UNIVERSITY OF CALIFORNIA SAN DIEGO

# Role of Microglia in Rett Syndrome Pathogenesis using induced-Pluripotent Stem Cells

A Thesis submitted in partial satisfaction of the requirements for the degree
Master of Science

in

Biology

by

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2019

The Thesis of Jacob John Jeziorski is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Co-Chair

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Chair

University of California San Diego

2019

# DEDICATION

I dedicate this thesis to my family and friends who were there for me. This journey has allowed me to realize how much I value and am grateful towards the meaningful connections and relationships in my life. These connections and the people a part of them are what made this achievement possible. I love you all.

TABLE OF CONTENTS

# LIST OF ABBREVIATIONS

BBB: blood brain barrier

bFGF: basic fibroblast growth factor

BMP4: bone morphogenetic protein 4

BSA: bovine serum albumin

CD14: cluster of differentiation 14

CNS: central nervous system

CSF-1R: colony stimulating factor 1 receptor

CTRL: control

DPBS: Dulbecco's phosphate-buffered saline

ELISA: enzyme-linked immunosorbent assay

FLT3L: Flt-3 ligand

GFP: green fluorescent protein

GM-CSF: granulocyte-macrophage colony-stimulating factor

GSN: gelsolin

IGF-1: insulin-like growth factor 1

IL-1β: interleukin 1β

IL-3: interleukin 3

IL-34: interleukin 34

IL-6: interleukin 6

iPSC: induced-pluripotent stem cell

ITGB2: integrin subunit beta 2

LPS: lipopolysaccharide

MAP2: microtubule associated protein 2

MCP-1: monocyte chemoattractant protein-1

M-CSF: macrophage-colony stimulating factor

*MECP2*: methyl-CpG-binding protein 2

MIP-1α: macrophage inflammatory protein 1-alpha

NPC: neural progenitor cell

P/S: penicillin-streptomycin

PPARG: peroxisome proliferator activated receptor gamma

RTT: Rett syndrome

SCF: stem cell factor

TPO: thrombopoietin

TREM2: triggering receptor expressed on myeloid cells 2

VEGF: vascular endothelial growth factor

VGLUT1: vesicular glutamate transporter 1

LIST OF SCHEMAS

LIST OF FIGURES

# ACKNOWLEDGEMENTS

I would like to acknowledge Alysson R. Muotri for allowing me to be a part of his lab. I am fortunate to work under such a visionary and ambitious scientist and innovator. The same visions he has in his projects, he shares in the visions he has in the members of his lab. I look forward to upholding those visions led by the example of my future actions. Thank you for your support.

I would like to acknowledge Susan Ackerman and Ashley Juavinett for the time and effort to be on my committee. The encouragement and support were greatly appreciated to help guide me to this point.

I would like to acknowledge Pinar Mesci for allowing me to work on such a novel and ambitious project alongside her. Her daily mentorship has shaped me into the scientist I am today. Thank you for your guidance and patience.

The results shown in this thesis are part of a project conceived and designed by Dr. Pinar Mesci under the supervision of Professor Alysson Muotri. Mesci, Pinar; Jeziorski, Jacob John; Nizet, Victor; LaRock, Christopher; Alysson, Muotri. They will be included in a future submission for publication, in which the thesis author would be recognized as a co-author.

ABSTRACT OF THE THESIS

Role of Microglia in Rett Syndrome Pathogenesis using induced-Pluripotent Stem Cells

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Jacob John Jeziorski

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Professor Alysson R. Muotri, Chair
Professor Susan Ackerman, Co-Chair

Microglia, the tissue-resident macrophages of the central nervous system (CNS), are the first line of defense against injury and disease. Given their unique ontology and context-specific functions, they might play key roles during neurodevelopment. Rett syndrome (RTT) is a progressive neurodevelopmental disorder characterized by a mutation in the methyl-CpG-binding protein 2 (*MECP2*) gene, whereupon affected patients can develop a range of behavioral and neurological symptoms. Although *MECP2* is expressed in many different cell types, RTT is attributed to cell autonomous effects from a primary neuronal dysfunction. The role of microglia in RTT pathology has been studied with an ongoing controversy in mouse models. Here, we aim

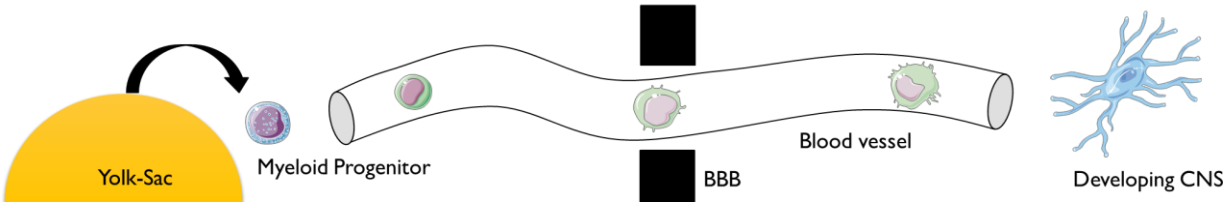
to decipher if human microglia have an impact in RTT pathogenesis by using induced-pluripotent stem cells (iPSCs). We utilized high throughput, multiplex experiments in a holistic approach to investigate the inflammatory signature of RTT microglia in gene expression and cytokine release. Consequentially, we observed genes differentially expressed in RTT microglia with no clear inflammatory phenotype. Additionally, we observed a significant decrease in RTT microglia's phagocytic ability, congruent with our gene expression analysis. Finally, we increased the complexity of our model by establishing a neuron-microglia co-culture system to study the impact of microglia on neuronal synaptogenesis in both healthy and RTT conditions. Altogether, our data suggests that the lack of *MECP2* in microglial cells alters its functions, which might be implicated in RTT pathogenesis and that they can potentially be targeted to develop novel therapies for RTT patients for whom there is currently no cure.

# INTRODUCTION

## 1. Microglial Cells – Tissue-Resident Macrophages of the CNS

### 1.1 Embryogenesis

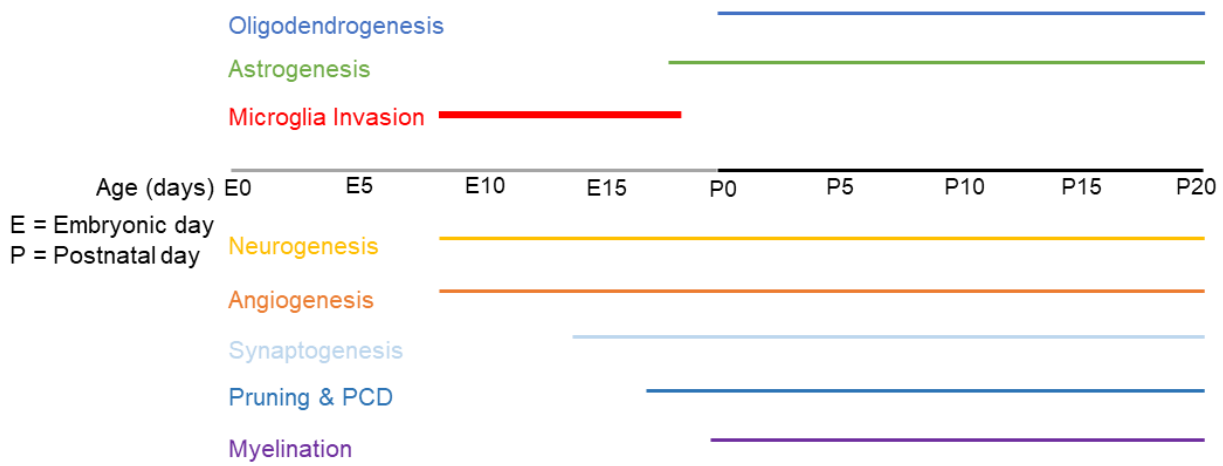
Microglia, known as the tissue-resident macrophage of the central nervous system (CNS), have a unique ontogeny and development aside from their cellular counterparts of the CNS, which may contribute to their distinct, physiological role in early embryonic neurodevelopment and overall brain homeostasis. Cell-lineage tracing in mouse models have shown that microglia are the only myeloid cells to be derived from primitive macrophages originating in the yolk-sac during embryogenesis (Perdiguero et al. 2018; Sheng, Ruedl, and Karjalainen 2015). From the yolk-sac, these erythromyeloid precursors migrate through the blood vessels into the newly developing CNS before the formation of the blood brain barrier (**Schema 1.**; Ginhoux et al. 2010).




**Schema 1**. Schematic showing the emergence of microglial cells and their CNS invasion during embryogenesis.

Consequentially, microglia are the only cell type of the CNS to be derived from the unique mesodermal ontogeny, rather than being derived from the ectoderm germ layer such as the other cell types of the CNS. Furthermore, the microglial invasion of the CNS has been shown to occur during key neurodevelopmental timepoints starting from neurogenesis to synaptogenesis and network maturation and preceding gliogenesis, speculating that microglia could have a rather crucial regulatory role during the maturation of the brain (**Schema 2.**; Reemst et al. 2016). A

global gene expression analysis of microglia using mouse models has revealed evidence of distinct stepwise developmental phases during microglia maturation, suggesting that microglia may have specific time-dependent characteristics and functions on different stages throughout early neurodevelopment (Matcovitch-Natan et al. 2016).




**Schema 2.** Timeline of embryogenesis in mouse, highlighting microglia invasion into the CNS overlaps and precedes important neurodevelopmental stages.

After the blood brain barrier is formed, there is minimal cell traffic to travel into the CNS leaving the regulation and maintenance of microglia to be solely-dependent on their capacity for self-renewal from cells of the CNS throughout the lifespan of the brain (Ajami et al. 2007). The self-organization and regional differences observed by microglial cells using a multicolor fate mapping system to monitor microglial dynamics has provided more evidence of their turnover rates and capacity for self-renewal in the CNS (Tay et al. 2017). With more advancements in technology and research, the additional role that microglia play in early embryonic development and maintenance is becoming progressively more understood. This insight can be used to discover the impact of microglia not only on a healthy brain, but on a diseased one. These observations of microglial migration and invasion of the CNS shown in mice have become a

catalyst towards developing and optimizing new models to study microglia in a human context and better understand their impact in embryogenesis. These new observations in a human context can additionally help identify the role microglia may have in the progression of neurodevelopmental diseases and become a selective target for cell therapy and disease treatment.

### 1.2 Context-Specific Differentiation and Function in the CNS

There are current challenges and interest to characterize the numerous subsets of heterogenous macrophage populations in different tissues and distinguish which molecular elements are necessary towards the nature of their context-specific differentiation and function. Tissue-resident macrophages are a heterogeneous population of immune cells found in most tissues and have crucial regulatory components to influence tissue homeostasis in development and maturation (Davies et al. 2013). These subsets of macrophages typically have a strong overlap in their overall development and function, however it is important to better distinguish what influences the specificity of their role in particular tissues. It has been shown that the niche microenvironment where tissue-resident macrophages reside drive their context-specific differentiation and function (Gosselin et al. 2017; Butovsky et al. 2014; Gosselin et al. 2014). Specifically, when transferring *ex vivo* human primary microglia samples to an *in vitro* culture, researchers observed a noticeable downregulation of microglia-specific genes to provide evidence for how the expression of microglia are strongly driven by their microenvironment. However, they provided additional evidence that suggests specific transcription factors can partially rescue this dysregulation to establish microglia identity and function within an *in vitro* culture (Gosselin et al. 2017). Furthering our knowledge of which specific molecular elements within the microenvironment of the CNS are responsible for the differentiation and maintenance

of microglia will assist in optimizing *in vitro* culture conditions to more accurately study their role in brain development and disease pathology.

Notably, the transmembrane tyrosine kinase receptor, known as colony stimulating factor 1 receptor (CSF-1R), has been shown to be of great importance in overall brain health and microglial survival (Erblich et al. 2011; Elmore et al. 2014). By using a CSF-1R-GFP reporter mouse strain, researchers were able to discover that the expression of CSF-1R was observed solely in microglial cells compared to other cell types of the CNS. Furthermore, the absence of CSF-1R in mice resulted in the ablation of microglia found in the CNS and a significant decrease in mouse survival toward adulthood (Erblich et al. 2011). The growth factor macrophage-colony stimulating factor (M-CSF; also known as CSF-1) is known to primarily target CSF-1R to promote proliferation, differentiation, and function of multiple different tissue-resident macrophage cells (Elmore et al. 2014). To investigate if the physical interaction between CSF-1 and CSF-1R is necessary for these phenotypes seen in microglial differentiation and function, researchers generated a mouse deficient in CSF-1 and were still able to observe the presence of microglia in the CNS, suggesting that alternative signaling molecules must be sufficient to promote microglia differentiation and survival (Wang et al. 2012). Remarkably, the team of researchers discovered that the presence of the CSF-1R alternative ligand, interleukin 34 (IL-34), is also responsible for the survival of microglia (Wang et al. 2012). They were able to pinpoint that neurons and other glial cells within in the CNS were the source to release IL-34 into the microenvironment to influence the differentiation of myeloid cells in the CNS (Wang et al. 2012). A mouse model looking at individual mRNA expression patterns of both CSF-1 and IL-34 transcripts in myeloid cells from the embryonic brain of mice showed different spatiotemporal activity to suggest that the ligands roles in CSF-1R activation is potentially

complementary rather than solely redundant *in-vivo* (Wei et al. 2010). These findings from mouse models are crucial towards optimizing the conditions necessary for an *in vitro* culture environment that would allow for the successful generation and maintenance of microglia for future human *in vitro* studies.

As the immune cells of the CNS, it is no surprise microglia have a necessary role as a defensive mechanism for CNS protection, however there is a striking amount of evidence which shows microglia having additional roles in the development of neuronal circuits and constant synaptic modulation in the developing and adult brain in mouse models. Microglia have the typical function of a tissue-resident macrophage as the primary immune response to an invading pathogen through their phagocytic capability, but additionally have context-specific functions in neuronal health and synaptic plasticity not observed in roles acquired in a tissue-specific manner as observed in mouse models. Microglia have morphological characteristics, such as their ramified branches and motile processes, which allow them to be vigilant scavengers of the CNS parenchyma in order to come into physical contact and phagocytose any harmful foreign materials or cellular debris that could come from damage or disease (Nimmerjahn, Kirchhoff, and Helmchen 2005; Davalos et al. 2005). Interestingly, this morphological dynamic of surveying the CNS parenchyma has an auxiliary role to monitor the functional state of synapses, suggesting there are salient interactions between microglia and synapses that can modulate the neuronal circuitry (Wake et al. 2009). During postnatal development in mice, these highly motile phagocytic cells have been observed to engulf synaptic material and have major impact in synaptic pruning, further linking microglia surveillance to synaptic maturation (Paolicelli et al. 2011). Discovering the context-specific functions of microglia that play a role in neurodevelopment is important in the field of neuroscience. However, these findings from

previous literature are typically made from mouse models, limiting the full extent of how these observations could derive reliable and effective human therapeutics. New discoveries using human models can provide a deeper understanding of the context-specific function of microglia and if their dysfunction could be a predominant instigator in neurodevelopmental diseases.

## 2. Rett Syndrome

### 2.1 Background

In 1966, Rett syndrome (RTT) was characterized as a rare neurodevelopmental disorder that occurs in about 1 in 10,000 females with a range of symptoms manifesting over several developmental stages in early life (Haas 1988). Initially, postnatal neurological and behavioral development seem typical until about 6 to 18 months when signs of subtle developmental stagnation in motor and language ability start to emerge. This is followed by a rapid regression and a late motor deterioration phase characterized by a lack of purposeful hand skills, motor impairment, and hypotonia, progressing in severity in affected children over the age of 10 (Haas 1988). Individuals with RTT may also show autistic-like behaviors and experience seizures, along with a broad range of other behavioral phenotypes. There is currently no cure for RTT patients, only limited treatments to control specific symptoms.

### 2.2 *MECP2*

In 1999, a loss-of-function mutation in the X-linked gene encoding methyl-CpG binding-protein 2 (*MECP2*), located at Xq28, was identified as the biological cause in the majority of RTT cases, by using a systemic gene screening approach (Amir et al. 1999). *MECP2* functions as a global transcriptional regulator in methylation regions of DNA by binding to two distinct domains, a methyl-CpG-binding domain and a transcriptional repression domain, to

epigenetically modify transcriptional activity and influence CNS maturation. Since *MECP2* is inherited on the X chromosome, there is phenotypic variation in RTT due to random differences in X chromosome inactivation and attributes the syndrome's specificity towards predominantly heterozygous female carriers of the *MECP2* mutation. Cases of hemizygous male carriers of the mutation are rare to survive past birth and if so observe more severe phenotypes.

### 2.3 Neuronal Phenotypes

The expression of *MECP2* has been observed at varying levels in every mouse and human tissue with an abundance of expression found within neuronal cells in the brain (Shahbazian 2002). In 2010, Marchetto and colleagues observed striking differences in neuronal phenotypes *in vitro* when comparing neurons derived from RTT patients using induced-pluripotent stem cells (iPSCs) to their parental control. Specifically, iPSC-derived RTT neurons were observed to have smaller soma sizes, fewer synapses, reduced spine density, altered calcium signaling, and electrophysiological defects when compared to their parental control (Marchetto et al. 2010). While this suggests the dysregulation of *MECP2* in neurons as a main driver towards the pathology of RTT, the biological mechanism for how these neuronal deficits transpire is still up for debate. Interestingly, *MECP2* expression has also been observed in glia cells in the CNS, and RTT studies in mice with *MECP2*-deficient glia have been shown to affect neurons in a non-cell autonomous fashion (Ballas et al. 2009; Lioy et al. 2011). As a potential drug therapeutic, administration of insulin-like growth factor 1 (IGF1) has been shown in mouse and human iPSC models to partially rescue RTT phenotypes, such as increasing glutamatergic synapse numbers in iPSC-derived RTT neurons and increasing life spans in RTT mice (Tropea et al. 2009; Marchetto et al. 2010). Clearly, by better understanding the biological components and

cellular mechanisms behind the pathogenesis of RTT, more effective drug and cell therapies can be discovered for RTT patients.

### 2.4 Microglial Influence

The biological mechanisms and pathways which impact RTT neuronal phenotypes during early neurodevelopment are still under debate and investigating the other cell types of the CNS has become an increasing interest for researchers to provide additional insight into more effective cellular therapies for RTT. With their phagocytic capability within the CNS, microglia have been observed to play a major role in the formation of synapses on the axons of neurons during early neurodevelopment and begs the question if disruption of the aforementioned microglia-mediated synaptic pruning in diseased conditions contributes to neurodevelopmental disorders (Wake et al. 2009). Transplantation of wild-type bone marrow into irradiation-conditioned *Mecp2*-null mice was observed to arrest RTT pathology with successful engraftment and differentiation of microglia in brain parenchyma, suggesting bone marrow transplantation as a feasible approach for RTT therapy (Derecki et al. 2012). Additionally, targeted *MECP2* expression in microglia in *Mecp2*-null mice attenuated disease symptoms with striking improvements in breathing, lifespan, body weight, and locomotor activity (Derecki et al. 2012). These results were not observed when phagocytic activity was blocked via pharmacological inhibition, suggesting these benefits are contingent on microglial phagocytic activity (Derecki et al. 2012). However, there is an ongoing controversy with the effectiveness of the experiments and mouse models used to produce these impressive findings. Several different groups of researchers were unable to reproduce this arrest in RTT pathology or attenuate disease symptoms when using similar experiments on *Mecp2*-null background mice (Wang et al. 2015). Briefly, these experiments consisted of three different RTT mouse models for bone marrow

transplantation (one of which was from the same background as the previous publication) and used a different transgene for targeted gene expression of *MECP2* which they showed had an increased efficiency and specificity and, as a result, these experiments were unable to reproduce the same attenuation in RTT pathology as reported by Derecki et al. (Wang et al. 2015). This controversy in mouse models shows limitations of these models towards understanding the role of microglia in RTT. Studying RTT by switching to a model within a human context could provide more reliable and accurate findings to further the field in RTT research and remedy these conflicting results found in mouse models.

## 3 Induced-Pluripotent Stem Cell Disease Modelling

The discovery of four specific transcription factors—Oct4, Sox2, Klf4 and c-Myc (known as the Yamanaka factors)—to reprogram somatic cells back into their early embryonic state has revolutionized the field of research by allowing scientists to study cells and diseases more accurately in a human context (Takahashi and Yamanaka 2006). Providing a robust *in vitro* cell culture environment to study in a human context allows stem cell research to provide additional insight when certain challenges and limitations are found in animal models. Particularly, in the field of neuroscience, stem cell research has allowed scientists to study the development of different cell types of the CNS and model neurodevelopmental diseases by reprogramming somatic tissue from patients already predisposed to the disease, thus conserving the genetic background and harvesting diseased cells in a less invasive manner. While this new approach has already pushed the field of neuroscience forward dramatically, there are still shortcomings within this methodology to overcome. *In vitro* cell culture conditions must appropriately recapitulate the microenvironment of the CNS and studying a single cell type within a dish does not appropriately model the complexity of interactions involved within the

developing and adult brain. Optimizing these *in vitro* conditions and increasing the complexity of these iPSC disease models to more accurately recapitulate the human brain will continue to advance the research and development in the field of neuroscience and could yield to the discovery of new altered pathways and novel therapies.

Isolating and studying primary human microglia has been a major challenge in the field of neuroscience with difficulty in characterizing primary human microglia and observing major differences when compared to primary mouse microglia *ex vivo* (Gosselin et al. 2017). As such, there has been a need to optimize stem cell differentiation protocols to mimic the microenvironment of the CNS and reliably establish the identify and function of iPSC-derived microglia *in vitro*. Recently, different models for efficient derivation of microglia-like cells from iPSCs have been successfully produced with comparable characterization, and effectively used to model diseases of the CNS, such as Alzheimer's disease (Muffat et al. 2016; Abud et al. 2018). With this exciting new ability to effectively derive microglia-like cells from human iPSCs, we set forth to better understand and study the expression, function, and neuro-immune interactions at play of iPSC-derived RTT microglia.

# MATERIALS AND METHODS

**Patient Consent**

Donated CTRL and RTT fibroblasts were obtained via skin biopsies from patients after informed consent was appropriately given under protocols approved by the University of California, San Diego Institutional Review Board (#141223ZF).

**Differentiation of iPSCs into microglial cells and cortical neurons**

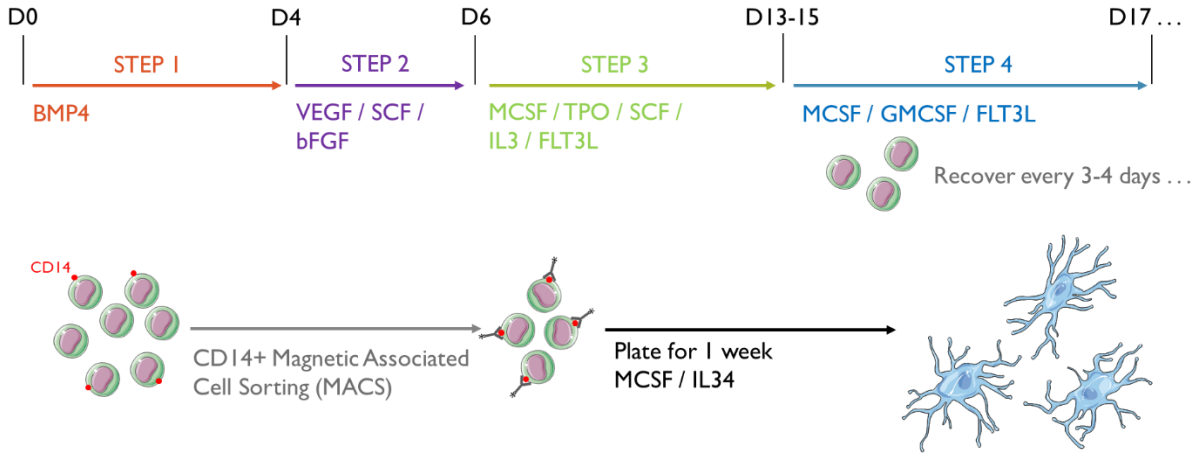
Human iPSCs were thawed and cultured on Matrigel (Corning, 1:100) coated 6cm cell culture plates (Fischer Scientific). iPSCs were then expanded weekly via mechanical passaging and fed daily with mTeSR1 (StemCell Technologies). Multiple cell lines were used for cell differentiation and data was combined into two categories characterized as control (CTRL) and RTT patient (noted as RTT) cell lines to increase the sample size and validity of our results (unless stated otherwise). We used two RTT cell lines: a RTT patient with a stop codon mutation ($MECP2^{Q83X}$) and a isogenic line to a patient with *MECP2* duplication syndrome, where both copies of the *MECP2* gene were knocked out via CRISPR/Cas9 gene editing technologies. We used four CTRL cell lines: two parental controls from RTT patients, two isogenic lines to either the RTT patient cells or the patient cells with *MECP2* duplication syndrome, where the mutation on the *MECP2* gene in the RTT patient cell line was corrected and one *MECP2* gene from the *MECP2* duplication syndrome patient cell line was knocked out via CRISPR/Cas9 gene editing technologies.

Microglial cells were differentiated from hiPSC based on a protocol developed in the Muotri Laboratory (Mesci et al. 2018), which was modified using two previously published papers (Yanagimachi et al. 2013; Douvaras et al. 2017). In the first step, BMP4 (80 ng/mL) was introduced in mTeSR media (StemCell Technologies) when individual iPSC colonies were

approximately 1mm in diameter and had a seeding density of about 30 colonies per plate, typically around 3-4 days after iPSC passaging. Step-1 media was prepared and changed daily for four days. For step two, mTeSR media was switched on day 4 to StemPro-34 SFM (Gibco, 2mM Glutamax and 0.1% Penecillin) introduced with a cocktail of cytokines including: basic fibroblast growth factor (bFGF, 25 ng/mL), stem cell factor (SCF, 100 ng/mL), and vesicular endothelial growth factor (VEGF, 80 ng/mL). Step two media was prepared and changed daily for two days. Using the StemPro-34 SFM medium, the cytokines were switched for the step-3 cocktail of factors including: thrombopoietin (TPO, 5 ng/mL), interleukin-3 (IL3, 50 ng/mL), SCF (50 ng/mL), macrophage-colony stimulating factor (MCSF, 50 ng/mL) Flt-3 ligand (FLT3L, 50ng/mL). After day 6, the media was changed on day 10 for a total of two media changes with the step-3 cytokine cocktail. On day 13-15, cytokines in StemPro-34 medium were changed to the step-4 cocktail of factors including: FLT3L (50 ng/mL), MCSF (50 ng/mL), GMCSF (25 ng/mL). The StemPro-34 SFM medium with step-4 cytokine cocktail was changed every 3-4 days. After these four steps of differentiation, the monocytic lineage-derived cells can be recovered from the supernatant of the conditioned medium every 3-4 days and positively sorted via Magnetic Associated Cell Sorting (autoMACS pro, Miltenyi Biotec) with $CD14^+$ labeled MicroBeads (Miltenyi Biotec). The membranes of $CD14^+$ sorted iPSC-derived microglia were then labelled with a red membrane-labelling dye, PKH26 (Sigma-Aldrich), diluted in a 2X solution of Diluent C (Sigma-Aldrich) following the instructions of the manufacturer.

Human Neural Precursor Cells (NPCs) were thawed and cultured on 10cm cell culture plates coated with 10 µg/mL poly-L-ornithine (Sigma-Aldrich) and 2.5 µg/mL laminin (Life Technologies). Media was changed every two days with NG Media consisting of DMEM/F12 50/50 1X (Corning Cellgro) prepared with 0.5% Penicillin Streptomycin (P/S) (Life

Technologies), 1% HEPES (VWR International), 1% Gem21 NeuroPlex (Gemini Bio-Products), 0.5% N2 NeuroPlex (Gemini Bio-Products), and 1X GlutaMAX (Life Technologies), and supplemented with 20 ng/mL human fibroblast growth factor (noted as NGF medium) (R&D Systems). NPCs were split in a 1:3 ratio onto poly-L-ornithine and laminin coated 10cm plates when confluent, using StemPro Accutase (Life Technologies) for 5 minutes at 37ºC, centrifuged and re-plated in NGF media.




**Schema 3.** Differentiation protocol for CD14$^{+}$ magnetically sorted iPSC-derived human microglia.

**Neuron and microglia co-culture**

Previously characterized NPCs and iPSC-derived microglia after magnet cell sorting and membrane labelling were used for co-culture experiments to be able to track the microglial cells in culture. Glass coverslips (Fisher Scientific) were placed individually onto 24-well cell culture plates coated with 100 µg/mL poly-L-ornithine and 5 µg/mL laminin. NPCs were plated on glass coverslips at 5 x $10^4$ cells per well and NGF media was changed every other day until cells reached 80% confluency. 5 x $10^4$ iPSC-derived microglia after magnet cell sorting and membrane labelling were plated on top of NPCs. To induce neuronal differentiation, bFGF was

withdrawn as a supplement from the NG media and changed every other day. Cells were co-cultured for 6 to 8 weeks until fixation with an observed microglia-to-neuron cell ratio of 1:20 per glass coverslip.

**Immunocytochemistry**

Cells were fixed in 4% paraformaldehyde for 20 minutes and washed three times in Dubecco's Phosphate Buffer Solution (DPBS) (Gibco). Cells were permeabilized and blocked in a diluted DPBS solution (denoted as blocking buffer) with 0.1% Triton X-100 (Promega) and 3% bovine serum albumin (BSA, Gemini Bio-Products) for 30 minutes in 25ºC. Cells were incubated overnight at 4ºC with added primary antibodies diluted in blocking buffer. Primary antibody dilutions were used as follows: anti-Homer1 (Synaptic Systems, 1:500), anti-VGLUT1 (Synaptic Systems, 1:500), anti-MAP2 (Abcam, 1:2000). After three DPBS washes for 5 minutes each, cells were incubated with secondary antibodies for 1 hour at room temperature. Secondary antibodies were used respectively conjugated to Alexa Fluor 488, 555, and 647 at a dilution of 1:1000 (Life Technologies). After 2 DPBS washes for 5 minutes each, nuclei of cells were stained using 4′,6-diamidino-2-phenylindole (DAPI, VWR International, 1:5,000) for 10 minutes. After 2 DPBS washes for 5 minutes each, glass coverslips (Fisher Scientific) were mounted using Prolong Gold Antifade Reagent (Life Technologies).

**Synaptic puncta quantification**

Previously characterized neurons by immunofluorescence and iPSC-derived microglia after magnet cell sorting and membrane labelling were fixed at 6 to 8 weeks and imaged using an Axio Observer Z1 Microscope with Apotome (Zeiss) using compiled z stack images at an objective resolution of 63X. Co-localization of pre- (VGLUT1) and post-synaptic (HOMER1)

markers were quantified manually when in contact with MAP2 at a length of 50µm. Each co-culture combination had at least 10 to 15 images processed from which 10 to 20 MAP2-postive neurons were used for co-localized synaptic puncta quantification from a total of two co-cultured batches.

**Phagocytosis Assay**

After magnetic cell sorting, iPSC-derived microglia were plated on 96-well cell culture plates (Corning) at a cell density of 2 x $10^4$ cells per well. A 0.5 mg/mL solution of pHrodo™ Red zymosan A particles (Life Technologies) diluted in live imaging solution (Life Technologies) was prepared with 2 drops added of NucleoBlue (Life Technologies) and briefly vortexed and sonicated before being incubated with cells for 2 hours at 37ºC. Cells were imaged on an Evos FL Imagine System (ThermoFisher) using a magnification of 4X. Cells were analyzed with a spectrophotometer to measure absorbances of pHrodo Red Zymosan A Particles and NucleoBlue at a wavelength of 589nm and 460nm, respectively. Percentage of cells involved in phagocytosis of zymosan particles was represented as a ratio of the measured absorbances between pHrodo Red Zymosan A Particles and NucleoBlue. Data was obtained in three separate batches and normalized to each individual batch control to limit experimental variations between different batches of experiments. Measured absorbances with no cells plated were subtracted to account for noise.

**RNA Extraction**

Total RNA was extracted from cells using RNeasy Micro and Mini kit for iPSC-derived microglia (Qiagen), following the instructions of the manufacturer. RNA concentrations and purities were recorded using the Nanodrop instrument (Thermo Fisher).

**Gene Expression Analysis**

50ng of total RNA per sample was prepared and subjected to gene expression analysis using the nCounter *SPRINT* Cartridge (Nanostring Technologies) and NanoString nCounter SPRINT Profiler (Nanostring Technolgies, San Diego, CA) following the instructions of the manufacturer. A Human Myeloid Innate Immunity Panel v2 was used to analyze the gene expression of 770 genes in 19 different pathways to incorporate all facets of the myeloid innate immune response. All samples were normalized to a batch of endogenous housekeeping genes. Nanostring results were analyzed using an R-program based nSolver software (Nanostring Technologies, San Diego, CA).

**Enzyme-linked Immunosorbent Assay (ELISA)**

$CD14^+$ sorted iPSC-derived microglia were seeded at 2 x $10^4$ cells per well on 96-well cell culture plates and kept in RPMI-1640 media (Gibco) prepared with 10% fetal bovine serum (FBS, Gemini) and 0.1% P/S. Cells were stimulated with or without treatments of 1 µg/mL Lipopolysaccharide (LPS, Sigma-Aldrich) for one day before 100uL of conditioned media was recovered from each well. Protein levels were quantified using a customized V-PLEX Human Cytokine 30-Plex Kit (Meso Scale Discovery, Rockville, Maryland, USA), consisting of three V-PLEX panels on a 10-spot, 96-well plate format coated with primary antibodies directed against multiple different chemokines and cytokines of interest and were treated with the appropriate detection antibodies, prelabeled with SULFO-TAG™ (Meso Scale Discovery, Rockville, Maryland, USA). Chemokines and cytokines measured from custom multiplex panels consist of: eotaxin, eotaxin-3, IL8 (HA), IP-10, MCP-1, MDC, MIP-1α, MIP-1β, TARC, GM-CSF, IL-12/IL-23p40, IL-15, IL-16, IL-17A, IL-1α, IL-5, IL-7, TNFβ, VEGF-A, IL-10, IL-12p70, IL-13, IL-1β, IL-2, IL-8, TNFα, IL-6. Samples with or without LPS stimulation underwent a 10-fold or

2-fold dilution, respectively, and all samples were run in three or four replicates in three separate batches following the instructions of the manufacturer.

**Statistical Analysis**

Results were analyzed using Prism Software (version 6, GraphPad, USA). Statistical significance was determined using one-way ANOVA tests followed by Tukey multiple comparisons tests to compare different groups and Welch's t-test to compare means of two groups using a $p < 0.05$. The reported values are means ± SEM, as mentioned in relevant figure captions. Sample sizes, n, reported in relevant figure captions.

# RESULTS

**Differential Gene Expression of RTT vs CTRL Microglia**

Currently, there is limited gene expression analysis on iPSC-derived microglia from patients with neurodevelopmental diseases in a human context. Therefore, we performed a comprehensive gene expression analysis using a human myeloid innate immunity panel from Nanostring Technologies to investigate how RTT microglia may have a different inflammatory signature on a gene expression level when compared to CTRL microglia. As previously stated, the nCounter human myeloid innate immunity panel is a custom multiplex panel which contains 770 genes associated with all aspects of the immune response in 19 different pathways and processes across 7 different myeloid cell types.

When we compared the inflammatory signature of RTT vs CTRL microglia on a gene expression level, from the 770 genes in the custom multiplex human myeloid panel, a total of 34 statistically differentially expressed genes were observed, whereupon 14 were significantly upregulated and 20 were significantly downregulated (**Fig. 1A-B**). Extending this analysis to gene ontology, we further identified the top six altered pathways and biological processes involved in innate immunity associated with these significantly dysregulated genes including pathways involved with vascular endothelial growth factor signaling, regulation of macrophage derived foam cell differentiation, phagocytosis and engulfment, etc. (**Fig. 1C**). Specifically, of the 12 genes from the custom myeloid panel associated with phagocytosis and engulfment, 4 were statistically downregulated (**Fig. 1C**). These downregulated genes from RTT microglia were identified as *TREM2*, *PPARG*, *GSN*, and *ITGB2* with their respective fold change and p-values compared to CTRL microglia measured and reported (**Fig. 1D-E**). These results suggest that the lack of *MECP2* in microglia might alter its normal function, particularly phagocytosis, on a gene expression level. However, further analysis of other dysregulated genes and associated

pathways is needed to more comprehensively understand how the inflammatory signature on a gene expression level differ between RTT vs CTRL microglia and, more importantly, how these differences may attribute towards the genesis or progression of RTT.

**Inflammatory profile of RTT vs CTRL microglia using a multiplex ELISA**

As a defense mechanism for CNS protection, activated microglia secrete various chemokines and cytokines to help initiate immune responses and support tissue repair and recovery (Hanisch 2002). Furthermore, the excessive or restricted activation from the dysregulation of microglial inflammatory secretion can cause neurotoxicity and influence the progression of neurodegenerative diseases. Thus, we aimed to investigate if there is an inflammatory phenotype observed in RTT microglia which could be attributed to the progression of RTT pathology. We performed a multiplex ELISA designed with multi-spot detection panels to analyze various chemokines and cytokines released by RTT and CTRL microglia with or without lipopolysaccharide (LPS) stimulation. LPS is found on the cell walls of gram-negative bacteria and is used as an effective tool to activate microglia and study neurotoxicity. As validation to prove the iPSC-derived microglia from the differentiation protocol we used respond appropriately to LPS stimulation, we observed that CTRL microglia stimulated with LPS have an expected increase in various inflammatory molecules (Mesci et al. 2018) (**Fig. 2A**). When we further analyzed the differences in inflammatory cytokine release of CTRL vs RTT microglia with or without LPS stimulation, we observed no significant differences in inflammatory secretion, suggesting that RTT pathology may not be influenced by the neurotoxicity from RTT microglia (**Fig. 2B**). However, from our panel of inflammatory molecules, we were able to identify two chemokines, GM-CSF and MIP-1α, which were significantly decreased and increased, respectively, after LPS stimulation in RTT microglia

compared to CTRL microglia (**Fig. 2C**). While these two chemokines could be of particular impact, overall, our multiplex ELISA experiments resulted in no conclusive inflammatory phenotype in RTT microglia and should be further studied to better understand if microglial neurotoxicity attributes RTT pathology.

**Phagocytosis is impaired in RTT vs CTRL Microglia**

As the tissue-resident macrophages of the CNS, microglia survey the parenchyma of the CNS scavenging for cellular debris and harmful foreign material to engulf and eliminate (Nimmerjahn, Kirchhoff, and Helmchen 2005). Furthermore, this dynamic has been observed to engulf synaptic material and have a major impact in synaptic pruning during early neurodevelopment (Paolicelli et al. 2011). This unique phagocytic capability within the CNS is a key characteristic of an effective immune response, major influence in healthy neurodevelopment, and consequentially may have more of a crucial role in the pathology of neurodevelopmental disorders when normal microglial function is threatened. Therefore, we hypothesized that the lack of *MECP2* in microglia could potentially result in a functional defect in their ability to effectively phagocytose foreign material in the CNS. To test for differences in the phagocytic capability of RTT vs CTRL microglia, we performed a phagocytosis assay on RTT and CTRL microglia by adding a solution of zymosan particles conjugated to a novel pHrodo dye and a live nuclear staining dye, nucleoBlue, to the media with RTT and CTRL microglia for a total of 2 hours and the dye-based fluorescence quantified via spectrophotometry (**Fig. 3A**). Zymosan is a ligand located on the surface of fungi and the conjugated pHrodo red dye was designed to dramatically increase in fluorescence when the pH decreases to become acidic as observed inside the phagosome, making this an effective tool to visualize and study phagocytosis. The ratio of measured absorbances between the zymosan particles conjugated to

pHrodo and the live nuclear staining was used to represent the percentage of microglia undergoing phagocytosis with each experiment normalized to their respective control. As a result, we observed a significant decrease in the percentage of RTT microglia that were able to phagocytose the zymosan particles conjugated to pHrodo (**Fig. 3B-C**). This indicates that RTT microglia have a significant functional impairment in their primary role as immune cells to combat pathogens and harmful material in the CNS. Furthermore, this impairment of phagocytosis in RTT microglia could be an influence towards the pathology of RTT, given the impact microglia have on regulating early neurodevelopment by synaptic pruning and should be further investigated in a more complex model in order to corroborate these findings.

**The impact of microglia on neuronal synaptogenesis in RTT**

Due to the role microglia have in synaptic maturation during early neurodevelopment by engulfing synaptic material (Paolicelli et al. 2011) and the decrease in glutamatergic synapses observed in iPSC-derived neurons from RTT patients (Marchetto et al. 2010), we aimed to further investigate the impact of microglia on neuronal synaptogenesis in RTT. In order to study the impact of microglia on synaptogenesis, neurons and microglia derived from iPSCs were co-cultured in several combinations using two cell lines: the RTT patient with a stop codon mutation (noted as RTT) and a parental control to the RTT patient (CTRL). These cells were co-cultured for 6-8 weeks until maturation and immunostained for neuronal and synaptic markers with images taken on an Apotome microscope (Zeiss) for the quantification of co-localized synaptic puncta (**Fig. 4A**). To confirm the presence and identity of microglia co-cultured with neurons throughout the experiment, we labeled microglia with a red membrane-labelling dye, PKH26, imaged at beginning and end timepoints of the experiment to be able to track them (**Fig. 4B**). As previously mentioned, neurons were immunostained with pre-synaptic marker,

VGLUT1 (red), post-synaptic marker, HOMER1 (green), neuronal marker, MAP2 (white), and DAPI (blue). After imaging the immunofluorescence, we counted co-localized synaptic puncta in contact with MAP2-positive neurons of 50µm in length from the different combinations of co-cultured cell lines (**Fig. 4C**). Neuronal phenotypes of co-localized synaptic puncta counted in CTRL and RTT conditions were reproduced (**Fig. 4C-E**) in iPSCs-derived neurons without co-cultured microglia as validation from previously made observations (Marchetto et al. 2010). Interestingly, we observed an increase in the number of co-localized synaptic puncta for both instances when co-culturing CTRL microglia on CTRL and RTT neurons compared to CTRL and RTT neurons without microglia, respectively (**Fig 4C-E**). When co-culturing RTT microglia on CTRL neurons, we observed a significant decrease in the co-localization of synaptic puncta, suggesting a toxicity of RTT microglia towards synaptogenesis in a healthy neuron (**Fig 4C-E**). Additionally, we observed a significant increase in the amount of co-localized synaptic puncta when comparing the addition of CTRL microglia vs RTT microglia on RTT neurons, suggesting that the synaptic defects observed in RTT neurons could be rescued with addition of healthy microglia (**Fig 4C-E**). Altogether, our data suggests that microglial cells might have a role in synaptogenesis during the human neurodevelopment.

# DISCUSSION

Neuronal phenotypes of RTT in a human context using iPSCs have been previously observed with morphological and functional defects characterized, in part, by a decrease in glutamatergic synapses and alterations in their electrophysiological activity (Marchetto et al. 2010). However, besides the loss-of-function mutation of *MECP2*, the biological mechanism within the CNS which drives these neuronal phenotypes—whether it be of more genetic, epigenetic, or cellular influence—is not fully understood and could provide a novel approach for effective therapeutics for RTT. While the role of other cell types within the CNS has been studied in RTT, the significance that microglia have on RTT is controversial in mouse models (Derecki et al. 2012; Wang et al. 2015). Fortunately, in recent years, robust and reproducible protocols to generate microglia derived from iPSCs have been achieved (Muffat et al. 2016; Abud et al. 2018; Mesci et al. 2018) and can be implemented to study how the lack of *MECP2* in microglia may influence RTT pathology in a human context. Therefore, we used human iPSCs to decipher whether microglia are implicated in the genesis or progression of RTT.

Pharmacological inhibition of phagocytosis in *Mecp2*-null background mice did not result in an arrest in RTT pathology after targeted gene expression of *MECP2* of microglia as previously shown when phagocytosis was functional in microglia (Derecki et al. 2012). These findings suggest that not only do microglia have a role in the progression of RTT, but additionally imply that the neurotypical development of the brain is contingent on the phagocytic ability of microglia. As such, we wanted to further investigate the salience and reproducibility of these results by using iPSC-derived microglia to study RTT in a human context and potentially amend conflicts originated from previous mouse models. Interestingly, our data supported the findings previously observed by providing more insight on the detrimental effects the lack of *MECP2* in human microglia have on their primary function as the immune cells in the CNS,

phagocytosis (Derecki et al. 2012). Here, we observed a significant impairment of phagocytosis in RTT microglia after introducing zymosan particles conjugated with pHrodo. This phenotype we observed was further supported when we investigated the inflammatory signature on a gene expression level of RTT microglia and identified statistically downregulated genes, *TREM2*, *PPARG*, *GSN*, and *ITGB2*, associated to microglial cell's ability to phagocytose and engulf biological material in the CNS. With this promising evidence gathered and its potential link to altering synaptic pruning in neurodevelopment, we speculated that the defects we observed in phagocytosis of RTT microglia could be of consequence to particular RTT neuronal phenotypes, such as decreased synapses and electrical activity, as previously shown due to their impact on neuronal synaptogenesis (Marchetto et al. 2010). We demonstrated this phenomenon by co-culturing microglia and neurons in different cell combinations of healthy and diseased conditions and quantifying the amount of co-localized synaptic puncta. As such, we provided evidence to suggest that the lack of *MECP2* in microglia can detrimentally alter the formation of synapses on healthy neurons and, inversely, that healthy microglia can partially ameliorate this neuronal phenotype observed in RTT. Our findings extend off previous literature observing only cell-autonomous effects that influence the pathogenesis of RTT and provides evidence that RTT pathology could be dependent on a non-cell autonomous mechanism governed by microglia in human models.

Even though we observed significant phenotypes in neuronal synaptogenesis when co-cultured with microglia, more evidence is required to more definitively understand the biological mechanism for how these phenotypes come about from microglia and further link these phenotypes more directly to microglial influence. The functional and genetic defects we observed in phagocytosis of RTT microglia can be interpreted as synonymous to deficits in their

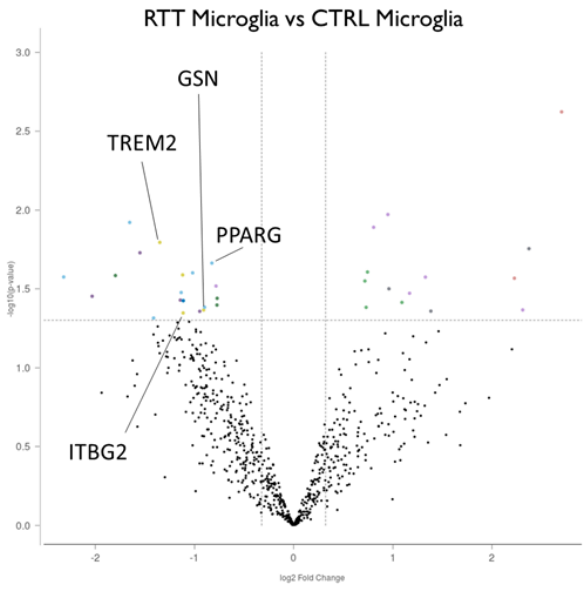
ability to engulf synaptic material during neurodevelopment, known as synaptic pruning. Interestingly, our co-culture results on the impact of microglia on neuronal synaptogenesis translates that the occurrence of synaptic pruning can increase in neuronal synaptogenesis when without synaptic pruning. This dynamic is more apparent when we additionally compared the amount of co-localized synaptic puncta on CTRL or RTT neurons co-cultured with or without CTRL microglia and observed a significant increase in the amount of co-localized synaptic puncta in both CTRL and RTT neurons when co-cultured with CTRL microglia, suggesting that healthy microglia might be engulfing non-functional or faulty synaptic material resulting in the formation of additional functional and healthy synapses. Therefore, we speculate off our findings that microglia's ability to engulf redundant or faulty synapses in early neurodevelopment is necessary overall to form more salient synaptic connections, and future research should be conducted to provide additional support.

While our findings provide additional evidence that microglia have an impact in RTT pathogenesis, they also present new questions to be addressed and provide more traction towards future research. To attribute more of a direct link between microglia influences these phenotypes we observed in neuronal synaptogenesis and is not an artifact of adding any different cell type in culture, we performed identical synaptogenesis experiments of neurons co-cultured with fibroblasts, hypothesizing that there will be no significant difference in the amount of co-localized synaptic puncta counted in CTRL and RTT neurons when co-cultured with fibroblasts. Furthermore, our results on neuronal synaptogenesis provide no direct evidence that the phenotypes we observed are due to the physical interaction or engulfment of synaptic material from microglia and could rather be due to the secretion of inflammatory molecules from microglia. To address this controversy, we gathered the conditioned media from RTT and CTRL

microglia and introduced it to the media of RTT and CTRL neurons in another identical synaptogenesis experiment to quantify the differences in the amount of co-localized synaptic puncta. Our results from the multiplex ELISA provided no conclusive evidence of any inflammatory phenotype from RTT microglia besides a significant decrease in the secretion of GM-CSF and a significant increase in the secretion of MIP-1α after LPS stimulation when compared to CTRL microglia with no clear or direct evidence to link towards phagocytosis. Based off these results, we hypothesize that there will be no significant differences in the amount of co-localized synaptic puncta counted when compared across the different combinations even though we cannot exclude a role of other cytokines or molecules that could be differentially released between RTT and CTRL microglia.

FIGURES

A




B

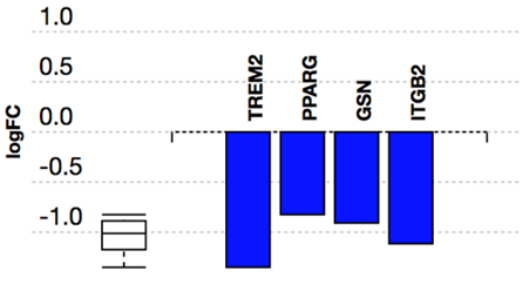
| Differentially Expressed | Genes |
|---|---|
| Total | 34 |
| Upregulated | 14 |
| Downregulated | 20 |

C

Biological Processes

| Name | # Genes (DE/ALL) | p-value |
|---|---|---|
| Vascular endothelial growth factor signaling pathway | 3/5 | 9.00E-04 |
| Regulation of macrophage derived foam cell differentiation | 4/11 | 0.001 |
| phagocytosis, engulfment | 4/12 | 0.002 |
| membrane invagination | 4/12 | 0.002 |
| plasma membrane invagination | 4/12 | 0.002 |
| regulation of sequestering of triglyceride | 2/2 | 0.002 |

D




E

| Gene | Fold Change | p-value |
|---|---|---|
| **TREM2** | -1.350 | 0.016 |
| **PPARG** | -0.825 | 0.022 |
| **GSN** | -0.948 | 0.043 |
| **ITGB2** | -1.115 | 0.045 |

**Figure 1. Differential Gene Expression of RTT vs CTRL Microglia. (A)** Volcano Plot of statistical significance (p-value, -$\log_{10}$) against fold-change (FC, $\log_2$) between RTT vs CTRL Microglia, demonstrating significantly differentially expressed genes of interest, TREM2, PPARG, GSN, and ITGB2. Horizontal bar on y-axis represents p-value = 0.05 (genes in grey underneath bar did not reach statistical significance). Vertical bars on x-axis represent FC<-0.25 and FC>0.25. **(B)** Table representing total statistically differentially expressed genes, and total upregulated and downregulated genes, respectively. **(C)** Table of differentially expressed (DE) genes vs all genes associated with biological process and averaged statistical significance (p-value) of DE genes from the human myeloid innate immunity panel, respectively. **(D)** Bar graph representing FC ($\log_2$) of downregulated genes involved in phagocytosis between RTT vs CTRL Microglia. Box plot of average statistical information from averaged genes shown. **(E)** Table representing raw values of calculated FC and p-value of DE genes of interest involved in phagocytosis. CTRL: n = 4; RTT: n = 2, n, represents amount of individual cell lines used. Data measured in three individual batches. Volcano plot was analyzed and generated by Rosalind with a HyperScale architecture developed by OnRamp BioInformatics, Inc. (San Diego, CA). Bar Graph was analyzed and generated by Advaita Corporation 2019.

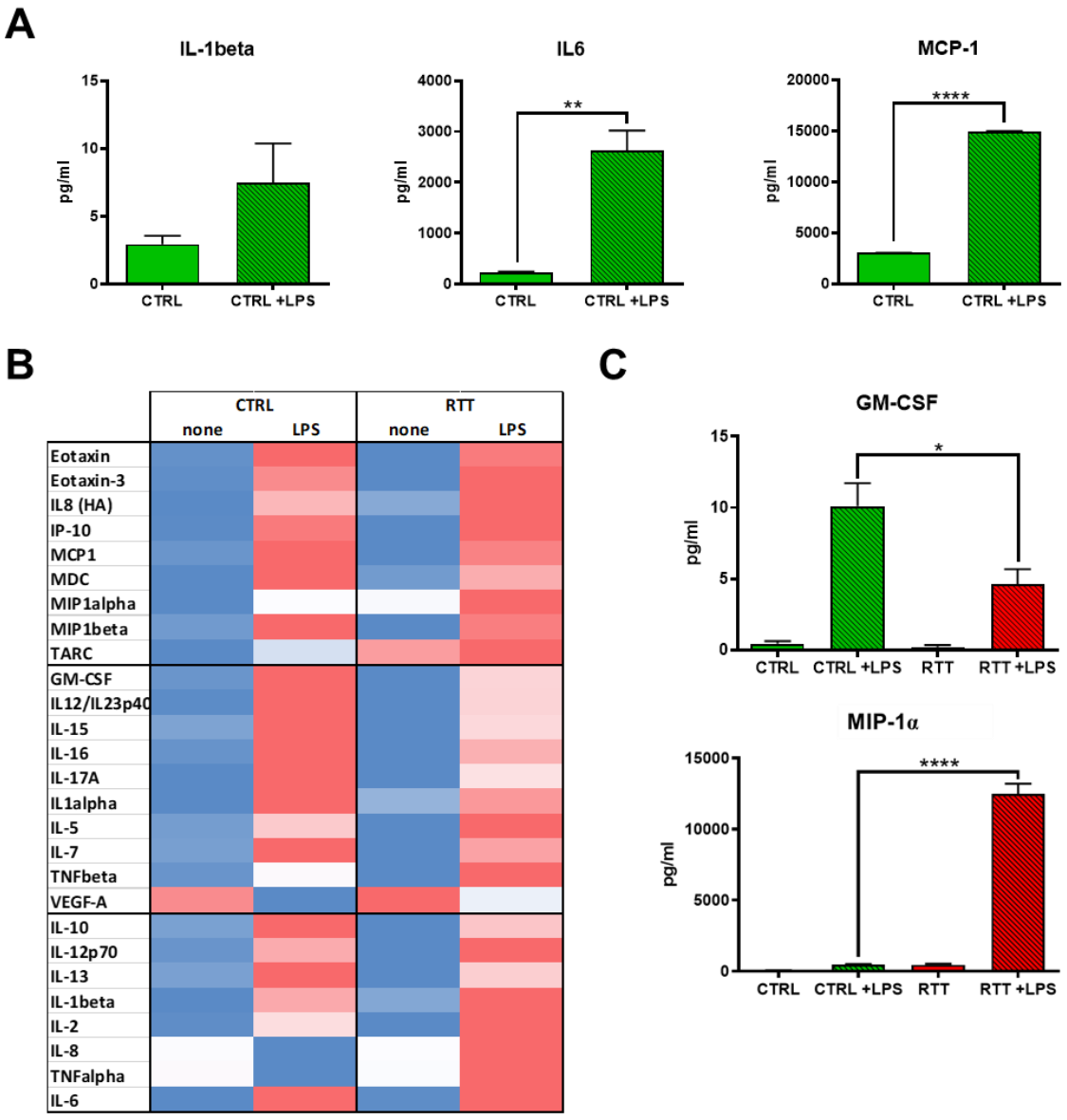



**Figure 2. Inflammatory cytokine release by RTT and CTRL Microglia using multiplex ELISA.** **(A)** Bar graphs represent the inflammatory molecules, IL-1β, IL-6, and MCP-1, secreted from CTRL microglia stimulated with or without lipopolysaccharide (LPS). **(B)** Heatmap represents the mean concentrations of secreted chemokines and cytokines from CTRL and RTT microglia with or without LPS stimulation, red to blue is more concentrated to less concentrated (pg/mL). **(C)** Bar graphs represent the cytokines, GM-CSF and MIP-1α, secreted from CTRL and RTT microglia stimulated with or without LPS stimulation. CTRL: n = 4; RTT: n = 2, n, represents amount of individual cell lines used. Data measured in three individual batches and was run in three to four replicates. Different groups were compared using one-way ANOVA tests with Tukey multiple comparisons tests. The presented values are means of secreted inflammatory molecules (pg/mL) ± SEM, *P<0.05, ****P<0.0001. Statistical significance provided by Welch's t-test to compare mean of two groups, **P<0.01, ****P<0.0001.

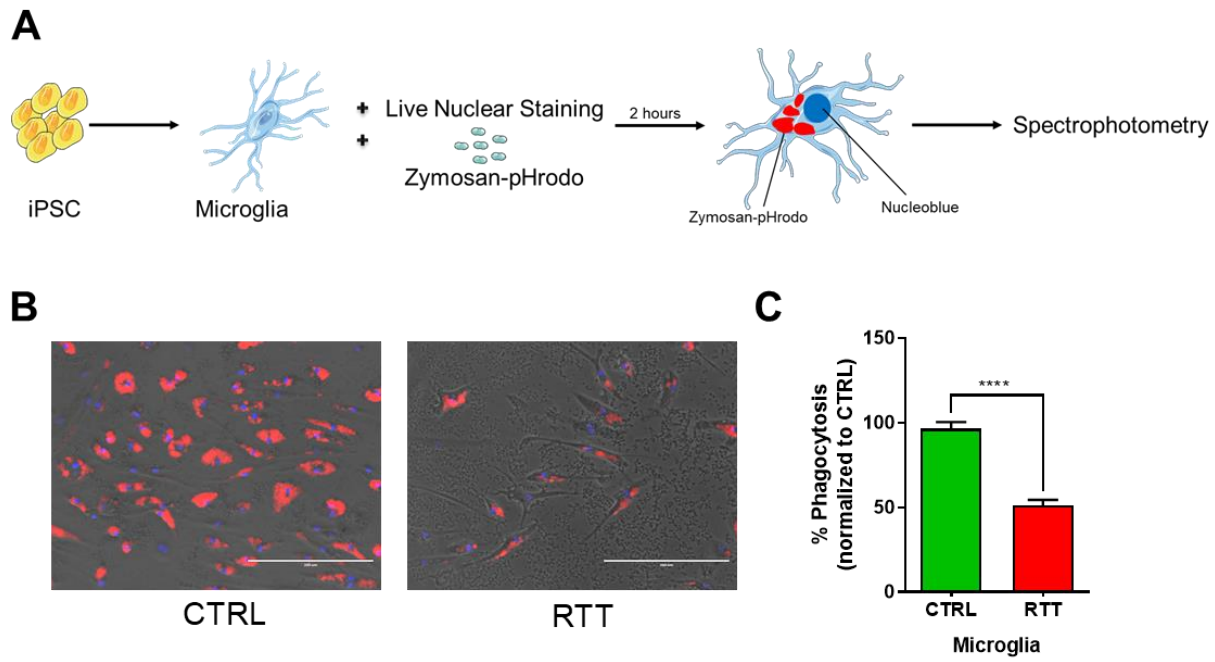
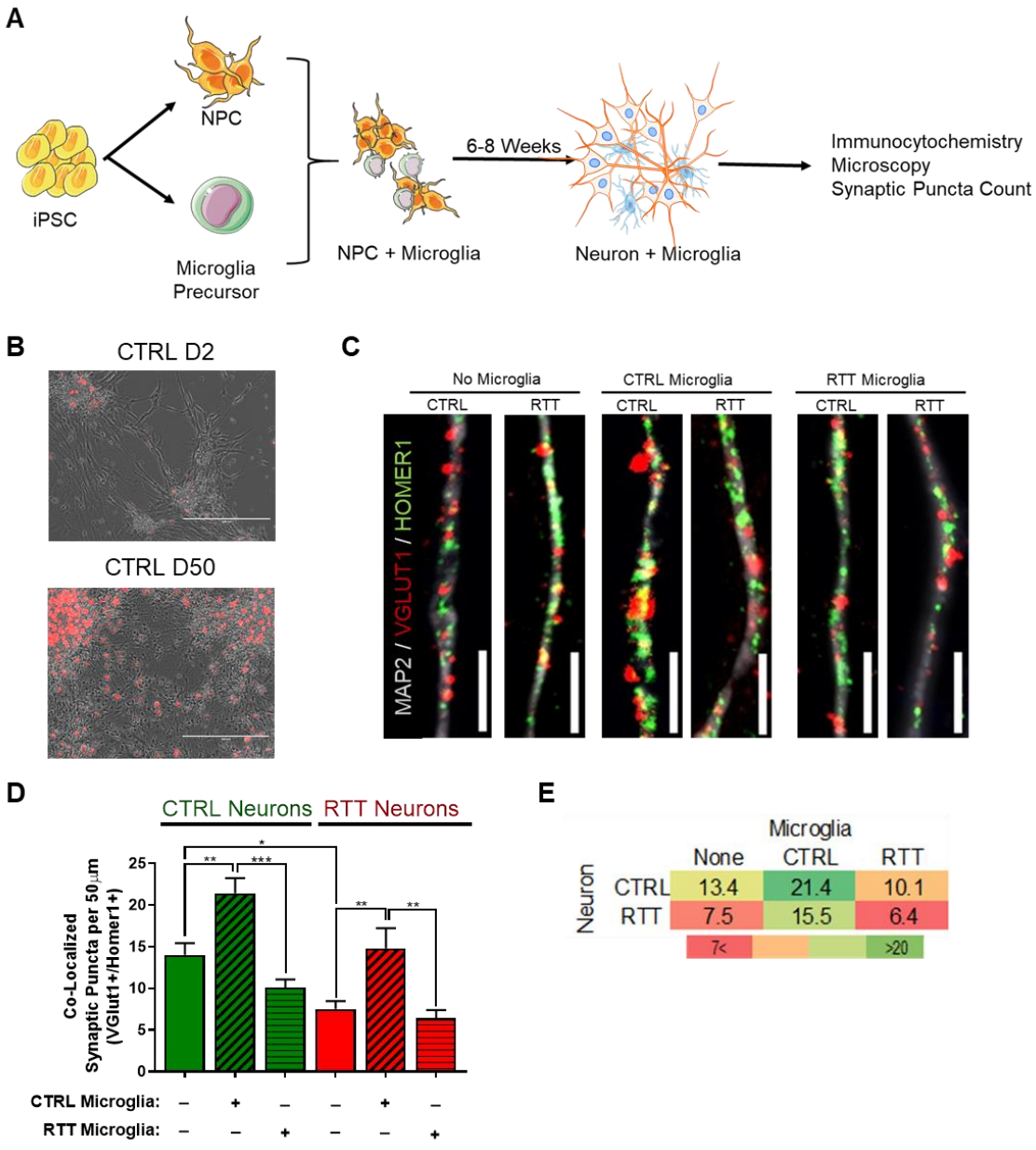



**Figure 3. Phagocytosis is impaired in RTT vs CTRL Microglia. (A)** Schematic of experimental design for pHrodo™ and NucleoBlue dye-based detection of phagocytosis. pHrodo™ Zymosan A Particles and NucleoBlue live nuclear staining solution were added to iPSC-derived Microglia for 2 hours and fluorescence was detected using a Spectrophotometer. **(B)** Images demonstrating dye-based fluorescence of zymosan particles phagocytosed (red) and live nuclear staining (blue) by CTRL and RTT microglia. **(C)** Bar graph represents percentage of CTRL vs RTT microglia phagocytosing zymosan particles, normalized to CTRL across six individual experiments. The presented values are means of the ratio between absorbances of zymosan-pHrodo™ and nucleoblue as a percentage ± SEM. CTRL, n=9; RTT, n= 7. n, number of wells measured by spectrophotometry for each cell line. Statistical significance provided by Welch's t-test to compare mean of two groups, ****P<0.0001.

**Figure 4. The impact of microglia on neuronal synaptogenesis in RTT. (A)** Schematic of the synaptic puncta co-localization co-culture experimental design. Microglia precursor and NPCs derived from iPSCs are co-cultured and differentiated to mature neurons and microglia over 6-8 weeks, followed by an analysis of synaptogenesis measured by immunofluorescent co-localized synaptic puncta from images captured by an apotome microscope. **(B)** Images of CTRL microglia stained with a red membrane-labelling dye, PKH26, co-cultured with CTRL neurons at day 2 and day 50 post co-culture. **(C)** Images of co-localized synaptic puncta from CTRL and RTT neurons without microglia and co- cultured with a combination of CTRL and RTT microglia, stained with pre- and post-synaptic markers, VGLUT1 (red) and HOMER1 (green) respectively, neuronal marker, MAP2 (white), and DAPI (blue). Scale bar = 10µm. **(D)** Quantification of co-localized synaptic markers on neurons without microglia and with microglia co-cultured in multiple cell line combinations. **(E)** Table representing the mean values of the number of co-localized synaptic puncta counted from neuron and microglia co-culture combinations of parental control (CTRL) and $MECP2^{Q83X}$ patient (RTT) cell lines. Neuron/Microglia combinations: CTRL/none, n= 20; CTRL/CTRL, n=10; CTRL/RTT, n=20; RTT/none, n=20; RTT/CTRL, n=20; RTT/RTT, n=20. n, number of $MAP2^{+}$ neurons measured for synaptic puncta co-localization. Different groups were compared using one-way ANOVA tests with Tukey multiple comparisons tests. The presented values are means of VGLUT1+/HOMER1+ co-localization ± SEM, *P<0.05, **P<0.01, ***P<0.001.




The results shown in this thesis are part of a project conceived and designed by Dr. Pinar Mesci under the supervision of Professor Alysson Muotri. Mesci, Pinar; Jeziorski, Jacob John; Nizet, Victor; LaRock, Christopher; Alysson, Muotri. They will be included in a future submission for publication, in which the thesis author would be recognized as a co-author.

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