DECIPHERING THE CONTRIBUTION OF MICROGLIA TO NEURODEGENERATION IN FRIEDREICH’S ATAXIA

A Thesis
presented to
the Faculty of California Polytechnic State University,
San Luis Obispo

In Partial Fulfillment
of the Requirements for the Degree
Master of Science in Agriculture with a Specialization in Animal Science
and a Focus in Regenerative Medicine

by
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June 2024
COMMITTEE MEMBERSHIP

TITLE: Deciphering The Contribution of Microglia to Neurodegeneration in Friedreich’s Ataxia

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Friedreich’s ataxia (FRDA) is the most prevalent inherited ataxia, affecting one in every 50,000 individuals in the United States. This hereditary condition is caused by an abnormal GAA trinucleotide repeat expansion within the first intron of the frataxin gene resulting in decreased levels of the frataxin protein (FXN). Insufficient cellular frataxin levels results in iron accumulation, increased reactive oxygen species production and mitochondrial dysfunction. Tissues most heavily impacted are those most dependent on oxidative phosphorylation as an energy source and include the nervous system and muscle tissue. This is evident in the clinical phenotype which includes muscle weakness, ataxia, neurodegeneration and cardiomyopathy. However, there has been a lack of data regarding the cell type specific contributions in FRDA pathogenesis. We generated a cohort of induced pluripotent stem cells (iPSCs) consisting of FRDA patient lines, CRISPR-Cas9 edited controls, carriers and non-related controls. Our preliminary data identified a hyperinflammatory microglial phenotype with extensive defects in mitochondrial function; since microglia are the primary innate immune cell of the brain, we hypothesized microglia may decrease neuronal viability which contributes to FRDA pathology. To investigate this, the iPSC cohort was utilized to generate microglia (iMGs) and neurons to better understand microglia-mediated neurodegeneration and how this contributes to pathology. An in vitro co-culture model composed of neurons, astrocytes and microglia was employed to better understand microglia-neuronal communication in FRDA. Healthy neurons co-cultured with FRDA iMG or with FRDA iMG-conditioned media demonstrated higher incidences of caspase-3 mediated apoptosis. These findings were recapitulated in vivo as xenotransplantation of FRDA microglia progenitors into a murine model resulted in reduced Purkinje cell survival in the cerebellum. Previous research has demonstrated the therapeutic potential of wildtype microglia to rescue the FRDA phenotype in the Y8GR mouse model of FRDA. To further explore the potential mechanisms behind this rescue, the delivery of mitochondria and FXN to FRDA microglia and neurons was investigated. CRISPR-Cas9 edited microglia demonstrated transfer of healthy mitochondria to FRDA microglia and neurons in an in vitro co-culture model. To investigate the transfer of frataxin protein, an FRDA iPSC line was transduced with an FXN-GFP lentivirus. Restoring FXN expression was demonstrated to rescue the FRDA microglial morphological phenotype. FXN-GFP microglia demonstrated transfer of frataxin protein to FRDA microglia suggesting the potential role of microglia as a therapeutic vehicle in FRDA. Together these findings show that FRDA microglia have a deleterious effect on neuronal viability, while healthy microglia may work as a therapeutic vehicle through the delivery of mitochondria and frataxin to FRDA cells.

Keywords: Friedreich’s Ataxia, microglia, neurodegeneration
I would like to thank my amazing and supportive mentor, Dr. Nicole Coufal, for helping me to grow every day and always providing an optimistic point of view. I also want to thank my mentor, Carla Pernaci, for always being there for me and for helping me develop so many new skills. Also, everyone in the Coufal Lab for being so supportive and providing an amazing lab community to grow in. I also want to thank the Embryonic Stem Cell Core and Genomic Core at the Sanford Consortium for Regenerative Medicine for equipment access and support. Additionally, I want to thank Friedreich’s Ataxia Research Alliance for funding this project.

I also want to thank everyone in the Regenerative Medicine Program at Cal Poly, San Luis Obispo. Thank you to the Regen Med faculty especially Dr. Dan Peterson, Dr. Trevor Cardinal, Dr. Elena Keeling and Emily Neal for helping me develop not only my lab skills but also my ability to communicate and build connections within the scientific community. I especially want to thank my mentor and advisor, Dr. Dan Peterson, who is entirely responsible for my joining of the program. It was Dr. Peterson’s animal science biotechnology course that inspired my passion for cell culture and fostered my decision to pursue the field of regenerative medicine as a career. I also want to thank Dr. Peterson for his guidance and mentorship not only in lab but also in my life and career. Thank you to the CIRM class of 2024 and everyone in the Peterson Lab for their support and friendship. Finally, thank you to the California Institute of Regenerative Medicine for funding and providing this great learning opportunity.
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Friedreich’s ataxia (FRDA) is a progressive neurodegenerative disease that affects 1 in every 50,000 individuals in the United States. This autosomal recessive disorder is caused by a homozygous GAA trinucleotide expansion in the first intron of the frataxin gene which encodes for the frataxin protein (FXN). This expansion mutation hinders transcription initiation which results in decreased production of frataxin with levels ranging from 5-35% in affected patients compared to healthy adults. FRDA patients can carry between 66 and 1500 GAA trinucleotide repeat expansions compared to normal alleles which contain fewer than 33 GAA repeats. The number of GAA repeats correlates with clinical severity and disease onset where higher repeats result in earlier onset and faster progression, a phenomenon known as anticipation. The clinical phenotype of FRDA patients is broad and includes muscle weakness, ataxia, areflexia, sensory loss, cognitive decline, and cardiomyopathy. The neurological phenotype of FRDA patients is characterized by the degeneration of sensory neurons in the dorsal root ganglion (DRG), dentate nucleus of the cerebellum and motor cortex as well as the degradation of motor neurons of the corticospinal tract. Disease onset presents between 5 to 15 years of age and there is currently no targeted treatment or cure available for this neurodegenerative disease.

The pathogenesis of Friedreich’s ataxia is attributed to the significant decrease in the production of frataxin. Frataxin is a mitochondrial protein involved in iron metabolism and homeostasis, specifically involved in iron-sulfur (Fe-S) cluster biosynthesis. Therefore a reduction of frataxin levels has been associated with iron accumulation in the mitochondria ultimately resulting in the production of toxic free radicals, reactive oxygen species and mitochondrial dysfunction. Many of the tissues that present cellular degeneration in FRDA have been observed to display abnormal iron accumulation. Post mortem tissue samples from FRDA
patients demonstrate iron accumulation in myocardial cells which parallels the cardiac hypertrophy commonly observed\textsuperscript{10}. Additionally, abnormal iron deposition has been reported in the dentate nuclei and dorsal root ganglia of patients\textsuperscript{11} which mirrors the neurodegeneration observed in these tissues. Frataxin has also been shown to play a role in oxidative phosphorylation (OXPHOS) as decreased synthesis of iron-sulfur clusters results in deficiencies in mitochondrial respiratory chain complexes I and II/III in cardiac and skeletal muscle tissue\textsuperscript{12,13}. This deficiency results in reduced OXPHOS and ATP production\textsuperscript{13,14} which is the most damaging in tissues highly dependent on OXPHOS as an energy source including nervous system and muscle tissue. Overall, iron accumulation, increased reactive oxygen species production, and decreased oxidative phosphorylation have been attributed to perpetuating a cycle of DNA damage and mitochondrial dysfunction which ultimately contributes to FRDA pathophysiology (Figure 1).

\textbf{Figure 1.} Overview of Pathogenesis in Friedreich’s Ataxia\textsuperscript{15}
The cell type-specific contribution to FRDA pathophysiology is not well understood. Neurons are known to be susceptible to FXN loss\textsuperscript{16,17}, however, whether microglia exacerbate and/or cause pathogenesis is still under investigation. Microglia are the resident macrophages of the central nervous system and play both an immune and neuroprotective role as they provide constant surveillance of the environment and contribute to neurogenesis and synaptic homeostasis\textsuperscript{18,19}. In normal physiological conditions, microglia are found in their resting state and are characterized by long and ramified branches that help to surveille the surrounding environment\textsuperscript{20}. When activated, their morphology becomes ameboid with fewer and shorter processes to phagocytose cellular debris, antigens or prune immature or excess synapses\textsuperscript{19–21}. This activated state is also associated with the release of pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-\(\alpha\)) or reactive oxygen species (ROS) that can trigger caspase-3-mediated neuronal apoptosis\textsuperscript{22,23}. Microglia-neuron interactions are crucial to the homeostasis of the CNS with interactions taking place indirectly via soluble messengers or intermediate cells as well as directly via tight membrane to membrane contacts\textsuperscript{24}. These interactions are crucial for regulating synaptic plasticity, clearance of excess or apoptotic neurons and the release of trophic and anti-inflammatory factors to enhance neuronal survival\textsuperscript{20,22,24}. However, CNS insults can cause microglia to become overactivated and dysfunctional, negatively impacting neurons via the release of neurotoxic factors or dysfunctional phagocytosis\textsuperscript{22} ultimately resulting in neurodegeneration and chronic inflammation. Microglia have been shown to contribute to disease progression in many neurodegenerative disorders including Alzheimer’s and Parkinson’s disease\textsuperscript{25–27}. There has been little research regarding microglia contribution to FRDA pathogenesis, however, it has been shown that hyperactive microglia enhanced oxidative damage and induced upregulation of DNA repair proteins in a FRDA mouse model\textsuperscript{28}, as well as in postmortem human tissues\textsuperscript{29}. 
Microglia play a critical role in the CNS as they are able to rapidly activate and converge on the site of brain injuries to provide repair and regeneration which has gained them recognition for their therapeutic potential in neurodegenerative diseases \(^{30,31}\). Our collaborators have demonstrated the potential of macrophages to serve as a therapeutic agent in the lysosomal storage disorder, cystinosis, that is characterized by a deficiency in the cystinosin protein \(^{32}\). After wildtype hematopoietic and progenitor stem cell (HPSC) transplantation into the bone marrow, a rescue of cystinosis pathology was observed in an *in vivo* model of cystinosis \(^{32-34}\). It was concluded that HPSC-progeny had differentiated into macrophages which delivered cystinosin-bearing lysosomes to diseased cells through tunneling nanotubes \(^{32,35}\). Based on this discovery, it was proposed that healthy microglia could provide similar therapeutic support in FRDA via the transfer of healthy mitochondria and frataxin to FRDA cells \(^{36}\). Based on this hypothesis, hematopoietic stem cells (HPCs) were xenotransplanted into the bone marrow of an FRDA mouse model and successfully differentiated into microglia-like cells in the brain and spinal cord and macrophages in the DRG, heart and muscle \(^{36}\). This transplantation was correlated with a rescue of the FRDA phenotype suggesting a putative contribution of microglia in dampening the hallmark features of FRDA \(^{36}\). Additionally, wildtype HPCs transduced with FXN-GFP demonstrated the transfer of mitochondrial proteins including FXN to diseased fibroblasts and neurons both *in vitro* and *in vivo* \(^{36}\), a mechanism that could be responsible for the rescue of the FRDA phenotype.

To investigate the cell-type specific contribution to FRDA pathology, our lab in conjunction with our collaborators, Dr. Cherqui’s lab, have been utilizing iPSC-derived microglia (iMG) and neurons from FRDA patients, related carriers, and healthy controls. We have developed a CRISPR/Cas9 mediated GAA repeat excision approach to correct the pathogenic GAA repeat in FRDA \(^{37}\) to generate an isogenic cohort of induced pluripotent stem cells (iPSCs)
that can be utilized as a potential therapeutical approach. This cohort of iPSCs is being utilized to characterize the neuronal and microglial phenotype in FRDA and to explore the impact the CRISPR/Cas9 GAA excision has on this phenotype. iPSC-derived FRDA neurons have been characterized as having increased caspase-3 expression and higher incidence of dendritic blebbing, both distinct indications of apoptotic neurons\(^3\). Additionally, FRDA neurons were demonstrated to have higher levels of mitochondrial superoxides as well as abnormal mitochondrial structure and number, indicating mitochondrial dysfunction. CRISPR gene editing was demonstrated to alleviate both the apoptotic phenotype and mitochondrial dysfunction\(^3\).

We have discovered a distinct inflammatory phenotype in FRDA microglia which is being further characterized and explored to better understand the mechanism behind this dysfunction as well as the potential to alleviate this by frataxin restoration via gene editing. While it is important to characterize microglia in isolation, it is critical to investigate how they contribute to neuronal homeostasis and survival as this provides a clearer picture of pathogenesis in FRDA patients.

Therefore, the objective of this research is to investigate whether microglia derived from FRDA patients contribute to neurodegeneration using a 2D co-culture model composed of microglia, neurons and astrocytes. Additionally, we will evaluate the therapeutic potential of CRISPR/Cas9 corrected microglia in dampening neurodegeneration in 2D culture. We will also investigate the potential for microglia-mediated delivery of healthy mitochondria and frataxin to diseased FRDA cells.
Chapter 2.

MATERIALS AND METHODS

2.1 Cell Lines

In this study, we utilized iPSCs derived from three FRDA patients, two of whom were isogenically corrected to generate their respective isogenic controls, two familial carriers and two unrelated healthy donors. FRDA iPSCs lines were generated from fibroblast derived from FF1 (GM03816) and the FF2 (#GM23913) patient lines purchased from Coriell Institute. 223 FRDA and 264 unrelated healthy control lines were generated from patients’ lymphoblast cell lines (LCLs) #GM16223 and #GM22264 respectively and were purchased from Coriell Institute as well. A previously established healthy control iPSCs line was also utilized together with two familial carriers, 236 and 849 cell lines. 223 FRDA patients LCLs and FF2 iPSCs lines underwent gene editing for FXN pathological expansion mutation utilizing our established CRISPR/Cas9 protocol\(^3\).

2.2 Reprogramming of Patient Lymphoblast Cell Lines

Patient lymphoblast cell lines (LCLs) were reprogrammed via the Epi5 Episomal iPSC Reprogramming Kit (Life Tech Cat# A15960) following the manufacturer’s protocols. Cells were counted and 2\times10^6 LCLs were resuspended in Nucleofector Solution to prime cells for nucleofection. The cell suspension was combined with either pmax GFP Vector to evaluate nucleofection efficiency or Epi5 reprogramming vectors (Sox2, Oct4, Lin28, Klf4, L-myc) and EBNA to reprogram the cells. After nucleofection, cell solution was added to pre-equilibrated media containing B27 Supplement (Gibco CAT#17504044), N2 Supplement (Gibco CAT#17502048) and seeded at various densities on Cultrex Basement Membrane Matrix (Trevigen, CAT#3432-001-01) coated 6 well plates. iPSCs were then plated at low density to
allow growth of individual colonies. iPSCs were maintained in mTESR1 plus medium (STEMCELL Technologies CAT#100-0274).

2.3 PCR for GAA repeat excision

QuickExtract kit (Lucigen, Middleton, W1, USA) was used to extract genomic DNA from FRDA, gene Edited, carrier and control iPSCs. Gene Editing (GE) fwd: 5’-GGT GTA GGA TTA AAT GGG AAT AA-3’; Rev: 5’ GGA TGC ACA GGA GCT TATT-3’, and Non-Gene Editing (NGE) fwd: 5’- GGA CCT GGT GTG AGG ATT AAA-3’; rev: 5’-CTA ATA CAT GCG GCG TAC CA-3’ primers were used to test the efficacy of our CRISP/Cas9 approach and to select the clones that has been used in this study. PCR reaction was carried out with GoTaq Green Master Mix 2X (Promega, GmbH) and the following settings were used: 95°C for 3min (95°C for 30sec, 52°C (GE) and 56 °C (NGE) for 30sec, 72 °C for 1min) x 35, 72 °C for 10min. PCR products were loaded and made then run on a 1% agarose gel at 120V for 30min. GE and NGE bands were detected at 401 bp and 313 bp, respectively using a 1Kb DNA ladder.

2.4 Transduction of iPSCs with LV-FXN-GFP

Frataxin conjugated to green fluorescent protein (FXN-GFP) was introduced into FRDA cell lines via lentiviral transduction. Lentivirus was provided by the Cherqui lab36. TrypLE Express (Gibco CAT#12604-013) was added to cells to enzymatically lift from the plate. The cell suspension was diluted with DMEM/F12 (Gibco CAT#11330-032) and centrifuged at 500g for 2 minutes. After aspirating the supernatant, the cell pellet was mixed with 5 microliters of the lentivirus and incubated for 30 minutes at 37C to allow for viral entry into the cell. Finally, cells were resuspended in mTESR1 Plus with 1 µM Rock Inhibitor (STEMCELL Technologies CAT#72308) to prevent cell death and plated onto a well of a Cultrex-coated 6 well plate.
2.5 Generation of hematopoietic progenitor cells from iPSCs

Induced hematopoietic progenitor cells (iHPCs) were produced using the StemDiff HPC differentiation kit. iPSC lines were cultured in mTESR1 Plus and on Cultrex-coated 6 well plates. Cells were enzymatically treated with ReLeSR (StemCell Technologies, CAT# 05872) into media containing 1 μM Rock inhibitor onto 6-well plates. The following day, media was changed to STEMdiff Media A (STEMdiff Hematopoietic Basal Medium CAT#05311) with STEMdiff Hematopoietic Supplement A (STEMCELL technologies CAT#05312) at a 1:200 dilution. One Day 3, 1mL STEMdiff Media A was added to each well. On either Day 4 or Day 5, the media was changed to STEMdiff Media B (STEMdiff Hematopoietic Basal Medium with Hematopoietic Supplement B (STEMcell Technologies CAT#05313) at a 1:200 dilution. On Day 6 or 7, 1 mL of STEMdiff Media B was added and Media B was added every other day from this point until collection. On Days 11-13, non-adherent cells were collected by tapping off of the plate and utilized for microglia differentiation.

2.6 Microglia Differentiation

After collection, iHPCs were resuspended in DPBS (Gibco, Cat. 14190-144) and cell counts were performed. Cell suspension was centrifuged at 300xg for five minutes and the cell pellet was resuspended in microglia complete media composed of DMEM/F12 (Gibco CAT#11330-032) with 1X Insulin-Transferrin-Selenium (Gibco CAT#41400045), 1% B27 Supplement (Gibco CAT#17504044), 0.5% N2 Supplement (Gibco CAT#17502048), 400uM monothioglycerol, 1X Glutamax (Gibco CAT#35050-061), 1X MEM NEAA (Gibco CAT#11140-050), 2.5 μg/mL Insulin (Sigma CAT#11061-68-0), 25ng/ml M-CSF (Humanzyme CAT#HZ-1039), 100ng/ml IL-34 (Humanzyme CAT#HZ-7064), and 50ng/ml TGFb-1 (Humanzyme CAT#HZ-1087). iHPCs were seeded at approximately 3x10^5 per well of a Cultrex-coated 6-well plate for differentiation. For
the next 28 days, 1mL of microglia complete media was added per well every 48 hours. Microglia (iMG) were mature after 28 days of culturing in complete media.

2.7 Neural Progenitor Cell Production

iPSC colonies of approximately 50-100 cells in size were lifted off from the plate with collagenase diluted in DMEM/F12. The colonies were then transferred onto a 6 well ultra-low attachment plates (Corning Ref#CLS3471) and cultured in neural induction media (NIM) which was composed of DMEM/F12, 1% N2 Supplement, 1% B27 Supplement, 100 ng/mL Noggin (R&D Systems Cat. #6057-NG), 100 nM LDN193189 (Stemgent #04-0074-02) and 10 µM uSB431542. They were placed on a shaker with gentle agitation for embryoid body formation. On Day 17, embryoid bodies were plated onto polyornithine and laminin (PLO) coated 10cm plates for rosette formation in NIM supplemented with fibroblast growth factor 2 (20ng/mL) and laminin (1µm/mL). On Day 24, the rosettes were mechanically selected and enzymatically dissociated in accutase (Stemcell CAT#07920). The resulting cell solution was subsequently plated onto PLO coated plates for neural progenitor cell expansion and maintained as a monolayer at high density.

2.8 Neuronal Differentiation

Neuronal Precursor Cells (NPCs) were maintained in NPC D24+ media containing DMEM/F12, 1% B27 Supplement, 1% N2 Supplement, 1X Laminin (ThermoFisher CAT#A25607) and 1X FGF2 (Proteintech CAT#HZ-1285). NPCs were cultured in neuron differentiation media contained DMEM/F12 with Glutamax, 1% B27 Supplement, 1% N2 Supplement, 10 µg/mL BDNF (Peprotech Cat. #450-02-1mg), 10 µg/mL GDNF (Peprotech Cat. #450-10-1mg), 1mM dibutryl cyclicAMP (cAMP) (Tocris Bioscience Cat #1141) and 200nM ascorbic acid (StemCell Technologies Cat. #72132) for 14 days to generate neurons.
2.9 Zymosan and NPC Phagocytosis Assay

Microglia were plated on 96-well corning Primaria plates and labeled with lectin at a concentration of 1:1000. Zymosan beads conjugated to pHrodo (ThermoFisher CAT# P35364) were spiked in at a concentration of 1:100. Alternatively, NPCs were labelled with 1mM staining solution (1ul of pHrodo beads in 10mL of NaC\textsubscript{2}O\textsubscript{4} (pH 8.5)) for 40 minutes at 37C. pHrodo-labeled NPCs were resuspended in co-culture media and added to microglia. Plate was imaged on IncucyteS3 Live-Cell Analysis System every 20 minutes for 4.5 hours.

2.10 Ramification Quantification

Characterization of induced-microglia (iMG) ramifications was carried out using an IncucyteS3 Live-Cell Analysis System. Phase images of iMGs were obtained at 10x and 20x magnification and analyzed using NeuroTrack software that allowed the detection of the cell body and the analysis of iMG branches. iMG ramification was quantified by ramification length (mm) per cell body cluster and number of ramification branch points per cell body cluster.

2.11 Agilent Seahorse Mito Stress Test

iMGs were plated onto Cell-Tak coated Agilent plate 2-3 days prior to assay. Oxygen consumption rate was measured by utilizing the XF Cell Mito Stress Test Kit following the manufacturer’s protocols (Agilent, CAT#103015-100).

2.12 Co-Culture

To co-culture NPCs and iMGs, NPCs were plated in glass bottom 8 well slides (Millipore Sigma, Cat. #PEZGS0816) at a density of 60k/0.7 cm\textsuperscript{2} and cultured in NPC D24+ media for 24 hours. Media was then exchanged for neuron differentiation media for 7 days. Mature iMG were resuspended in co-culture media which consisted of neuron differentiation media supplemented with 25ng/ml M-CSF (Humanzyme CAT#HZ-1039), 100ng/ml IL-34 (Humanzyme...
CAT#HZ-7064), and 50ng/ml TGFb-1 (Humanzyme CAT#HZ-1087). Microglia were then added to neurons at a ratio of 1:10 and co-cultured for either 2 or 7 days. To co-culture iMG, acceptor microglia were plated in glass bottom 8 well slides at a density of 50k/well. Acceptor microglia were either labeled with Qtracker Cell Labeling Kit (ThermoFisher, CAT#Q25021MP) according to manufacturer’s instructions or with CellTrace Violet (ThermoFisher, CAT#C34557) according to manufacturer’s instructions. Microglia were then washed twice with microglia media. Donor microglia were added at a ratio of 1:1 and were co-cultured for 48 hours.

2.13 Conditioned Media
To generate conditioned media iMG were plated at a density of 150k cells/well in a 24 well plate. The media was collected 48 hours later and was centrifuged at 300 rcf for 5 min. The supernatant was collected and the conditioned media was added to neuron media at a ratio of 1:2 or 1:4 and was added to neurons for 48 hours.

2.14 Labeling Mitochondria with MitoTracker
Donor microglia were incubated with MitoTracker Green (ThermoFisher, CAT#M7514) or MitoTracker Deep Red (ThermoFisher, CAT#M22426) for 30 minutes at 37C. Microglia were then washed twice in PBS by centrifugation and added to acceptor microglia or neurons at a ratio of 1:1 for 5 hours.

2.15 Immunocytochemistry
Cells are fixed in 4% paraformaldehyde (PFA) for 30 min and washed thrice with PBS. Permeabilization and blocking was performed with 10% normal donkey serum and 0.5% Triton X-100 (Sigma Aldrich) in PBS for 1 hr at room temperature. Cells were then incubated in primary antibody diluted in 3% NDS, 0.5% Triton X-100 overnight at 4C. Next, PBS was used to wash the primary antibodies, followed by incubation with secondary antibodies diluted in 3% NDS, 0.5%
Triton X-100 for 1 hour at room temperature. Next, slides were rinsed with PBS with 1 µL/mL DAPI (Thermo Fisher, CAT#D1306) for 5 minutes to counterstain nuclei. Slides were rinsed with PBS 3 times for 5 minutes and then a coverslip was applied with Immu-Mount (Thermo, CAT# 9990402). Images were acquired using the Andor Dragonfly spinning disc confocal microscope and were processed and analyzed using the Imaris Software (Oxford Instruments).

2.16 Mice
Mice utilized in the present study were maintained at the Sanford Consortium for Regenerative Medicine. Humanized immunodeficient mice purchased from the Jackson Laboratory (Strain #017708) (CSF1h/h) and subsequently depleted of murine microglia (CSF1RΔFIRE/ΔFIRE) by Drs. Clare Pridans and David Hume[39]. Mice were maintained in accordance with the University of California, San Diego (UCSD) research guidelines for the care and use of laboratory animals.

2.17 Intracerebroventricular injection (ICV) of iHPCs
iHPSCs from 246 healthy donor, 223 FRDA and 223 Edited were collected as previously described and a density of 100k was resuspended in 4 µl of 1x PBS/pups. Intracerebroventricular injection (ICV) was performed in neonatal mice (P0-P2) by bilateral injection of 2 µl of PBS containing 50k of iHPCs per side per mice targeting the lateral ventricle.

2.18 Tissue processing
Mice were allowed to mature to adults and on day 60 were anesthetized and intracardially perfused with 0.1M PBS (pH 7.4) followed by 4% paraformaldehyde (PFA) solution. The brain was post-fixed with 4% PFA solution over night at 4°C and subsequently cryoprotected in 30% sucrose solution (in PBS) for 48 hr at 4°C prior to embedding in Tissue-Tek® O.C.T. Tissue was then sliced at 40 µm tick sections and free-floating sagittal cerebellar slices were cut using a cryostat and collected in PBS + 0.8% sodium-azide (AxonLab) at 4C.
2.19 Immunohistochemistry of free-floating cerebellar sections

Cerebellar sections were blocked using 10% of normal donkey serum (NDS) + 0.5% of Triton-X100 (diluted 1:10 in PBS) for 2 hours at RT, followed by overnight incubation with primary antibody in 3% BSA + 0.5% of Triton X-100 in PBS at 4°C. Following incubation with primary antibody, the sections were washed thrice with PBS and incubated for 2hr at RT with appropriate secondary antibodies. The sections were then rinsed again with PBS, stained with DAPI and subsequently mounted on glass slides using Immuno-mount mounting medium (Thermo, CAT# 9990402).

2.20 Confocal microscopy

Confocal images were acquired from lobule V-X using an Andor Dragonfly spinning confocal microscope, fitted with 10x, 20x, 40x and 63x objective. Identical confocal settings were maintained during acquisition and all the images were processed and acquired simultaneously.

2.21 Statistics

Data was analyzed with GraphPad Prism (Version 10) and represented as Mean ± SEM.

Comparison between two groups was performed with unpaired, 2-tailed Student’s t test and among three groups with one way ANOVA. p-value < 0.05 was considered statistically significant where * corresponds to < 0.05, ** to < 0.01, *** to < 0.001 and **** to < 0.0001.
Chapter 3.
RESULTS

3.1 Characterization of the Microglial Phenotype in FRDA

3.1.1 Generation of an isogeneic iPSC cohort from FRDA patient lines

To investigate microglia and neuronal phenotypes in FRDA, FRDA iPSC lines were purchased (FF1 and FF2) or reprogrammed from patient lymphoblast cells (223 and 850). CRISPR/Cas9 gene editing was utilized to excise the pathogenic GAA repeat mutation in intron 1 of the FXN gene to generate isogenic edited lines (Figure 2A). Once edited and reprogrammed to iPSCs, subcloning was utilized to generate clonal gene edited or unedited lines and designed PCR primers were utilized to verify the gene editing. NGE primers could only bind when gene editing did not occur while GE primers flanked the expected deletion and could only amplify the intronic region when gene editing occurred (Figure 2B). PCR and gel electrophoresis was then utilized to verify iPSC clones that had been biallelically edited or unedited for further expansion (Figure 2C). iPSCs were also generated from relatives of the FRDA patients who were asymptomatic carriers with one allele containing the mutation. This resulted in an iPSC cohort that consisted of four FRDA lines, three isogenic edited lines, two related carriers and two unrelated controls (Figure 2D). iPSC generation was confirmed with immunostaining for pluripotency markers (Figure 2E).
3.1.2 FRDA microglia display impaired morphology, phagocytosis and oxidative phosphorylation

Induced microglia (iMG) were generated from iPSC lines to characterize the microglial phenotype in FRDA and the potential to rescue this phenotype by CRISPR/Cas9 deletion of the GAA repeat expansion. First, iMGs were investigated for an inflammatory and activated phenotype as this is a common characteristic of microglia in neurodegenerative disease \(^{23,25,29,40-42}\). The lysosomal membrane protein, CD68, is commonly utilized as a marker of microglial activation as it is upregulated in phagocytic and hyperinflammatory microglia \(^{21,43}\).
Immunostaining for CD68 in mature iMGs revealed increased expression of CD68 in FRDA microglia lines compared to edited, control and carriers (Figure 3A). Another indication of microglial activation is morphology, such that when microglia are activated they enter a “primed” state that is characterized by shorter and less branching ramifications\textsuperscript{44}. Incucyte NeuroTrack Analysis Software was utilized to analyze microglia ramification length per cell body. FRDA and carrier lines demonstrated significantly shorter ramifications when compared to unrelated control and edited lines (Figure 3B-C). Activated and inflammatory microglia can subsequently result in a hyper phagocytic phenotype\textsuperscript{22,45}. To analyze iMGs for phagocytic activity, a phagocytosis assay with zymosan bioparticles was utilized. Zymosan, a yeast cell wall glucan, that was conjugated to a pH-sensitive dye was spiked into microglia cultures followed by imaging of phagocytic activity every 20 minutes over a 4-hour period. FRDA iMGs were demonstrated to be hyper phagocytic as more zymosan bioparticles were phagocytosed by FRDA microglia over the 4-hour period as well as by the final endpoint compared to all other lines (Figure 3D-E).

FRDA cells have been characterized by mitochondrial dysfunction resulting from improper iron homeostasis and reduced oxidative phosphorylation\textsuperscript{12-14}. To determine if this disease phenotype also extends to FRDA microglia, the Seahorse XF Cell Mito Stress Test was utilized to measure cellular bioenergetics. This assay involves the administration of drug treatments to specifically target different aspects of the electron transport chain to test its efficiency by measuring the oxygen consumption rate (OCR)\textsuperscript{46}. OCR was first measured at basal conditions followed by treatment with the ATP synthase inhibitor, oligomycin, to inhibit mitochondrial respiration to determine ATP production. The mitochondrial uncoupler carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone (FCCP) was then injected to stimulate an energy source to determine maximal respiration. Finally, a mixture of rotenone and antimycin was
injected to inhibit complex I and III to determine the contribution of non-mitochondrial respiration. FRDA iMGs exhibited impaired mitochondrial oxidative phosphorylation as indicated by significantly decreased basal respiration, ATP production and maximal respiration compared to all other lines (Figure 3G-H). These results demonstrate that FRDA microglia display an activated and inflammatory disease phenotype as well as mitochondrial dysfunction which was shown to be rescued with removal of the repeat expansion by gene editing.
Figure 3. FRDA microglia present an inflammatory phenotype and mitochondrial dysfunction. A) Quantification of CD68 integrated density demonstrates a significant increase in CD68 expression in FRDA iMGs. B) Representative images of FRDA and edited microglia ramifications using Incucyte S3 NeuroTrack analysis software. Scale bar = 20 µm. C) Quantification of ramification length per cell body. FRDA and carrier iMGs demonstrate shorter ramification length. D) Time course of phagocytosed zymosan bioparticles normalized to cell count. Images were taken every 20 minutes for 4.5 hours. E) Quantification of phagocytosed zymosan by area under the curve (right) and at the final endpoint (left). FRDA iMGs demonstrate significantly higher phagocytosis of zymosan bioparticles. F) Seahorse XF Cell Mito Stress Test of microglia lines to measure cellular bioenergetics. Various drug treatments were utilized to modify the electron transport chain to measure basal respiration, ATP production, maximal respiration, proton leak and non-mitochondrial respiration via oxygen consumption rate. G) Basal OCR, ATP production, proton leak, maximum OCR and non-mitochondrial OCR were significantly decreased in FRDA iMGs. n = 3 FRDA, 2 edited, 2 carrier and 2 non-related controls with 3 biological replicates for each cell line. Data is represented as mean +SEM and analyzed with one-way ANOVA where *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

3.2 The Impact of FRDA Microglia on Neurodegeneration

3.2.1 Generation of in vitro co-culture model to study microglia-neuron interactions

As FRDA microglia demonstrated key markers of activation and inflammation, we next wanted to investigate how this disease phenotype would impact neuronal viability. In order to study microglia-neuronal interactions in vitro, a co-culture model was generated using neural progenitor cells (NPCs) and iMGs. Non-related control iPSCs were differentiated into NPCs which were subsequently differentiated into a heterogeneous neuronal culture for 7 or 14 days. Simultaneously, FRDA, edited and non-related control iPSCs were differentiated into induced hematopoietic progenitor cells (iHPCs) and then differentiated into iMGs for another month. iMGs were then added to the neuronal culture on day 7 or 14 at a ratio of 1:5 and subsequently cultured for 2 days (Figure 4A-B). The co-culture was then characterized for different cell types by immunostaining for β-tubulin III (TUJ1) for neurons, glial fibrillary acidic protein (GFAP) for astrocytes and ionized calcium binding adaptor molecule 1 (IBA1) for microglia (Figure 4C).
Figure 4. Generation of co-culture model to study microglia-neuronal interactions. A) Schematic overview of co-culture workflow. B) Overview of protocol for neural progenitor cell (NPC) differentiation to neurons and subsequent co-culture with microglia. NPCs were differentiated to neurons for 7 or 14 days depending on the experiment. Microglia were added in a 1:5 ratio on day 7 or 14 and were co-cultured for 48 hours. C) Representative images of co-culture, staining includes TUJ1 for neurons, GFAP for astrocytes and IBA1 for microglia. Scale bar represents 40 µm.

3.2.2 FRDA microglia induce apoptosis in co-cultured neurons

Once the co-culture model was generated, the impact FRDA microglia have on the viability of healthy neurons could be investigated and quantitated. FRDA, edited, control or no microglia were added to healthy neuronal cultures for 48 hours at which neuronal apoptosis was measured. Cells undergoing apoptosis experience degradation of DNA by endonucleases which produces double stranded DNA breaks. The TUNEL assay was utilized to identify these DNA breaks via the enzyme, terminal deoxynucleotidyl transferase (TdT), which identifies and labels double stranded DNA breaks with fluorescent dUTPs. The percentage of β-tubulin III positive cells that were also positive for TUNEL were quantified across all conditions. Healthy neurons that were cultured with FRDA iMGs demonstrated a higher percentage of TUNEL+ neurons.
compared to those cultured with no iMGs, however, there was no significant difference between FRDA and edited iMG conditions (Figure 5A). Neuronal apoptosis was also measured by the expression of caspase-3 which is a key executioner of apoptosis and is responsible for mediating the cleavage of autophagic proteins. FRDA, edited, control or no microglia were added to healthy neuronal cultures for 48 hours. The percentage of β-tubulin III positive cells that were also positive for caspase-3 was quantified across all conditions. Healthy neurons that had been cultured with FRDA iMGs demonstrated higher numbers of caspase-3 positive or apoptotic neurons compared to all other conditions (Figure 5B-C).

To investigate if neuronal apoptosis occurred independently of direct cell to cell, microglia-conditioned media (iMG-CM) was added to the neuronal cultures. Microglia media was conditioned by iMGs for 48 hours and then diluted 1:1 with neuronal media and added directly to healthy neuronal cultures for 48 hours. Healthy neurons that had been cultured with FRDA iMG-CM demonstrated higher percentages of caspase-3 positive neurons when compared to those treated with edited iMG-CM (Figure 5D). To further investigate what factors may be responsible for inducing apoptosis, cytokine panels were completed for FRDA and edited iMGs. Two anti-inflammatory chemokines were queried; both tumor necrosis factor receptor 2 (TNF RII) and CC motif chemokine ligand 17 (CCL17) were found to be significantly lower in FRDA microglia compared to edited (Figure 5D), suggesting loss of homeostatic anti-inflammatory modulation in FRDA.

As FRDA microglia have been demonstrated to be hyper phagocytic of zymosan particles, we wanted to investigate the impact this might have on neural progenitor cells (NPCs). Microglia have previously been implicated in modulation of the neural progenitor pool both during development and related to adult neurogenesis, a pathway which could be overly active in FRDA. NPCs were labeled with a pH-sensitive dye and were spiked into microglia cultures
which were then imaged for phagocytic activity every 20 minutes over a 4-hour period. FRDA and carrier microglia tended to phagocytose more NPCs compared to control and edited lines over the 4-hour period (Figure 5E). The impact of microglia on neuronal viability was also explored in vivo using the Csf1r\^AFIRE/AFIRE murine line, a humanized immunodeficient line that are depleted of native mouse microglia\(^50\). FRDA, edited and control iHPCs were xenotransplanted into the ventricles of Csf1r\^AFIRE/AFIRE neonates where they engrafted throughout the brain and differentiated into microglia (xMGs) (Figure 5F). To investigate neuronal loss, calbindin was utilized to stain for Purkinje cells of the cerebellum as the cerebellar cortex and dentate nucleus have been characterized by neurodegeneration in postmortem FRDA tissue\(^8,51\). Mice with FRDA xMGs demonstrated significant loss of Purkinje cells in the cerebellar cortex when compared to those injected with edited or control xMGs (Figure 5G). Overall, FRDA microglia were shown to induce caspase-3 mediated neuronal apoptosis in healthy neurons. This microglia-mediated apoptosis may be caused by a combination of soluble factors or cytokines as well as by the direct phagocytosis of viable neurons. This phenotype was replicated in vivo as demonstrated by Purkinje cell loss in the cerebellum of the Csf1r\^AFIRE/AFIRE murine model.
Figure 5. FRDA microglia reduce neuronal viability in co-culture and in vivo. A) Quantification of β-tubulin III positive neurons expressing TUNEL after 48 hours of co-culture with microglia. Co-culture with FRDA iMGs results in a significantly higher ratio of TUNEL positive neurons
compared to no iMG. B) Quantification of β-tubulin III positive neurons expressing caspase-3 after 48 hours of co-culture with microglia. Co-culture with FRDA iMG results in a significantly higher ratio of caspase-3 positive neurons compared to all other conditions. C) Representative images demonstrating greater proportion of caspase-3 positive FRDA neurons compared to edited neurons. Scale bar = 50 μm D) Cytokine panel demonstrates that CCL17 and TNF RII are significantly higher in edited iMGs compared to FRDA. E) Quantification of β-tubulin III positive neurons expressing caspase-3 after addition of microglia-conditioned media (iMG-CM) for 48 hours. Exposure to FRDA iMG-CM resulted in more caspase-3 positive neurons compared to edited iMG-CM. F) Time course of pHrodo-labeled NPC phagocytosis, images taken every 20 minutes for 4.5 hours (left). Quantification of NPC phagocytosis by area under the curve (right). G) Schematic of ICV injection of iHPCs into Csf1rΔFIRE/ΔFIRE mice. H) Representative images (left) and quantification (right) of Purkinje cell number in the cerebellum after xenotransplantation. Injection with FRDA microglia results in decreased Purkinje cell numbers. n = 2 FRDA, 2 edited, and 1 non-related control with 3 biological replicates for each cell line. Data is represented as mean ±SEM and analyzed with one-way ANOVA where *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

3.3 Therapeutic Potential of Microglia in FRDA

3.3.1 Edited microglia transfer healthy mitochondria to FRDA cells

Wildtype hematopoietic progenitor cells transplanted into the bone marrow of an FRDA mouse model were demonstrated to differentiate into microglia in the brain and spinal cord and to alleviate some of the FRDA disease phenotype. It has been hypothesized that the mechanism of this rescue could stem from the transfer of healthy frataxin-bearing mitochondria from wildtype microglia to diseased FRDA cells. To investigate this hypothesis, the transfer of mitochondria from CRISPR edited microglia to FRDA microglia and neurons was explored. First, transfer between microglia was analyzed by utilizing a microglia co-culture model composed of acceptor and donor microglia cultured in a 1:1 ratio. To differentiate the two populations of iMGs, “acceptor” iMGs were labeled with a Qtracker cell labeling kit (CellTracker) that delivers fluorescent nanocrystals into the cytoplasm of live cells. The mitochondria of “donor” iMGs were then labeled with MitoTracker and subsequently co-cultured with “acceptor” iMGs for 5 hours. Acceptor iMGs that had received mitochondria from donor iMGs could be identified as double positive for both CellTracker and MitoTracker (Figure 6A).
Edited iMGs (donors) were co-cultured with either FRDA or control iMGs (acceptors) to compare the transfer of healthy mitochondria to diseased vs control iMGs. The percentage of acceptor cells that were positive for MitoTracker were quantified and it was concluded that edited iMGs were more likely to transfer healthy mitochondria to FRDA iMGs over control iMGs (Figure 6B-C). To investigate mitochondrial transfer from edited iMGs to FRDA neurons, the previously established co-culture model was utilized. Edited iMGs with MitoTracker-labeled mitochondria served as the donor cells and were added to FRDA neuronal cultures at a ratio of 1:1 for 5 hours (Figure 6D). β-tubulin III positive neurons (acceptors) that were positive for MitoTracker were identified indicating healthy mitochondria had been transferred from edited iMGs to FRDA neurons (Figure 6E).
Figure 6. Edited microglia transfer mitochondria to FRDA cells. A) Workflow of mitochondrial transfer experiment between edited iMGs (donors) and FRDA or control iMGs (acceptors). B) Representative images of MitoTracker-labeled mitochondria (red) transferred to FRDA or control iMGs labeled with CellTracker (green). Scale bar represents 10µm. C) Quantification of percentage of acceptor cells that are positive for MitoTracker. A significantly higher proportion of FRDA microglia received healthy mitochondria compared to control. D) Workflow of mitochondrial transfer experiment between edited iMGs (donors) and FRDA neurons (acceptors). E) Representative images of MitoTracker-labeled mitochondria (red) transferred to FRDA neurons (green). Scale bar represents 10µm. n = 1 FRDA, 1 GFP-FXN. Data is represented as mean +SEM and analyzed with one-way ANOVA where *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

3.3.2 Generation of FXN-GFP iPSC line to investigate FXN transfer

After demonstrating that edited iMGs will transfer healthy mitochondria to FRDA cells, we wanted to confirm that FXN was also being transferred to rescue FXN deficient cells. FF1 FRDA iPSCs were transduced with a lentivirus expressing hFXN-GFP to generate a corrected FXN-GFP iPSC line that could be utilized to track FXN transfer (Figure 7A). After transduction, FXN-GFP iPSCs were subcloned to generate a homogenous FXN-GFP line and were screened with flow cytometry. Two FF1 FXN-GFP clones were generated, clone 1 with 98.2% FXN-GFP expression and clone 9 with 96.4% FXN-GFP expression (Figure 7B). FXN-GFP iPSCs were then differentiated to iMGs and immunostaining confirmed the retention of FXN-GFP expression (Figure 7C). FXN-GFP was also confirmed to co-localize with human mitochondria in mature microglia (Figure 7D). To investigate if transduction rescued some of the FRDA phenotype, morphological analysis was completed with Neurotrack analysis software. FF1 FXN-GFP microglia were demonstrated to have longer ramifications when compared to the original FF1 FRDA line (Figure 7E). Therefore, FRDA microglia transduced with FXN-GFP were rescued from the FRDA morphological phenotype.
Figure 7. Generation of FXN-GFP iPSC line to investigate the transfer of FXN. A) Workflow for generation of FXN-GFP iPSC line. B) Flow cytometry of subcloned FXN-GFP iPSCs confirming homogeneous expression of FXN-GFP. C) Immunostaining to confirm FXN-GFP expression is retained in mature microglia. Scale bar represents 20 µm. D) Immunostaining to confirm FXN-GFP co-localizes with the mitochondria. Scale bar represents 5 µm. E) Representative images of ramification length per cell body in FF1 FXN-GFP and FF1 FRDA microglia using Incucyte NeuroTrack analysis. Scale bar = 200 µm. F) Quantification of ramification length demonstrates that FXN-GFP iMGs display longer ramifications than FRDA iMGs. n = 1 FRDA, 1 FXN-GFP. Data is represented as mean ±SEM and analyzed with one-way ANOVA where *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001
3.3.3 FXN-GFP corrected microglia transfer FXN to FRDA cells

Once generated, the FXN-GFP iPSC line was utilized to explore the therapeutic potential of microglia-mediated delivery of FXN to FRDA microglia and neurons. To explore FXN transfer between microglia, FRDA iMGs (acceptors) were labeled with CellTracker and co-cultured for 48 hours with FXN-GFP iMGs (donors) (Figure 8A). FXN-GFP was detected within Celltracker-positive microglia confirming that FXN was transferred to FRDA microglia (Figure 8B). To investigate if FXN was being transported within the mitochondria, FXN-GFP iMGs were incubated with MitoTracker. They were subsequently co-cultured with FRDA iMGs labeled with CellTrace, a dye that binds to amines on the surface and inside of cells. FRDA iMGs were identified that stained positive for both MitoTracker and GFP (Figure 8C) demonstrating that FXN may be transferred within the mitochondria and not independently. Additionally, staining for F-actin revealed the formation of tunneling nanotubes between microglia which could serve as a potential mechanism for mitochondria and FXN transfer between cells (Figure 8D).

FXN-GFP iMGs were also cultured with FRDA neurons to determine if this transfer also occurred between microglia and neurons (Figure 8E). Immunostaining revealed the presence of FXN-GFP outside of microglia suggesting the transfer of FXN to other cell types. FXN-GFP was also identified to occasionally co-localize with β-tubulin III indicating that there may be FXN transfer to FRDA neurons (Figure 8F). FXN-GFP iHPCs were xenotransplanted into Csf1r\textsuperscript{ΔFIRE/ΔFIRE} mice where they retained FXN-GFP expression and can be utilized to identify transfer in vivo (Figure 8G).
Figure 8. FXN-GFP corrected microglia transfer FXN to FRDA cells. A) Workflow of FXN transfer experiment between FXN-GFP iMGs (donors) and FRDA iMGs (acceptors). B) Representative images of CellTracker-labeled FRDA iMG (white) containing FXN-GFP (green). Scale bar represents 20µm. C) Representative images of CellTrace-labeled FRDA iMGs (white) containing both transferred mitochondria (red) and FXN (green). Scale bar represents 7µm. D) F-actin staining confirms formation of tunneling nanotubes between microglia. Scale bar represents...
15µm. **E)** Workflow of FXN transfer experiment between FXN-GFP iMGs (donors) and FRDA neurons (acceptors). **F)** Representative images of FXN-GFP outside of microglia (white) and co-localization with FRDA neurons (red). Scale bar represents 20µm. **G)** Representative images of FXN-GFP xMGs in the brainstem of Csf1r\(^{ΔFIRE/ΔFIRE}\) mice. xMGs retain FXN-GFP expression which colocalizes with human mitochondria (red). Scale bar represents 10µm.
Chapter 4.
DISCUSSION

Friedreich’s ataxia (FRDA) is the most commonly inherited ataxia and affects 1 in every 50,000 individuals in the United States\(^1\). The genetic basis for FRDA is a GAA trinucleotide repeat expansion in the \textit{frataxin} gene which hinders transcription initiation and ultimately results in reduced levels of the frataxin protein (FXN)\(^4\). The clinical phenotype of FRDA patients consists of muscle weakness, ataxia, areflexia and sensory loss with patients eventually experiencing more severe symptoms such as neurological dysfunction, cardiomyopathy and diabetes\(^7\). Neurological dysfunction stems from neurodegeneration in the dorsal root ganglion and corticospinal tract as well as in the dentate nucleus and cerebellar cortex of the cerebellum\(^8\). There is currently no targeted treatment or cure available for this neurodegenerative disease\(^7\). FRDA pathogenesis is attributed to the significant decline in frataxin, a mitochondrial protein responsible for iron homeostasis and iron-sulfur cluster biosynthesis\(^2,3\). This deficiency results in mitochondrial iron accumulation, increased reactive oxygen species production and decreased oxidative phosphorylation that drives a cycle of DNA damage and mitochondrial dysfunction\(^9,12-14\). While the cell type-specific contribution to pathophysiology is not well understood, neurons have been demonstrated to be susceptible to FXN loss. However, whether microglia exacerbate and/or cause this neuronal dysfunction has yet to be investigated.

Microglia are the resident macrophages of the central nervous system where they play crucial roles in both immune functioning and neurological support. At homeostasis, microglia are responsible for releasing regulatory cytokines, phagocytosing cellular debris and pathogens, pruning immature or excess synapses and maintaining the neural progenitor cell pool\(^19-22,24\). However, in many neurological diseases, microglia have been demonstrated to become overactivated and inflammatory contributing to neurodegeneration and chronic inflammation\(^25-\).
There has been little research regarding the contribution of microglia to FRDA pathology therefore the goal of our research is to characterize the FRDA phenotype in microglia and investigate how this phenotype impacts neuronal survival in FRDA. Additionally, we strive to explore the potential of healthy microglia to serve as a therapeutic vehicle in FRDA.

4.1 Characterization of the Microglial Phenotype in FRDA

To characterize microglia function in FRDA and advance our understanding regarding microglia-neuronal interactions, an iPSC cohort was generated composed of FRDA patient lines, CRISPR-Cas9 gene edited isogenic controls, asymptomatic related carriers and non-related controls. iPSC-derived microglia or induced microglia (iMGs) were subsequently generated and characterized for common markers of activation and inflammation. FRDA iMGs demonstrated a characteristic activated morphology consisting of an ameboid shape with shorter and less branched ramifications. Additionally, FRDA microglia demonstrated higher expression of lysosomal membrane protein, CD68, which is upregulated in microglia in response to inflammatory stimuli and tissue damage. Therefore, changes in morphology and higher CD68 expression in FRDA microglia indicate an activated and inflammatory phenotype which is often associated with the release of pro-inflammatory cytokines such as TNF-α, IL-6 and IL-1β as well as nitric oxide and reactive oxygen species which can induce neuronal apoptosis. Activated and inflammatory microglia have also been associated with hyper phagocytic activity which was measured through the utilization of a zymosan phagocytosis assay. FRDA microglia displayed greater phagocytosis of zymosan bioparticles when compared to control microglia indicating a hyper phagocytic phenotype that could present as excessive synaptic pruning or phagocytosis of viable cells.
Mitochondrial dysfunction and reduced oxidative phosphorylation have been identified as key contributors to FRDA pathogenesis and have been identified in other FRDA cell types\textsuperscript{12–14}. To determine if this mitochondrial phenotype also extends to FRDA microglia, the Seahorse Mito Stress Test (Agilent Technologies) was utilized to measure cellular bioenergetics. FRDA microglia exhibited decreased oxygen consumption during basal respiration, decreased ATP production, a lower maximum respiration and decreased non-mitochondrial respiration. These results point to a dysfunctional electron transport chain (ETC) that is unable to match the efficiency achieved in control microglia. This impairment may stem from the reduction of iron-sulfur clusters that are necessary for ETC enzyme activity\textsuperscript{15}. Ultimately, decreased efficiency can result in the generation of mitochondrial reactive oxygen species contributing to cellular oxidative stress and DNA damage\textsuperscript{15}. Overall, changes in morphology, upregulation of inflammatory markers, increased phagocytosis and a dysfunctional electron transport chain support the fact that FRDA microglia demonstrate a dysfunctional phenotype that has the potential to impact FRDA pathology.

### 4.2 The Impact of FRDA Microglia on Neurodegeneration

At homeostasis, microglia play key roles in neurogenesis and neuronal survival as demonstrated by the release of key neurotrophic and anti-inflammatory factors such as IGF1, NGF and BDNF, phagocytosis of damaged or apoptotic neurons and the maturation and refinement of neural circuits\textsuperscript{22,23}. These indispensable physiological roles of microglia suggest that microglial dysfunction can alter and damage neuronal functioning in neurodegenerative diseases. Therefore, to better understand the role of microglia in FRDA, we investigated the impact FRDA microglia have on neuronal viability. To study microglia-neuron interactions, a 2D \textit{in vitro} co-culture model was developed composed of neurons, astrocytes and microglia. To investigate microglia-mediated neuronal apoptosis, the TUNEL assay was utilized to identify and
label double stranded DNA breaks as an indicator of apoptosis. Healthy neurons co-cultured with FRDA microglia demonstrated higher incidences of DNA damage when compared to neurons cultured without microglia, however, there was no significant impact of FRDA microglia when compared with edited microglia. Some limitations of the TUNEL assay is that DNA damage is not unique to apoptosis but is also a result of necrosis, or in cells undergoing DNA repair, and therefore can also be reversed. Therefore, cleaved caspase-3 was also utilized as a measure of apoptosis. Caspase-3 is an enzyme responsible for mediating the cleavage of autophagic proteins at the end of the apoptotic cascade and therefore serves as a key marker of irreversible apoptosis. Co-culturing healthy neurons with FRDA microglia resulted in a higher percentage of caspase-3 positive or apoptotic neurons when compared to those co-cultured with isogenic or non-related controls. To investigate the role cytokines and soluble factors play in the initiation of neuronal apoptosis, microglia-conditioned media (iMG-CM) was added to healthy neurons. Media conditioned by FRDA microglia resulted in higher incidences of apoptotic neurons indicating that FRDA microglia may release cytotoxic factors which induce caspase-3 mediated neuronal apoptosis or alternatively may fail to supply the necessary neurotrophic factors to support neuronal survival. Cytokine panels of iMG-CM were completed and two anti-inflammatory chemokines, tumor necrosis factor receptor 2 (TNF RII) and CC motif chemokine ligand 17 (CCL17), were demonstrated to be present in significantly lower concentrations in FRDA iMG-CM compared to edited iMG-CM. TNF RII is a receptor for TNF-alpha and is related to the immunosuppressive ability of many immune cells specifically by modulating the release of anti-inflammatory cytokines. The chemokine, CCL17, has been recognized for its role in regulating inflammatory responses but specially has been shown to ameliorate neuroinflammation and neuronal apoptosis after brain injury. These results suggest the loss of homeostatic anti-inflammatory cytokines normally released by healthy microglia.
Another key aspect of homeostatic immune functioning, is the responsibility of microglia to actively monitor the brain environment with pattern recognition receptors (PRRs) to identify and remove toxic stimuli through internalization and phagocytosis\textsuperscript{25}. However, in neuroinflammatory conditions, PRRs have been identified to mediate neuronal damage in the absence of a pathogen indicating dysfunctional phagocytosis\textsuperscript{25}. Additionally, microglia have previously been implicated in modulation of the neural progenitor pool both during development and related to adult neurogenesis, a pathway which could be overly active in FRDA\textsuperscript{49}. When NPCs were added to microglial cultures, FRDA microglia tended to demonstrate higher phagocytic activity than control microglia indicating the potential for dysfunctional phagocytosis of NPCs in FRDA pathology. This decreased neuronal viability mimics the neuronal loss observed in the cerebellar cortex, dentate nucleus and dorsal root ganglion of FRDA patients\textsuperscript{8,51}. Therefore, to investigate the microglial impact on neuronal loss in the cerebellum, iHPCs were xenotransplanted into the Csf1r\textsuperscript{AFIRE/AFIRE} murine line, a humanized immunodeficient line that are depleted of native mouse microglia\textsuperscript{50}. Mice xenotransplanted with FRDA iHPCs demonstrated a significant loss of Purkinje cells in the cerebellar cortex implicating a role of microglia in FRDA neurodegeneration. This suggests the tendency of FRDA microglia to overly phagocytose viable neural lineage cells, leading to neuronal loss.

4.3 Therapeutic Potential of Microglia in FRDA

Microglia have been recognized for their ability to rapidly respond to stimuli throughout the CNS as well as their tendency to make frequent contacts with neurons and other cells to recognize injured cells and shape neuronal activity\textsuperscript{31}. This rapid activation and frequent communication with other cell types highlights microglia as a potential therapeutic agent in neurodegenerative diseases. One potential microglia-mediated therapeutic mechanism is the
bidirectional transfer of organelles, proteins and nucleic acids to other glial and neuronal cells. Microglia have been demonstrated to transfer functional mitochondria to alpha-synuclein burdened microglia and neurons to restore cellular homeostasis. Additionally, mitochondrial transfer has been observed between microglia and astrocytes, affecting the phagocytic function of microglia or inhibiting neuroinflammation in astrocytes. Multiple active processes have been implicated to facilitate this transfer. One process includes the release of extracellular vesicles or microvesicles into the extracellular space where they can be internalized by other cell types. Another is the transfer of organelles, proteins and nucleotides through cytoskeletal protrusions known as tunneling nanotubes (TNTs) which have been observed to form between microglia, astrocytes and neurons.

This cell-to-cell communication and organelle transfer has been shown to serve various purposes including to enhance cell viability, enhance degradation of dysfunctional organelles and modulate glial-mediated neuroinflammation which may contribute to a rescue of disease pathology in various diseases. Macrophages have been demonstrated to serve as a therapeutic agent in a lysosomal storage disorder called cystinosis, as transplantation of macrophage-progenitors into cystinosis mice demonstrated a rescue of the disease phenotype. When macrophages were co-cultured with cystinosis-deficient fibroblasts, GFP-tagged cystinosin was visualized being transferred from macrophages to cystinosin fibroblasts via tunneling nanotubes. A similar rescue was observed in the Y8GR murine model of FRDA after transplantation of wildtype hematopoietic stem cells (HSCs) dampened the hallmark features of FRDA. Therefore, it has been proposed that microglia could provide similar therapeutic support in FRDA via the transfer of healthy mitochondria and frataxin to FRDA cells.

After co-culturing CRISPR/Cas9 edited microglia with FRDA microglia, mitochondria from edited microglia were detected within FRDA microglia suggesting mitochondrial transfer.
between the cells. FRDA microglia were more likely to receive healthy mitochondria when compared to healthy microglia suggesting the delivery of mitochondria to these mitochondrial dysfunctional cells may serve a rescue purpose. To also investigate the transfer of frataxin protein, an FRDA iPSC line was transduced with a lentivirus expressing the FXN protein fused to the fluorophore, GFP. Transduction of FRDA iPSCs with FXN-GFP resulted in a rescue of the FRDA morphological phenotype. When FXN-GFP microglia were co-cultured with FRDA microglia, FXN-GFP was observed within FRDA microglia demonstrating FXN transfer between microglia. FRDA microglia were also demonstrated to simultaneously receive both mitochondria and FXN indicating that FXN-bearing mitochondria are transferred rather than the independent transfer of FXN protein. The transfer of mitochondria and FXN to neurons was also queried using an in vitro co-culture model. Mitochondria from edited microglia were observed within FRDA neurons demonstrated a possible delivery of healthy mitochondria to FRDA neurons. When FXN-GFP microglia were co-cultured with FRDA neurons, FXN-GFP was observed outside of microglia indicating the potential of FXN transfer to other neuronal or glial cell types. While requiring further investigation, microglia have demonstrated the potential to transfer healthy frataxin-bearing mitochondria to frataxin deficient FRDA cells and therefore present the ability to serve as a therapeutic vehicle in FRDA. The mechanism of this transfer stands to be further investigated with tunneling nanotubes serving as a potential mechanism.

4.4 Limitations and Future Directions

Further experimentation is required to further characterize the impact microglia have on neuronal viability and the consequences this has in FRDA pathology. NPCs were utilized to generate a heterogenous neuronal culture to study microglia-neuronal interactions, however, this does result in the generation of a variety of neuronal cell types including neurons,
astrocytes and oligodendrocyte precursors. Therefore, the generation of induced neurons from iPSCs could allow for a co-culture model of strictly neurons and microglia to eliminate the potential influence of other cell types on microglia-neuronal interactions. Additionally, the impact of FRDA microglia on astrocytes has yet to be characterized and therefore a co-culture of microglia and astrocytes could allow for further study of these interactions in FRDA. Media conditioned by microglia at homeostatic conditions was isolated and added to neuronal cultures to study the impact of cytokines and soluble factors on neuronal apoptosis. However, a more physiological relevant model of microglia cytokine release could utilize a transwell culture system to allow microglia and neurons to share culture media while preventing direct cell-to-cell contacts. Additionally, Purkinje cell survival in the cerebellum after FRDA iHPC transplantation was characterized, however, the mechanism of this microglia-mediated neurodegeneration stands to be further studied. Microglia-mediated clearance activity is vital to the establishment of cerebellar circuits and Purkinje cell development\textsuperscript{18} therefore the impact FRDA microglia have on synaptic remodeling and on the excitability of cerebellar neurons requires further investigation.

Preliminary experiments to investigate mitochondrial and frataxin transfer were completed, however, these experiments served as proof of concept and require more biological replicates to draw significant conclusions. Additionally, a control was only utilized in mitochondrial transfer to microglia and therefore all other experiments require a condition with healthy cells serving as acceptors to better understand if organelle and frataxin transfer is truly dependent upon frataxin deficiency and mitochondrial dysfunction. Additional limitations of mitochondrial transfer experiments include the unreliability of MitoTracker as it has been shown to accumulate in other membrane structures and signal can be influenced by mitochondrial membrane potential and oxidative stress\textsuperscript{61}. Therefore, a more reliable indicator of mitochondrial
transfer could be the utilization of a fluorescently tagged mitochondrial iPSC line that could be utilized for transfer experimentation *in vitro* and *in vivo*. Additionally, frataxin transfer was difficult to detect and confirm in microglia-neuron co-cultures and *in vivo*, therefore, utilization of a mitochondria marker such Tomm20 or MitoTracker in conjunction with FXN-GFP transfer experiments could help to differentiate and confirm true transfer from GFP precipitates. GFP was also identified outside of microglia and TUJ1-positive neurons indicating potential transfer to other unidentifiable cell types. Staining with additional cell markers including glial fibrillary acidic protein (GFAP) for astrocytes, nestin for NPCs and myelin basic protein (MBP) for oligodendrocytes could reveal if transfer occurs to other neuronal and glial cell types. Finally, the mechanism of mitochondria and frataxin transfer is yet to be investigated. To determine if tunneling nanotubes are responsible for this transfer, confocal timelapse can be utilized to identify the movement of FXN-GFP within F-actin and Connexin43 positive projections. Additionally, electron microscopy can be utilized to visualize TNTs and the specific organelles transferred between cells.
Chapter 5.
CONCLUSION

Overall, FRDA microglia have been characterized by an inflammatory and hyperphagocytic disease phenotype as well as defects in oxidative phosphorylation. Investigating microglia-neuron interactions in a co-culture model revealed that healthy neurons co-cultured with FRDA microglia or FRDA microglia conditioned media demonstrated higher incidences of caspase-3 mediated apoptosis. These findings were recapitulated in vivo as xenotransplantation of FRDA microglia progenitors into a murine model resulted in reduced Purkinje cell survival in the cerebellum. Overall, FRDA microglia were implicated to have a detrimental effect on neuronal viability which could contribute to pathophysiology in FRDA patients. The therapeutic potential of microglia was also established as CRISPR-Cas9 edited microglia demonstrated transfer of mitochondria to FRDA microglia and neurons and FXN-GFP microglia demonstrated transfer of frataxin to FRDA microglia. Therefore, healthy microglia have demonstrated the potential to serve as a therapeutic vehicle by delivering mitochondria and frataxin to FRDA cells.
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Appendix A.

PROTOCOLS

A1. iHPC Differentiation

iHPCs from iPSCs/hES using STEMdiff Hematopoietic Kit (StemCELL Technologies Cat#05310)

(Estimating HPC yield $0.6 \times 10^6$ - $1 \times 10^6$/well)

Day 0 → Ideal culture is 70-80% confluent, larger colonies are better

Media: 14mL mTESR, Rocki (1:1000) / 6-well plate

☐ Wash cells with dPBS x1 (no calcium or magnesium)

☐ Add 1mL/ReLeSR well, aspirate immediately

☐ Incubate at 37 C for 3 min

☐ Add 1mL media and tap plate several times until colonies come off

☐ Transfer colonies with p1000 to a 15mL conical

☐ Wash well with additional 1mL media and add to conical

☐ In 15mL conical, take 5-10μL (depending on starting confluency) from ~ top $\frac{1}{3}$ of cell suspension and plate onto each well of 6-well plate

Goal is aggregates of 100-200 um diameter (50-200 cells clumps),

(Achieving ~40 colonies/well in 24 hours as day 0

If colonies are too small and wait one more day)

Day 1 → Picking wells, changing to Media A

Media: 5μL of Supplement A per 1mL of basal media (1:200), 2mL of media A per well of a 6 well

☐ Choose wells that are sparse with colonies that are a “good size” (see photo above)
Want ~3-5 colonies per field of view, colonies should have plenty of space to grow.
  - Don’t want the colonies to be too crowded! Sparser is typically better but may be line dependent

**Day 3** → Add 1mL/well of Media A

**Day 4** → Changing to Media B

  Media: 5μL of Supplement B per 1mL of basal media (1:200), 2mL of media B per well of 6 well

  - Aspirate media A and change to media B
  - Want the colonies to look like a “fried egg” (see photo)
  - If the colonies do not look ready, can wait an extra day (day 5) to change media to B
    - Do not wait past the extra day (day 5) to switch to media B!

**Day 6**: Add 1mL/well of Media B

**Day 8**: Add 1mL/well of Media B

**Day 10**: Add 1mL/well of Media B

**Day 11-14**: Collect cells day 11-13 or 14

  Collect iHPCs and plate at 200-300k/well of 6 well in 2mL of iMG media with cytokines
A2. iMG Differentiation and Culture

Microglia Culture

- Feed with 1mL/well M/W/F (don’t remove any media)
- Once wells become very full then remove some of the media (can take off, centrifuge, and add back in 1mL of old media (never want to remove ALL the media), or I find that the cells settle to the bottom so you can aspirate off the top a fair bit and then start adding 1mL/well again.
- Use cells on Day 28 for sort or end application.

Passaging Microglia

1. Collect majority of microglia media from well and add to 15 mL conical (leave ~1 mL)
2. Scrap cells with cell scraper and add to 15 mL conical
3. Centrifuge for 5 minutes at 0.3 rcf
4. Aspirate off media and resuspend in 2 mL PBS to count
5. After counting, centrifuge and resuspend in iMG media to plate

A3. NPC and Neuron Culture

Passaging NPCs to 8-well chamber slides

NOTE: Pass at 90-100% confluency and at 1:4-1:6 ratio

Beforehand: Prepare 3x Matrigel coated slides

a. 1 mg of Matrigel in 8 mL of DMEM
b. 200 uL/well

1. Warm accutase (1 mL/well), DMEM-F12 (2 mL/well), NPC D24+ media
2. Aspirate off Matrigel from slides and add D24+ with 75 uL/well
3. Aspirate off media from wells

4. Add accutase (1 mL/well)

5. Incubate 3-5 minutes in 37°C (depending on when cells detach, can tap gently)

6. Add 2 mL/well of DMEM to accutase and move cell solution to 15 mL conical

7. Spin for 2 min at 0.5 rcf

8. Aspirate supernatant and resuspend in NPC D24+ media
   a. For 1:5 split: resuspend in 5 mL of D24+, take 1 mL and divide between 8 wells of chamber slide with 125 μL/well

9. If they look good and are 30% confluent then change to neuron media the next day to start differentiation

**NPC to Neuron Differentiation**

**Day 0:** Change to neuron media

- Feed M/W/F
- When feeding do not tilt the slide! Keep flat and leave some media when aspirating

**Day 7:** Change media to co-culture media and add mature iMG

- Feed M/W/F
- Don’t aspirate off media from here on out, just add on top!

**Day 14:** Neuron differentiation is complete and ready to use for assays
A4. Adding iMG to Co-Culture

1. Exchange neuron media in wells for co-culture media
   a. Remove 100-150 uL leaving a good amount of neuron media (~100-150 uL)
   b. Add 100 uL of co-culture media by adding media to side of well (not dropwise)

2. Collect media from iMG wells and add to 15 mL conical, leave 2 mL

3. Scrape iMG and add media to 15 mL conical

4. Spin at 0.3 rcf for 5 min

5. Aspirate off supernatant and resuspend in 2 mL PBS

6. Count

7. Determine # of cells / 1 uL

8. Determine the amount of cell solution to collect that contains the desired total amount of cells

9. Add this volume to a 0.5 mL tube (easier to see pellet)

10. Spin 0.5 mL tube and original 15 mL conical at 0.3 rcf for 5 min

11. Determine the volume of co-culture media to resuspend in

12. Aspirate off PBS and resuspend in co-culture media according to math above

13. Distribute to wells gently

14. Aspirate off PBS from 15 mL conical and resuspend in iMG media and replate back into a new well
A5. NPC Phagocytosis Assay

1. Day before – plate iMG in 96-well primeria plate in quadruplicate (10k/well)

2. Stain iMG with lectin
   a. Dilute lectin 1:1000 in iMG media – enough to make 100 uL/well
   b. Remove iMG media from 96-well and replace with lectin solution (100 uL/well)
   c. Incubate plate for 40 min at 37C
   d. Wash twice with PBS
   e. Add 100 uL of iMG media/well

3. Stain NPCs with pHrodo (Start as soon as iMG are incubating with lectin)
   a. Aspirate NPC media from well and add 1 mL of accutase
   b. Incubate for 3 min at 37C and then tap lightly to dissociate cells from plate
      (should see them come off bottom of plate)
   c. Add 2 mL of DMEM to well and collect and spin at 0.5 rcf for 2 min
   d. Make pHrodo solution
      i. Make 10 mL of 0.1 M NaHCO$_3$
      ii. Add 1 uL of pHrodo
   e. Aspirate supernatant and resuspend in 10 mL of pHrodo solution
   f. Incubate in tube at room temperature on shaker for 40 min
   g. Wash twice with PBS
   h. Resuspend in iMG media so 100 uL/well

4. Take single image of plate prior to adding NPCs

5. Spike in NPC solution (100 uL/well) and image on incucyte every 20 min for 4 hours
A6. Immunocytochemistry

Day 1

1. Fix in 4% PFA for 30 minutes
2. Rinse with PBS 3 times, 5 min each
3. Block with 10% Normal Donkey Serum (NDS) + 0.5% Triton for 1 hour
4. Incubate with primary antibodies diluted in 3% NDS + 0.5% Triton overnight at 4C

Day 2

1. Wash with PBS 3 times, 5 min each
2. Incubate with secondary antibody diluted in 3% NDS + 0.5% Triton for 1 hour at room temperature
3. Rinse with PBS with 1:1000 DAPI for 10 minutes
4. Wash with PBS 3 times, 5 min each
5. Mount with ImmunoMount