Role of TrkB.T1 in Glial Inflammatory Response Elicited by MHV

By

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A thesis submitted to the Faculty of Graduate and Postdoctoral Affairs in fulfillment of the requirements for the degree of

Master of Science

In

Neuroscience

Carleton University,

Ottawa, Ontario

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Abstract

It has become clear that the SARS-CoV-2 virus can enter the central nervous system (CNS) and impact glial cell and neuronal functioning. In the present thesis, we used a rodent analogous coronavirus, murine hepatitis virus (MHV), in culture to directly assess its impact on astrocytic and microglial cells. Given the increasing importance of the brain neurotrophic factor (BDNF)-tyrosine kinase receptor B (TrkB) signaling system in glial functioning, we also assessed whether the unique truncated TrkB receptor isoform (TrkB.T1), the only BDNF receptor on astrocytes, would modulate glial reactivity to MHV viral infection. Our results largely support the notion that MHV readily infects astrocytes and cause a degree of toxicity to these cells. The addition of microglia to the astrocytic culture modulated the magnitude of this effect and greatly increased pro-inflammatory cytokine release. Furthermore, TrkB.T1 deficiency appeared to greatly reduce astrocyte viability and microglial morphology. These data may have useful implications for better understanding the nature of glial responses to coronaviral infection and the importance of TrkB in such responses.
Acknowledgements

Over the past two years, I have continuously felt thankful to be able to continue my studies during the pandemic. Although the circumstances in which I worked on my masters were not the most ideal, a few individuals made it the best possible experience for me. Namely, Natalie Prowse, Teresa Fortin, and Ashley McFee. I am so appreciative of their knowledge, guidance, and support. Not to mention, the patience and effort that my supervisor, Shawn Hayley, put into making my thesis the best it can be is something I will always be grateful for.

There are many others who provided me with moments of support and advice that I will always appreciate. Having spent almost 4 years with some of these amazing people inspired me to always stay motivated and humble. I would also like to thank my friends and family for their continuous support and patience. I can only hope to provide the same love and support you have provided to me during this time.

Lastly, I would like to thank my thesis committee for being encouraging and offering valuable insights into how to enhance my thesis.
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Introduction

BDNF-TrkB signaling

As part of the neurotrophin family, BDNF is involved in many neurological mechanisms including the formation of new synapses, plasticity, and dendritic branching (De Vincenti, Rios, Paratcha, & Ledda, 2019). In the brain of humans and rodents, BDNF is found in relatively high levels in the hippocampus, amygdala, cerebellum, and cerebral cortex (Miranda, Morici, Zanoni, & Bekinschtein, 2019). Within the periphery, BDNF can also be detected in the heart, gut and spleen and recent evidence has suggested it may have anti-inflammatory effects and act as an immunomodulator (Haba, et al., 2022; Song, et al., 2022; Gómez-Lázaro, et al., 2011; Lommatzsch, et al., 2005).

The primary BDNF receptor, TrkB, has several isoforms, the most common being the full-length TrkB (TrkB.FL) and secondarily, being the truncated TrkB (TrkB.T1) form. As the TrkB.T1 form of the receptor on neurons normally acts as a negative inhibitor buffering the impact of the TrkB.FL form, it can restrain neuronal gene expression induced by BDNF (Offenhäuser, Muzio, & Biffo, 2002), as well as neural calcium efflux (Eide, et al., 1996).

The mature TrkB.FL plays a role transmitting BDNF signals to different pathways in order to regulate nervous system plasticity, including synaptogenesis, neurogenesis, myelination, and cell survival (Cao, et al., 2020). There has also been evidence that overexpression of TrkB.FL has neuroprotective effects that were associated with reduced memory impairment and depressive-like behavior (Karpova, et al., 2014; Koponen, et al., 2004) and that decreased expression of TrkB.FL is linked to neurodegenerative processes (Vidaurre, et al., 2012). The reverse can also been
seen in a study where decreased BDNF and TrkB expression contribute to the cognitive deficits and neurotoxicity (Chiu, et al., 2022). On the other hand, TrkB.FL agonists such as LMDS-1 and -2 can offer neuroprotective effects in rodent models of neurodegenerative diseases (Chiu, et al., 2022).

The truncated TrkB.T1 receptor is most robustly expressed on astrocytes and in fact, is thought to be the only BDNF receptor found on these cells at physiologically relevant levels. TrkB.T1 plays a crucial role in regulating astrocyte morphology (Holt, et al., 2019) and calcium homeostasis into astrocytes (Rose, et al., 2003). It was noted that the levels of TrkB.T1 were higher compared to the full-length TrkB isoform, with advanced aging or in response to traumatic brain injury (Fenner, 2012). Further, a study by Saba et al. (2018), found that through TrkB.T1 signalling, BDNF protected astrocytes from apoptosis, reduced reactive oxygen species (ROS) and promoted antioxidant release. Hence, there is some evidence, albeit sparse, that TrkB.T1 modulates glial response to pathological stimuli and it might be particularly important to determine how BDNF-TrkB.T1 signaling might regulate glial phenotypes in response to infectious viral challenges.

Glial cells and TrkB

Besides the role of BDNF signaling in neuroplasticity and neuronal survival, this trophic factor has also been recently implicated in playing a role in the regulation of neuroinflammatory processes by way of its impact on glial cells (Kwon & Koh, 2020; Ding, et al., 2020). Some recent data does provide evidence that BDNF may protect the brain from the damaging effects of an inflammatory response due to a bacterial challenge (Zhao, Zhang, Xu, Wang, & Li, 2020). Yet,
although the role of BDNF-TrkB signaling in neuronal outcomes in response to a wide range of stressors has been well established (Guo, Nagappan, & Lu, 2018), far less is known of the role of this trophic pathway in microglial and astrocyte signaling. Furthermore, very little is known concerning the mechanistic impact of BDNF-TrkB signaling with regards to glial responses in the face of infectious agents. This is particularly important in light of the prominent role that the inflammatory immune system might play in neurodegeneration and stressors related illness (Marrie, et al., 2017). It is possible that a bi-directional link exists between trophic BDNF signaling and pro-inflammatory factors, such that their mutual regulation is key to host defenses.

**Astrocytes**

Astrocytes act in the CNS to help ensure homeostasis by controlling homeostasis, encouraging synaptic plasticity, maintaining protection of the nervous system, and regulating inflammatory factors such as cytokines and ROS (Kim, Park, & Choi, 2019; Chiareli, et al., 2021). In cases of tissue injury or inflammatory distress, astrocytes may contribute to the progression of various neurodegenerative diseases by adopting a toxic phenotype (Liddelow, et al., 2017; Liddelow & Barres, 2017). There are two general categories of reactive astrocytes, A1 which is known to release neurotoxins and be detrimental to synapses (Liddelow & Barres, 2017), and A2 which encourage the survival and development of neurons or synaptic repair (Li, Chen, Zhang, Zhang, & Yao, 2019). Liddelow et al. (2017), indicated that the reactivity of A1 astrocytes is influenced by microglial release of cytokines, most notably interleukin (IL)-1α, tumor necrosis factor alpha (TNF-α) and compliment component 1q (C1q). Following activation, the morphology of an astrocyte alters to become more elongated which matches the bipolar and stellate forms (Acaz-Fonseca, Ortiz-Rogriguez, Azcoitia, Garcia-Serguela, & Arevalo, 2019). If astrocytes
continue to be activated due to inflammation or disease, their morphology changes to have less complex branching as well as shorter and thicker processes and during this state, they are particularly reactive (Sun & Jakobs, 2012).

TrkB.FL expression is observed in many different cells throughout the nervous system (Deinhardt & Chao, 2014), while the TrkB.T1 isoform is most commonly found in astrocytes, and to a lesser extent in neurons (Fenner, 2012). The TrkB.T1 form is expressed in neurons of the cerebellar cortex (Ohira, Shimizu, & Hayashi, 1999), whereas in the prefrontal cortex (Ohira, Shimizu, Yamashita, & Hayashi, 2005) and subcortical white matter (Ferrer, et al., 1999), TrkB.T1 is expressed only on astrocytes. A decrease in morphological complexity and reduced volume exists in astrocytes that lack the TrkB.T1 receptor (Holt, et al., 2019), indicating its importance for astrocytic homeostasis. Also of importance, deletion of TrkB.T1 impairs astrocyte ability to proliferate and migrate in case of a CNS trauma (Wu, Renn, Faden, & Dorsey, 2013; Matyas, et al., 2017).

**Microglia**

Being the first line of immune defense in the CNS, microglia play a highly significant role in homeostasis and protection against microbial invaders (Colonna & Butovsky, 2017). Microglia are able to protect the CNS from threats by having a number of functional states, that involve shifting into different morphologies (Cuadros & Navascues, 1998), releasing cytokines and chemokines (Kettenmann, Hanisch, Noda, & Verkhratsky, 2011) and regulating phagocytotic activity (Sierra, Abiega, Shahraz, & Neumann, 2013). In their active state, the morphology of microglia transforms from their resting, ramified form into an ameboid shape, resembling a fried egg where the cell body is enlarged, and the cell processes are close to non-existent (Crews &
Vetreno, 2015). It is in such an “activated” state that microglia release pro-inflammatory cytokines and the oxidative radical, superoxide, as a defensive mechanism against immunological threats.

Like astrocytes, microglia also have two different polarized active states, M1 and M2. The M1 activated microglia is involved in pro-inflammatory functions including the production and release of ROS, nitrogen reactive species (NRS) and other cytokines like TNF-α, IL-1β, IL-6 and IL-12 (Bachiller, et al., 2018). In contrast, M2 activated microglia are known for their anti-inflammatory state due to its role in production and release of transforming growth factor β (TGF-β) and BDNF (Tang & Le, 2016). Microglia in an M2 phenotype can also protect the CNS by releasing BDNF, which can then signal the TrkB pathway (Hardeland, 2021).

The expression of all forms of the TrkB receptor within microglia are significantly lower than that of neurons and astrocytes (Holt, et al., 2019). But is still believed to be physiologically relevant and like astrocytes and neurons, microglia are also able to synthesize and release BDNF (Pöyhönen, Er, Domanskyi, & Airavaara, 2019). In cultured murine microglial cells, BDNF was found to bind to TrkB receptors and cause an increase of intracellular calcium levels (Mizoguchi, et al., 2009). Further evidence supporting the expression of TrkB on microglia comes from a study that reported that pre-treatment with BDNF reduced the inflammatory impact of LPS upon pure microglial cultures (Wu, et al., 2020). Our own preliminary evidence found that primary microglial cells did take up BDNF into their cytoplasm and that this influenced their phenotype (Prowse et al. unpublished; Paquette et al., unpublished).

**Interaction between Astrocytes and Microglia**

Recent studies have shown evidence of communication between microglia and astrocytes, with regard to the regulation of inflammation and neural development (Matejuk & Ransohoff,
2020). Such inter-glial communication is dependent on growth factors, neurotransmitters, cytokines, ROS, chemokines, and glutamate to name a few (Matejuk & Ransohoff, 2020). One study investigating the interaction between astrocytes and microglia found that the release of TGF-β by astrocytes promoted microglial phagocytosis (Bialas & Stevens, 2013). When considering the role of neurotransmitters in astrocyte and microglia communication, a few studies noted the activation of microglia triggered glutamate release from astrocytes (Pascual, Ben Achour, Rostaing, Triller, & Bessis, 2012; Bezzi, et al., 2001; Kim & Na, 2016).

In terms of trophic factors, the secretion of BDNF by astrocytes mediates the signaling of neuroprotective factor erythropoietin and its mediator sonic hedgehog in microglia (Lai, et al., 2018). Conversely, there is evidence that activated microglia inhibit astrocytic release of BDNF (Araki, Ikekaya, & Koyama, 2021). Microglia have also been found to augment the activity and promote changes in chromatin of astrocytes that were previously activated by brain injury (Villarreal, Vidos, Monteverde Busso, Cieri, & Ramos, 2021).

Exposure to infectious agents or environmental toxins appears to mobilize microglia and astrocytes but with different time courses (Karthikeyan, Patnala, Jadhav, Eng-Ang, & Dheen, 2016; Norden, Trojanowski, Villanueva, Navarro, & Godbout, 2015; Du Preez, et al., 2021; Kirkley, Popichak, Azali, Legare, & Tjalkens, 2017). In the case of a traumatic injury, microglia are the first responders (Hanisch & Kettenmann, 2007), followed later by astrocytes that coordinate secondary measures including forming glial scars (Sofroniew, 2014). Ultimately, microglia and astrocytes likely have some degree of coordinated actions in addition to their obvious cell autonomous roles. They also have coordinated synaptic effects with neurons that have been
referred to as “quad-partite synapses” (Macht, 2016), wherein both glial cell types have the capacity to shape synaptic plasticity.

Immune Stimulators

As this thesis focuses on the downstream effects of virally infected astrocytes and microglia, reviewing previous research about immunostimulants is of importance. In this regard, lipopolysaccharides (LPS) and polyinosinic:polycytidylic acid (Poly I;C) are common immunostimulants in research where their ability to induce an immune response is used to investigate diseases that incorporate inflammatory pathways. The most commonly used bacterial agent is LPS, which is a bacterial surface glycoprotein found in Gram-negative bacteria that is recognized by toll-like receptor 4 (TLR-4) as a pathogen-associated molecular pattern (PAMP) (Bertani & Ruiz, 2018). In contrast, Poly I;C is a synthetic analogue of dsRNA used to resemble intermediate replication that exists in cells infected by an RNA virus and is commonly used to stimulate viral infections. Poly I;C activates toll-like receptor 3 (TLR-3) and initiates inflammatory responses (Stowell, et al., 2009). LPS exposure was reported to reduce the expression of BDNF and the number of dendritic spines (Fang, et al., 2020; Li, et al., 2021), whereas Poly I;C treatment decreased levels of serotonin in the medial prefrontal cortex (Katafuchi, Kondo, Take, & Yoshimura, 2006) but increased activity of norepinephrine within the paraventricular hypothalamus and hippocampus (Gandhi, Hayley, Gibb, Merali, & Anisman, 2007). In another study, exposure to Poly I;C triggered increased levels of IL-1β, IL-6, TNF-α and CD11b in frontal cortex and hippocampus (Gibney, et al., 2013) indicating neuroinflammation.
In comparison to the numerous physiological discoveries involving Poly I;C and LPS, far fewer studies have utilized live microbial agents to orchestrate neuroinflammatory responses. Among the relevant microbial agents, coronaviruses might be particular germane in light of the current COVID-19 pandemic and the fact that these are fairly ubiquitous viruses. Moreover, there are emerging reports that besides the pulmonary system, the COVID-19 causing agent, SARS-CoV-2, has been proven to gain entry into the brain and have a high affinity to infecting astrocytes (Andrews, et al., 2021, Andrews, et al., 2022) which may lead to fatigue (Noda, Ifuku, Hossain, & Katafuchi, 2018), depressive symptoms (Maeng & Hong, 2019) and neurodegeneration via mitochondrial inflammatory response (Denaro, et al., 2022). Thus, there is reason to suppose that the virus might impact biological underpinnings of neurodegenerative disorders. Unfortunately, SARS-CoV-2 does not readily infect the mice, however another coronavirus, the murine hepatitis virus (MHV) is often used in mice to replicate the effects that SARS-CoV-2 may have in humans (Körner, Majjouti, Alcazar, & Mahabir, 2020). In particular, the MHV-JHM strain readily infects murine neurons and glia (Miura, et al., 2008).

The MHV-JHM strain is a neurotropic, single-stranded RNA virus that has been found to be more neurovirulent compared to another popular strain, MHV-A59 (Buchmeier, Lewicki, Talbot, & Knobler, 1984; Lavi, Gilden, Highkin, & Weiss, 1986). Infection with MHV-JHM occurs through a glycoprotein receptor located in the murine CNS, known as murine CEACAM1a (Compton, Stephensen, Snyder, Weismiller, & Holmes, 1992). But there is also evidence indicating that murine CEACAM1a is not necessarily always required for the spread of MHV-JHM (Miura, et al., 2008), indicating that other mechanisms are involved in the process of infection.
MHV-JHM infection produces profound expression of pro-inflammatory cytokines IL-1 and IL-6 (Rempel, Murray, Meisner, & Buchmeier, 2004) and a large number of studies indicate that MHV-JHM can induce demyelinating effects on neurons throughout the CNS (Perlman, Sun, Barnett, & E.M, 1995; Wang, Stohlman, & Fleming, 1990; Morris, Tieszer, Mackinnon, & Percy, 1989; Tirotta, Carbajal, Schaumburg, Whitman, & Lane, 2010). Yet, there is a paucity of data concerning the impact of the virus upon non-neuronal cells, such as astrocytes and microglia (Joseph, Grun, Lublin, & Knobler, 1993; Perlman & Ries, 1987; Sun, Grzybicki, Castro, Murphy, & Perlman, 1995). In fact, astrocytes seem to be the target cell type when it comes to MHV-JHM infection (Sun, et al., 1995; Perlman & Ries, 1987). Finally, at a practical level, MHV-JHM is able to easily grow in cell culture (Leibowitz, Kaufman, & Liu, 2011) and in light of the current pandemic, it is of particular importance to better understand at a cellular level the impact of coronaviral infections.

Experimental prospectus

This thesis is focused upon the potential role of the BDNF-TrkB trophic pathway in glial responses to a coronaviral challenge. In particular, we speculate that the truncated form of the receptor, TrkB.T1, is playing a role in astrocyte reactivity to viral exposure, with it modulating the morphological state and release of soluble trophic and inflammatory factors by these cells. It is of further interest to study the role of TrkB.T1 on astrocytes, when also in the presence of microglia. To this end, the experimental study specifically aims to investigate the difference in morphology and reactivity between TrkB.T1 receptor knockout (T1KO) and wildtype (WT) mice astrocyte cell cultures when introduced MHV-JHM. An additional examination will be regarding the presence
of microglia in the cell cultures, allowing evaluation of both individual astrocyte cultures, as well as co-cultures of both microglia and astrocytes.

It is hypothesized that MHV-JHM will be a robust glial activator and that it will have a dramatic impact on samples of astrocytes when microglia are also present. This is expected to also be modulated by TrkB.T1 KO, such that deficiency in the receptor will result in a further shift towards a pro-inflammatory state.

We have three primary aims: 1. To assess the toxic effects (as per changes in morphology, cell coverage and cytokine release) of MHV infection in astrocytes and 2. How co-culturing these cells with microglia impacts these outcomes and 3. How specific TrkB.T1 deficiency in astrocytes and/or microglia also influences these outcomes.

Methods

Animals

The subjects in the current research study are laboratory mice strain C57BL/6J of either WT genotype or T1KO genotype. The T1KO transgenic strain was a generous donation from the University of Maryland by Dr. Susan Dorsey. This transgenic strain was generated by conditionally removing the TrkB.T1 coding exon through a recombinant cloning strategy with the resulting targeting vector propagated to C57BL6 blastocysts to produce a heritable mutation (Dorsey et al., 2006). Validation via both western blot and fluorescence confirmed that TrkB.T1 was not expressed in full knockouts while protein expression of TrkB.FL remained unaltered.
(Dorsey et al., 2006). Heterozygote breeders for our colony were initially derived by breeding heterozygous males with C57BL/6 WT females. Colony mice were genotyped by extracting genomic DNA from mouse ear punches using standard techniques. PCR for TrkB.T1 genotyping was performed using genomic DNA template primers TrkBT1-5s: (5'-CCAGCTATTGAGTAATGAATGAGTC-3'), TrkBT1-2(5'-CTACCCATCCAGTGATCTT-3'), and TrkBT1-4a (5'-CCACCGCGGTGGTCCATAACTTCG-3'), where the absence of TrkB.T1 results in a product at 350bp, and the presence of TrkB.T1 results in a product at 600bp. Thus, full knockouts present at 350bp, WT at 600bp, and heterozygotes display products at both locations. This transgenic strain has been used in a variety of other studies involving chronic pain and trisomy but have not been used to study the impacts of chronic stress and immune stimulators (Cao, et al., 2020; Dorsey, et al., 2006).

Samples were obtained from post-natal day 1-3 murine pups were cultured. Mixed glia cultures will be grown in T75 flasks with 3 biological replicates.

Murine pups were taken from their mothers on postnatal Day 1-3 and sacrificed via rapid decapitation. Once the brain was taken out of the skull, it was placed into a culture petri dish filled with 6 mL of dissection media to be dissected. After removing the meninges, the cortex was separated from the other brain regions, cut into smaller pieces, and transferred into 1 ml of dissection media in a 5 mL tube for cell culturing. Each brain was dissected and transferred into separate 5 mL tubes in order to maintain individual differences.

Each 5 mL tube, now containing 2.5 mL of dissection media including the cortical pieces, had 2.5 mL of warm TrypLE added and set on a shaker inside a 37°C incubator for 30 minutes. After the 30 minutes, the 5 mL tubes were placed in a centrifuge to run at 1200 RPM (300g) for 5
min in order to pellet the cortex tissue pieces. The supernatant was then decanted to leave only the pellet which will be resuspended in 1 mL of complete media. The tissue pieces were titrated in order to become dissociated into single cells. The suspension was then strained by a prewet (by 1 mL of complete media) 70 μm pore cell strainer that was placed on top of a 50 mL falcon tube. The strainer was rinsed 3 times with 1 mL of complete media before the 5 mL of cell media in the 50 mL falcon tube was drawn up by a 10 mL stripettor and added to a PDL-coated T75 flask with 5 mL of warmed complete media, giving a total of 10 mL of media in the T75 flask.

Experimental treatments

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Genotype</th>
<th>Culture</th>
</tr>
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<tbody>
<tr>
<td>MHV / Control</td>
<td>WT</td>
<td>Astrocyte</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Astrocyte + Microglia</td>
</tr>
<tr>
<td>T1KO</td>
<td></td>
<td>Astrocyte</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Astrocyte + Microglia</td>
</tr>
<tr>
<td>WT Astrocyte + KO Microglia</td>
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<td></td>
</tr>
<tr>
<td>KO Astrocyte + WT Microglia</td>
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</tr>
</tbody>
</table>

Table 1. Layout of treatment groups.

Pure astrocyte and astrocyte-microglia co-cultures were treated with vehicle or MHV-JHM from mice with differing genetic compositions (T1KO and WT). The presence of microglia can encourage additional inflammation and reactivity; therefore, it would be significant to analyze how the presence of microglia changes the reactive state of astrocytes.
For flasks intended to be purified for astrocytes, mixed glia cultures were grown for approximately 1-1.5 weeks until 95% confluency was reached and then went through passaging procedures to remove as many microglia and oligodendrocytes as possible. One to two weeks after replating, passaged astrocyte flasks went through microbeading to obtain pure astrocyte cultures. During the intervening weeks, astrocyte flasks went through half media changes every 3-4 days with AWESAM solution. Once microbeaded, astrocyte cells were counted to approximately 20,000 cells per well for MHV experiments. Flasks intended for microglia remained in mixed glia cultures 3-3.5 weeks after harvest date with media changes of complete media solution every 5-6 days.

Cell culturing

**Poly-D-Lysine (PDL) coating**

T75 flasks and Greiner 24 well plates were PDL coated by leaving 5 mL of a 1:1 mixture of PDL and sterile distilled water in a T75 flask for at least 2 hours. After the 2 hours, each flask was rinsed it 3 times with sterile distilled water before adding 5 mL of complete media and storing in an incubator.

**First passage of astrocytes**

After 1-1.5 weeks or when mixed glia achieves 95% confluency, one flask of mixed glia cells was used to produce 2 flasks of astrocytes, wherein flasks are prepared at least 2 hours in advance with PDL coating. The neck of each flask was covered with parafilm to prevent gas exchange and each flask was placed on an orbital shaker, that was inside a non-CO₂, 37°C
incubator, to shake for 4-6 hours at 240 RPM. Next, one flask was taken into a biosafety cabinet with ventilation while manually shaking. The flask was tapped 5 times on a hard surface to facilitate the removal of oligodendrocyte progenitors before quickly removing the media and rinsing twice with pre-warmed sterile phosphate-buffered saline (PBS). Trypsin was then added to the flask at 4 mL for 5 minutes and was then neutralized by 4 mL of pre-warmed complete media for 1 minute. To dislodge the cells from the flask once more, the flask was tapped on a hard surface a few times before drawing up all the media and putting it in a 50 mL falcon tube to be centrifuged for 5 minutes at 1200 RPM (300g). After the 5 minutes, the supernatant was decanted and the pellet that remains was resuspended in 1 mL of AWESAM media. The 1 mL pellet was added to 10 mL of prewarmed AWESAM where 5 mL was added to each of the 2 new T75 flasks and stored in an incubator. AWESAM half media changes occurred every 3-4 to maintain cell growth and survival.

**Microbeading of Astrocytes**

After the first passage just described, a microbeading procedure was used to obtain further purified astrocytes. Before starting the microbeading procedures, a Degas buffer was made. First, 210 mL of PBS and 1.25 grams of BSA crystals were mixed then sterile filtered. An additional 40 mL of PBS was added to the sterile filter. The sterilized mix was poured into a 250 mL Erlenmeyer flask that has a spout. A stir rod was added into the Erlenmeyer flask which was placed it on top of a stir plate that was set to 100 RPM speed. A suction device was placed on the spout and the solution was left in the Degas buffer for approximately 2 hours. Glass cover slips were added to Greiner 24 well plates, PDL coated then left with 500 μl of AWESAM media in each well.
To begin preparing for the microbead extraction, 4 mL of trypsin was added to the chosen flasks and left in the incubator for 5 minutes before tapping the backside of the flask to dislodge the cells. Once cells lifted, 4 mL of Dulbecco's Modified Eagle's Medium (DMEM) was added to neutralize the mixture. The media was then removed and added to a sterile 50 mL falcon tube to be centrifuged for 5 minutes at 1200 RPM (300g) and 4°C. The supernatant of the tube was discarded, and the remaining pellet was resuspended in 160 μl of Degas buffer and titrated to achieve a single-cell suspension. The suspension was then separated into two 1.5 mL Eppendorf tube with 80 μl of suspension in each.

An anti-ACSA-2 microbead kit was used that contains a small bottle of blocking reagent and microbeads where 10 μl of the blocking reagent was added and mixed to each of the 1.5 mL Eppendorf tubes for 10 minutes in the fridge. After, 10 μl of the microbeads were added to each tube, mixed, and left in to sit at 4°C for 15 minutes. Next, 1 mL of degas buffer was added to each tube and titrated before having placed them in a centrifuge for 4 minutes set at 1 G and 4°C. The supernatant was carefully decanted, and the remaining pellet was resuspended in 500 μl of degas buffer. The magnetic columns for the microbead extraction were pre-wet with 500 μl of buffer and then 500 μl of the resuspended pellet was drawn through the magnetic column. The column was rinsed with 500 μl of degas buffer 3 times before adding a final 1 mL of Degas buffer to the pure astrocyte suspension and transferring the solution to a 5 mL tube where cells were counted and plated at 20,000 cells per well, with 3 technical replicates.

**Cell counting**

To count cells, a 1:1 ratio of 15 μl of Tryphan blue and 15 μl of cell solution was added to a 500 μl microcentrifuge tubes to attain a dilution factor of two. Then, a hemocytometer was loaded
with the mixture and moved to an EVOS microscope to count cells within the two grids. The counts were averaged and then the following calculations were used:

\[(\text{Average cell count}) \times (\text{dilution factor}) \times 10^4 = “A” \text{ cells/mL}\]

The desired cell count was determined and calculated by dividing the number of cells that have been counted by the number of cells needed for the experiment and then multiplied by the desired total volume. The equation looked like the following:

\[\frac{\text{Desired cell count “B”}}{\text{Current cell count “A”}} \times 4 = “C” \text{ amount of cell solution required (mL)}\]

\[\text{Total volume needed - C = “D” amount of media needed to add to C}\]

\[D + C = \text{Desired total volume including the desired cell concentration}\]

**Saura Procedure for Microglia**

In separate mixed glial flasks, microglia were obtained for co-culture experiments, this involved a Saura procedure. Once the optimal time for microglia development was reached (3-3.5 weeks), a 1:1:1 ratio of Trypsin, DMEM, and Versene was added to each T75 flask, with the total volume of solution in each flask being made up to 6 mL. The solution was incubated in the flask for 30 minutes in a 37°C incubator. After the 30 minutes, a layer of cells lifted from the flask, leaving only microglia at the bottom of the flask. The 6 mL of solution was drawn out and rinsed twice with DMEM. To plate microglia in the Grenier 24 well plates, 6 mL of MGM media was added to the flask and a cell scraper was used to remove the microglia attached to the flask. After scrapping, the flask was checked on the EVOS system to ensure the majority of cells were scraped,
and then the media was drawn out, set in a 50 mL falcon tube and 500 μl of microglia solution was added to the appointed wells.

Exposure to MHV

To develop an appropriate MOI for the MHV experiments of this research, the following equation was used and formulated by Poisson distribution (Phuong, 2011):

\[
\text{Dilution factor} = \frac{0.7 \times N \times V}{m \times X}
\]

Where 0.7 represents an equation formulated by Poisson distribution where 1 TCID50 = 0.69 PFU/ 0.7 PFU. N represents the titer of the MHV strain (JHM) where 5 x 10^6.25 is equal to TCID50/0.2ml in 1 day on NCTC clone 1469 cells (ATCC® CCL-9.1™) at 37°C with 5% CO₂ by CPE. The V represents the total number of virus solution that will be added to each well, whereas m represents the MOI and X represents the cell count of cells in each well. For astrocytes, MOI testing at 0.5, 1, 3 and 5 took place on a cell count of 22,500, and the optimal level of infectivity was determined to be with an MOI of 0.5.

MHV

In Greiner 24 well plates with glass coverslips inside the wells, purified astrocytes were plated at 20,000 cells per well with 500 μl of cell solution in each well, with three biological replicates and three technical replicates. Astrocyte cells were left to settle and grow for 3-4 days in an incubator at 37°C and 5% CO₂ and then were infected with MHV strain JHM at an MOI of 0.5 (where a number of MHV stock was mixed into AWESAM media solution beforehand). After 24 hours of incubation, the MHV media was removed and normal AWESAM media was added.
onto the cells. In addition to this, microglia cells in MGM media were added to the appropriate coculture wells, such that half the wells included infected astrocytes alone and the other half included infected astrocytes with untreated microglia. At 48 hours, plates were taken out of the incubator and samples of supernatants were taken from each well and stored at -80 Celsius. The glass coverslips left in the well plates were fixed with paraformaldehyde (PFA) solution.

**Cell fixing**

In order to fix all astrocyte and microglial cells, a mixture containing a ratio of 1:1 of PFA and PBS was prepared and 500 µl of the mixture was added to wells that had their previous media removed. The PFA mix was left to incubate for 15 minutes at room temperature before being washed with PBS. The final wash of PBS was left on, the well plates were sealed with parafilm and moved to store at 4°C.

**Genotyping**

To prepare the tissue, 100 µl of extraction solution and 25 µl of tissue preparation solution were added to each sample that had been stored in 1.5 mL Eppendorf tube and incubated for 10 minutes at room temperature. The samples were then spun down for 1 minute on a Scientific Industries Genie SI-D238 Disruptor Shaker. Then, the samples were taken to a 95°C dry bath thermostat to be incubated for 3 minutes. Then, the samples were spun down for 1 minute on the Genie SI-D238 Disruptor Shaker. In each sample, 100 µl of REDExtract-N-Amp kit Neutralizing B solution was added before being spun down for another minute on the Genie SI-D238 Disruptor
Shaker. The final product was placed in a centrifuge set at 10 G for 10 minutes at 4°C. During the wait period, a PCR master mix was made up with the following volumes per sample:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (μl)</th>
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<td>PCR-grade Water</td>
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<tr>
<td>REDExtract-N-Amp PCR reaction mix</td>
<td>10</td>
</tr>
<tr>
<td>TrkB.T1-5s primer</td>
<td>0.1</td>
</tr>
<tr>
<td>TrkB.T1-2 primer</td>
<td>0.05</td>
</tr>
<tr>
<td>TrkB.T1-4a primer</td>
<td>0.1</td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
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</tr>
</tbody>
</table>

Table 2. Volumes (μl) of reagents required for PCR master mix.

The PCR master mix was added to each PCR tube at 16 μl per tube and in addition to that, 4 μl of the prepared tissue sample was added. Once added, the PCR tubes went through a brief centrifuged and then added to the PCR machine set with a T1-KO PCR program that runs for about 2 hours. During this time, an agarose gel was prepared for electrophoresis. To create a mini gel, 60 mL of TAE buffer and 1.2 grams of Agarose powder were mixed in a 250 mL bottle and microwaved for 1 minute. After the minute, the solution was gently swirled before being placed back into the microwave to heat for another 30 seconds. Once the 30 seconds was up, the bottle was rinsed with cold water until the bottle was cool to touch. Before pouring the mixture into a gel stand with combs, 3 μl of redsafe was added and mixed well. Once the gel was set and the tissue samples were ready, 8 μl of tissue sample was added into each lane, with the first lane being 5 μl of DNA extraction ladder. The gel was then placed in an Electrophoresis tank to run at 100 V for about 25 minutes and then was visualized on a LICOR machine at 600 nm for 3 minutes.
Figure 1. Genotype validation. (A) Genotype validation via PCR for T1KO tissue used for culture. Note all samples produced a single band which migrated to 350bp, indicating they are full knockouts. (B) Example genotype gel image showing WT (single band at 600bp), KO (single band at 350bp) and Heterozygote (2 bands, at 350 and 600bp).

Immunohistochemistry

On Day 1, fixed cells were permeabilized in 0.1% Triton X in PBS for 15 minutes with 200 µl each well. After permeabilizing, the cells were washed with Dubecco’s PBS twice for 5 minutes and then washed with PBB wash twice for 5 minutes. The cells were then incubated with a Blocker solution for 45 minutes, during which the solution of primary antibody was made up. In this trial, 1:1000 of S100 calcium-binding protein B (S100β), 1:200 of M-Dectin-1 and 1:2000 of anti-MHV-JHM, was produced in 5 mL of primary antibody solution in a 15 mL tube. After the Blocker, the wells were washed twice for 5 minutes with PBB wash and 200 µl of antibody solution was gently applied to wells for an overnight incubation.
On Day 2, the well plates were washed three times with PBB wash for 5 minutes. Secondary antibodies consisted of 1:1000 of Alexa Fluor® anti-mouse 647, 1:1000 of Alexa Fluor® anti-rabbit 488 and 1:1000 of Alexa Fluor® anti-rat 594. The secondary antibody solution was added to each well at 200 µl per well. Well plates were incubated in the dark for 60 minutes. After 60 minutes, the wells were washed three time with PBB wash for 5 minutes and then stained with 1:15,000 Hoescht stain (DAPI) in 10 mL of PBS for a precise three minutes. The well plates were then washed 3 times with PBB wash for 5 minutes and 2 times with PBS wash for 5 minutes. After the last wash, the cover slips containing the stained cells were carefully lifted from the wells and plated onto glass slides using Gelvatol. The mounted slides were left in a black box overnight and imaged once dry.

Proquantum Immunoassay

Cytokine levels were analyzed by using Thermofisher Scientific: Invitrogen ProQuantum Immunoassay kits for TNF-α, IL-6 and IL-4. Reagents including the protein standard (TNF-α, IL-6, or IL-4), assay dilution buffer, antibody-conjugate A, antibody-conjugate B, and antibody-conjugate dilution buffer were set out to thaw at room temperature. The lyophilized protein standard was reconstituted with the assay dilution buffer, inverted gently 5 times, and set to incubate at room temperature for 15 minutes. During the incubation period, 6 µl of antibody-conjugate A and antibody-conjugate B were added to a 1.5 mL Eppendorf tube along with 348 µl of antibody-conjugate dilution buffer. Once the mixture was prepared, 40 µl of it was transferred to each well of a setup plate. The setup plate was prepared on ice. Unknown samples were then diluted 1:3 fold. Next, 14 µl assay dilution buffer and 7 µl of the unknown samples were added to
assigned wells and mixed by pipetting. A standard curve was then created via serial dilution where 80 µl of assay dilution buffer was added to wells of the last column. In the first well (S1), 5,000 pg/ml was created by adding 20 µl of protein standard to the existing 80 µl of assay dilution buffer. Next, a serial dilution was carried out where 20 µl of S1 is added to the next well, S2 and so on. Wells were mixed via pipettes and pipette tips were changed after every well. The setup plate was then sealed with an adhesive seal and struck on both sides of the plate by the hand 3 times. The well plate was then centrifuged for 1 minute at 3,000 g. After, using a multichannel pipette, 2 µl of the antibody-conjugate mixture and 2 µl of the diluted samples were moved from the setup plate to the final PCR plate and were mixed thoroughly via pipetting. The PCR plate was sealed with an adhesive seal and were mixed via striking the sides of the plate by hand 3 times. The plate was then placed in a centrifuge for 1 minute at 3,000 g. After centrifuging, the plate was incubated at room temperature for 1 hour. During this hour, two reagents, ligase and master mix were set to thaw in ice. After the hour, 2.5 mL of master mix and 15 µl of ligase were mixed in a 5 mL tube via pipetting to make the qPCR reaction mixture. This mixture was then poured into a reagent reservoir where a multichannel pipettor was used to add 16 µl of it to all assay wells. The PCR plate was then sealed with an optical plate seal and mixed by the previously described striking method and centrifuged for 1 minute at 3,000 g. The PCR plate was then run using a qPCR instrument with settings below.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Settings</th>
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<tbody>
<tr>
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</tr>
<tr>
<td>Reagents</td>
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</tr>
<tr>
<td>Reporter dye</td>
<td>FAM</td>
</tr>
<tr>
<td>Quencher</td>
<td>NFQ-MGB</td>
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<tr>
<td>Passive reference</td>
<td>ROX</td>
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<tr>
<td>Assign wells</td>
<td>Define all wells of 96-well plate as Unknown</td>
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Role of TrkB.T1 in Glial Inflammatory Response Elicited by MHV

<table>
<thead>
<tr>
<th>Threshold</th>
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<tbody>
<tr>
<td>Baseline</td>
<td>3-15</td>
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</table>

Table 3. Settings to run qPCR instrument for Proquantum Immunoassay.

Microscopy/Imaging

All phase contrast images were taken with Incucyte® Live-Cell Analysis with a 20x objective, in a 24 well plate including coverslips, with 9 images per well. A video and images from 4 specific time points (30 minutes, 24 hours, 24.5 hours, and 48 hours) were captured. The 30-minute marker represents 30 minutes after MHV was applied to the culture. The 24-hour time stamp represents the state of the culture right before MHV media was removed and fresh media was applied (astrocyte only wells) or MHV media was removed and fresh media, as well as microglia was applied (astrocyte and microglia co-culture wells). At 24.5 hours, the changes discussed previously were made. Finally, the 48-hour mark represents the state of the cells by the end of the experiment. Images were exported out of Incucyte® Live-Cell Analysis software in tiff. format and analyzed with ImageJ. To identify cell density, the default thresholding program on ImageJ was used. All area measurements for cell density are in microns squared (µm²).

Immunofluorescent imaging was taken with Zeiss AxioObserver Z1 at 20x (air) magnification with the support of Cell Biology and Image Acquisition Core (RRID: SCR_021845) funded by the University of Ottawa, Ottawa, Natural Sciences and engineering Research Council of Canada, and the Canada Foundation for Innovation.
Statistics

Quantitative experimental data was analyzed using a 2 Immune challenge (control vs MHV) X 3 Microglial condition (WT microglia vs T1KO microglia vs no microglia) X 2 Astrocyte genotype (WT astrocytes vs T1KO astrocytes) between-subjects ANOVA design. The data were analyzed on IBM SPSS Statistic Version 25 (1.0.0.1406) with a p < 0.05 taken as statistically significant. GraphPad Prism 5 was used to generate a 2x3x2 interaction graph. Bonferroni post-hoc comparison and Tukey’s corrected comparisons were performed to determine significance between factors of interest.
Results

Morphology: Incucyte® Live-Cell Analysis
Figure 2. Time course for WT and T1KO knockout astrocyte only cultures. Incucyte® Live-Cell Analysis Live images were taken from high resolution movie frames over 48 hours. WT (A-D) and T1KO (E-H) astrocytes were untreated or infected with MHV for 24 hours. Then media was changed, MHV removed, and cells further imaged until a total of 48 hours had elapsed. The media change alone caused some very modest clumping of WT astrocytes by 48 hours (B, yellow arrow). The MHV treatment induced a dramatic loss of cell density and astrocyte morphological changes and clumping (D, red arrows). The higher magnification image also reveals viral induced projections between astrocyte aggregates and obvious cellular debris. T1KO profoundly reduced astrocyte density and caused changes in morphology (E-F). As show in the zoomed in image, astrocytes lost their projections and adopted a flattened, hypertrophic shape with marked intracellular vacuoles, consistent with cellular distress (yellow arrow). The T1KO astrocytes that were infected with MHV (G-H) displayed further signs of cellular distress including vacuolation and cell fusion forming syncytia like structures (purple arrow) and extreme cell loss and necrotic cellular debris and viral like fragments (green arrow and high magnification image). (Scale bar is 10 µm).
Over 48 hours, WT control astrocytes displayed typical morphology (Fig 2, A-B), wherein confluency generally increased over time. In contrast, within 24 hours of MHV treatment, the WT astrocytes begin to display reactive phenotypes and developed vacuoles with clear signs of cellular degradation by 48 hours (Sup Fig 1, E-H; Fig 2, D). In contrast to WT astrocytes, T1KO astrocytes did not propagate as rapidly as WT, showing a precipitous decline in cell density (Fig 8, B). They also displayed basal morphology consistent with a reactive phenotype, which lacked branching complexity and had a flattened appearance (Fig 2, E-G). With MHV treatment, T1KO astrocytes further developed signs of distress, with intracellular vacuoles appearing and cells forming large syncytia with multiple cells fusing together by 48 hours (Fig 2, H; Fig 5, T).
Figure 3. Time course for common WT and T1KO knockout astrocyte-microglia co-cultures. Co-cultures were genotype matched, such that both astrocytes and microglial were derived from the same genotype. Incucyte® Live-Cell Analysis Live images were taken from high resolution movie frames over 48 hours. WT (A-D) and T1KO (E-H) astrocytes were untreated or infected with MHV for 24 hours. Then media was changed, MHV removed and microglial cells were added, and cultures were further imaged until a total of 48 hours had elapsed. The immediate (Sup Fig 2, C) addition of microglia and the later time (B, 48 hours) had little impact on cells. The MHV treatment had little effect at the early time (C.), but once again caused marked pathology by 48 hours (D.) and as shown in the zoomed image, was associated with ameboid microglia and “sheets” of necrotic cellular debris (yellow arrows). Cell density was again reduced in T1KO derived astrocyte culture (E) and interestingly, the added microglia in the non-treated condition resulted in a virtual complete loss of astrocytes (F). The T1KO astrocytes responded vigorously to the MHV infection, with a loss of density and cell aggregate formation at 24 hours (G. and high magnification image with red arrow). By 48 hours, the only remaining cells were highly ameboid microglia, rod-shaped microglia or condensed bushy...
hypertrophic microglia (H. and high magnification image with green arrows). (Scale bar is 10 µm).

The addition of microglia to the astrocyte cultures prompted dramatic morphological changes in both WT and T1KO astrocytes (Sup Fig 2, C, D, G, H, K, L, O, P). In the WT control group, a brief decrease of cell area can be seen when microglia are added; however, by the end of the 48 hours the cells mostly regained confluency (Sup Fig 2, A-D). However, in the WT MHV treatment groups, increased reactive astrocyte and microglia morphology can been seen by the 48-hour marker, along with a clear reduction in cell density (Fig 3, C-D). There are also indications of reactive cell morphology and loss of cell coverage after the addition of microglia in the T1KO control group (Fig 3, F). When treated with MHV, T1KO cultures exhibited greater loss of cell density and indications of cell damage compared to the controls (Fig 3, E-H). Overall, visualizations of the T1KO astrocytes and microglia indicate much more reactive astrocyte phenotypes, decreases cell confluency, with dramatic cell loss evident by 48 hours.
Role of TrkB.T1 in Glial Inflammatory Response Elicited by MHV
Figure 4. Time course for mixed WT and T1KO knockout astrocyte-microglia co-cultures.
Co-cultures were mixed, such that astrocytes and microglia came from different genotypes. Incucyte® Live-Cell Analysis Live images were taken from high resolution movie frames over 48 hours. Astrocytes again were untreated or infected with MHV for 24 hours. Then media was changed, MHV removed and microglial cells were added, and cultures were further imaged until a total of 48 hours had elapsed. The top four panels are from T1KO astrocytes that were co-cultured with WT microglia. In the absence of viral treatment (A-B), cell density was low, and signs reactive cell phenotypes and clustering appeared. (A. and high magnification image, yellow arrow). By 48 hours, the few remaining cells appeared flattened ameboid or hypertrophic microglia (B. red arrow). With MHV infection, once again cells had a flattened appearance with intracellular vacuoles and very dramatic long protrusions from cell to cell (D. and zoomed image, orange arrow). The bottom four panels are WT astrocytes (E, G) that were co-cultured with T1KO microglia (F, H). Cell density and morphology were relatively unchanged and not overtly showing signs of distress in controls (E-F) or at the 24-hour time with MHV infection (G). However, by 48 hours there were signs of marked cellular pathology (H. green arrow). As shown in the zoomed area image with the green arrow, the culture was largely showing cellular swelling with “soapy liquified” cellular material and debris clearly consistent with necrotic degeneration. Yet, unlike
some of the other MHV infected groups there were still pockets of astroglia looking cells still alive. (Scale bar is 10 µm).

Similar microglial induced reactivity can be seen when genotypes were mixed between the two cell types, wherein T1KO astrocytes were paired with WT microglia (T1KO-WT) and WT astrocytes were paired with T1KO microglia (WT-T1KO). Overall, the astrocyte morphology of the T1KO-WT group appeared to be less mature, with far less process complexity (Fig 4, A-D). By the end of the experiment, the control T1KO-WT displayed morphological signs of cell reactivity and degradation resulting in a lower cell density (Fig 4, D; Fig 8, B). Reactive astrocyte phenotypes were particularly extensive in response to the MHV-treatment in the T1KO-WT cultures (Fig 4, C-D). Furthermore, at the final 48-hour mark the MHV-treated T1KO WT cultures presented astrocytic syncytia-like morphology (Fig 4, D).

The WT-T1KO group had more complex morphology and developed confluency more rapidly than the aforementioned T1KO-WT group (Fig 4, E & G). In both of the control and MHV-treated groups, the addition of KO microglia caused the WT astrocytes to display increased intracellular vacuoles and more activated astrocyte morphology (Sup Fig 3, K, L, O, P). The MHV treatment generally augmented the manifestation of these pathological cellular features (Fig 4, H).
Morphology: Immunohistochemistry

Figure 5. Cultured WT and T1KO astrocytes in culture exhibit morphological differences to MHV infection. Astrocyte only cultures fixed at 48 hours after MHV application. The figure shows individual immunofluorescence for DAPI (cell nucleus; blue), S100β (astrocytic marker; green), M-Dectin-1 (microglial marker; purple), MHV (viral detection antibody; red), as well as a merged image. Untreated WT astrocytes (A-E) displayed modest S100β immunoreactivity (green). After MHV infection, the astrocytes also showed an obvious intra-cellular signal for MHV (F-J; red). The T1KO astrocytes treated with MHV (P-T) had increased S100β immunoreactivity (green), together with a remarkable increase in MHV spread (S-T, red) It appeared that T1KO facilitated cell to cell fusion to create multinucleated syncytia. (Scale bar 10 µm).

In WT astrocyte only cultures, MHV-treated astrocytes showed signs of MHV infection and tended to aggregate in small syncytia-like shapes (Fig 5, E & J). Untreated T1KO astrocytes displayed minimal branching compared to WT cells, and MHV-treated T1KO astrocytes had increased S100β expression. These cells also aggregated together in large multinucleated syncytia-like forms with increased MHV immunoreactivity (Fig 5, O & T).
Figure 6. WT and T1KO astrocyte-microglia co-cultures display differential immunostaining. Astrocytes were untreated or infected with MHV for 24 hours. Then media was changed, MHV removed and microglial cells were added, and cultures were further imaged until a total of 48 hours had elapsed. The figure shows individual immunofluorescence for DAPI (cell nucleus; blue), S100β (astrocytic marker; green), M-Dectin-1 (microglial marker; purple), MHV (viral detection antibody; red), as well as a merged image. There was obvious immunofluorescence for astrocytes (green) and microglia (purple) in WT control cultures (A-E). The MHV infection had little impact on S100β (G) or M-Dectin-1 (H) and only modest MHV (I) labeling was observed. The T1KO cells displayed increased S100β labelling (L, green) which was further augmented with MHV infection (Q, green). Microglia appeared to adopt a more rounded morphology with diminished processes in T1KO cultures (M, R; purple). The T1KO also increased overall anti-MHV labelling (S-T; red), suggestive of facilitated infection. (Scale bar 10 µm).

The WT control cultures displayed small, ramified astrocytes and microglia (Fig 6, B-C), consistent with a basal or “resting” phenotype. The MHV infection had little impact on the density or morphology of these cells (Fig 6, G-H) and MHV labeling was observed after infection but was minimal (Fig 6, I-J). The T1KO cells displayed signs of increased astrocytic and microglial
reactivity (Fig 6, Q-R), as indicated by condensed S100β and M-Dectin-1 labelling, with aggregating cells that had thickened soma and reduced fiber projections. Microglia in particular, essentially appeared to adopt a more rounded morphology with diminished processes in T1KO cultures. Importantly, the MHV treated T1KO cells showed increased MHV labelling, which is consistent with augmented viral spread. (Fig 6, S-T).

Figure 7. Mixed WT and T1KO knockout astrocyte-microglia co-cultures. Co-cultures were mixed, such that astrocytes and microglia came from different genotypes. Astrocytes were untreated or infected with MHV for 24 hours. Then media was changed, MHV removed and microglial cells were added, and cultures were further imaged until a total of 48 hours had elapsed. The figure shows individual immunofluorescence for DAPI (cell nucleus; blue), S100β (astrocytic marker; green), M-Dectin-1 (microglial marker; purple), MHV (viral detection antibody; red), as well as a merged image. Robust astrocytic S100β labelling was evident in both T1KO astrocyte-WT microglia and WT astrocyte-T1KO microglia mixed control cultures (B, L; green). But this was reduced with MHV infection in both genotypes (G, Q; green). Microglial M-Dectin-1 labelling was very low, with only sparse small fibrous cell evident in T1KO astrocyte-WT microglial mixed
cultures (C, H; purple), although it was moderately increased in WT astrocyte-T1KO microglial mixed cultures (M, R; purple). The level of MHV infection was particularly evident in the T1KO astrocyte-WT microglia culture (I-J, red), compared to the opposite mixed culture (S-T, red). (Scale bar 10 µm).

When co-culturing T1KO astrocytes with WT microglia or the reverse WT astrocytes with T1KO microglia, the astrocytes and microglial signals were similar, with cells having generally spindly, branched appearance (Fig 7, E & O). There was greater S100β labelling evident, compared to the M-Dectin-1, suggestive of a greater astrocyte presence (Fig 7, B, L, C, M). The microglia that were present had small cell bodies with very fine fibrous projection, indicative of a “resting” state (Fig 7, C & M). Interestingly, MHV infection similarly reduced both of the astrocytic and microglial markers in both mixed culture conditions (Fig 7, H & R). The one aspect in which the two mixed cultures that did differ was with regards to MHV infection. Indeed, the T1KO astrocyte-WT microglial culture showed greater MHV immunofluorescence, consistent with viral spread, compared to the WT astrocyte-T1KO microglial mixed culture (Fig 7, I-J & S-T).

**Cell Density**

![Figure 8](image)

**Figure 8. Reduced overall cell density in T1KO cultures and with MHV treatments.** The overall cell density (which includes both astrocytes and microglia) of cultures was assessed in WT and T1KO astrocytes as a function of percentage presence and genotype of microglia and exposure
to MHV or control media. Cell density was assessed at 24.5 hours (A) and 48 hours (B) after the initial exposure to MHV/control media. *p < 0.05, relative to control WT Astro, #p < 0.05, relative to WT Astro. n = 3/group.

Following 24 hours of astrocyte exposure to MHV, the virus was removed, and microglia or control media added and after a further 24 hrs cell density was determined. At the 24.5-hour timepoint of culture conditions (30 min following MHV removal and microglial addition), there was a genotype main effect such that of T1KO astrocytes had significantly lower overall density than did their WT counterparts F(1,24)=32.3, p< 0.0001(Fig 8, A). There were no significant effects for MHV treatment or microglial addition at this early time.

At the 48-hour time point, there was a significant two-way treatment (control vs MHV) x Astrocyte genotype (WT vs T1KO) interaction F(1,24)= 7.45, p< 0.01 (Fig 8, B). The follow up Tukey’s corrected comparisons revealed that the MHV treatment significantly reduced overall cell density only in the context of WT astrocytes alone (no microglia added) or with WT microglia present in the culture (p < 0.05). Also, at the 48-hour time, there was a significant Astrocyte genotype (WT vs T1KO) x Microglial presence (none vs WT vs T1KO) interaction F(2,24)= 3.86, p< 0.05 (Fig 8, B). In this regard, the T1KO astrocytes provoked an overall reduction in cell density, relative to WT astrocytes (p < 0.05). Furthermore, the presence of microglia (either WT or T1KO) increased cell density in WT astrocyte cultures but had the reverse effect in astrocyte T1KO cultures, wherein reductions were evident (p < 0.05).
Cytokine Release

Figure 9. Cytokine changes in T1KO cultures and with MHV treatments. Using a highly sensitive pro-quantum immunoassay, cytokine analyses were conducted on WT and T1KO astrocytes as a function of the presence and genotype of microglia and exposure to MHV or control media. Cell supernatant was collected 48 hours after the initial exposure to MHV/control media. *p < 0.05, relative to No MG; #p < 0.05, relative to WT Astro. n = 3/group.

At the end of the 48-hour time period, there was a significant main effect of astrocyte genotype, with T1KO astrocyte cultures having significantly greater overall TNF-α levels $F_{(1,24)}=4.43$, p<0.05 (Fig 9, A). There was also a significant main effect for the microglial factor, with concentration of TNF-α varying between the three different microglial conditions $F_{(2,24)}=17.1$, p<0.001 (Fig 9, A). The follow up comparisons revealed that the addition of WT microglia to the culture significantly elevated TNF-α levels, relative to the remaining groups (i.e., no microglia and T1KO microglia) (p < 0.05).

There was also a main effect for levels of IL-6 with regards to the microglial condition $F_{(2,24)}=42.5$, p<0.0001 (Fig 9, B). Further comparisons revealed significantly higher IL-6 levels in response to the addition of WT microglia to the culture, relative to the two other microglial conditions (p<0.05). Finally, the levels of the anti-inflammatory cytokine, IL-4, were significantly impacted by the MHV treatment $F_{(1,24)}=4.95$, p<0.05 (Fig 9, C). Although the effect was modest,
MHV did induce a statistically significant reduction of overall IL-4 levels (p < 0.05), irrespective of the genotypes of astrocytes or microglia.

Discussion

Much attention has been devoted to the involvement of BDNF and TrkB.FL in neuropsychiatric and neurodegenerative diseases, but little has been done to assess the importance of the TrkB.T1 receptor. Furthermore, although accumulating data have correlated BDNF levels with anti-inflammatory outcomes, there have been no studies that have interrogated the TRkB.T1 receptor with regards to immunological challenges. To date, there has been no prior work examining how the TrkB.T1 influences astrocytic responses to viral insults, nor how TrkB.T1 influences microglial interactions with astrocytes in this context. Our novel approach examining the role of TrkB.T1 on these two different glial cell types in the context of a viral insult may help broaden our understanding of astrocyte-microglial mechanisms involved in viral-induced neuropathology.

Prior studies have reported phenotypic changes in glial cells when exposed to immune insults such as LPS, Poly I:C and HIV (Koss, Churchward, Tsui, & Todd, 2019; He, Taylor, Yao, & Bhattacharya, 2021; Steiner & Humpel, 2022; Edara, Ghorpade, & Borgmann, 2020). Accordingly, we presently found that exposure to the coronavirus, MHV, induced dramatic fundamental morphological changes, over 48 hours, in WT astrocytes that were infected. Most notably, after 48 (but not 24) hours, there was a dramatic degeneration and loss of astrocytes, and
clumping that formed aggregates that were associated with cellular debris and outstretched viral processes.

Not surprisingly, the addition of WT microglia added to a more complex overall mix of differing cell morphologies over time but did not appreciably influence MHV induced loss of cells. However, the added WT microglial cells did cause a significant elevation of the pro-inflammatory cytokines IL-6 and TNF-α at the 48-hour time point. The microglia also appeared more like typical ameboid M1-like when combined with the MHV infected astrocytes. It is known that such inflammatory ameboid microglia contribute to neuroinflammation, as well as neurodegenerative disease, by playing a role in apoptosis and instigating the release of cytotoxins (Giulian, 1987; Meyer, Lima, Deniselle, & De Nicola, 2022).

Perhaps the most notable finding in the present thesis was the fact that TrkB.T1 deficient astrocytes were profoundly different from their WT counterparts. Indeed, threshold analyses of T1KO astrocyte isolated images revealed an overall lower cell density when compared to WT astrocytes at both 24.5-hour and 48-hour time points, suggestive of reduced viability and increased susceptibility to infection. This is consistent with the only existing report that found sign of an immature morphology in T1KO astrocytes (Holt et al, 2019). The present T1KO astrocytes also were morphologically very different from their WT counterparts, with a hypertrophic appearance and the tendency to aggregate together. These groups had clear signs of cellular distress, including many intracellular vacuoles and signs of cellular swelling with a “soapy-like” appearance. They also had few cellular branches and showed signs of essentially fusing together in multi-nucleated syncytia. Curiously, the addition of TrkB.T1 deficient microglia appeared to modestly reduce this pathology, whereas addition of the WT microglia had the opposite impact. However, in all cases,
by 48 hours there was substantial cell death and the only existing cells appeared to be highly activated M1-like inflammatory amoeboid microglia.

In all groups of T1KO astrocytes (regardless of the presence of microglia or the particular microglial genotype), MHV caused a number of cell nuclei to fuse together and create pathological syncytia. This effect seems most apparent in cultures of pure T1KO astrocytes (Fig. 8). Notably, this phenomenon was previously reported to be caused by MHV in lung tissues (Zhang, et al., 2021; Lin, Li, Wang, & Shi, 2021). The formation of syncytia may increase chances of viral spread, and cytopathic effects, leading to cell death (Rajah, Bernier, Buchrieser, & Schwartz, 2022). The fact that the formation of syncytia was most apparent in T1KO astrocytes, suggests the importance of the receptor in coronoviral spread. In fact, these astrocytic cells often had long projections from one aggregate to another. This is typical of what is observed during viral spread between multinucleated cellular aggregates.

We assessed the pro-inflammatory cytokines IL-6 and TNF-α since they are prominently involved in neurodegeneration following immune challenges and they have recently been identified as potential biomarkers of COVID-19 severity (Lu, et al., 2021). In the presence of viral insults, glial cells, specifically microglia, normally release pro-inflammatory cytokines such as, TNF-α, IL-6, and IL-1β (Tran, Lee, & Cho, 2022; He, Taylor, Yao, Bhattacharya, & al., 2021; Liu, et al., 2017). In the present study, increased levels of IL-6 and TNF-α were induced by the presence of WT microglia, but this effect was absent when T1KO microglia were added to the astrocyte culture. This suggests that TrkB.T1 may be having a pro-inflammatory role in microglia. Yet, since TNF-α levels overall were greater in the T1KO astrocytes (compared to their WT counterparts), the TrkB.T1 receptor may have an anti-inflammatory role in astrocytes. This intriguing finding
raises the possibility that TrkB.T1 has opposing actions on cytokine release in astrocytes vs microglia. It is however noteworthy that in the “no microglia” conditions, little to no IL-6 or TNF-α was detected, which is consistent with the role microglia play in releasing inflammatory factors when activated by viruses such as SARS-CoV-2 (Mishra & Banerjea, 2021; Frank, et al., 2022) and MHV-JHM (Wege, et al., 1998). Taken together, these results reinforce the hypothesis that microglia are mediating the release of pro-inflammatory cytokines and that TrkB.T1 may modulate this response.

In contrast to IL-6 and TNF-α, the anti-inflammatory cytokine, IL-4, was reduced (albeit modestly) by MHV treatment but unaffected by genotype. This finding is consistent with the general notion that viral treatments, such as MHV-JHM, suppress anti-inflammatory cytokine release. While there is a lack of data concerning MHV-induced decrease of IL-4 within the CNS, there is evidence of viral suppression of IL-4 release within the spleen (de Souza, Smith, & Bottomly, 1991). It is also important to note that while this finding is statistically significant, its biological implications are unclear given the magnitude of the effect.

Overall, the addition of WT microglia to astrocytes of either genotype induced a pro-inflammatory response through upregulation of IL-6 and TNF-α, while MHV induced the downregulation of IL-4. An important limitation in this analysis is the timing of the assay. Indeed, cytokine release can be rapidly upregulated and has varied time courses (Fernández-Albarral, et al., 2021; Ozger, et al., 2021; Sullivan, et al., 2000). In our study, cell media supernatant samples were taken at the 48-hour timepoint, and thus future studies should analyze cytokine release at earlier time points of the experiment. By the time of our cytokine analysis, there was already
significant pathology and cell loss, so we likely missed earlier inflammatory cytokine changes that might have been induced by MHV.

At present, there is a lack of literature on the role TrkB.T1 plays in neuroinflammation initiated by a viral insult. Although further research is needed to make conclusive claims, we have demonstrated that in the presence of coronaviral infection, astrocytes lacking the TrkB.T1 receptor rapidly display activated phenotypes and form cell aggregated syncytia. Conversely, microglia lacking the TrkB.T1 receptor may have an opposing impact on inflammatory processes. These novel findings have provided new insights into how the truncated Trk receptor mediates inflammatory responses between microglia and astrocytes. These data enhance our knowledge with regards to viral impact on glial cells and how the trophic BDNF-TrkB axis might control glial viability and resilience in the face of such challenges. Future studies should assess the mechanistic underpinnings responsible for microglial-astrocyte crosstalk following a viral infection, as the nature of this crosstalk may be influenced by genotypic variations that impact the BDNF-TrkB axis.
Appendix A

Formulations of significant media used in cell culture procedures

<table>
<thead>
<tr>
<th>Solution</th>
<th>Ingredients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Awesam media</td>
<td>48 mL Neurobasal Medium, 5 µl of HBEGF, 500 µl Glutamax, 500 µl Pen-strep, 1 mL B27 supplement</td>
</tr>
<tr>
<td>Complete media</td>
<td>445 mL DMEM-high glucose, 50 mL of fetal bovine serum, 5 mL of Pen-strep</td>
</tr>
<tr>
<td>Dissection media</td>
<td>490 mL HBSS, 3g of glucose, 5 mL of HEPES, 5 mL of Pen-strep (final solution was sterile filtered)</td>
</tr>
<tr>
<td>MGM</td>
<td>48.5 mL of DMEM/F-12, 500 µl Pen-strep, 500 µl Glutamax, 500 µl of TNS stock, 50 µl COG stock, 50 µl TCH stock</td>
</tr>
</tbody>
</table>

Appendix B

Formulations of various solutions used in immunohistochemical procedures

<table>
<thead>
<tr>
<th>Solution</th>
<th>Ingredients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blocker</td>
<td>0.75 mL NGS in 6.75 mL of 2% BSA</td>
</tr>
<tr>
<td>2% BSA</td>
<td>0.15g BSA in 7.5 mL Dubecco’s PBS</td>
</tr>
<tr>
<td>PBB</td>
<td>0.75g BSA in 150 mL Dubecco’s PBS</td>
</tr>
<tr>
<td>PBB wash</td>
<td>250 µl of 10% Triton X in 50 mL of PBB</td>
</tr>
<tr>
<td>Permeabilizer</td>
<td>75 µl of 10% Triton X in 7.5 mL of Dubecco’s PBS</td>
</tr>
</tbody>
</table>
Supplementary Information

Supplementary Figure 1. Complete time course for WT and T1KO knockout astrocyte only cultures. Incucyte® Live-Cell Analysis Live images were taken from high resolution movie frames over 48 hours. WT (A-H) and T1KO (I-P) astrocytes were untreated or infected with MHV for 24 hours. Then media was changed, MHV removed, and cells further imaged until a total of 48 hours had elapsed. (Scale bar is 10 µm).
Supplementary Figure 2. Complete time course for common WT and T1KO knockout astrocyte-microglia co-cultures. Co-cultures were genotype matched, where both astrocytes and microglia were derived from the same genotype. Incucyte® Live-Cell Analysis Live images were taken from high resolution movie frames over 48 hours. WT (A-H) and T1KO (I-P) astrocytes were untreated or infected with MHV for 24 hours. Then media was changed, MHV removed and microglial cells were added, and cultures were further imaged until a total of 48 hours had elapsed. (Scale bar is 10 µm).
Supplementary Figure 3. Complete time course for mixed WT and T1KO knockout astrocyte-microglia co-cultures. Co-cultures were mixed, such that astrocytes and microglia came from different genotypes. Incucyte® Live-Cell Analysis Live images were taken from high resolution movie frames over 48 hours. T1KO (A-H) astrocytes and WT (I-P) astrocytes again were untreated or infected with MHV for 24 hours. Then media was changed, MHV removed and microglial cells were added, and cultures were further imaged until a total of 48 hours had elapsed. (Scale bar is 10 µm).
Bibliography


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