as 1.

Transcriptome profiling by RNA sequencing:

Total RNA was quantified via a fluorescence dye-based methodology (RiboGreen, Thermo Fisher, cat. R11490) on a Spectramax Gemini XPS plate reader (Molecular Devices, Mountain View, CA). RNA integrity was assessed using automated capillary electrophoresis on a Fragment Analyser (Advanced Analytical Technologies, Inc, Santa Clara, CA). Total RNA input of 200 ng was used for library preparation using the TruSeq Stranded mRNA Library Preparation Kit (Illumina, San Diego, CA, cat 20020594). Sequencing libraries were quantified by PCR using KAPA Library Quantification Kit for NGS (Roche, Wilmington, MA, cat KK4854) and assessed for size distribution on a Fragment Analyser. Sequencing libraries were pooled and sequenced on a NextSeq. 500 Desktop Sequencer (Illumina) using a NextSeq. 500 High Output Kit v2 with paired-end reads at 75 bp length. Raw sequencing data was demuxed using bcl2fastq2 Conversion Software 2.17 before alignment using TopHat Alignment v1.0 and differential expression analysis using Cufflinks Assembly and DE v1.1.0 on BaseSpace Onsite (Illumina). Functional enrichment analysis was performed using the PANTHER Classification System. Significantly overrepresented gene ontology biological processes at $p < 0.01$ were adjusted using a Bonferroni correction for multiple testing.

Western Blots:

Fresh ventricular tissue was homogenized (Polytron) in RIPA lysis buffer (Sigma, cat R0278). Isolated proteins were separated by SDS-PAGE (4–15%, BioRad, cat 456-1084) and transferred onto PVDF membranes (BioRad, cat BR20191004). Blots were

probed with the following specific antibodies: anti-SERCA2a antibody (1:1000, Cell Signaling Technology, cat 9580), anti-SMN (1:1000, BD Transduction Laboratories, cat 610647), anti- α -actin (1:5000 mybiosource.com, cat MBS477269) anti-BNP (1:1000, Abcam, cat ab236101), and GADPH (1:5000, Abcam, cat ab8245). Bound antibodies were detected with SuperSignal West Dura ECL substrate (Pierce, cat 34075).

Cell contractility and calcium transients measurements in mouse cardiomyocytes:

Unloaded cell shortening and intracellular calcium (Ca^{2+}) transients were measured in freshly isolated ventricular myocytes, prepared as described previously (82, 83). Isolated myocytes were transferred into a recording chamber mounted on an Olympus X51 inverted microscope and superfused with normal Tyrode solution (composition in mM: NaCl, 137; KCl, 5.4; NaH_2PO_4 , 0.16; glucose, 10; CaCl_2 , 1.8; MgCl_2 , 0.5; HEPES, 5.0; NaHCO_3 , 3.0; pH 7.3–7.4.). All experiments were performed at room temperature. Video images were acquired using a Myocam camera (IonOptix). Cells were field stimulated at 1 Hz in all experiments. In experiments aimed at measuring intracellular Ca^{2+} , isolated cells were incubated in Wittenberg Isolation Medium (WIM, composition in mM: NaCl, 116; KCl, 5.3; NaH_2PO_4 , 1.2; glucose, 11.6; MgCl_2 , 3.7; HEPES, 20; L-glutamine, 2.0; NaHCO_3 , 4.4; KH_2PO_4 , 1.5; 1X essential vitamins (GIBCO cat 12473-013); 1X amino acids (GIBCO cat 11120-052); pH 7.3-7.4.) solution containing fluo-3-AM (1 μM) for 30 min at room temperature. Fluo-loaded cells were transferred into the recording chamber and perfused with normal Tyrode solution supplemented with 500 μM probenecid to inhibit dye export. Cells were stimulated to contract at 1 Hz in all cases and fluorescence was captured with a photomultiplier tube

(IonOptix). Intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) was estimated using the Maravall equation(164):

Where K_d was assumed to be 600 nM {Dilly, 2006 #267} and $R_f = F_{\max}/F_{\min}$. $F_{\min}$ and $F_{\max}$ were measured in each cell by incubating the cell in modified Tyrode solutions supplemented with 20 mM 2,3 butadione monoxime, 10 mM A23187 and either 0 mM CaCl_2 + 10 mM EGTA, or 100 mM CaCl_2 , respectively.

Cell lines

To study the effects of SMN-deficiency on human cardiomyocytes, we utilized iPSCs from a spinal muscular atrophy patient (GM23240; Coriell). These iPSCs were reprogrammed from fibroblasts using lentiviral constructs encoding OCT4, SOX2, NANOG, and LIN28. NCRM-1 iPSCs were used as a control line and obtained from XCell Sciences (Novato CA). NCRM-1 iPSCs were obtained from XCell Sciences and reprogrammed by episomal plasmid from CD 34+ human cord blood cells. To grow and maintain iPSC lines, standard 6-well tissue culture plates were coated with growth factor reduced Matrigel (Corning; cat 354277) diluted 1:100 in DMEM/F12 (Gibco, cat 11330-032) same day as iPSC plating. Frozen stocks of iPSCs were thawed and plated on Matrigel coated plates in TeSR E8 (StemCell, cat 05990) supplemented with ROCK inhibitor (Y-27632, 10 μ M, Tocris, cat 1254). After 24 h, media was replaced with TeSR E8 (without ROCK inhibitor) and culture media was replaced daily until fully confluent. iPSC lines were passaged using 0.5 mM EDTA (Life Technologies, cat 15575-038) in PBS without CaCl_2 and MgCl_2 (Hyclone, Slt30256.01). Cells were maintained at 37 °C, 5% CO_2 .

Generation and maintenance of cardiomyocytes

We began the cardiomyocyte differentiation protocol when cells were 80% confluent using the protocol developed by Feaster et al (76). Briefly, differentiation was initiated (day 0–2) by replacing TeSR E8 medium with RPMI 1640 medium (Lonza, cat 12-702F) supplemented with B27 (minus insulin, Gibco, cat A1895601) and CHIR99021 (6 μ M, LC Laboratories, cat C-6556), a GSK3 inhibitor. On days 3–4, CHIR99021 was removed and replaced with RPMI 1640 medium supplemented with B27 (minus insulin) and IWR-1 (500 μ M, Sigma, cat I0761), a Wnt signaling inhibitor. On days 5–9, cells were maintained in RPMI 1640 medium supplemented only with B27 (minus insulin). From days 10–15, a metabolic selection protocol was employed using RPMI 1640 without glucose (Life Technologies, cat 11879) plus B27 without insulin. Following metabolic selection, cells were maintained in RPMI 1640 supplemented with B27 (Gibco, cat 17504-044) and 1% pen strep (Gibco, cat 10378-016). Beating cardiomyocytes were fed daily until day 20 when functional assays were carried out as described below.

Transfection

To increase SMN expression in patient iPSC-derived cardiomyocytes, we transfected cells with SMN-GFP cDNA plasmid (1 μ g) using lipofectamine 3000 (Invitrogen, cat 100022052) and P3000 (Invitrogen, cat 100022058) for 48 h according to the manufacturer protocols. Transfection was performed at day 18 of differentiation. Generation of SMN-GFP plasmid was previously described (273) and a gift from Greg Matera (University of Carolina at Chapel Hill). To reduce SMN expression in control iPSC-derived cardiomyocytes, cells were transfected with 40 pmol of pre-validated siRNA (IDT, cat 37206943) and RNAi Max (Life Technologies, cat 56532) per manufacturer protocol at the 18 day time point. Opti-MEM (Gibco, cat 11058-021)

diluent was used in both protocols. Successful transfection was confirmed in western blot analysis.

Flow cytometry

SMA and Control patient iPSC-derived cardiomyocytes were dissociated using TrypLE (Gibco, cat 12605-010). Disaggregated cells were centrifuged and resuspended in fixation buffer (Biolegend, cat 554655) for 15 min at room temp, washed 3 times in 1X permeabilization wash buffer (Biolegend, cat 421002, and resuspended in permeabilization wash buffer containing FITC conjugated anti- Cardiac Troponin (1:20, Miltenyi Biotec, cat 130-106-689) for 1 h at room temp protected from light. Cells were read in cell staining buffer (Biolegend, cat 420201) on BD Accuri C6 Flow Cytometer CFlow Plus software. Unstained cells were used as a negative control.

Calcium Imaging

SMA and Control patient iPSC-derived cardiomyocytes wells were washed with PBS and incubated in normal Tyrode's solution (composition in mM: NaCl, 137 mM; KCl, 5.4 mM; NaH₂PO₄, 0.16 mM; glucose, 10 mM; CaCl₂, 1.8 mM; MgCl₂, 0.5 mM; HEPES, 10.0 mM; NaHCO₃, 3.0 mM; pH 7.3–7.4.) supplemented with cell permeant Fluo-4 AM, fluorescent Calcium indicator (1 μ M, Invitrogen, cat F14201) and probenecid (500 μ M, Sigma, cat P8761). Cells were incubated for 15 min at 37 °C, 5% CO₂. Calcium transients were recorded on a Nikon eclipse Ti2 inverted microscope equipped with a large view CMOS camera on a $\times$ 20 objective with NIS Elements AR software (Nikon). Decay at T_{10} , T_{50} , T_{75} , T_{90} , and T_{100} were analyzed using Clampfit software (Axon).

Data Analysis

All data were analyzed using IonWizard, Clampfit, and Microsoft Excel software and, except where noted, results are presented as means $\pm$ SEM (standard error of the mean). In all cases, $p < 0.05$ was considered significant. Statistical tests used and resultant p values are given in the figure legends. Statistical analysis was performed in GraphPad Prism 7.

RESULTS

Heart failure markers are elevated in SMN-deficient mice

There is growing evidence suggesting that peripheral tissues, including the heart, may be affected by the loss of SMN function in SMA patients. Heart failure is associated with the reactivation of fetal genes, including atrial natriuretic peptide (*ANP*; *Nppa*), brain natriuretic peptide (*BNP*; *Nppb*), and skeletal α -actin (*Acta1*), associated with structural and functional remodeling of the heart (86). The activation of ANP and BNP in particular has been shown to correlate well with the clinical severity and prognosis of heart failure (136, 251, 289). ANP and BNP mRNA expression was markedly increased in whole heart tissue from SMN deficient mice modeling a severe form of the disease (145) at both post-natal day 5 (P5; early in the disease progression) and post-natal day 10 (P10; closer to end-stage) when compared to unaffected littermates (Fig. 3). Similarly, we found increased expression of skeletal α -actin at P5. Together, these results imply that mechanical function of the heart may be altered early in the disease progression of this severe SMA mouse model.

Diastolic function is impaired in SMN-deficient mouse cardiomyocytes

To directly test cardiac function, we compared unloaded sarcomere shortening in paced (1 Hz) ventricular cardiomyocytes isolated from unaffected and SMN-deficient mice at P12–P13 (Fig. 4). The mean amplitude of sarcomere shortening and fractional shortening were similar in myocytes from control and SMN deficient littermates. While the extent of sarcomere shortening was unaffected by SMN deficiency, the rate of contraction and relaxation were both markedly slowed in SMN deficient cells. Both time-to-peak shortening (131 ± 5 vs. 105 ± 3 ms, $*p < 0.01$, t test) and time-to-50% re-lengthening (97 ± 5 vs. 68 ± 2 ms, $*p < 0.01$, t test) were markedly increased in SMN-deficient cells compared with cells from unaffected littermates. We also noted a significant reduction of resting (diastolic) sarcomere length in SMA myocytes (1.60 ± 0.02 μm vs. 1.73 ± 0.02 μm in control, $n = 36$ and 37 , respectively, $*p < 0.01$, t test). Thus, as predicted by the elevated heart failure markers, cardiomyocyte function, particularly during diastole, is compromised in SMN-deficient mice.

Ca²⁺ handling gene expression is altered in SMN-deficient mouse heart

To gain an understanding of how SMN deficiency might affect cardiac function, we performed transcriptome expression profiling of whole heart tissue from SMA model mice at P10. Differential expression analysis identified 637 transcripts that were upregulated and 541 transcripts that were downregulated in heart tissue of SMA mice as compared to unaffected littermates (Fig. 5A). Subsequent analysis of the set of 1178 differentially expressed transcripts yielded significantly enriched gene ontologies for biological functions in regulation of muscle cell and fiber development (3.8-fold enrichment, p value = $3.9\text{E}-04$). Additionally, specific key genes involved in muscle function and calcium-ion handling (1.8-fold enrichment, p value $1.6\text{E}-04$) are lower

(*Atp2a2*, *Casq1*, *CaBP1*, *S100a10*, *Rcan1*, and *Hrc*) and higher in transcript level (*Scin*, *Efh2*, and *Myom2*) in heart tissue of SMA mice as compared to unaffected mice (Fig. 5B). Transcript expression of all identified calcium handling-related genes were significantly different prior to disease end-stage, suggesting that SMN-deficiency alters the cardiac transcriptome during disease progression.

SERCA2 expression is reduced in SMN-deficient cardiomyocytes

It is notable that *Atp2a2*, the gene encoding the sarcoplasmic reticulum (SR) Ca^{2+} ATPase or SERCA2, is found among the major Ca^{2+} handling related genes altered in the SMN-deficient heart. Impaired contractile function of the failing heart is often associated with dysregulation of intracellular Ca^{2+} cycling and reduced expression of SERCA2 protein (24, 70, 165). As predicted from transcriptome data, we found that SERCA2 protein expression is reduced in SMN-deficient cardiomyocytes as early as P5 (Fig. 6). There are two major splice variants of SERCA2—SERCA2a and SERCA2b (304). SERCA2a is considered the muscle specific isoform expressed in both cardiac muscle and slow twitch skeletal muscle, while the SERCA2b isoform is ubiquitously expressed. Given the role of SMN in regulating spliceosome assembly and function, we examined the relative expression of the two major splice variants in SMN-deficient and control hearts. Interestingly, there appears to be an isoform switch, with reduced SERCA2a and increased SERCA2b mRNA expression, but overall, there is a significant reduction of SERCA2 protein regardless of the isoform.

Ca^{2+} reuptake kinetics are slowed in SMN-deficient mouse cardiomyocytes

SERCA2 is the major determinant of Ca^{2+} sequestration from the cytoplasm in murine cardiomyocytes (150, 156, 230); therefore, it is predicted that SMN-deficient cells

exhibit slowed removal of cytosolic Ca^{2+} following a triggered release. To test this, we estimated $[\text{Ca}^{2+}]_i$ during triggered Ca^{2+} transients in fluo-3-AM loaded ventricular myocytes isolated from unaffected and SMN-deficient littermates at P12-P13 (Fig. 7). Although resting $[\text{Ca}^{2+}]_i$ tended to be elevated prior to stimulation, there were no significant differences in diastolic or peak systolic $[\text{Ca}^{2+}]_i$ between control and SMA cardiomyocytes. Consistent with reduced expression of SERCA2, however, there was marked slowing of Ca^{2+} removal from the cytoplasm with T_{50} significantly ($*p < 0.01$, t test) increased in SMN cardiomyocytes (146 ± 4 ms) compared with control cells (126 ± 4 ms). These data are consistent with the conclusion that SMN-deficiency is associated with reduced SERCA2 expression, causing slowed Ca^{2+} reuptake and impaired cardiomyocyte mechanical function.

SMN deficiency slows $[\text{Ca}^{2+}]$ reuptake kinetics and reduces SERCA2a expression in iPSC-derived cardiomyocytes

Human-induced pluripotent stem cells (iPSCs) have emerged as a powerful system to model human cardiac disease and study Ca^{2+} handling (59, 115, 217, 266). To confirm that our findings are not restricted to the mouse model, we assessed Ca^{2+} transients in spontaneously contracting clusters of cardiomyocytes generated from patient-derived (SMA) and unaffected control iPSCs. As expected, SMN protein expression was markedly reduced in SMA patient-derived cells. In agreement with data obtained from mouse cardiomyocytes, there was also marked slowing of Ca^{2+} reuptake kinetics in SMA cells with T_{50} (556 ± 49 ms) increased compared with control (348 ± 21 ms) (Fig. 8A). Moreover, transfection of patient-derived cells with SMN to increase SMN levels, increased both SERCA2 protein expression and Ca^{2+} reuptake rate (Fig. 8B). Conversely, using siRNA to reduce SMN expression in control cells, reduced both

SERCA2 expression and the T_{50} for Ca^{2+} reuptake (Fig. 8C). However, we did not observe the isoform switch from SERCA2a to SERCA2b, as observed in the mouse tissue, in the SMN-deficient iPSC-derived cardiomyocytes. Taken together, these results demonstrate that SMN regulates SERCA2 expression and intracellular Ca^{2+} cycling kinetics in cardiomyocytes that may impair cardiac function and lead to elevation of heart failure markers, as observed in mice (Fig. 3) and patients with SMA (135).

DISCUSSION

The major pathological feature of SMA is neuromuscular degeneration; however, there have been reports that cardiovascular function is impaired in both SMA patients and mouse models of the disease (25, 46, 107, 248, 267). The data in the present study demonstrate increased expression of heart failure molecular markers, reduced expression of SERCA2, and impairment of cardiomyocyte contraction in a severe mouse model of SMA. Moreover, these findings are reversibly recapitulated in cardiomyocytes generated from patient-derived iPSCs, indicating that SMN deficiency likely causes a similar phenotype in human cardiac muscle as well. Taken together, these results add support to the conclusion that cardiovascular function is compromised by loss of SMN function and indicate that dysregulation of SERCA2 expression and Ca^{2+} homeostasis lies at the center of cardiac pathophysiology.

Heart function and failure in SMA

Efforts to treat SMA are largely focused on preventing or reversing neuromuscular degeneration. We showed previously that daily injections of the HDAC inhibitor, trichostatin A, improves motor function and modestly extend the lifespan of SMA mice (13). Interestingly, however, these mice ultimately die from cardiovascular

causes (13). Here, we show that BNP and skeletal α -actin are elevated as early as P5 in SMA mice, prior to the development of profound neuromuscular degeneration.

Moreover, even though cell shortening and Ca^{2+} transient amplitude are preserved in SMN-deficient cells, we show that the rate of relaxation and diastolic sarcomere length are markedly reduced in SMA cardiomyocytes, suggesting that SMN deficiency may be associated with diastolic heart failure or heart failure with preserved ejection fraction (302). It is interesting to note, that SMA patients often require artificial ventilation as a result of lung congestion and respiratory distress even though the diaphragm muscle is relatively spared from degeneration (102). Although speculative, heart failure resulting from SMN deficiency as evidenced here may contribute to the respiratory pathology in SMA patients in addition to neuromuscular degeneration. Nevertheless, the present findings support the conclusion that cardiac function is compromised in SMN deficient animals, even though the major pathological feature is neuromuscular degeneration.

Effects of SMN deficiency appear to be cell autonomous

Because neurodegeneration is central to SMA pathology, it is reasonable to consider the influence of innervation of the heart as a cause of pathology. Indeed, several studies suggest that sympathetic denervation and sympatho-vagal imbalance are evident in SMA patients and mouse models of the disease (25, 102, 107, 268). Bradycardia and slowed conduction have been reported in patients, consistent with sympathetic denervation of the heart (73, 311). We cannot rule out that sympathetic denervation contributes to cardiovascular pathology in SMA; however, here we show clear evidence of impaired cardiac function with slowed intracellular Ca^{2+} dynamics and reduced SERCA2 expression in a model of severe, type I SMA. It is likely that the degree of

cardiac involvement varies with disease severity and a study in patients with the less severe type II/III SMA variant reported no evidence of cardiac dysfunction (46). Importantly, the reduction in SERCA2 expression and slowing of Ca^{2+} reuptake was recapitulated and reversible in cardiomyocytes generated from SMA patient-derived iPSCs. When coupled with the observation that evidence of heart failure emerges as early as P5 in the mouse, this result provides strong evidence that the impairment of cardiomyocyte function is a result of cell autonomous loss of SMN function, and not secondary to neurodegeneration.

Altered Ca^{2+} handling as a common disease mechanism in SMA

It remains unclear why SMN deficiency so potently targets motor neurons for degeneration. The principal finding in this study is that SMN deficiency causes a decrease in the expression of SERCA2 protein in both SMA mouse heart and cardiomyocytes generated from patient-derived iPSCs. SMN is known to play a role in snRNP biogenesis and spliceosome assembly (176, 177, 226). It is not known how SMN deficiency inhibits SERCA2 biosynthesis, but we did observe an apparent isoform switch, with reduced SERCA2a and increased SERCA2b mRNA expression albeit with a significant reduction in total SERCA2 protein regardless of the isoform. One possibility is that disruption of alternative splicing mechanisms in cardiomyocytes leads to reduced SERCA2 expression. However, the SERCA2 alternative splicing was not observed in the human cardiomyocytes when SMN was knocked down even though total SERCA2 expression was reduced. This suggests that alternative splicing cannot fully account for the reduced SERCA2 expression in SMN-deficient cells.

Interestingly, SMN deficiency also affects intracellular Ca^{2+} handling in motor nerve terminals. Similar to the observations in the present study, Ca^{2+} removal from the cytoplasm is significantly impaired in motor neuron terminals following repeated stimulation (250). Elevated basal Ca^{2+} has also been observed in SMN-deficient iPSC-derived astrocytes (173), and there is also a report showing reduced SERCA1a protein in hindlimb muscles of SMN-deficient mice (34). These findings coupled with the data in the present study suggest that dysregulation of Ca^{2+} may be a common disease mechanism in SMA.

Conclusion

Collectively, these results show for the first time that SMN deficiency alters intracellular Ca^{2+} signaling and cardiac excitation-contraction coupling in a model of SMA. These findings are recapitulated in SMA patient iPSC-derived cardiomyocytes suggesting that this is a cell autonomous outcome of SMN deficiency and suggest that dysregulation of intracellular Ca^{2+} may be a common pathological mechanism in the disease. Finally, while neuromuscular degeneration remains the hallmark feature of the disease, impaired heart function may be a contributing factor in disease progression that will require monitoring in light of new therapies that are improving motor function and extending survival.

CHAPTER 3: Characterizing Neuroinflammation in SMA.

ABSTRACT

Survival motor neuron (SMN) protein deficiency causes loss of alpha motor neurons and subsequent muscle atrophy in patients with spinal muscular atrophy (SMA). Restoration of SMN in motor neurons of SMA mice does not fully rescue the disease phenotype, suggesting that other cells contribute to the disease. Microglia, the resident CNS immune cells, display a reactive morphology in SMA model mice. Moreover, altered spleen and thymus development have been reported in SMA mice, suggesting potential system-wide changes in immune cell function. The role of immune cells in SMA pathology, particularly in the CNS, remains unresolved. In this study, we sought to characterize the reactive profile of microglia in SMA model mice. We generated microglia from control and SMA patient-derived induced pluripotent stem cells. Microglia were validated by flow cytometry, immunocytochemistry and transcriptome profiling. SMN-deficient patient iPSC derived microglia display a unique transcriptome profile, an aberrant migration pattern and an altered complement and TREM2-dependent phagocytosis compared to control. Additionally, conditioned media from SMA iPSC microglia alter motor neuron morphology and physiology. Current available treatments for SMA are not effective in all patients, suggesting other disease modifying therapeutics are required. This finding shows a role of microglia in SMA pathology.

INTRODUCTION

Spinal muscular atrophy (SMA) is a debilitating inherited neurological disease resulting in muscle weakness and subsequent atrophy. SMA is considered one of the leading genetic causes of infant mortality (193, 275). SMA patients have difficulty meeting developmental milestones and in the majority of cases, do not survive to their second birthday if left untreated. SMA is characterized by deletions or mutation in the *SMN1* gene,

resulting in insufficient production of the survival motor neuron (SMN) protein (194). Current FDA approved therapeutics, Risdiplam (Evrysdi™), Nusinersen (Spinraza®), and Onasemnogene ebeparvovec (Zolgensma®) aim to increase levels of SMN protein (80, 100, 114, 178, 183, 185, 193, 222, 240, 263, 272, 283). However, effectiveness and toxicity vary among patients, suggesting additional avenues for therapy are needed.

The SMN protein is ubiquitously expressed in all tissues, and has a well-established role in the assembly of small nuclear ribonucleoproteins (snRNPs) (137, 193, 194). snRNPs are essential for pre-mRNA splicing, and have the potential to alter gene transcription (23, 137, 275). Motor neurons in the ventral horn of the spinal cord are most vulnerable to reduced levels of SMN protein (42, 193). Furthermore, rescuing SMN in motor neurons alone does not completely resolve the symptoms associated with SMA disease progression (113). Interestingly, systemic SMN replacement has been shown to ameliorate the SMA phenotype in a SMA mouse model, suggesting the contribution of non-neuronal cells (63, 64, 167, 216, 242, 275). There is growing evidence supporting SMA as a multi-system disorder with peripheral and CNS immune cells contributing to SMA disease progression (63, 64, 212, 216, 316).

Within the CNS, glia maintain homeostasis and assist in neuronal survival through pH buffering, ion exchange, glutamate recycling, stabilizing synapse structure, and clearance of debris (207, 218, 221, 264, 298). Oligodendrocyte development and CNS myelination were found to be normal in SMA mice suggesting oligodendrocyte function is likely unimpaired in SMA (216, 313). Astrocytes contribute toward SMA physiology through increased GFAP expression and altered morphology due to SMN loss (242). Moreover, SMN replacement in astrocytes alone partially attenuated their pro-

inflammatory profile, improved neuromuscular circuitry, and extended life span of SMA model mice. Together, these findings suggest that immune cells may contribute to the SMA disease phenotype (17, 39, 45, 85, 103, 253, 299).

As the main immune cell of the CNS, microglia have been shown to influence the outcome of many neurodegenerative diseases (151, 159). Microglia are highly dynamic cells that continually survey their environment for potential threats. Alterations in the pathological condition can cause a rapid response (8, 151). Microglia function primarily through release of cytokines and trophic factors, recruitment of adaptive immune cells, as well as clearing of cellular debris and programmed elimination of neuronal cells (295). Interactions between adaptive immune cells and antigen presenting cells, are critical when responding to damage associated-molecular patterns released after degenerating tissue and cell damage (131, 260, 305). The adaptive immune response is generated by the presence of lymphocytes, either T cells or B cells (50, 166). Although both classes of lymphocytes are derived from bone marrow progenitor cells, T cells travel to the thymus, where they undergo further maturation and specialization (63, 166, 297). Once mature, lymphocytes express specific antigen pattern recognition receptors that dictate highly specialized cell-mediated responses and will travel to the spleen and other lymph nodes. Conditions where development is impacted in the spleen or thymus can thus impact the production of lymphocytes (129). In SMA model mice, hypoplasia of both the spleen and thymus was observed (58, 129). The hypoplasia coincided with an increased production of B and T lymphocytes compared to controls, potentially contributing to the increased inflammatory cell interactions in SMA (129).

Previously, microgliosis was observed in the ventral horn of severe SMA mice (295). Iba1, a microglia marker, was upregulated during late stages of disease progression in SMA model mice (295). Interestingly, pharmacological depletion of microglia was shown to rescue proprioceptive synapses on motor neuron proximal dendrites, improving SMA mouse motor function (308). However, the reactive state of microglia during SMA disease progression is unknown. Microglia can be neuroprotective or neurotoxic, depending on the stimuli and other spatiotemporal factors. Previously, complete microglia ablation in an SMA mouse model did not stop motor neuron death (308). While eliminating microglia could help clarify if microglia are necessary for SMA disease progression, it is possible, given the spectrum of immune functions of microglia in health and disease, that we could potentially be missing the full spectrum of properties of microglia in SMA (124, 291). In addition, what happens to microglia after SMN is restored is still unclear. In this study, we investigate the role of lymphocytes on SMA pathology and characterize the reactivity profile of iPSC-derived SMN-deficient microglia.

METHODS

Generation of transgenic mice

The generation and phenotypic characterization of the SMN-deficient transgenic animals (Jackson, *SMN1*⁷;*SMN2*;*Smn*^{-/-}) has been described previously. Transgenic and wild type littermate mice were used at postnatal day 5 (P5) and 10 (P10). Since no significant gender difference has been observed in SMA disease, our study will exclude gender as a factor (74, 249). Tail snips from offspring will be collected at P2 and genotyped using PCR assays. The primers used for genotyping were 5'-

CAAACACCTGGTATGGTCAGTC-3' and 5'-GCACCACTGCACAACAGCCTG-3' for the *SMN2* transgene, 5'-GCCTGCGATGTCGGTTTCTGTGAGG-3' and 5'-CCAGCGCGGATCGGTCAGACG-3' for the mouse knockout allele, and 5'-GTGTCTGGGCTGTAGGCATTGC-3' and 5'-GGCTGTGCCTTTTGGCTTATCTG-3' for the *smn* gene. All procedures complied with the standards for the care and use of animal subjects as stated in the *Guide or the Care and Use of Laboratory Animals* (NIH publication No. 85-23, revised 1996) and all protocols were approved by the IACUC at the Uniformed Services University of the Health Sciences. *Smn*^{2B/2B} mice with a mixed genetic background were mated with BL6 wild type female mice. Litters were genotyped for the *Smn*^{2B} allele (forward primer: 5'-AAC TCC GGG TCC TCC TTC CT-3' and reverse primer: 5'-TTT GGC AGA CTT TAG CAG GGC-3'). Male *Smn*^{2B/+} mice were mated to wild type BL6 female mice for subsequent generations. The backcrossing was continued as previously described (72).

Flow Cytometry

To assess immune cell populations, spinal cords were extracted from wild type and SMN Δ 7 mice at P10. Using an SMN Δ 7 breeding colony, mice pups were genotyped at P2. Neural Tissue Dissociation Kit P (Cat No. 130-097-628, Miltenyi) was used for isolating a single cell suspension for analysis. Tissue was flushed with HBSS without Ca²⁺ and incubated with enzymes provided in the kit. The gentleMACS OctoDissociator protocol 37C_ABDK_01 was used to carry out the mechanical and enzymatic digestion (Miltenyi). Cell suspension was filtered over 70 μ m filters and washed with cold HBSS with Ca²⁺. Sample was centrifuged (300g/10 min/4C) and pellet was re-suspended in PBS supplemented with 0.5% BSA buffer. Afterwards, magnetic myelin removal beads

II (Miltenyi) were used for negative selection of myelin from the single cell suspension. Cells were blocked with TruStain fcX (Cat. 101319, Biolegend) CD11b (Cat. 101207, Biolegend), CD45 (Cat. 100312, Biolegend), and CD3 (Cat No. 100312, Biolegend) were used for positive fluorescent staining of monocytes, microglia, and T-cells, respectfully. Cells were read in cell staining buffer on BD Accuri C6 Flow Cytometer CFlow Plus software. Cells were gated on live cells, CD45⁺, and further gated on CD11b⁺ and CD3⁺. Unstained cells were used as a negative control. OneComp eBeads were used for positive controls (Cat. 01-1111-42, Invitrogen). All analysis was done using the Flow Jo software (FlowJo, LLC, Ashland, OR).

Behavior Assessment

Righting Reflex

To assess motor coordination, pups were tested for their ability to right themselves. Righting time is defined as the average of two trials of the time required for a pup to turn over after being placed on its back (maximum 30 seconds). Pups were given time between the trials to eliminate the possibility of exhaustion. Weight was recorded daily. Mice that lost 30% of their body weight and were unable to right themselves were euthanized.

Pen Test

To assess balance, mice were placed on a wide pen at a height of about 30-40cm. Time until they fall was recorded. Tests were performed at P19 until P25.

Inverted Mesh GripTest

To assess their ability to hold on against their body weight, mice were put on a mesh until a good grip was maintained. The mice were then oriented upside down at that time and time spent holding to the mesh was recorded (maximum 60 seconds). The testing began on P13 and was performed every two days.

Immunohistochemistry

To analyze microglia morphology throughout the SMA disease progression, spinal cords (N=3 for both WT and SMA) were extracted from wild type and SMN Δ 7 mice at P10. Mice pups were genotyped at P2. Lumbar regions were sectioned into 7- μ m axial segments and probed with primary antibodies for Iba1 (Wako), followed by 3, 3'-diaminobenzidine (DAB) staining. Outcome measures, including branching, image fluorescence and soma diameter were assessed using ImageJ.

Cell lines

BV2: The BV2 microglia cell line (a gift from Carol Colton, Duke University) was cultured in a humidified incubator at 37°C 5% CO₂/95% oxygen. The cells were maintained with Dulbecco's Modified Eagle Media (DMEM, Invitrogen) with 10% fetal bovine serum (FBS, Hyclone, Logan, UT, USA) and 1% penicillin/streptavidin (Fisher, Pittsburgh, PA, USA).

Induced Pluripotent Stem Cells: To study the effects of SMN-deficiency on microglia, we utilized SMA iPSCs (Type I SMA Male, age two, CS77iSMA1-nxx from Cedar Sinai) reprogrammed with episomal plasmid from SMA Type I patient fibroblasts. Control iPSCs from (ATCC-BYISO112 Human [Non-Hispanic Caucasian Male, age 31 ATCC® ACSTM-1026ATCC] were used. Dox inducible motor neuron iPSCs were a generous donation from the Michael Ward lab. iPSC lines were maintained in standard

6-well tissue culture plates coated with growth factor reduced Matrigel (Cat. 354277, Corning) diluted 1:100 in DMEM/F12 (Gibco) on the same day as iPSC plating. Frozen stocks of iPSCs were thawed and plated on Matrigel coated plates in E8 and E8 Flex (Gibco). iPSC lines were passaged using 0.5mM EDTA in PBS without CaCl₂ and MgCl₂ (Life Technologies). Cells were maintained at 37°C, 5% CO₂.

Transfection

Plasmid transfection of SMN-GFP (1µg) on microglia was performed using Lipofectamine 3000 (Invitrogen, cat 100022052) and P3000 (Invitrogen, cat 100022058) for 48 hours according to the manufacturer protocols. The SMN-GFP was generously donated by Greg Matera. SMN siRNA knock-down was done using 40pmol of pre-validated siRNA (IDT, cat 37206943) and RNAi Max (Life Technologies, cat 56532) per manufacturer protocol for 48 hours. Opti-MEM (Gibco, cat 11058-021) diluent was used in both protocols. Transfection was validated by western blot.

iPSC Microglia Differentiation

To differentiate microglia from SMA and control patient iPSCs, the previous protocol from Haenseler et al (103) was applied. In 6 well low adhesion plates (Thermo Scientific), iPSCs were harvested using Accutase and resuspended at 1x10⁶ cells/ml in stem cell media supplemented with BMP4 (50ng/ml, R&D Systems), VEGF (50ng/ml, R&D Systems), and SCF (20ng/ml, R&D Systems). Cells were allowed to incubate at 37°C in a humidified incubator with 5% CO₂ for four days to allow for the formation of embryoid bodies (EBs). EBs were transplanted to matrigel coated flasks with XVIVO15 media (Lonza) supplemented with M-CSF (100ng/ml, R&D Systems) and IL-3 (25ng/ml, R&D Systems) and incubated for a week at 37°C, 5% CO₂. EBs were embedded and

allowed to extend into surrounding adherent stromal cells to develop structures similar to cystic yolk-sac. After one week, microglia precursors were collected from the supernatant and plated on Matrigel-coated plates in microglia medium consisting of Advance DMEM with N2 (1:100), IL34 (100ng/ml, R&D Systems), and GM-CSF (10ng/ml, R&D Systems). Microglia-like cells were fed every 3-4 days with microglia media. Cell based assays were carried out after 7 days of incubation.

Motor Neuron differentiation

Immunocytochemistry

To analyze the effect of SMN deficiency on cell morphology and phenotype in vitro, SMA and control cells were fixed with 4% paraformaldehyde on round coverslips. Standard immunocytochemistry (ICC) was performed. Cells were blocked for 30 minutes in PBS/ 10% Goat serum/0.1% Triton X, at 4°C. Cells were then stained for primary antibodies to Iba1 (Wako) overnight at 4°C. Appropriate secondary antibodies linked to AlexaFluor dyes (Invitrogen, Carlsbad, CA) were incubated on the fixed cells for 1 hour at room temperature. Round coverslips were mounted onto slides using mounting media containing DAPI (Vector Labs, Burlingame, CA). Cells were imaged at 20x using a Leica DM4000 B LED and corresponding software (Leica, Wetzlar, Germany).

Phagocytosis Assay

To assess phagocytic activity, we used uncoated carboxylate-modified microspheres with an excitation emission spectrum of 580/605 (FluoSpheres, F8821, ThermoFisher Scientific). FluoSpheres were prepared in a 3% BSA, 25 mM Na₂HPO₄, pH 6.0 solution as previously described (196). 5×10^5 concentration of control and SMA microglia precursor cells were plated per well of a six-well plate. Phagocytosis assay

was carried out after a week of differentiation. Briefly cells were washed with 1xPBS, FluoSpheres (1:67 in Optimem) were added directly to each well, and incubated for 15 min at 37°C. Unstained cells received Optimem only. Cells were then washed three times with 1xPBS and gently scraped and centrifuged at 300g for 10 min. Pellets were resuspended in FACS buffer in polyethylene tubes and submitted to flow cytometry (BD Accuri C6, BD Biosciences, Franklin Lakes, NJ). Unstained cells were used for gating control. 5,000 cells were collected per group with the threshold of 1,000,000 to exclude debris. The mean fluorescence of all viable cells was measured, and data were analyzed using unpaired Student's *t* tests.

Western Blot

To assess protein level, cells were lysed and combined with Laemmli sample buffer containing β -mercaptoethanol (1:1, Biorad, Cat. 1610737). Combined samples were boiled at 95°C for 5 min. 35 μ g samples were loaded on 4-12% Novex WedgeWell Tris-Glycine Gels, and run according to manufacturer's instructions. Blots were transferred to PVDF membranes using Biorad Trans-blot Turbo. Antibody for SMN (1:1000, BD Biosciences, Cat. 610646,) was used.

PCR

RNA was isolated using the Qiazol and chloroform extraction method, then purified with the Qiagen RNeasy kit (Qiagen, cat 74104). cDNA conversion was performed using a Veriti thermal cycler and a high-capacity cDNA conversion kit (Applied Biosystems). Gene expression analysis was performed using pre-validated Taqman probes for C1q, C3, TREM2, TMEM119, CHAT, and PGK1 (Cat. Hs00706358_s1, Hs00163811_m1, Hs00219132_m1, Hs01938722_u1,

Hs00252848_m1, Hs00943178_g1, respectfully, Life Technologies). Quantification occurred in a StepOnePlus qRT-PCR machine (Applied Biosystems) using Universal Probes master mix (BioRad) per manufacturer instructions.

Sequencing

Samples were prepared as previously described (196). Total RNA was quantified via a fluorescence dye-based methodology (RiboGreen) on a Spectramax Gemini XPS plate reader (Molecular Devices, Mountain View, CA). RNA integrity was assessed using automated capillary electrophoresis on a Fragment Analyser (Advanced Analytical Technologies, Inc, Santa Clara, CA). Total RNA input of 200 ng was used for library preparation using the TruSeq Stranded mRNA Library Preparation Kit (Illumina, San Diego, CA). Sequencing libraries were quantified by PCR using KAPA Library Quantification Kit for NGS (Roche, Wilmington, MA) and assessed for size distribution on a Fragment Analyser. Sequencing libraries were pooled and sequenced on a Novaseq 6000 (Illumina) using a 200SBS kit with paired-end reads at 101 bp length.

RNA-seq read alignment, quantification, and analysis

Raw sequencing reads were demuxed using bcl2fastq2 (v2.20) and aligned to the reference genome with MapSplice (v2.2.1) [1]. Gene-level quantification was performed with HTSeq (v0.9.1) [2] against UCSC gene annotations (obtained February 21, 2016). Read alignment statistics and sample quality features were calculated with SAMtools [3] and RSeQC [4]. Sequencing quality was verified by manual inspection of sample-wise characteristics: total reads, mapping percentages, pairing percentages, transcript integrity number (TIN), 5' to 3' gene body read coverage slopes, and ribosomal RNA content. DESeq2 (v1.16.1) [5] was used to identify differentially expressed genes

(DEGs) between pairwise conditions from raw gene read counts. Significant DEGs were identified as those with an FDR q-value ≤ 0.05 , an absolute fold change > 2 (i.e. $|\log_2(\text{fold-change})| > 1$), and mean transcripts per million (TPM) > 1 across samples.

Migration Assay

Cellvis 8-Chamber Glass Preparation

To assess resting migration, Matrigel (Corning) ECM was prepared at 1:100 concentration in DMEM/F12 media. 400 μL of ECM was added to a chamber. The chamber glass was then incubated at room temperature for 30 minutes, followed by 37°C for one hour. The chamber glass was stored in an incubator until cells were ready to be transferred to the chambers.

Cell Transfer to Chamber Glass

Media was aspirated off cells, and 3 mL of 1:6000 Invitrogen CellTracker Green CMFDA dye in 1X PBS was added onto the dish. The CellTracker dye was allowed to incubate on the cells at room temperature for 5 minutes before it was aspirated. Then, 3 mL of cold 0.5mM EDTA (MediaTech, Inc.) was added to the dish. The dish was placed at 4°C for 10 minutes. After, EDTA was aspirated, and 1 mL of macrophage media was added to the dish. The dish was placed at an angle, then a Corning Cell Lifter was used to scrape cells into the media. The cells in media were then transferred to a 15 mL NEST conical and vortexed. A cell count was taken with a Marienfeld hemocytometer. Cells were plated at a concentration of 5,000 cells per well in the Cellvis 8-chamber glass slide.

Live Cell Microscopy

The chamber glass was transferred to an environmental chamber attached to an Olympus IX83 Inverted Light Microscope. The environmental chamber is INU (Incubation System for Microscopes) Tokai Hit, and the stage conditions were set to 40°C Top Heater, 39.5°C Stage Heater, and 38°C Bath Heater. DI water was used for the bath. A Hamamatsu digital camera C13440-20CU (Hamamatsu, Japan). Excelitas Technologies X-cite 120 LED Boost (Olympus, Tokyo, Japan) was used for fluorescent imaging. The chamber glass was left in the environmental chamber for two hours to allow the macrophages to spread and settle on the ECM components. Olympus CellSens software was then used to select positions on the chamber glass. Both Relief Contrast and GFP images were taken for each position. Each ECM condition had seven positions selected, one picture was taken every ten minutes, over a 16 hour time course.

Migration Assay Analysis

Olympus video files were opened in ImageJ with the OlympusViewer Plugin. The GFP and Relief Contrast channels were split and only the GFP channel was utilized. The TrackMate Plugin was then used for automated tracking of the macrophages, which are recognized by their contrast from the background. Each frame the cell moves, the program creates a track throughout all 97 frames. From this, the data output includes cell number, frame number, and X-Y position. These values are saved and utilized by the Chemotaxis Tool plugin, which calculates the velocity of each cell based on the track generated by TrackMate, as well as persistence (d/T), which is the propensity of a cell to continue in the direction that it is already moving instead of changing directions. Velocity and Persistence values for all of the cells in each condition are put into an excel file and the mean is calculated for each category.

Scratch Wound Assay

Cells were plated on the previously prepared Cellvis 8-Chamber Glass to 100 percent confluency. Cells were serum and cytokine starved 24 hours prior to running the assay. On the day of an experiment, 1:1000 Invitrogen CellTracker Red dye was administered in cell media. The CellTracker dye was allowed to incubate on the cells at 37°C for one hour before. After incubation, media was aspirated, and a scratch wound was made using a 200µm pipette (Thermo Fisher). The Hamamatsu digital camera C13440-20CU and Excelitas Technologies X-cite 120 LED Boost was used for fluorescent imaging. The chamber glass was left in the environmental chamber for 30 minutes prior to selecting positions (Olympus CellSens software). Both Relief Contrast and GFP images were taken for each position. Each condition had three positions selected, and one picture was taken every ten minutes, over a 16-hour time course.

Electrophysiological recordings

Motor neurons were differentiated on round glass coverslips in 24 well plates at a plating density of 250,000 cells/well for 14 days. Cells were pre-treated 24 hour before recording in desired media condition, and the coverslips were transferred to the recording chamber on a Zeiss 7 MP microscope system. (Zeiss, Oberkochen, Germany).

Electrophysiological recordings were performed with an Axon Multiclamp 700B amplifier and 1440A Digidata unit connected to a PC running pClamp10 software (Molecular Devices, San Jose, CA). Electrodes used for recording were pulled on a horizontal pipette puller (P-97; Sutter Instrument Company, Novato, CA) from thin wall borosilicate glass capillaries with filament (Warner Instruments, Holliston, MA) to a pipette resistance of 3-6 M Ohm when used with the solutions below. Cells were perfused

in the recording chamber with an extracellular bath solution (135 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 20 mM glucose (dextrose anhydrous) and 10 mM HEPES, , adjusted to pH 7.3 with 1 M NaOH) throughout the duration of the experiment. The pipette solution contained in mM: K-gluconate 130, KCl 15, HEPES 5, EGTA 1, Mg-ATP 4 and Na-GTP 0.3. The pH was adjusted to 7.3 with a 1M KOH solution. Additionally, 15 μ M Alexa Fluor 488 Hydrazide, sodium salt (Thermo Fisher Scientific) was added to the internal solution for visualization of recorded cells. Cells were recorded in whole-cell mode which was achieved by brief suction pulses after obtaining a giga Ohm seal. Cells with an access resistance over 50M Ohm were discarded. For current voltage relationship measurements cells were held at -70 mV and the membrane potential was stepped in 5 mV increments from -50 mV to + 45 mV for 100ms. To gauge cell excitability, cells were recorded under current clamp conditions with a current injection to hold the membrane potential at -70mV. A pulse protocol depolarized the cell successively by current injections in 2 to 5 pA steps to elicit action potentials (AP). An active voltage response was deemed an AP if it crossed 0 mV. The maximum number of APs per cell was determined. After recording electrophysiological measures, small z-stacks at optimal z-step size were acquired in ZEN software (Zeiss) with the infrared laser (Vision 2, Coherent, Santa Clara, CA) tuned to 800nm and utilizing a 40x, 1.0 NA water immersion objective. Maximum intensity projections of the acquired stacks were used to determine morphology of the recorded cells.

Motor Neuron Morphological Assessment

The number of dendrites branching from the soma was determined by Sholl analysis using the Image J software (NIH, Bethesda, MD). All tracings and analysis were

performed with the experimenter blinded to the condition. Images were acquired from the electrophysiology assessments for all groups. Conditions where the dye was not properly injected into the soma prior to imaging was discarded. Briefly, images were converted to grayscale and scaled to appropriate size bar. The Sholl analysis was used under the following parameters from the chosen soma center point: start radius 10 μ m, step radius 10 μ m. Branching was recorded up to 200 μ m for all groups.

Statistics

All data was analyzed using the GraphPad Prism software (GraphPad Version 8, San Diego, CA). All behavior data was analyzed with (ANOVA) tests. *Mantel-Cox* and *Gehan-Breslow-Wilcoxon* analyses were performed to determine significance between groups for all significant main effects and interactions. Flow cytometry and cell culture data between a control and SMA group was analyzed with a Student's *t*-test. Two-way ANOVA was used followed by post hoc Bonferroni for Multiple Comparisons to analyze the Sholl curves between groups. For all statistical tests, a *p* value of <.05 was considered significant.

RESULTS

Microglia exhibit a reactive profile in SMA mice

Previous studies have provided evidence that microglia migrate to and adopt a reactive profile at the sites of neuronal cell death in SMA model mice (295). What triggers this migration is unknown but there is some evidence that lymphocytes can cross the blood brain barrier during neurodegenerative conditions and activate microglia (159). Using flow cytometry we evaluated the overall number of microglia (CD11b⁺), monocytes (CD45⁺), and lymphocytes (CD3⁺) in the spinal cords of unaffected and SMA

mice (Fig. 9A). Experiments were carried out at post-natal day 10; near the disease end-stage in the SMN Δ 7 SMA mouse model where inflammation is expected to peak (280). The number of microglia, monocytes and lymphocytes were similar between control and SMN Δ 7 mouse spinal cords suggesting no increased of microglia proliferation or peripheral immune cell infiltration.

We next sought to determine the reactive state of microglia in SMA mice. Ramified quiescent microglia have a small soma and elongated processes. Once activated, the microglia adopt an amoeboid morphology with an enlarged soma and retracted processes (89, 142). We used 7 μ m thick spinal cord sections from the lumbar region (L3- L5), where motor neuron death is most prevalent in SMA mice, and analyzed the microglia soma size and processes (Fig. 9B) (154, 181). Soma diameters of Iba1+ microglia were measured in cross sections and was significantly increased in the microglia from SMA mice compared to controls (Fig. 9C). SMN-deficient microglia had significantly fewer processes compared to controls consistent with a more reactive state (Fig. 9C). Together, microglia in the SMA mouse spinal cord appear to adopt a reactive profile with an enlarged soma and retracted processes.

Lymphocyte ablation does not improve motor function or survival of SMA mice

Under neurodegenerative conditions, lymphocytes can infiltrate the CNS and modulate immunological responses (40, 186, 241). Additionally, lymphocytes in the periphery can release chemical messengers that cross the blood brain barrier (BBB) and interact with antigen presenting cells. Lymphocyte release messengers can cause microglia activation and influence the outcome of many neurodegenerative diseases, including amyotrophic lateral sclerosis (ALS), multiple sclerosis (MS), Alzheimer's

disease (AD), and Parkinson's disease (PD) (40, 162, 186, 241). Here we assessed if lymphocytes, including both T cells and B cells, specifically contribute to SMA disease progression.

T cells can be subcategorized into two main classes, including the T helper cells and cytotoxic T cells (228). In the SMN Δ 7 mouse, atrophy of motor neurons starts at approximately post-natal day 5, with a peak immune response and survival expectancy at only 14 days (145, 193). Due to the shortened life span, severe models of SMA may generate a hyper-responsive immune response, potentially missing any subtle underlying immune irregularities. To address this, we used the intermediate *Smn*^{2B/-} mouse model, which has an average life span of 28 days (72). Of the T cell classes, helper T cells, known as CD4⁺ cells, are considered one of the more important cells in the adaptive immune response (95). CD4⁺ cells can promote B cells to secrete antibodies to recruit additional immune cells to the site of infection as well as stimulate cytotoxic T cells to target degenerating cells (95, 161).

First, to assess if helper T cells impact the SMA phenotype, we used CD4 KO mice on the *Smn*^{2B/-} background and evaluated motor function and survival (Fig. 10A). We found that knocking out CD4 T cells did not improve motor function, as measured by the pen test, rotor rod test, and upside-down mesh test. Moreover, there was also no extension in survival compared to controls. As CD4 T cells are a subset of lymphocytes, we next addressed if all lymphocytes, including T cells and B cells, play a role in SMA pathophysiology. To do so, we used RAG1 knock out mice on an *Smn*^{2B/-} background. The recombinant activating genes (RAGs) encode for proteins that form a heterotetrametric complex critical in V(D)J recombination in T and B cells, necessary for

immune diversity (192). Thus, lack of RAG1 results in B and T cell deficiency due to their inability to mature (192). We found no motor function improvement when RAG1 was knocked out compared to controls (Fig. 10B). Additionally, lymphocyte deficiency did not extend the life span of the *Smn*^{2B/-} mice (Fig. 10B). Our findings suggest that lymphocytes may not be drivers of SMA disease progression, although we cannot rule out the possibility that they may have a more complex role that could not be uncovered by complete depletion.

SMA microglia have an altered profile compared to controls

We show here that microglia adopt a reactive morphology in the spinal cord of SMA mice (Fig. 9B, C). To further characterize how SMN-deficiency alters microglia in human cells, we generated microglia from SMA patient-derived iPSCs using a modified protocol from Haenseler et al (Fig. 18A) (103). Given that gene expression changes can occur during iPSCs differentiation we first show that SMN levels remained low in the iPSC-derived SMA microglia (Fig. 18D). We confirmed expression of microglia specific genes including TMEM119 and TREM2 and surface marker expression of Iba1, HLA molecules, CD45, and CD11b in the control and SMA microglia (Fig. 18B, C, E). Next, we examined cell soma size and number of processes to determine if human SMA microglia displayed the same alterations in cell morphology observed in microglia from SMA mice. We found that SMA microglia had a significantly reduced number of processes (Fig. 11B). However, control and SMA microglia had similar cell soma size, inconsistent with what we observed in mice spinal cords.

We performed an unbiased RNAseq experiment to assess global changes in gene expression in control and SMA microglia (Fig. 12). A gene set enrichment analysis

(GSEA) (195, 287) was conducted using the Reactome and Hallmark databases to identify representative pathways that are significantly induced by SMN deficiency. Transcriptome data confirmed expression of microglia specific genes (*p2ry12*, *csfl*, *trem2*, *hla*, *merlk*, *Clq*). Reactome analysis revealed 10197 differentially expressed genes between control and SMA microglia (5398 up, 5519 down). Pathways representing cytokine signaling in innate and adaptive immune response genes (458 and 278, respectively), cytokine and chemokine expression (*CCL2-5*, *CCR1*, *CCR5*, *CXCL10*, *CXCL8*), and activation of toll-like receptors (*MYD88*, *TICAM1*, *TIRAP*, *TLR1-6*) were enriched in SMA microglia compared to controls. The chemokine receptor CCR1 contributes to immune cell migration and select microglial function (53, 91). Together, our transcriptome analysis supports that SMA microglia adopt a reactive profile independent of contributions of dying motor neurons.

Genes involved in actin, myosin regulatory light chain interacting proteins, rho GTPases activating WASPS and WAVES pathways (*arpc2*, *myl1p*, *RAC2*, *WAS*, *WASF2*, *ROCK1*, *ROCK2*, *RHOA*) were also increased in SMA microglia, supporting a role for SMN in cytoskeletal dynamics (137, 247, 308). Changes in actin filaments and tubules impact migration, chemotaxis, recruitment, as well as phagocytosis in microglia (28, 233, 274). Damaged neurons emit extracellular ATP or ADP, which act as chemoattractants and cause microglia chemotaxis, which depends on both specific receptors and the cytoskeletal machinery (274). Our analysis shows an increase in the P2Y pathway-associated genes in SMA microglia (*P2RY1*, *P2RY11-13*, *P2RY6*). P2Y belongs to a purinergic G protein coupled receptor family that is critical in detecting extracellular ATP levels (274). Furthermore, degenerating motor neurons and synapses tagged with

complement proteins to be phagocytosed (160, 181, 308). SMA microglia were primed with increased complement cascade associated genes (*ClqA-C*, *C5*, *C2*), as well as apoptotic cell recognizing phagocytosis receptors (*CD36*, *FCGR3B*, *CD206*). (99, 180, 223). In summary, microglia differentiated from SMA patient iPSCs have a unique transcriptomic profile, indicating that SMN may be a critical regulator of multiple microglial functions, including migration and phagocytosis.

SMN deficient microglia display increased migration and phagocytic activity

Microglia are sensitive to changes in their environment and are constantly surveying their local surroundings. Cell migration is a fundamental feature of immune cells, including microglia, which is essential to their role in tissue protection, repair, and regeneration. Cell migration dynamics can be quantified by determining cell velocity and persistence. Velocity is defined as the total distance that cell migrates over a specific time and persistence is how long a cell migrates in any particular direction. To assess cell migration dynamics, both control and SMA microglia were fluorescently labeled with cell tracker dye and migration was tracked using time-lapse confocal fluorescent microscopy over 16 hours and velocity and persistence were determined using ImageJ software (Fig. 13). We found that SMA microglia displayed a significantly higher velocity compared to control microglia (Fig. 13A). To confirm that the difference in velocity was due to SMN, we transiently restored SMN into the SMA microglia and found cell velocity return to levels observed in control cells (Fig. 13B). We found no difference in persistence between SMA and control microglia (Fig. 13A,B)

The combined impact of SMN loss on migration speed and rate of response to injury was further investigated by the scratch wound healing assay (Fig 14A,B). Our

results indicate that SMA microglia closed more of the wound area, consistent with our migration findings (Fig. 14B).

A key microglia function is removing apoptotic cells, cell debris and pathogens by phagocytosis. A previous study showed increased expression of C1q tagged substrates co-localizing with microglia that have engulfed neuronal particles, suggesting exacerbated microglia phagocytosis in SMA model mice (308). To determine if SMA microglia display enhanced phagocytic activity, we incubated fluorescently-tagged beads with SMA and control microglia and measured the fluorescent intensity, indicative of bead internalization, by flow cytometry. We found that human SMA microglia have an enhanced phagocytic capacity compared to control microglia (Fig. 15A).

The complement system and TREM2 receptor pathways are two well documented pathways that elicit the phagocytic response in CNS immune cells (209, 314, 317). Both pathways have been implicated in neurodegenerative disease such as ALS, Alzheimer's, frontotemporal dementia, and multiple sclerosis (56, 93, 132, 153, 207, 308, 317). Here, we sought to determine if these pathways were activated in SMA microglia. We first investigated if C1q was differentially regulated in our human iPSC derived microglia. Indeed, C1q expression was elevated in the SMA microglia compared to controls (Fig 15B). Interestingly, there was no difference in the C3 gene expression between groups. C3 is a complement protein that when activated, cleaves into C3a and C3b, contributes to the tagging of material that needs to be eliminated (209).

We next explored whether TREM2, an immunoglobulin receptor, contributed to the SMA microglia phagocytosis. When TREM2 is stimulated, it initiates a downstream cascade through DAP12 receptor to induce phagocytosis (149). Here we show that

TREM2 is significantly upregulated in the SMA iPSC microglia compared to controls (Fig 15B). To determine if the increased phagocytic activity of SMA microglia compared to controls was due to TREM2 upregulation, we knocked down TREM2 using an siRNA and measured phagocytosis (Fig 15C). We found that knocking down TREM2 reduced phagocytic activity of SMA microglia to activity observed in control cells (Fig 15C) (27, 80, 309). Together, we show here that vital microglia functions, migration dynamics and phagocytosis, are enhanced in SMA microglia with potential implications on modifying the SMA disease phenotype.

Conditioned media from SMN-deficient microglia alters motor neuron physiology and morphology

We next sought to determine if SMA microglia release factors positively or negatively regulates motor neuron function. iPSC-derived control and SMA motor neurons (Fig 19) were incubated for 24 hours in media from control and SMA microglia (Fig 16B). Motor neurons were randomly chosen and electrophysiological recordings were performed under whole-cell patch clamp technique conditions. Alexa 488 was added to the internal solution to visualize the recorded cells to assess their morphology (processes are denoted by the white arrow, Fig 16B). The yellow arrow denotes a representative cell that was analyzed. The control iPSC motor neurons exposed to SMA iPSC microglia media for 24 hours showed increased numbers of action potentials compared to the motor neurons that were incubated in control microglia media (Fig 16C). The amplitude of the inward sodium and the potassium repolarization currents remained consistent throughout the groups (Fig 16D and 16E, respectfully). To determine if these findings remained consistent with SMN deficient motor neurons, motor neurons were differentiated as previously described and an siRNA was used to

knock down SMN. SMN expression levels were validated (Fig 14A). Importantly, consistent with the control motor neurons, SMN deficient motor neurons exposed to SMA microglia conditioned media had an increased number of action potentials compared to SMN-deficient motor neurons treated with control microglia conditioned media (Fig 16F). Sodium and potassium current amplitude were significantly higher in SMN deficient motor neurons treated with SMA microglia conditioned media compared to controls (Fig 16G and 16H, respectfully). While there was a difference in overall current amplitude with sodium and potassium, this significance was lost with current density, which is the current flow per unit area, in SMN deficient motor neurons compared to controls for all media conditions (Fig 16J, I).

Previously, motor neurons in a zebrafish model of SMA showed defects in branching bifurcations compared to controls (175). We next explored if SMA microglia media can induce morphological changes in control and SMN-deficient motor neurons (Fig 17). We observed an increase in the number of processes in control and SMN-deficient motor neurons treated SMA compared to control microglia media. Using Sholl analysis we found that both control and SMN-deficient motor neurons treated with media from SMA microglia media had significantly increased branching (Fig 17A,B). The data presented here show that media released by SMA microglia alters both the morphology and electrophysiological properties of motor neurons.

DISCUSSION

This study investigated the potential involvement of immune cells in cell and animal models of SMA. We found that loss of lymphocytes does not alter the survival or motor function of SMA model mice. To date, the effect of microglia on SMA has

predominantly carried out in mouse models. Our findings here in mice are consistent with previous reports that microglia are reactive, at least in the end stage of the disease.

However, it is unclear if microglia behave similarly when SMN is deficient in human cells. Our current findings indicate that SMN-deficient human microglia tend towards an anti-inflammatory state, while demonstrating enhanced migration velocity and phagocytic activity. In addition, SMA microglia non-autonomously altered the electrophysiological properties of motor neurons, suggesting that microglia likely play a complex role in SMA pathology that cannot be fully appreciated by complete ablation.

Lymphocytes do not drive SMA disease progression

We examined the impact of lymphocytes in SMA by using both *CD4^{-/-}* and *Rag1^{-/-}* mice on a *Smn^{2B/-}* background. Our study found that depleting T helper cells alone or complete lymphocyte ablation did not improve the life span of these animals or their ability to perform motor function assays. Although the role of lymphocytes may not have been prominent, it should be noted the spleen and thymus were already found to be significantly smaller in the SMA mice, potentially contributing to the lack of proper lymphocyte maturation and organ development previously reported. In fact, studies have shown that SMN deficiency impacts the development and spleen size of three major SMA model mice (129). By reducing the production of mature and functional lymphocytes from birth, we rule out these developmental abnormalities, such as immature T cell development, that could impact the immune response in SMA patients.

Microglia in SMA: helpful or harmful?

Although more literature is coming out illuminating microglia, little is still known of the exact role that they play with motor neurons and the SMA phenotype. As the

primary CNS line of defense, microglia are particularly sensitive to immediate changes in their surrounding milieu and can respond accordingly by either migrating to the site of injury, engulfing the imminent danger, or releasing factors to recruit additional aid. Others have found microglia in SMA mouse models to have an inflammatory phenotype, with an increase in reactive oxygen species (ROS), release of TNF α , as well as increased NF κ B activity (10). The transcriptome data for the human iPSC differentiated microglia showed an upregulation in signaling pathways for ROS, TNF α , and NF κ B in SMA microglia compared to controls. Additionally, SMN deficient microglia have an increased phagocytic behavior in SMA mice compared to controls (308). Although our findings show no overall increase in microglia population (Fig. 9A), morphological assessment indicates an increased soma and retracted processes, indicative of a reactive morphology in the SMA mouse spinal cord (Fig. 9B,C), consistent with previously published findings.

To test whether this pro-inflammatory phenotype is conserved in the human model, we differentiated microglia from unaffected and SMA patient iPSCs. We cannot rule out the genetic component to the individual control and SMA patient iPSCs, however we found that microglia differentiated from human cells also had the increased phagocytic phenotype previously reported in the mice models of SMA (Fig. 15A). The changes we see in migration and phagocytosis in SMN deficient human derived microglia is not surprising as previously it has been shown that the SMN protein plays a role in cytoskeletal dynamics (51). There are two main phagocytosis pathways that have become prominent in literature, including C1q and TREM2. Similar to the microglia in SMA mice, we saw an increase in C1q gene expression in our human iPSC microglia.

Surprisingly, we saw no change in C3 expression between control and SMA microglia (Fig. 15B). Perhaps the C3 increase previously documented in the SMA mouse models is not a result of microglia activation, but that of other immune cells. Other players such as astrocytes may be contributing to the activation of microglia and need to be further elucidated. Furthermore, the phagocytosis inducing TREM2 gene was also upregulated. This suggests that microglia in the SMA disease progression may have an exacerbated phagocytic response elicited by not only the complement pathway but also the TREM2 pathway. Due to such an increase in phagocytosis, the upregulated CD33 gene expression observed could be acting as a compensatory mechanism. Additionally, TREM2 has been associated with not only phagocytosis, but migration and an anti-inflammatory phenotype. Indeed, SMN deficient human iPSC derived microglia had an increased velocity, suggesting an increased migratory capability. Due to their response to the scratch wound, as well as their increased velocity, it is possible that microglia in the human SMA condition may be responding too quickly to the damaged motor neurons, and the increased phagocytosing of not only the degenerating motor neurons, but those that are trying to recover.

SMN deficient microglia release factors that influences motor neuron excitability

The signaling and interaction between motor neurons and microglia in SMA is not well understood. In the healthy condition, microglia phagocytose dying neurons and maintain homeostasis which allow for proper neuronal firing. We have shown that the phagocytic nature of microglia in SMA is consistently increased. One possibility is that SMA microglia are phagocytosing not only dying but also healthy motor neurons, impairing their activity. However, our co-culture assays using media from SMA patient

derived microglia show that treated motor neurons are having an increased excitability. Furthermore, SMN deficient motor neurons, when exposed to SMN deficient microglia media have increased generation of action potentials due in part by increased inward sodium current, as well as repolarization. The SMA iPSC microglia media is also increasing arborization in both control and SMN deficient motor neurons. This is a novel finding as it shows that microglia in SMA may be having a beneficial role in motor neuron physiology.

Conclusion

Overall, we have shown that loss of lymphocytes does not improve SMA disease phenotype and likely does not contribute to the microglia phenotype in SMA mice. SMN-deficient microglia however displayed an altered transcriptome profile, increased mobility and enhanced phagocytic activity, which could modify the SMA phenotype. Further studies using microglia and neuron co-cultures is warranted to further elucidate the role of SMA microglia in SMA disease progression.

CHAPTER 4: DISCUSSION

The overarching goal of this dissertation is to develop SMA patient-derived iPSCs to study the role of SMN in cardiomyocytes and microglia. Human iPSC models of pathological cell-types potentially provide a more accurate representation of the mechanisms underlying SMN deficiency. Current SMN restoring therapies are not enough to ameliorate all symptoms, with muscular weakness persisting even after treatment (27, 80, 309). The majority of the alternative therapies currently under clinical evaluation are targeted at restoring muscle size and reducing the threshold to muscle contraction, symptoms which occur later in the disease progression. Cardiac and immune dysfunction have been reported in severe cases of SMA prior to the onset of NMJ denervation (64), suggesting a critical role in disease pathogenesis. However, current therapies geared towards slowing motor neuron loss and stalling disease progression does not consider non-neuronal cells. Also, a majority of the SMN focused research relies heavily on rodent models or postmortem tissue, representing a hurdle to therapeutic translatability. Here, we generated human cardiomyocytes and microglia cells from unaffected and severe SMA patient iPSCs. The work uncovered novel functions of SMN in cell types not previously studied in SMA. Moreover, we have generated a human derived cell-based platform that could be applied to mechanistic analysis of disease-associated cellular pathways and amenable to high throughput drug screening.

Cardiac dysfunction in SMA

The first aim of my study was to understand the impact of SMN deficiency on cardiomyocyte function. SMA patients have functional and structural irregularities found in the heart (311). Congenital heart defects are observed during embryonic development in severe SMA mice (268), and BNP, FABP3, and CK heart failure markers have been reported in the early phase of the disease (135). Our findings of elevated heart failure markers in SMN Δ 7 mice are consistent with those observed in SMA patients. BNP and skeletal α -actin mRNA and protein levels are elevated prior to motor neuron degeneration and remain elevated during the course of the disease. Increased BNP levels are linked to a decrease in the left ventricle's systolic function (68). However, to the best of our knowledge, a thorough examination of cardiac function in SMA Type I patients has not been conducted. Results from the studies outlined here strongly support a closer examination of the role of cardiac dysfunction in SMA pathology.

SMA cardiomyocytes display impaired contractile dynamics

The rate at which the heart contracts and relaxes is a determinant of cardiac function (123). Cardiac performance is compromised when the heart contraction is compromised (123). SMA cardiomyocytes displayed altered contraction kinetics. While there was no marked difference in overall fraction shortening, the diastolic sarcomere length was significantly reduced. Moreover, experiments revealed a delayed time to peak shortening and 50% increase in relaxation time. Influx of calcium into the cell induces a conformational change in troponin, allowing for myosin heads to bind to actin, generating a contraction (75). Reuptake of calcium into the sarcoplasmic reticulum facilitates muscle relaxation (75). The inability for SMA cardiomyocytes to fully relax could

indicate irregularities in either calcium reabsorption, or in the contractile regulatory machinery (231, 285). In healthy cardiac tissue, expression of the myofibrillar protein, troponin, is low (3). Troponin expression is increased in severe SMA patients (117). Recently it has been shown that compounds that increase the sensitivity of troponin for calcium improves the SMA phenotype in mice and the implications of this is now under clinical consideration. While the rationale for these experiments was to reduce the threshold to muscle contraction, our studies would suggest that troponin sensitizing drugs may also help to restore contractile kinetics. One intriguing possibility is that troponin sensitizing drugs could reverse the contractile defects observed in SMA cardiomyocytes.

SMN alters SERCA expression

Our data shows cardiomyocytes from severe SMA mice have reduced calcium reuptake. The slow calcium reuptake prevents cardiac muscle relaxation, possibly explaining the reduction in diastolic length previously observed (75, 123). The slowed calcium reuptake was mirrored in cardiomyocytes derived from SMA iPSCs indicating that this phenomenon is not exclusive to mice. RNAseq transcriptome profiling showed a downregulation in *Atp2a2* (SERCA2), the principal gene regulating cardiac calcium dynamics (231). The SERCA pump oversees muscle contraction and relaxation as well as heartbeat frequency (123, 231). SMN protein is known to be involved in snRNP biogenesis, known machinery implicated in pre-mRNA splicing (193). Thus, altered SMN levels can cause splicing abnormalities (320). Previously, SERCA1a, an isoform found in skeletal muscle, was found to be reduced in SMA mice (34). There are more than ten isoforms of SERCA in humans (231). SERCA2a and SERCA2b isoforms are expressed in cardiac tissue, where SERCA2b is ubiquitously expressed at low levels in all

cells (231). In normal development, there is a greater expression of SERCA2a in cardiac tissue (231). Cardiomyocytes taken from postnatal day 10 SMA mice had a shift from SERCA2a to predominantly expressing the SERCA2b isoform. While we did not observe this shift in our differentiated human cardiomyocytes, it could be because the cells we differentiated were not fully mature. While our findings strongly correlate SERCA2 expression and the slow calcium reuptake in SMN-deficient cells, it is also possible that other calcium reuptake regulators account for the alterations in calcium kinetics. Ongoing experiments are underway to determine the intrinsic role of SMN in cardiomyocyte function. We show here that SMN deficiency impacts calcium homeostasis, potentially exacerbating the autonomic contribution to cardiac pathophysiology in SMA.

Microglia adopt a reactive profile in SMA

The second aim of this dissertation was to characterize the role of SMN in microglia (210). We confirm here a previous report that microglia appear morphologically reactive in a severe SMA mouse model (295). Immunohistochemical analysis of SMA mouse lumbar spinal cord sections showed enlarged soma size and retracted processes, indicative of a reactive state (232). Based on previous findings reported in the literature, we hypothesized that the microglia population would be elevated in spinal cords of SMA mice. However, flow cytometry analysis revealed no significant difference in the number of microglia between control and SMA spinal cord. These findings suggest that the increased presence of reactive microglia at the site of neuronal cell death in the spinal cord of SMA mice is most likely due to migration rather than proliferation. To confirm that the reactive morphology adopted by SMA mice

microglia is conserved in humans, we generated microglia from patient iPSCs. While there was no change in soma size, SMA microglia had retracted processes when compared to controls. One possible explanation for this discrepancy is that the reactive profile of SMA microglia in mice cannot be fully reconstituted *in vitro*, and requires extrinsic factors released by injured neurons or astrocytes to trigger a fully reactive profile. These findings suggest that both intrinsic and extrinsic factors regulate the reactive profile of SMN-deficient microglia.

Lymphocytes do not contribute to SMA pathology

Microglia are known to interact with infiltrating lymphocytes to contribute to neuroinflammation under neurodegenerative conditions (292). In SMA, the lymphocyte population is upregulated (64), setting up the intriguing possibility that microglia in SMA become reactive due to the presence of lymphocytes. While we observed no increase in lymphocyte infiltration in SMA mouse spinal cord, we could not rule out the possibility that lymphocyte involvement in SMA disease pathology. Our group, in collaboration with the Kothary lab, generated T helper (CD4) cells and lymphocyte (RAG1) knockout mice on a moderate SMA mouse background to determine the contribution of lymphocytes on motor function and survival of SMA mice. Ablation of T helper cells or all lymphocytes did not ameliorate the disease phenotype. These data indicate that lymphocytes are not critical modulators of the SMA pathology.

SMN regulates migration dynamics

For highly migratory and phagocytic microglia, changes in cellular morphology rely heavily on dynamic reorganization of actin (277). SMN is known to interact with cytoskeletal proteins (31, 67). Cytoskeletal dynamics, including actin network

stabilization, is modulated by the GTPase Ras homolog gene family member A/Rho-associated protein kinase (RhoA/ROCK) pathway (245, 258, 296). The RhoA/ROCK pathway increases when SMN is depleted (29, 30, 208). Systemic pharmacological inactivation of the ROCK pathway improved motor neuron survival, NMJ maturation and muscle fiber size (29). The ROCK pathway regulates migration, phagocytosis, and inflammatory cytokine release in microglial cells (245). Studies focusing on the cytoskeleton dynamics show that inhibiting ROCK activation blocks microglia morphological changes, reducing phagocytosis and microglia-neuron interactions (258). Type I SMA patient iPSC differentiated to microglia have significantly increased phagocytosis, consistent with studies in mice. SMN dependent alterations in the RhoA/ROCK pathway in microglia could account for these changes. Restoring SMN in iPSC microglia rescued the increase in velocity of SMA microglia to controls, supporting an intrinsic role of SMN in cell motility consistent with effects on cytoskeletal dynamics.

SMN regulates microglia phagocytosis

Here we report a dramatic upregulation of TREM2 and the complement system in human SMA microglia. While TREM2 has not been studied in the context of SMA, severe SMA mice had increased expression of complement associated proteins (308). In mice studies, elevated C1q expression is correlated with increased phagocytosis (308). Antibodies against C1q complement protein improved severe motor function deficits in SMA model mice (308). Here we show C1q expression is also upregulated in our iPSC SMA microglia. Surprisingly, there was no change in C3 complement protein expression in the iPSC-derived microglia as reported in the mice. We presume that this contradictory finding is likely due to the lack of astrocytes, a major producer of C3, in

our microglia cultures. Current studies are underway in our lab and others to determine the relative contributions and crosstalk between microglia and astrocyte in SMA (153).

TREM2 is a regulator of phagocytosis in microglia. Here for the first time, we show an upregulation of TREM2 expression in SMA microglia. Importantly, using an siRNA to TREM2, we show that reducing TREM2 expression reverses the increased phagocytic activity of SMN-deficient microglia. TREM2 has not only been implicated in microglial phagocytic activity but in cell migration (171). Here we show patient-derived SMN-deficient microglia display increased velocity in both a cell migration and scratch wound assay. While we did not investigate the role of TREM2 on migration, the results from the phagocytosis assays suggest that TREM2 may regulate converging pathways. TREM2 has recently been implicated in other neurodegenerative diseases, including Alzheimer's disease, where dysregulation of microglial activity is emerging as a pathological mechanism. Here we show that TREM2 expression is upregulated when SMN is deficient, potentially representing a molecular link between cytoskeleton dynamics, phagocytosis and cell migration.

Conditioned media from SMA microglia alters motor neuron morphology and electrophysiological function

Our data uncovers a potential neuroprotective role of microglia in SMA pathology. Control and SMN deficient iPSC motor neurons were treated with control and SMA microglia conditioned media for 24 hours. SMN deficient motor neurons treated with SMA microglia media displayed increase in number of action potentials, and amplitude of inward sodium current and potassium rectifying current. Moreover, Sholl analysis of motor neurons showed significant increase in dendritic arborization compared

to controls. Microglia release growth factors such as BDNF, to promote neuronal survival, and sustainment of newly formed synapses (84). Our data suggest that SMA microglia potentially release neurotrophic factors that promote motor neuron survival and recovery. The protective effect of SMN-deficient microglia could be enhanced by the increased phagocytic clearance of toxic cell debris. Nevertheless, we cannot rule out the possibility that the enhanced phagocytic activity of SMN-deficient microglia may drive unregulated synaptic pruning, which could lead to premature neuronal cell death. Further studies clarifying the relative contribution of neurotrophic factor release and phagocytic activity in SMA pathology is undoubtedly warranted.

IMPLICATIONS

Disease Model

Prior to this study, no research has been done in cardiomyocytes or microglia differentiated from unaffected and SMA patient derived iPSCs. Isolating cells from animal models causes cells to lose their intrinsic nature (199). Here we have differentiated and validated human cardiomyocytes and microglia cells that retain patient genetic background. Overall, we have generated human cells to research SMN impacted mechanisms.

Therapeutic application

Currently, there are only three FDA approved drugs for SMA (80, 101, 184, 235, 283). The cost of these therapeutics is high and the long-term effects of gene therapies is still unknown. While they have improved the SMA condition, these treatment delay symptom onset and are not a cure. A major obstacle for drug development is translation of research findings in animal models to human pathology.

Here we have generated human derived cardiomyocytes and microglia that allow for high throughput screening of potential SMA therapeutics.

FUTURE DIRECTION

Based on the findings presented in this dissertation, we propose the following experiments for future studies:

- 1.) To genetically or pharmacologically elevate SERCA2 expression to assess improvements of calcium dynamics, cardiomyopathies and survival in SMA animal models.
- 2.) To generate mature human cardiomyocytes to determine if reduced SMN dependent SERCA2 splicing defects are conserved.
- 3.) To genetically restore SMN levels in human-derived microglia to determine and assess microglia reactive profile.
- 4.) To perform proteomic analysis to determine what factors are released by microglia that promote motor neuron outgrowth and influence sodium and potassium currents.
- 5.) To identify pathways that can be targeted to enhance the neuroprotective properties of microglia in SMA.

SUMMARY

This body of work identified the importance of SMN expression in the functional development of cardiomyocyte and microglia. By using human iPSC derived models, we provide a human cell platform to identify and characterize SMN-dependent pathways. In cardiomyocytes, SMN dependent SERCA2 deficiency altered calcium signaling, contributing to improper cardiomyocyte contraction. In microglia, reduced SMN

expression resulted in a reactive morphology, unique transcriptome profile, as well as increased phagocytosis and migration. Together, this study has provided novel insight into the role of SMN in cells and tissues previously understudied in SMA. Combination therapeutics targeting these underlying mechanisms may improve SMA patient recovery, potentially moving us closer to a cure.

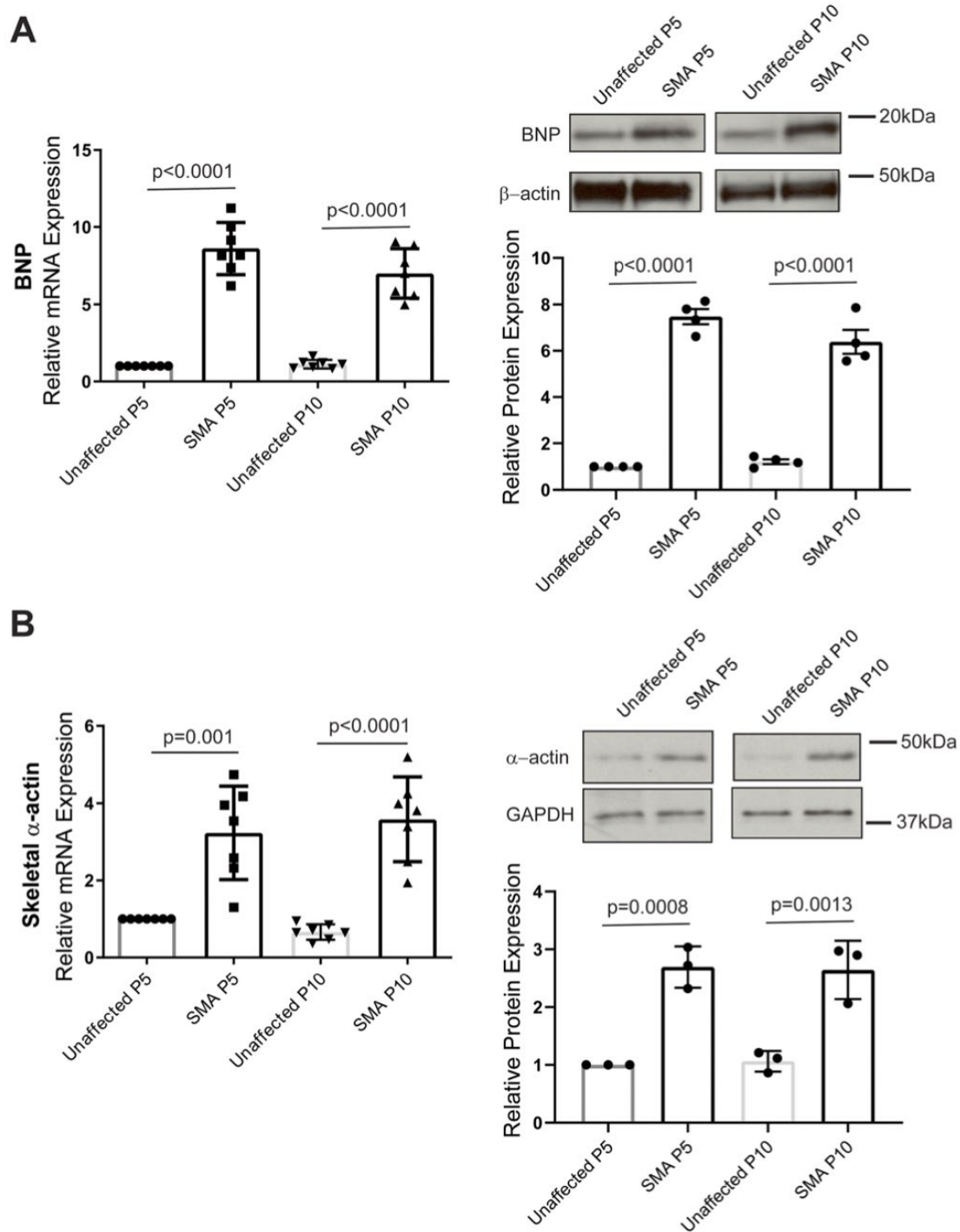


Figure 3. Elevated expression of heart failure markers in SMA model mice. qRT-PCR analysis of a BNP and b ACTA1 mRNA expression. Expression levels (mean $\pm$ SEM) in SMN-deficient (SMA) heart tissue are represented relative to unaffected controls at each time point. Statistical analysis: p values shown for one-way ANOVA, Tukey's post hoc test, $n = 7$.

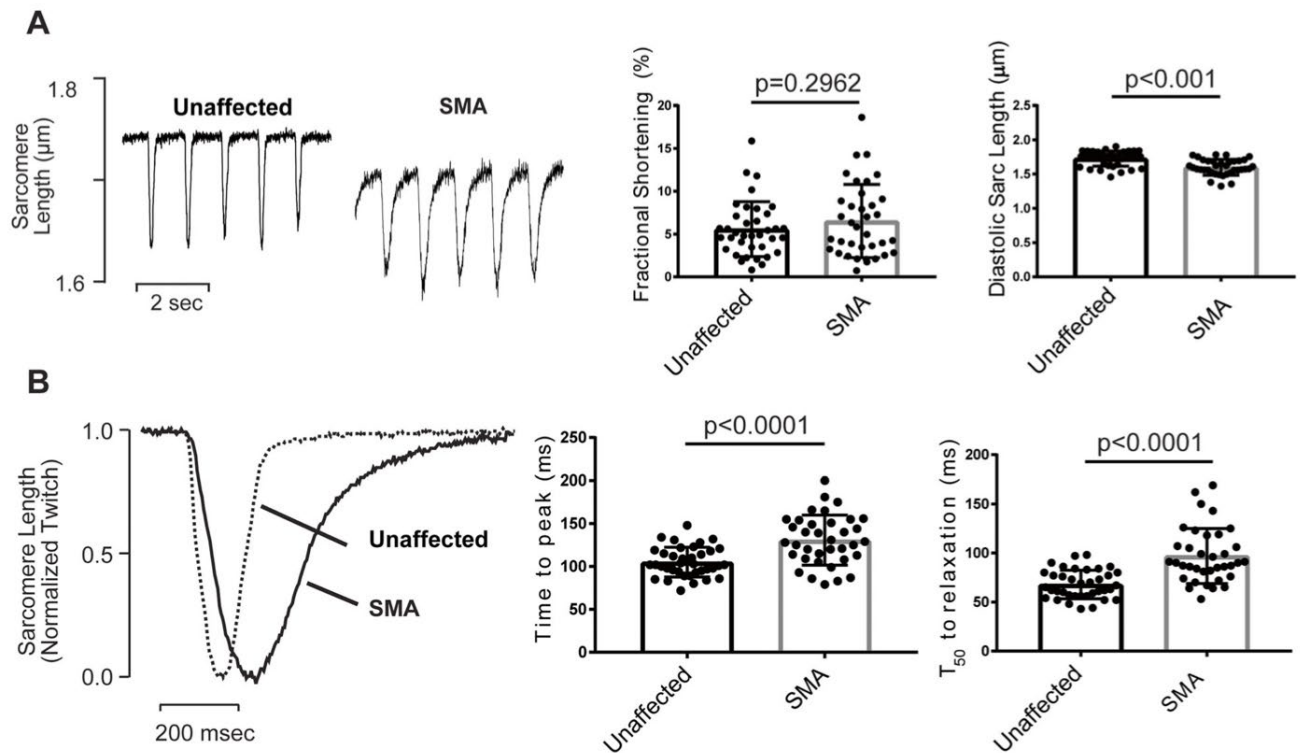


Figure 4. Contraction kinetics are slowed in SMA cardiomyocytes. **a.** Representative recordings of sarcomere length obtained from ventricular myocytes isolated from unaffected or SMA mice (aged P12–13). Cells were field stimulated to contract at 1 Hz. Fractional shortening was not markedly different; however, there was a significant decrease in the diastolic sarcomere length. **b.** Shown are normalized average record of a typical single twitch illustrating the slowed contraction and relaxation kinetics in SMA compared to control cardiomyocytes. Both the time to peak shortening and time to 50% relaxation (T_{50}) were significantly increased in SMA myocytes compared to control. Statistical analysis: p values shown for t test, $n = 36$ and 37 for SMA and control, respectively.

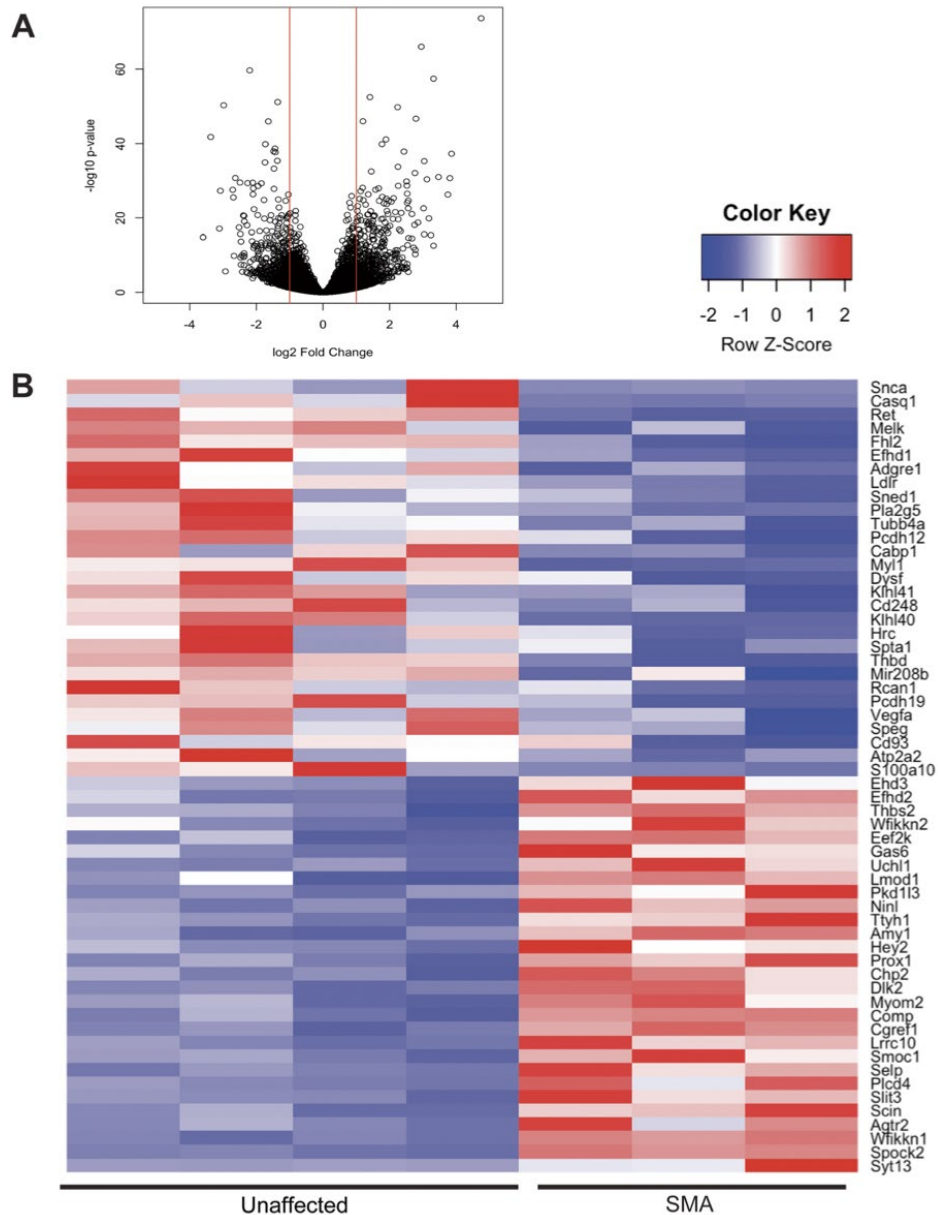


Figure 5. Transcriptome analysis suggests Ca^{2+} handling is altered in SMA heart. **a.** Shown is a “volcano plot” of statistical significance versus transcript fold change in SMA compared to unaffected control hearts. A total of 1178 transcripts were up- or down-regulated in SMA heart. **b.** Heat map illustrating a subset of genes that were significantly changed. Red denotes high expression, blue denotes low expression. Of note, the principal gene regulating Ca^{2+} uptake into the sarcoplasmic reticulum, Atp2a2 (SERCA2), was downregulated.

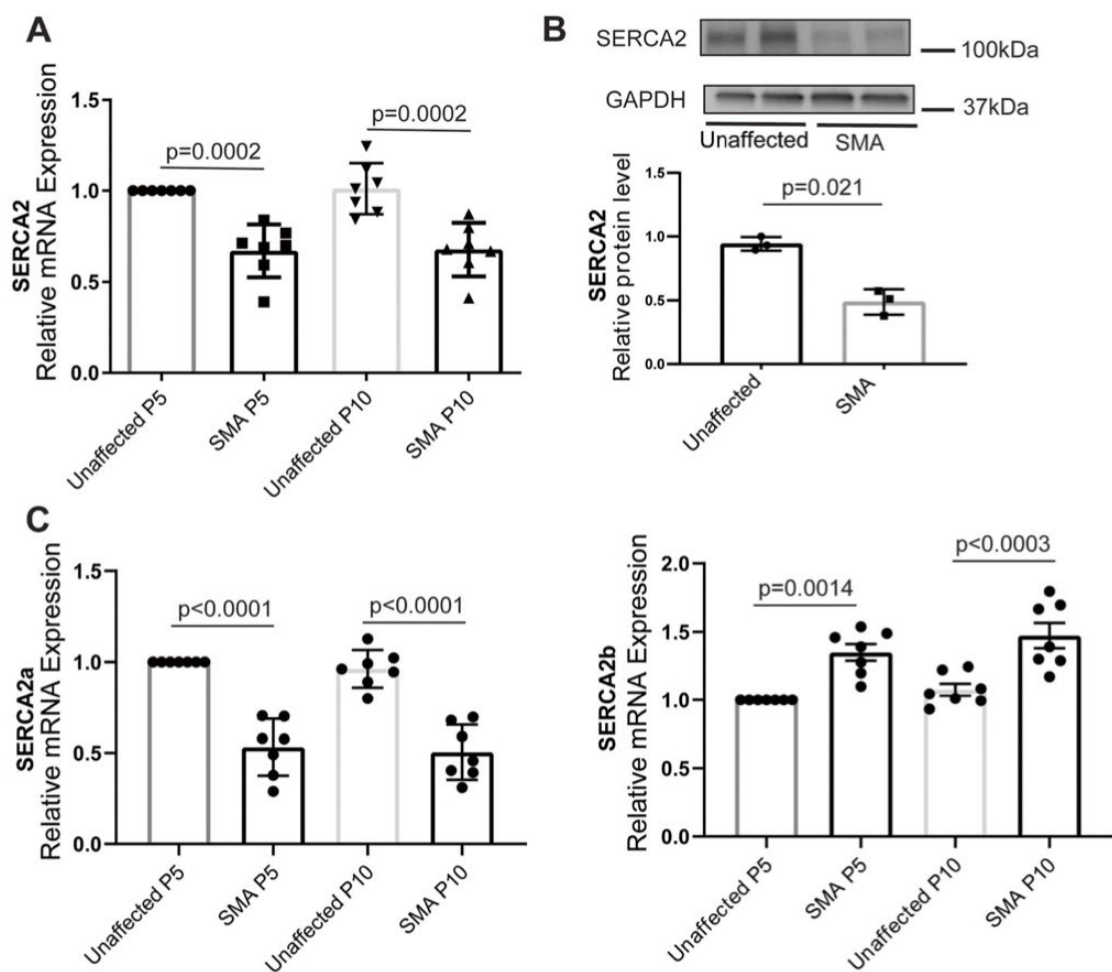


Figure 6. SERCA2a expression is reduced in SMA hearts. **a.** qRT-PCR analysis of SERCA2 mRNA. Expression levels in SMN-deficient cardiomyocytes are represented relative to unaffected controls (n = 3). **b.** Western blot analysis of SERCA2a protein levels. Quantification of SERCA protein level in SMA cells relative to controls at right. **c.** qRT-PCR analysis of SERCA2a and SERCA2b mRNA. Expression levels in SMN-deficient (SMA) cardiomyocytes are represented relative to unaffected controls. Statistical analysis: p values shown for one-way ANOVA, Tukey's post hoc test, n=7.

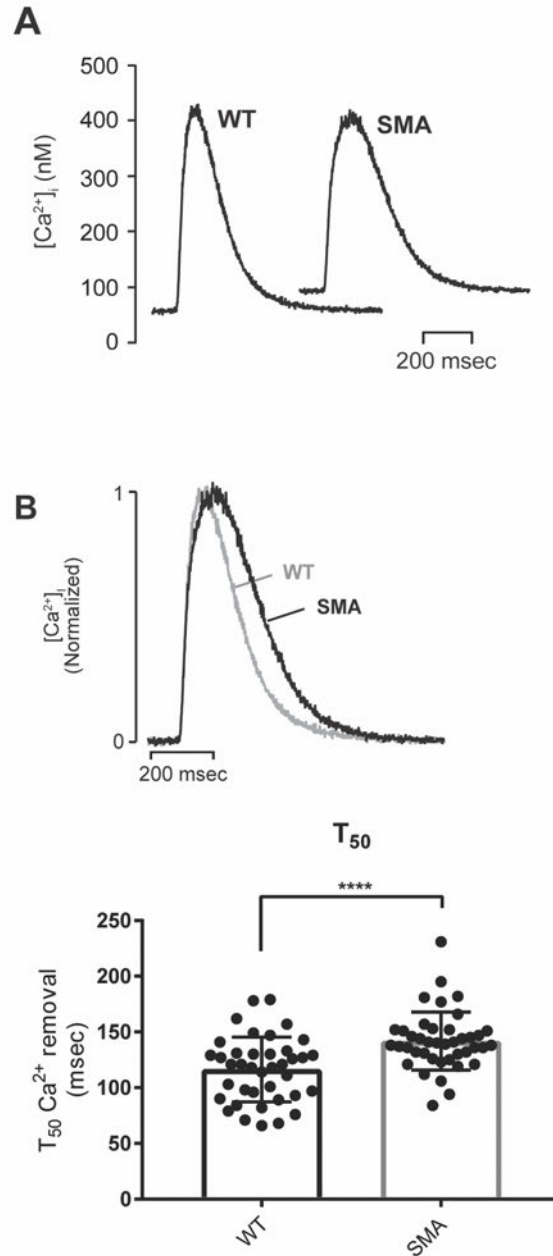


Figure 7. $[Ca^{2+}]_i$ transient kinetics are slowed in SMA cardiomyocytes. **a.** Representative $[Ca^{2+}]_i$ transient measured in ventricular myocytes isolated from SMA or unaffected hearts (aged P12–13). There was no significant difference in the estimated diastolic or peak systolic $[Ca^{2+}]_i$ ($n = 21$ for both SMA and control). **b.** Typical normalized $[Ca^{2+}]_i$ transients from SMA and control cells are shown illustrating that the time to 50% removal of cytoplasmic Ca^{2+} (T_{50}). **c.** was significantly increased in SMA myocytes compared to control in agreement with the slowed relaxation kinetics. Statistical analysis: p values shown for t test, $n = 41$ and 39 for SMA and control, respectively.

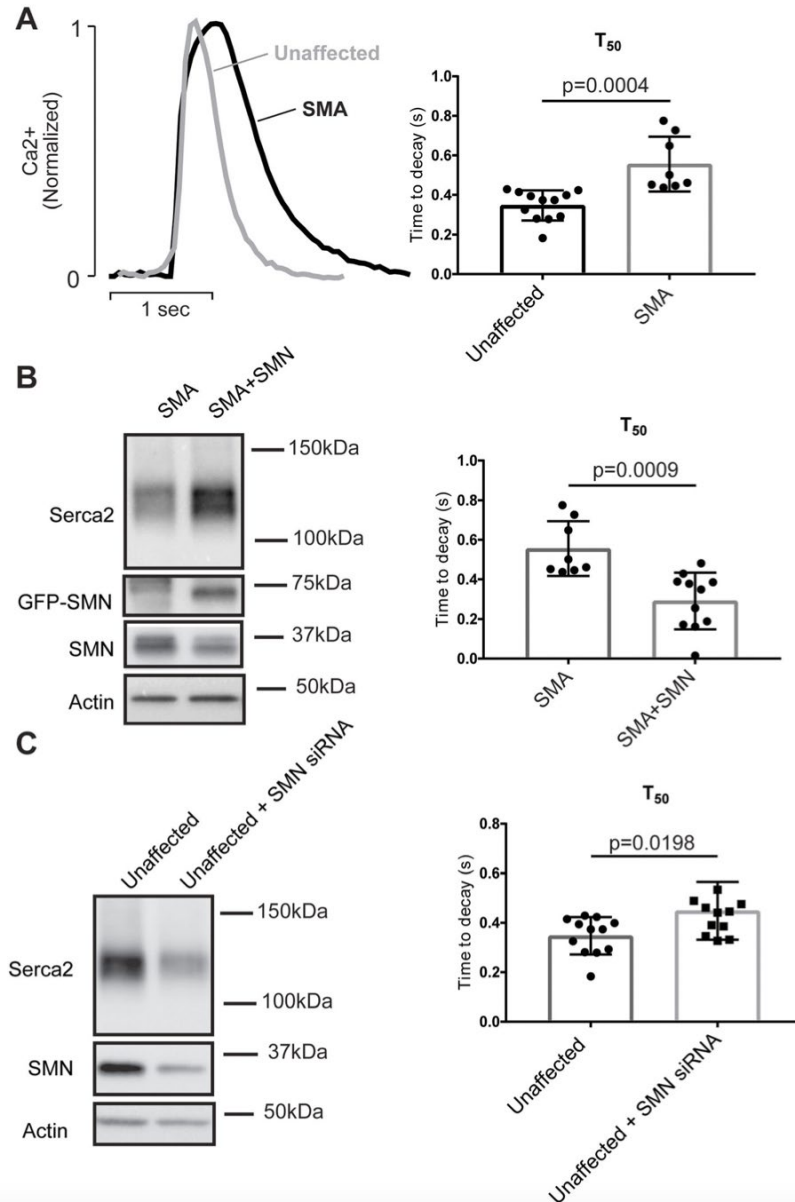


Figure 8. Defects in $[Ca^{2+}]_i$ handling are recapitulated in cardiomyocytes derived from SMA patient iPSCs. **a.** Representative $[Ca^{2+}]_i$ transients measured in iPSC-derived cardiomyocytes engineered from unaffected control (NCRM1) and SMA patient (GM23240) iPSCs illustrating the slowed removal of cytoplasmic Ca^{2+} . **b.** Expression of SERCA2a expression is increased (western blot, left) and T_{50} is decreased (right) in SMA patient iPSC- derived cardiomyocytes when SMN protein is restored. Cells were transfected on day 18 of differentiation and recordings were made on day 20. **c.** Similarly, expression of SERCA2a expression is reduced (western blot, left) and T_{50} is increased (right) in unaffected control iPSC-derived cardiomyocytes when SMN protein is reduced. Cells were transfected on day 18 of differentiation and recordings were made on day 20. Statistical analysis: p values shown for one-way ANOVA, Tukey's post hoc test, n = 7.

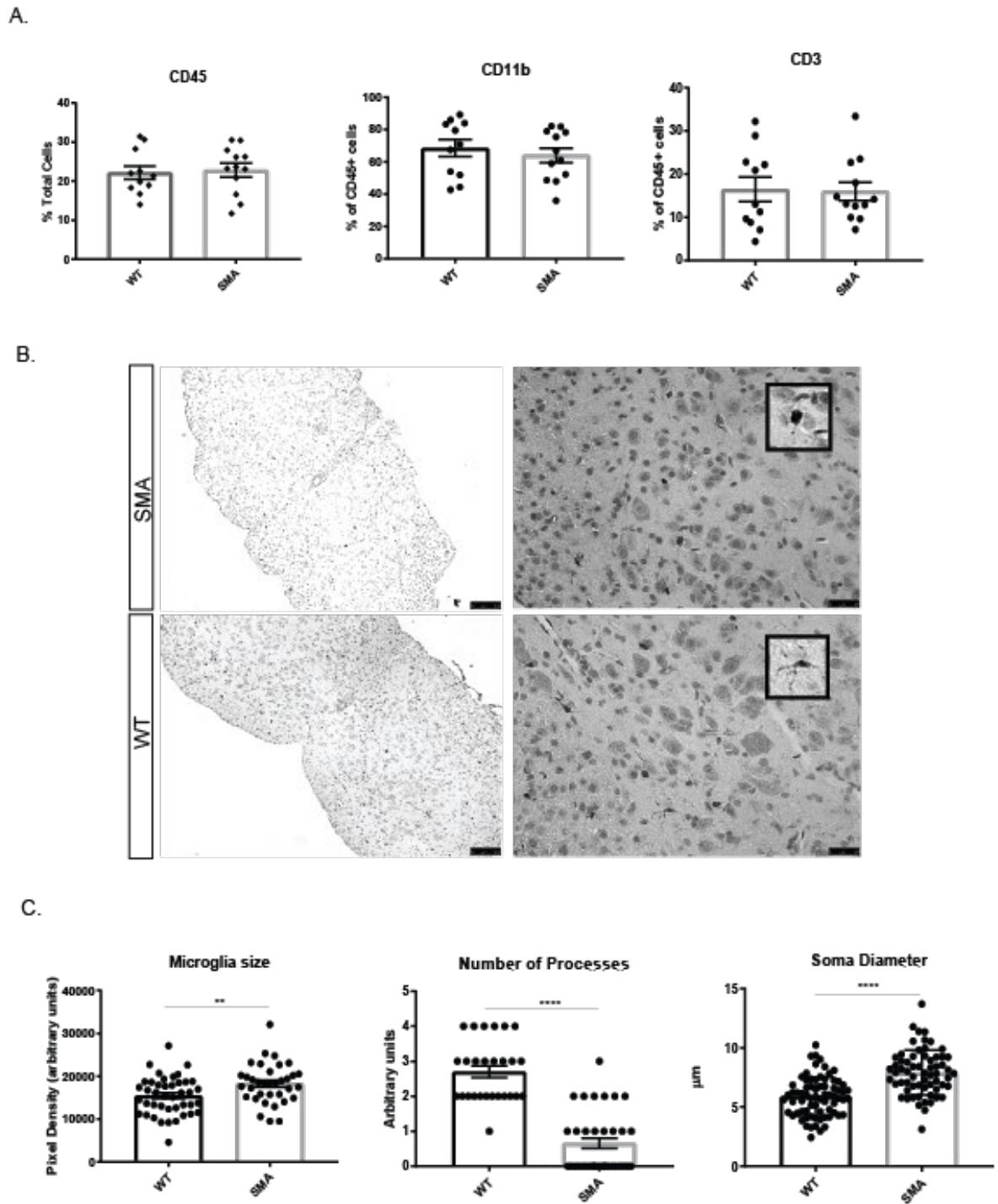


Figure 9. Microglia adopt a reactive morphology in SMA mouse spinal cords. **a.** Monocytes were isolated from entire P10 WT and SMA mouse spinal cords (WT N=11, SMA N=12). Flow cytometry analysis was used to quantify total

monocytes (CD45+), microglia (CD11b+), and T-cells (CD3+) populations. Bars represent mean $\pm$ SEM. **b.** Spinal cord sections from L1-L5 of P10 SMA and WT mice were immuno-stained for Iba-1. DAB staining was used to visualize antibody. Scale bars = 100 μ m (left panel) and 25 μ m (right panel). **c.** Iba1 pixel density, soma diameter, and number of branching were analyzed using Image J. Values represent mean $\pm$ SEM. **p=0.0081, **** p=<0.0001, t-test, (n = 42 and 35 for wild type and SMA, respectively).

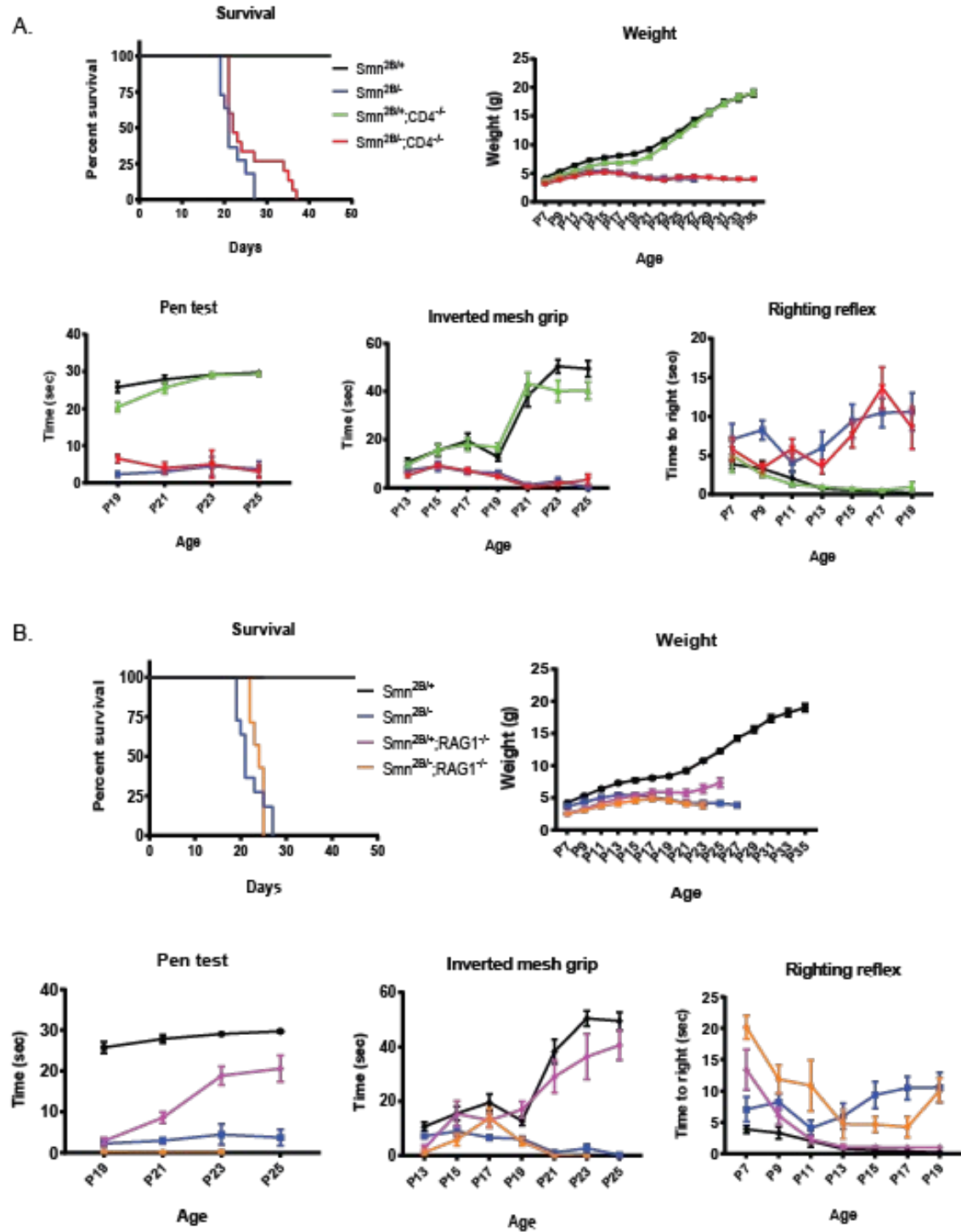


Figure 10. Depletion of mature lymphocytes does not improve survival or motor function in SMA mice. **a.** Survival and motor function was assessed in *Smn*^{2B/+} (black), *Smn*^{2B/-} (blue), *Smn*^{2B/+};*CD4*^{-/-} (green), *Smn*^{2B/-};*CD4*^{-/-} (red) mice (n≥11/group).

b. Survival and motor function was assessed in $Smn^{2B/+}$ (black), $Smn^{2B/-}$ (blue), $Smn^{2B/+};Rag1^{-/-}$ (pink), $Smn^{2B/-};Rag1^{-/-}$ (yellow) mice ($n \geq 7$ /group). Performance in inverted mesh grip (starting at P13), pen test (starting at P19), and righting reflex (starting at p7) was recorded. Survival and motor function was assessed every other day. Significance of behavior data was determined by one way ANOVA. *Mantel-Cox* and *Gehan-Breslow-Wilcoxon* analyses were performed to determine significance between groups for all significant main effects and interactions.

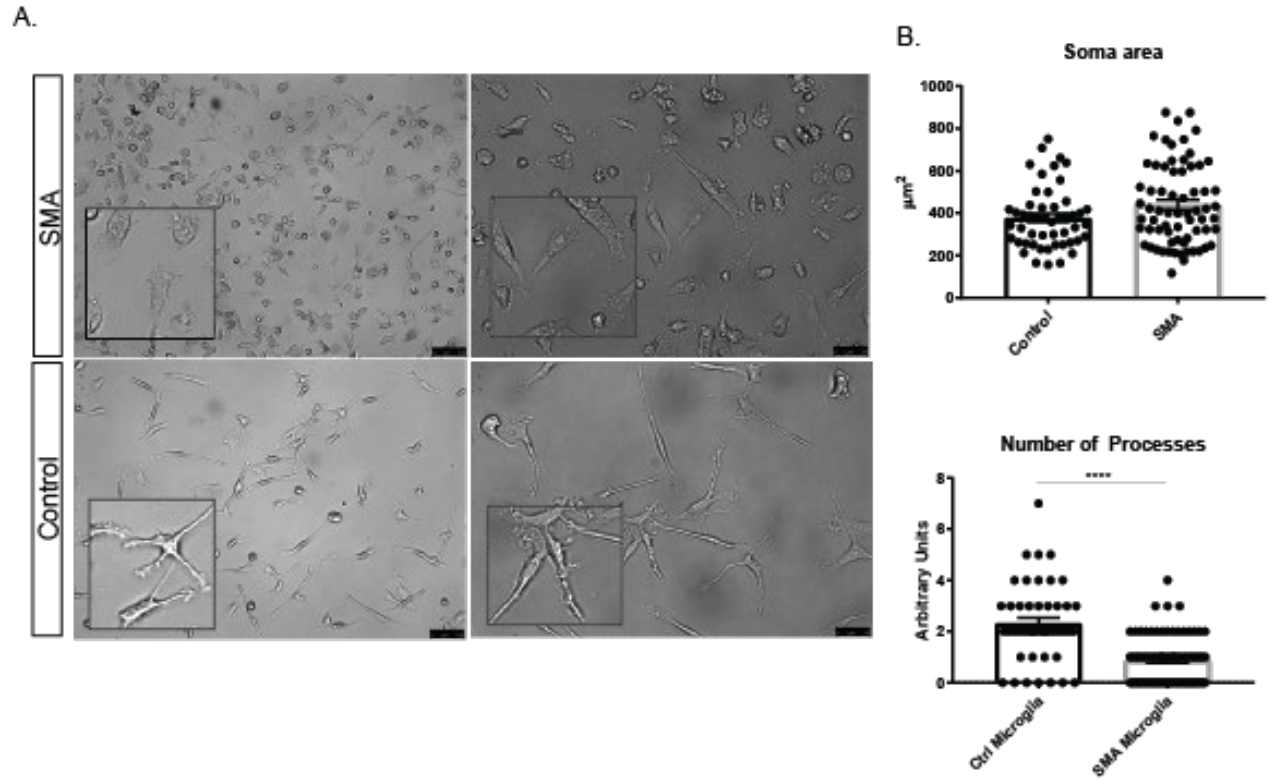


Figure 11. Processes are retracted in iPSC-derived SMA microglia. **a.** Representative bright field images show control and SMA microglia at 10 days post differentiation. Scale bars= 50 μ m **b.** Number of processes (control n=47, SMA n=80) and soma area (control n=51, SMA n=69) were analyzed using ImageJ. All groups were analyzed by unpaired t-test. ****p<.0001. Values represent mean $\pm$ SEM.

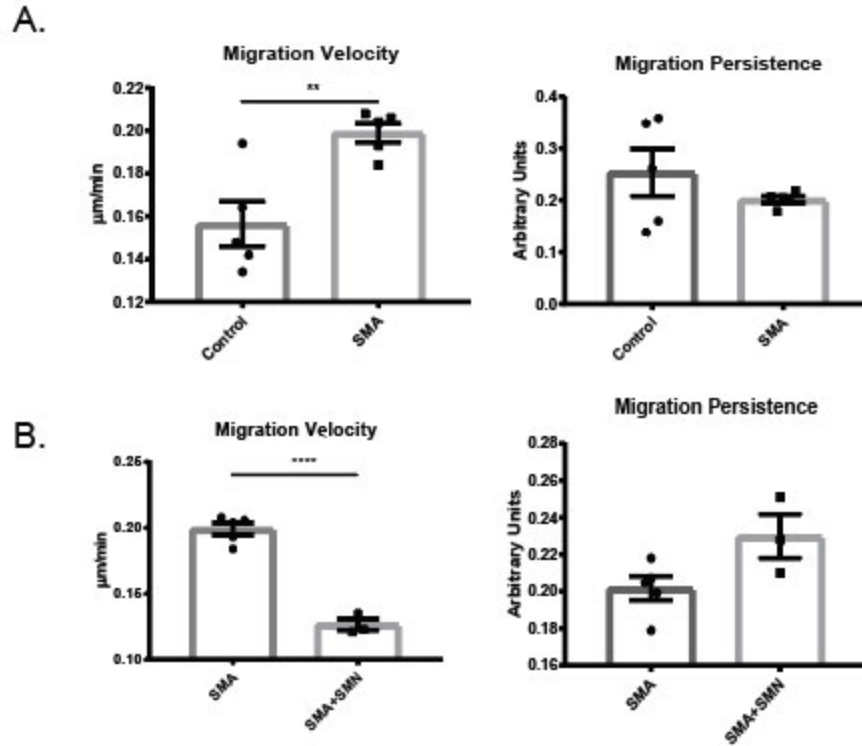


Figure 13. Cell velocity is increased in SMA microglia compared to controls. **a.** Video analysis of control and SMA microglia was performed over 16 hours, with images taken every 10 minutes. No change in persistence was detected. ** $p=0.0061$. **b.** SMN levels were restored in SMA microglia and velocity and persistence was analyzed. SMN rescued SMA microglia had reduced velocity to control levels. **** $p<0.0001$. All groups were analyzed by unpaired t-test. Values represent mean $\pm$ SEM.

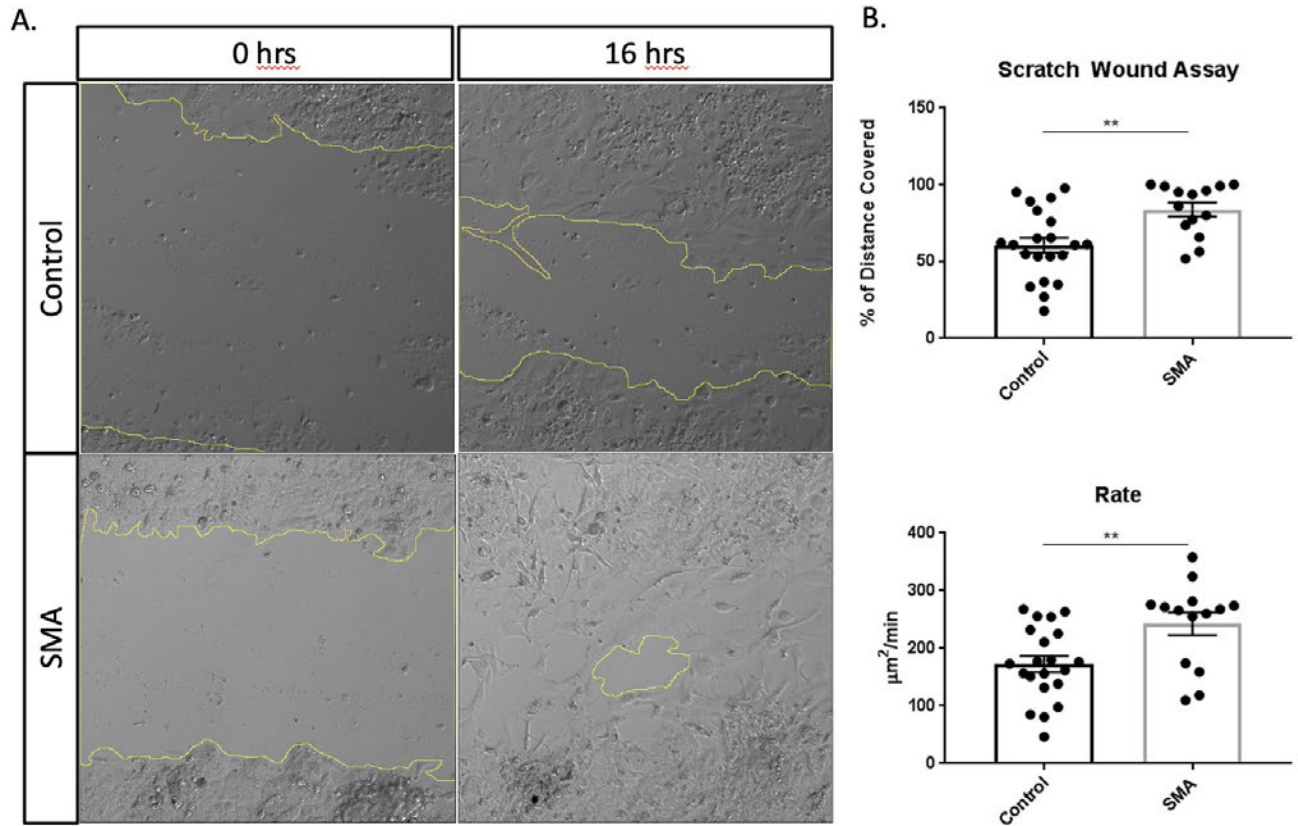


Figure 14. Migration is increased in SMA microglia compared to controls **a.** Control (n=21) and SMA Microglia (n=14) were plated in a monolayer. A single wound was administered and the chamber was imaged every 10 min for 16 hours. **b.** Area occupied and rate was analyzed and quantified using ImageJ. (% Distance covered **p=0.0024; Rate of **p=0.0051). All groups were analyzed by unpaired t-test. Values represent mean ±SEM.

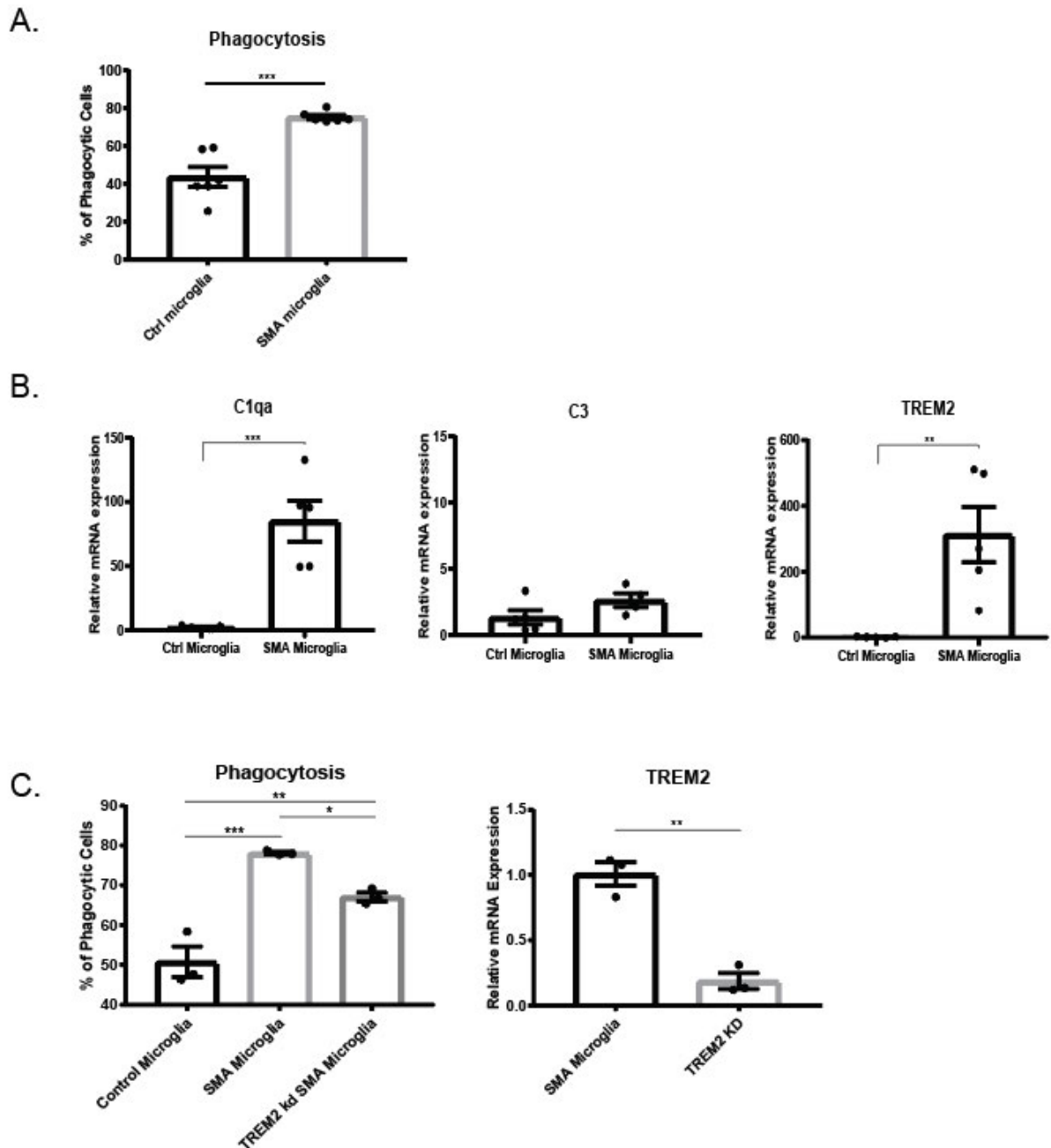


Figure 15. Phagocytosis is upregulated in SMA microglia. **a.** Phagocytosis was analyzed by flow cytometry. (n=6) *** p=0.002 **b.** Phagocytosis genes C1q, C3, and TREM2 were analyzed. (ctrl n=5, SMA n=5) ** p=0.0058, *** p=0.0008 **c.** TREM2 was knocked down using siRNA. Phagocytosis was analyzed by flow cytometry. *p=0.0443, ** p=0.0073, *** p=0.0005. All groups were analyzed by unpaired t-test. Values represent mean $\pm$ SEM.

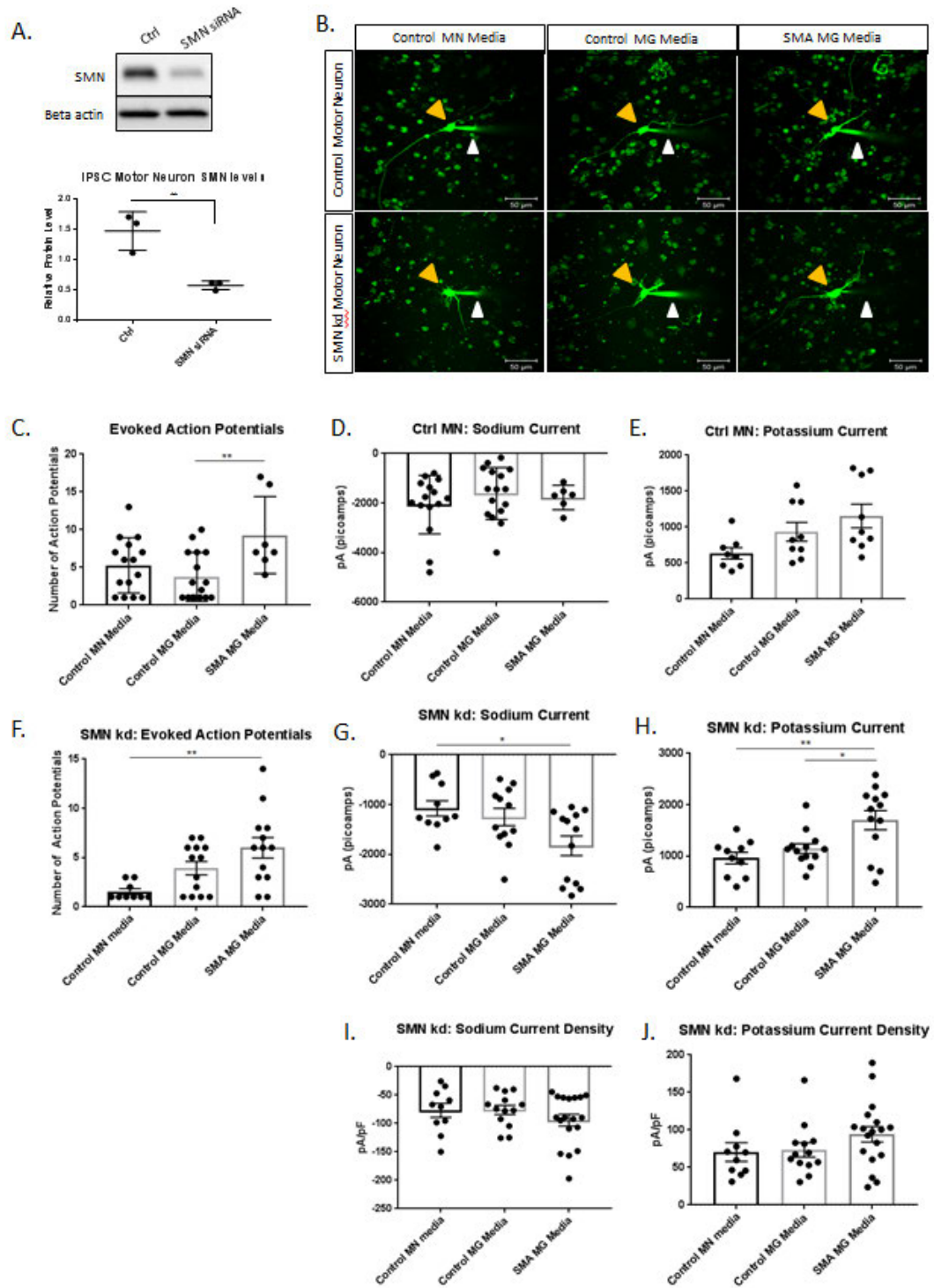


Figure 16. SMA microglia alter motor neuron excitability. **a.** SMN knockdown in iPSC motor neurons was validated by western blot (n=3). Analyzed by t-test, $**p=0.0087$. **b.** Control and SMN knock down motor neurons were exposed for 24 hrs to conditioned media. Representative images of each group were taken. Yellow arrow indicates the motor neuron that was patched. White arrow denotes

glass capillary used to inject visualization dye. Scale bar = 50 μ m. **c.** Number of evoked action potentials was recorded in control motor neurons (ctrl motor neuron media n=15, ctrl microglia media n=16, and SMA microglia media n=8). ** p=0.0053. **d.** Inward sodium current was recorded in control motor neurons exposed 24 hours to conditioned media (ctrl motor neuron media n=15, ctrl microglia media n=16, and SMA microglia media n=6). **e.** Potassium current was recorded in control motor neurons exposed for 24 hours to conditioned media (ctrl motor neuron media n=8, ctrl microglia media n=9, and SMA microglia media n=9). **f.** Number of action potentials were recorded in SMN knock down motor neurons (ctrl motor neuron media n=10, ctrl microglia media n=13, and SMA microglia media n=13). ** p=0.0026. **g.** Inward sodium current was recorded in SMN knock down motor neurons exposed for 24 hours to conditioned media (ctrl motor neuron media n=10, ctrl microglia media n=12, and SMA microglia media n=13). * p=0.0205. **h.** Potassium current was recorded in SMN knockdown motor neurons exposed 24 hours to conditioned media (ctrl motor neuron media n=10, ctrl microglia media n=13, and SMA microglia media n=13). * p=0.0213 ** p=0.0034. **i.** Current density for inward sodium current was calculated for SMN deficient motor neurons. **j.** Current density for potassium current was calculated for SMN deficient motor neurons. All groups analyzed by one-way ANOVA. Values represent mean $\pm$ SEM.

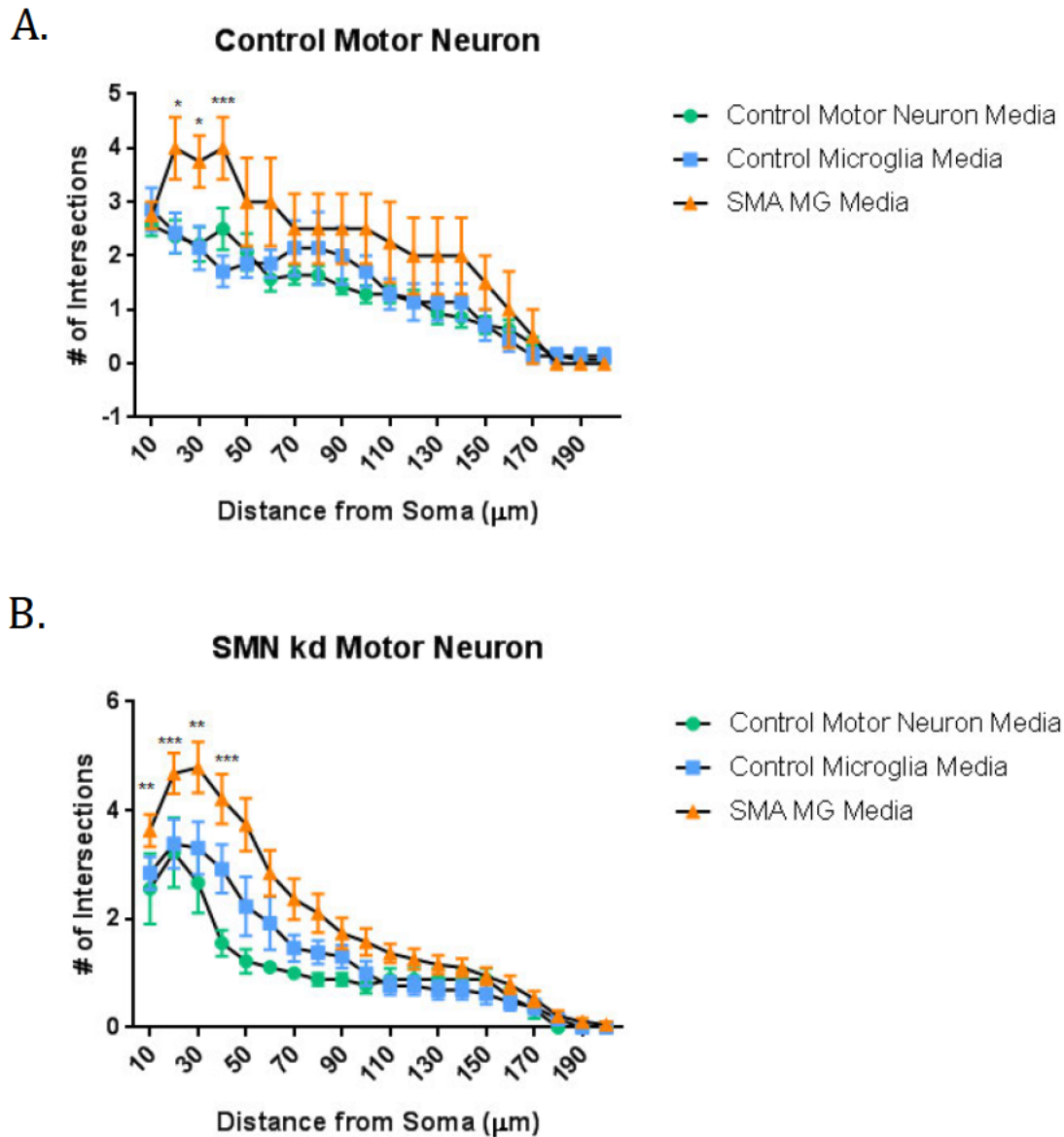


Figure 17. Motor neurons treated with SMA microglia conditioned media result in increased branching. Plot of Sholl analysis of motor neurons treated with microglia media. Total number of crossings was counted at start radius of $10\mu\text{m}$, with a step radius $10\mu\text{m}$. Branching was recorded up to $200\mu\text{m}$ for all groups. Control motor neurons (a.) and SMN deficient motor neurons (b.) were treated for 24 hours in control motor neuron media (green), control microglia media (blue), and SMA microglia media (orange). Two-way ANOVA was used followed by post hoc Bonferroni for Multiple Comparisons to analyze the Sholl curves between groups. Significance was determined when comparing control microglia media to SMA microglia media. a. $*p=0.0131$, $*p=0.0108$, $***p<0.001$ (left to right). b. $**p=0.0037$, $***p=0.0007$, $**p=0.0041$, $***p=0.0006$, left to right. Values represent mean $\pm$ SEM.

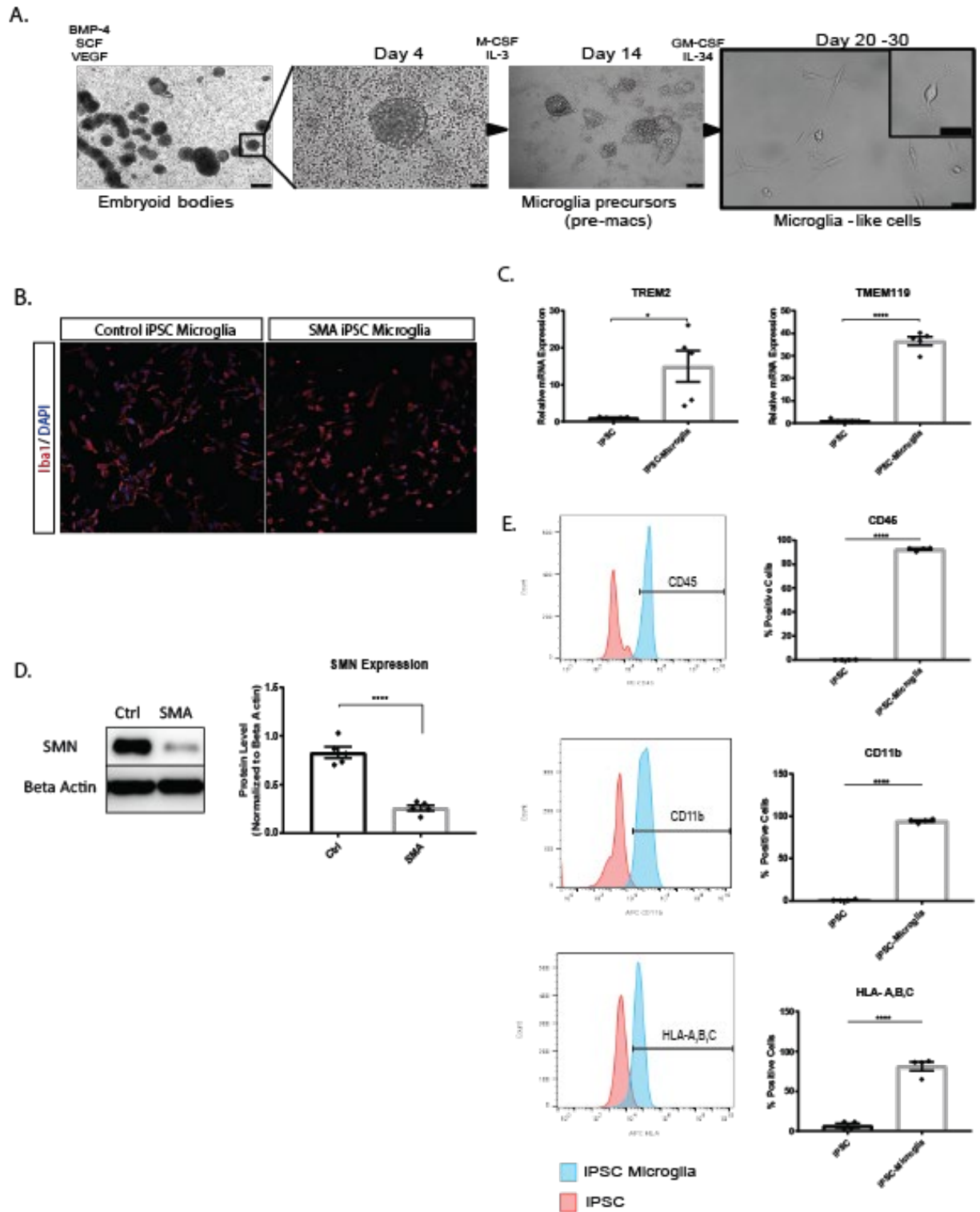


Figure 18. iPSC differentiated microglia were validated for CNS microglia specific markers **a.** Representative images shown of microglia differentiation steps adapted from Cowley et al. protocol. **b.** iPSC-microglia were immune-stained for Iba1, a marker for microglia. **c.** Gene expression for TREM2 and TMEM119

was analyzed in iPSCs and microglia 10 days after differentiation. (n=5/group) *p=0.0112 , ****p<0.0001. **d.** SMN protein levels in control and SMA patient iPSC lines were validated (n=5/group). ****p<0.0001. **e.** HLA-A,B,C, CD45, and CD11b surface marker expression was validated by flow cytometry (n=4/group). Representative histogram is shown per marker. ****p<0.0001. All groups were analyzed by unpaired t test. Bars show mean $\pm$ SEM.

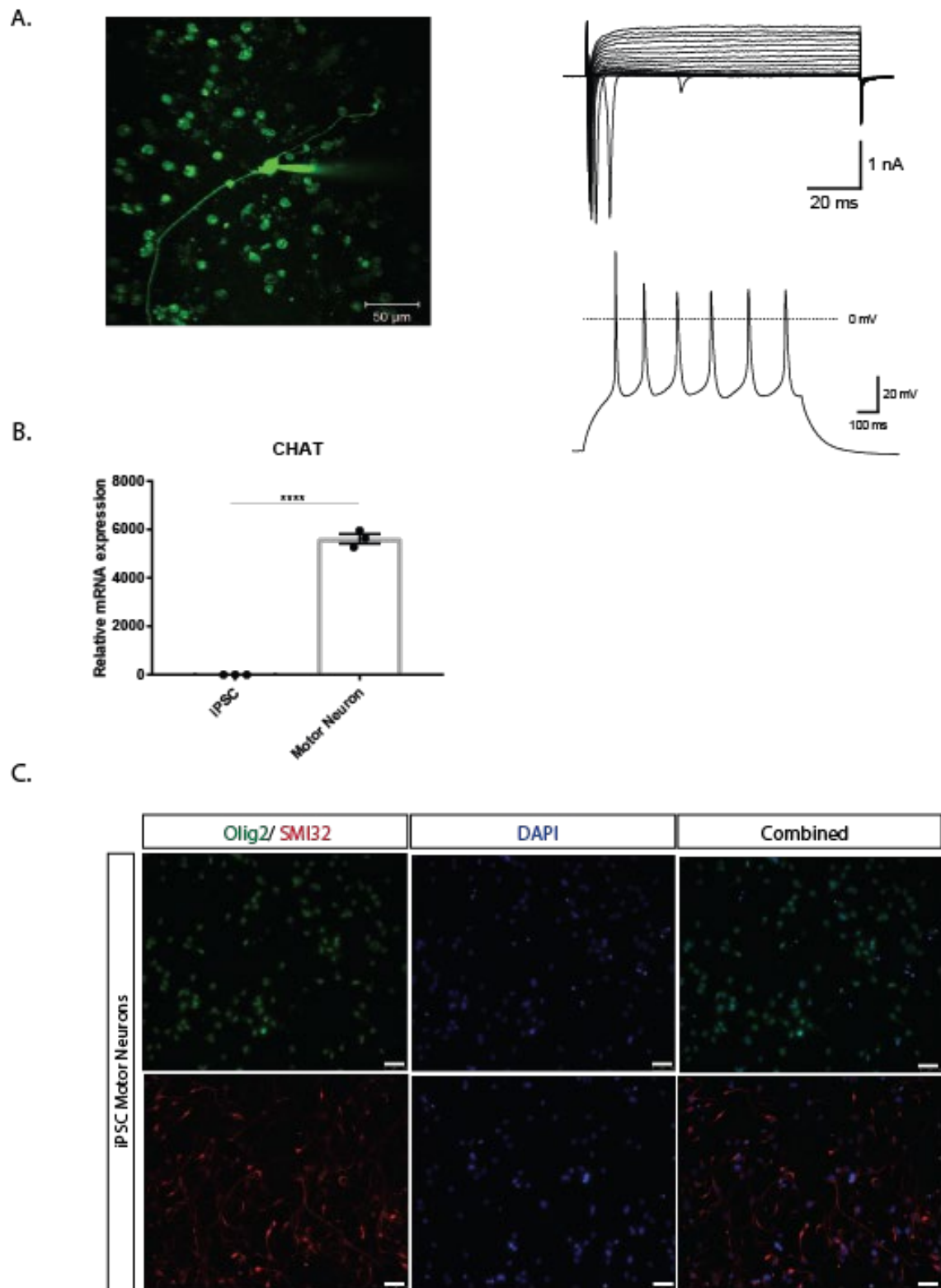


Figure 19. iPSC-derived motor neuron was validated at 10 days after differentiation. **a.** Representative image indicates a motor neuron that was patched. Scale

bar = 50 μ m. Recordings demonstrate inward sodium current and action potential propagation. Scale bar shows 1nA by 20ms and 20mV by 100ms, respectfully. Action potential threshold was set at 0mV. **b.** CHAT gene expression was validated. (n=3/group) ****p=<0.0001. Values represent mean $\pm$ SEM. **c.** Staining of motor neurons shows positive expression of Olig2 (green) and SMI32 (red). DAPI was used to visualize cell nuclei. Scale bar = 50 μ m.

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