Astrocyte-mediated immune modulation during mycobacterial infection

By

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Dissertation presented for the degree of

DOCTOR OF PHILOSOPHY

Division of Clinical Sciences and Immunology

Department of Pathology

Faculty of Health Sciences,

UNIVERSITY OF CAPE TOWN

April 2023

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PUBLICATIONS

Peer reviewed publications/abstracts in international journals:

1. **Geyer, S**., Jacobs, M. & Hsu, N.J. 2019. Immunity against bacterial infection of the central nervous system: an astrocyte perspective. *Frontiers in molecular neuroscience*, 12, 57.
2. **Geyer, S.,** Hsu, N.J., Keeton, R., Jacobs, M. Understanding host immune responses in astrocytes during CNS-TB infection [abstract]. In: Proceedings from the 17th International congress of immunology (IUIS 2019), Oct 17-23, 2019 Beijing, China. European Journal of Immunology, 2019. 49 (Suppl.3):1434. Abstract P0428

Manuscript in preparation:

Geyer, S., Nieuwenhuizen, N., Walters, A., Kaufmann S.H.E., Mollenkopf H.J., Lennard K. S., Mulder N., Hsu N.J., and Jacobs M. Transcriptomic profiling of Astrocytes following Mycobacterium tuberculosis infection.

Conference presentations:

1. Virtual Poster Presentation: **Geyer, S.,** Hsu, N.J., Keeton, R., Walters, A., Jacobs, M. Investigating host immune responses in astrocytes during CNS-TB infection. Tuberculosis: Science aimed at ending the epidemic, Keystone Symposia eSymposia, 2020
2. Virtual Oral Presentation. **Geyer, S.,** Hsu, N.J., Keeton, R., Walters, A., Jacobs, M. Investigating host immune responses in astrocytes during central nervous system-tuberculosis infection. SAMRC 14th Early Career Scientist Conference, 2020
3. Poster Presentation: **Geyer, S.,** Hsu, N.J., Keeton, R., Walters, A., Jacobs, M. Understanding host immune responses in astrocytes during CNS-TB infection. 17th International congress of immunology, IUIS. Beijing, China 2019
4. Oral Presentation: **Geyer, S.,** Hsu, N.J., Keeton, R., Walters, A., Jacobs, M. Understanding host immune responses in astrocytes during CNS-TB infection. Synergy in Immunology, 7th SAIS Congress, Durban, South Africa 2019.

DEDICATION

To my dearest children Razzúq and Aila.

May this inspire you to be like a river, carving its way through the mountains, persistent and unyielding in its pursuit of the sea. May you navigate the obstacles and forge new paths with unwavering courage, always moving forward towards your very own ocean of dreams.

ACKNOWLEDGEMENTS

I'd like to first and foremost give thanks to The Almighty for guiding me with infinite wisdom throughout my PhD journey. Thank you for bringing me peace and clarity, and for helping me navigate the most challenging moments when I felt lost, overwhelmed, or uncertain. I am grateful for the insights gained through reflection and for the blessings that enabled me to pursue my dreams.

It is a rare opportunity to be mentored on both professional and personal aspects of life by the same person, and I consider myself fortunate to have had Prof. Muazzam Jacobs as both my supervisor and mentor. Thank you, Prof. M., for believing in me and providing invaluable support, advice, and opportunities that helped me achieve my academic goals. Your expertise in the field, unwavering encouragement, and motivation pushed me to strive for excellence, and your continuous feedback greatly enhanced the quality of my research work. I am deeply grateful for your wise counsel and consistent guidance, which sustained me through many challenges and contributed to my personal growth and development. I cannot thank you enough for all you have done to help me realise my potential.

I also wish to convey my heartfelt appreciation to Dr Nai-Jen Hsu. Thank you for your incredible support, mentorship and teachings. Your extensive knowledge and comprehensive training were crucial in shaping my technical abilities and the direction of my research. Your astute feedback and constructive criticism helped me refine my ideas and approach my research from a fresh perspective. Dr Hsu, thank you for your continual encouragement and willingness to lend a listening ear. The time and effort you invested in me have not gone unnoticed, and I feel fortunate to have had the opportunity to learn from you. I will always remember the endless BSL3 hours and our shared love and appreciation for food!

To my Flow Cytometry guru, Dr Roanne Keeton. Thank you for the flow cytometry training and for always providing assistance especially during troubleshooting. I am also grateful for the extensive hours you put into my BSL3 training in preparation for accreditation. I

highly appreciate the opportunities you granted me as well as your continuous patience, support, and guidance over the years.

I would like to extend a special thank you to our collaborators' Dr Hans-Joachim Mollenkopf and Dr Natalie Nieuwenhuizen from the Core Facility Genomics/Microarray, Max Planck Institute for Infection Biology (Berlin, Germany) as well as Dr Katie S. Lennard from the Division of Computational Biology, Department of Integrative Biomedical Sciences and Institute of Infectious Disease and Molecular Medicine (Faculty of Health Sciences, University of Cape Town, South Africa). You each played an instrumental role in the microarray and transcriptomic analysis, and for this I am truly grateful. Thank you for your time, assistance and feedback.

To the Jacobs' group: I would like to extend my deepest gratitude to Mr. Faried Abbass for his invaluable assistance in the lab which has been instrumental in my research. I would also like to express my heartfelt appreciation to my colleagues, past and present - Antoinette Labuschagne, Avril Walters, Emily Tangie, and Petr Konecny, for their unwavering support, encouragement, and experimental assistance. Working alongside you has been a privilege, and I am grateful for the camaraderie and laughter we shared, which made the working space a pleasant and enjoyable place.

Furthermore, I would like to thank my SATVI office comrades, Claire Imbrattta, Agano Kiravu, and Tim Reid, for their continued support and assistance throughout my academic journey. Your encouragement and guidance have been invaluable, and I am grateful for the positivity and sense of community you brought to the office.

No science can be performed without technical support. To all members of the Division of Immunology and the various core facilities, thank you for your technical support and assistance. Many thanks to the UCT Research Animal Unit and its staff, particularly Mr Rodney Lucas who always made himself available to train and assist me when needed. To Prof. Dirk Lang and Ms Susan Cooper from the Confocal Facility, thank you for your time spent training me and helping me capture magnificent confocal images. Thank you to the Flow Cytometry core facility, and the manager Mr Tim Reid, for all the support,

advice and troubleshooting. Thank you to the UCT Histology Unit, particularly Lizette Fick for her contributions to the histology experiments. Thank you to Stellenbosch University for conducting the Luminex Multiplex Assay, particularly Candice Snyders (Immunology Research Group, Division Molecular Biology and Human Genetics, Faculty of Medicine and Health Sciences, University of Stellenbosch), for her assistance.

I would like to thank the National Research Foundation (South Africa), Deutscher Akademischer Austausch Dienst (Germany) and the South African Medical Research Council for the financial support received during my PhD.

To my parents, thank you for your unwavering love, support, and guidance throughout my academic journey. Your tireless efforts and sacrifices have taught me the true meaning of dedication, perseverance, and resilience. Your constant faith in me has been a driving force behind my success and helped me reach this significant milestone in my life. I will always cherish the lessons and values you have instilled in me, which shaped me into the person I am today and helped me overcome countless obstacles.

I want to take a moment to express my heartfelt gratitude to my husband and best friend, Faheem Benjamin, for being my pillar of strength, my supporter, and my partner throughout my PhD journey. You are the lighthouse that guided me through the turbulent waters of my PhD. Your unwavering belief in my abilities and steady encouragement illuminated my path and helped me navigate the stormy seas of academic challenges. Thank you for being my rock during this journey, for always being there for me and believing in me, especially when the waves of doubt and uncertainty threatened to overwhelm me. Your love and unwavering support made all the difference, and for that I am eternally grateful.

Throughout my PhD journey, my children were my biggest cheerleaders, always reminding me of my worth and my ability to conquer anything that came my way. Their love, support, and encouragement kept me going even when the research work became overwhelming. I deeply appreciate their patience during the times when my research work consumed most of my time and energy, leaving little room for family time. Thank

you Razzúq and Aila, for being my shining stars and guiding light, my motivation, and my greatest source of joy. Your understanding, support and massages allowed me to pursue my goals with a sense of peace and confidence, knowing that you were behind me every step of the way. You have made my PhD journey a richer, more meaningful experience, and I love you both all the way to the moon and back into my arms.

To my beloved grandmothers, Ma June and Ma Eeza, who always believed in me and supported me in pursuing my dreams. Although you are no longer with us, your love and guidance continue to inspire me every day. I want to express my deepest gratitude for your love and encouragement, and for the wonderful memories that we shared. You were always there for me, each in your own way. Your words of wisdom, your kindness, and your unwavering support have left an indelible mark on my life, and I am forever grateful to you both. I carry your love and guidance with me always, and I know that you are still with me, cheering me on as you always did. I love you and miss you both dearly.

To the rest of my family and friends. There are too many of you to mention, but each and every one of you played an integral part in this process. Whether it was a wellness check-in, offering me motivation and encouragement, looking after the kids, or just simple a chat. All your contributions are greatly appreciated.

ABSTRACT

Central nervous system tuberculosis (CNS-TB) is the severest clinical extra-pulmonary manifestation of tuberculosis (TB) disease and constitutes approximately 1% of global cases. Little is known about the cells that regulate immune responses during CNS-TB infection. Astrocytes is an important cell type demonstrated to regulate innate and adaptive immunity in CNS disease and injury. Astrocytes play a progressive role in maintaining the structural and functional integrity of the CNS while supporting neuronal function and participating in host protection during infection of the CNS. They exist as distinct populations with complex morphological identities and functional modifications suited to their micro-environment. The principal aim of this study was to elucidate the transcriptional profile and cellular immune responses of astrocytes to gain insight into their immunomodulatory potential during CNS-TB infection.

The study incorporated both *in vitro* and *in vivo* experimental methods. Mycobacterial internalisation was assessed both in primary astrocyte cultures and in mice. For the first time, internalisation of *M. bovis* BCG and *M. tuberculosis* bacilli were conclusively demonstrated, indicating that astrocytes are target cells for non-virulent and virulent mycobacterial strains. All transcriptional data sets were generated from primary astrocyte cultures and validated by protein expression in primary astrocyte cultures and in mycobacterial infected mice via flow cytometric and multi-analyte analysis.

The findings of this study are novel and collectively demonstrate the sophistication and complexity of astrocyte activity and functions during host immunity. Extensive transcriptomic analysis of primary cultured astrocytes revealed increased expression of multiple pathways in infected cells, particularly those involved in inflammation and immune regulation, and emphasised potential participation during innate immunity. Notably, various pro-inflammatory cytokines and chemokines essential for host defence during CNS-TB were upregulated by astrocytes following mycobacterial challenge. The enhanced expression and production of well-described pro- and anti-inflammatory factors as well as chemotactic factors following mycobacterial infection may potentially modulate host immune responses by regulating blood-brain barrier permeability and facilitating immune cell recruitment and activation at the

site of infection. Furthermore, increased expression of neurotrophic factors by astrocytes was observed; by supporting neuronal functions and modulating neuroinflammation, astrocyte-derived neurotrophic factors can help to limit infection-induced damage and promote the resolution of inflammation during CNS-TB. The dichotomous behaviour of astrocytes in which they contribute to the maintenance and protection of CNS function and host immune responses while potentially enhancing pathology during infection was evident in the transcriptional and translational profiles.

This study explored astrocyte contributions to host immunity during CNS-TB by examining their potential regulation of CNS inflammation in the presence of mycobacterial challenges and highlighted the intricate interplay of cytokines/chemokines that need careful modulation to achieve optimal outcomes. These findings demonstrate that astrocytes are crucial regulators of host immunity during mycobacterial infection and play a progressive role in maintaining the structural and functional integrity of the CNS.

ABBREVIATIONS

APC: Antigen-Presenting Cell

BBB: Blood Brain Barrier

BCG: Bacillus Calmette-Guerin

BCG-GFP: Bacillus Calmette-Guerin expressing Green Fluorescent Protein

β-Tub: Beta-III-Tubulin

BSA: Bovine-Serum Albumin

CNS: Central Nervous System

CNS-TB: Tuberculosis infection of the Central Nervous System

CSF: Cerebrospinal fluid

DC: Dendritic cell

DEG: Differentially Expressed Gene

DMEM-F12: Dulbecco's Modified Eagle's Medium F12

FACS: Fluorescent Activated Cell Sorting

FBS: Fetal Bovine Serum

HBSS: Hank's Balanced Salt Solution

hNSG: Humanised NOD-*scid* IL2Rgamma^{null}

GDNF: Glial Derived Neurotrophic Factor

ICAM: Inter-Cellular Adhesion Molecule

ICC: Immunocytochemistry

IFN: Interferon

IHC: Immunohistochemistry

IL: Interleukin

ISG: Interferon Stimulated Gene

LPS: Lipopolysaccharide

LTBI: Latent Tuberculosis Infection

MCP: Monocyte Chemotactic Protein

MDR: Multidrug-Resistant Tuberculosis

MFI: Mean Fluorescent Intensity

MHC: Major Histocompatibility Complex

MMP: Matrix metallometalprotease

MOI: Multiplicity of Infection

Mtb: *Mycobacterium tuberculosis*

NSG: NOD-*scid* IL2Rgamma^{null}

NSG-BLT: NOD-*scid* IL2Rgamma^{null}-Bone Liver Thymus

PenStrep: Penicillin Streptomycin

PBS: Phosphate-Buffered Saline

PFA: Paraformaldehyde

PRR: Pattern Recognition Receptor

RBC: Red blood cell; erythrocyte

RR-TB: Rifampicin-Resistant Tuberculosis

SPF: Specified-Pathogen Free

TB: Tuberculosis

TBM: Tuberculous Meningitis

TGF-β: Transforming Growth Factor-Beta

Th: T helper

TLR: Toll Like Receptor

TNF: Tumour Necrosis Factor

XDR-TB: Extensive Drug-Resistant TB

"*The teacher who is indeed wise does not bid you to enter the house of his wisdom but rather leads you to the threshold of your mind*."- Kahlil Gibran

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Introduction

1.1 Aetiology and Epidemiology of Tuberculosis

Tuberculosis (TB) is a devastating infectious disease that has burdened humans throughout history. While the source of this disease was previously unknown, its symptoms were familiar and recognised through the ages as consumption, phthisis, and the white death or great white plague. A book dating back to 1720 entitled “A New Theory of Consumptions” by English physician Benjamin Marten proposed that it could be caused by “wonderfully minute living creatures,” emphasizing the spread by living entities (Doetsch, 1978). In 1821 a noteworthy discovery of Rene´ The´ophile Hyacinthe Laennec was the illustrations of abnormalities and tuberculous cavities in his book, A Treatise on Diseases of the Chest. Today these cavities are identified as caseous necrosis (Schluger, 2005). Later, Jean-Antoine Villemin described the infectious nature of TB, but until then, the etiologic agent remained unknown. It was only in 1882 when Marten’s theory was confirmed by Robert Koch, through his discovery of *Mycobacterium tuberculosis*, that the bacterium responsible for TB was identified.

Despite a consistent downward trend in TB cases, it is still among the top 10 life-threatening communicable diseases worldwide, classed as one of the most fatal diseases from a single infectious agent, second only to Covid-19. TB is a prevailing concern that has claimed an estimated 1.4 million lives in 2021 of which 187 000 fatalities were associated with HIV (WHO, 2022). The problem is further compounded by incessant high levels of multidrug-resistant TB (MDR-TB), classified as bacterial resistance to both rifampicin and isoniazid. In 2019 there were an estimated 450 000 people who developed rifampicin-resistant TB (RR-TB) or MDR-TB (WHO, 2022). The burden of drug resistant TB is a hovering concern with global treatment success undesirably low at only 57%. This coursing epidemic is highlighted as a universal health prerogative and a key public health priority in South Africa. Demographic factors such as poverty, malnutrition, overpopulation, and the persistent high incidence of MDR-TB are

considerable elements in the coursing TB global epidemic (Corbett et al., 2006). Aside from the MDR-TB battle are the challenges with extensive drug-resistant TB (XDR-TB) (resistance to isoniazid, rifampin and at least one second-line drug) which is essentially an untreatable disease in developing regions. Effective TB control programs entailing efficient diagnosis, drugs, and vaccines are fundamental to managing this disease.

Amplifying the current TB challenges and straining an already overwhelmed healthcare system is the COVID-19 pandemic caused by the novel coronavirus 2 (SARS-CoV-2) (Gorbalenya et al., 2020). The impact of COVID-19 extended beyond the direct consequences of the virus, such as mortality and morbidity. One distressing instance of this pandemic was the disturbance of vital services for individuals with TB. It exemplified how the pandemic disproportionately impacted some of the most impoverished populations, who were already at a heightened risk of developing TB. The COVID-19 pandemic has negatively impacted the progress in reducing the global TB burden through the reallocation of staff, resources, and funding. Provisional data from 84 countries indicated a 21% decline in case notifications in 2020 compared to 2019. South Africa was identified as one of the top four countries with the greatest shortfalls displaying a 41% reduction in case notifications (Fig. 1.1). Economic impact, loss of income, and increased unemployment rates due to the COVID-19 pandemic are estimated to elevate TB cases by 1 million cases per year over the next six years, while disturbances in TB care could result in an additional half a million deaths (Mcquaid et al., 2021, Soko et al., 2021, Khan et al., 2021, Maurer et al., 2021). Furthermore, this has highlighted the importance of the TB regimen and how reduced access to diagnostic and treatment services disrupts essential TB care, especially in impoverished countries.

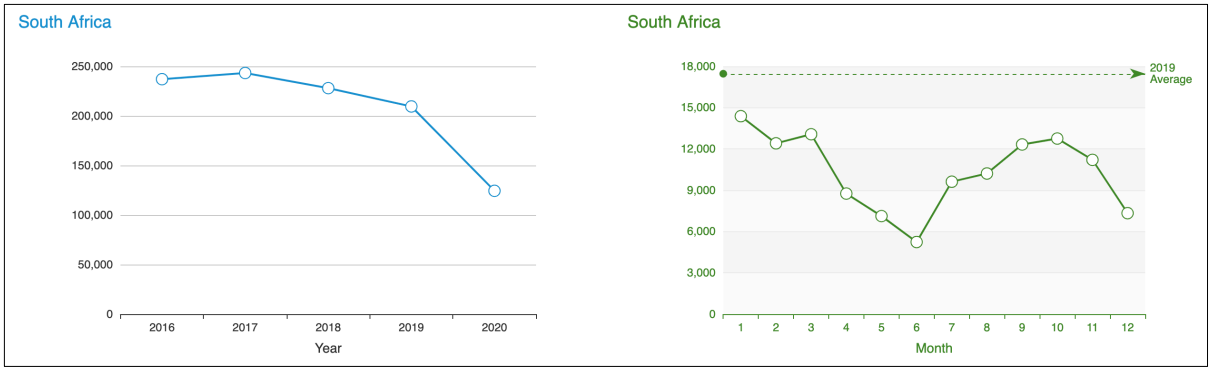


Figure 1.1 | South African case notifications of TB incidence per year from 2016-2020. Data shows a 41% reduction in reports of new and relapse TB cases in 2020 compared to 2019 as a result of the COVID-19 pandemic (WHO, 2020).

1.2 Pathogenesis of Tuberculosis

1.2.1 Pulmonary tuberculosis

Tuberculosis is typically a pulmonary disease with the lungs serving as both the entry point and primary site of infection. After inhalation of infectious droplets, the bacilli are engulfed by resident alveolar macrophages and contained within a phagosome. The maturation of the phagosome into a phagolysosome, through the subsequent fusion with a lysosome, creates an acidic bactericidal compartment within which the ingested bacillus can be destroyed. Distinct from other pathogens, *M. tuberculosis* has developed strategies for exploiting the host and evading bacterial lysis. Once internalised *M. tuberculosis* perturbs host function by arresting phagosome maturation and establishing a niche for intracellular life within an endosome-like compartment (Malik et al., 2003, Kyei et al., 2006, Kang et al., 2005, Nigou et al., 2001). Some studies suggest that maturation is blocked at an early endosomal stage (Appelberg, 2006, Russell, 2011, Ehrt et al., 2015), while mouse macrophage studies indicate that new compartments are established after temporary residence within phagolysosomes (McDonough et al., 1993, de Chastellier, 2009).

Following the phagocytosis of *M. tuberculosis* by macrophages, various cytokines, and chemokines are released attracting circulating immune cells to the site of infection, with neutrophils being the first to arrive. Monocytes recruited from the blood differentiate into macrophages once they reach the tissue and initiate phagocytosis. Dendritic cells (DCs),

another principal cell involved in *M. tuberculosis* infection, transport bacterial antigens to the draining lymph nodes and initiate adaptive immunity (Wolf et al., 2008). Granulomatous focal lesions are formed in response to perpetual stimuli and are critical for containing infection (Williams and Williams, 1983, Russell et al., 2009). Granuloma structures, however, inadvertently create a reservoir for bacilli persistence and may facilitate proliferation and dissemination (Davis and Ramakrishnan, 2009, Ramakrishnan, 2012, Flynn, 2004). Disease prognosis is determined by the host's ability to eradicate the bacilli. Mycobacteria that are not eliminated may be contained within these granulomas in a latent state for prolonged periods or may progress to active disease, usually during conditions of a compromised immunity (Fig. 1.2) (Modlin and Bloom, 2013, Achkar and Jenny-Avital, 2011). Latent TB infection (LTBI) is characterised as a clinically asymptomatic state and accounts for 90% of infections (Cadena et al., 2017); conversely, active TB patients display clinical symptoms of disease and represent a much smaller set of individuals. The traditional categorisation of infection into active TB and LTBI was criticised for being too facile due to the limited binary approach. Instead, they represent two extremities of a continuum of infection outcomes determined by interactions between the host and bacteria (Achkar and Jenny-Avital, 2011, Flynn et al., 2015). A fine balance between protection and pathogenesis is maintained with a shift in immune competency or bacterial replication influencing disease progression.

In the event of immune disruption, dormant bacilli can reactivate causing the death of phagocytic cells, the formation of a necrotic caseous core, termed the caseum, and the subsequent disintegration of the granuloma. Rupture of these structures releases bacilli, allowing it to spread within the surrounding lung parenchyma or disseminate via the lymphatic or blood circulatory systems to remote tissue (Fig. 1.2). This process is a hallmark of active TB disease (Coleman et al., 2014).

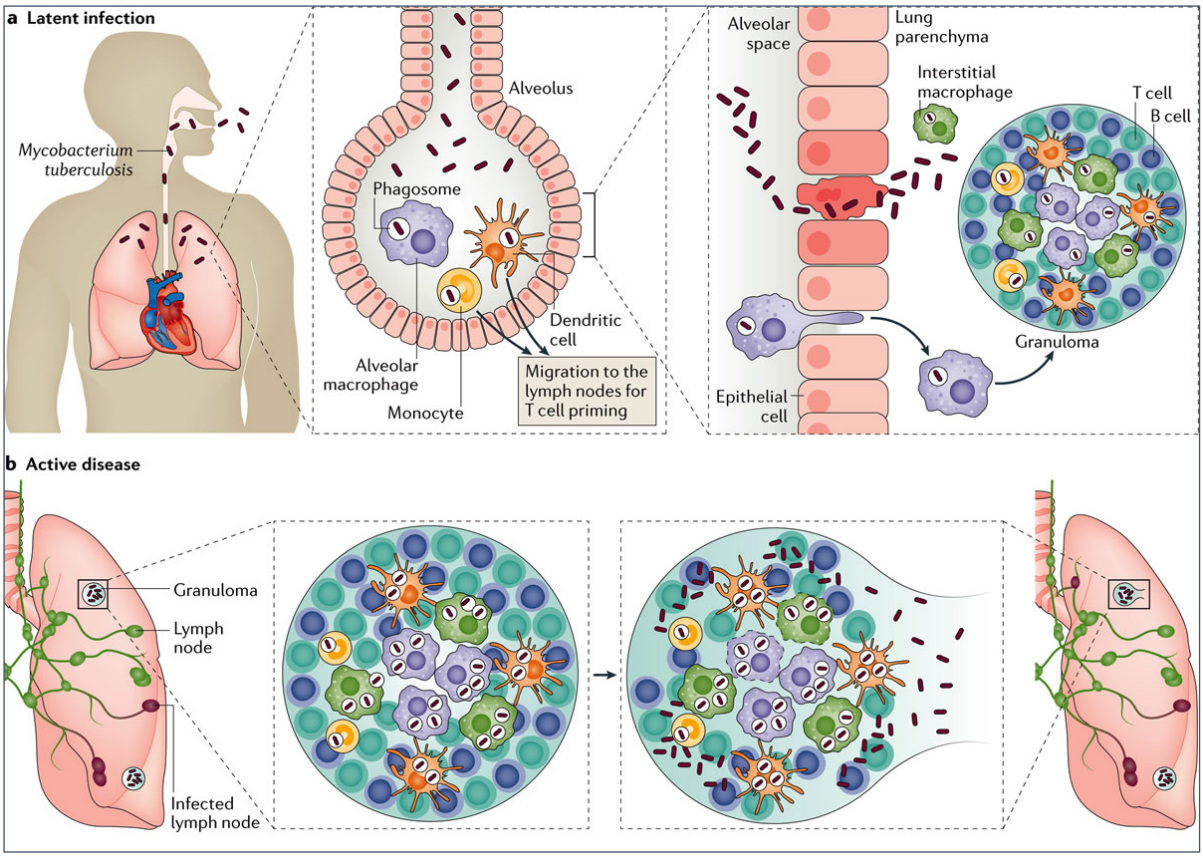


Figure 1.2 | Pathogenesis of tuberculosis. Following *M. tuberculosis* inhalation, resident alveolar macrophages engulf the bacilli and through the release of various cytokines and chemokines, recruit peripheral immune cells to the infection site. Dendritic cells migrate to the lymph nodes where they initiate an adaptive response by presenting antigen to T cells. The influx of various cell populations into the lung parenchyma creates an organised aggregation known as a granuloma. This structure is created to contain the infection and eliminate the bacteria. (a) The bacilli may persist within the granuloma for an extended period as a latent TB infection (LTBI). (b) In some cases, however, there may be a reactivation of infection due to necrosis of the granuloma centre causing their disintegration and eventual rupture leading to active disease. Bacilli are then free to invade the lung and environment, or to spread hematogenously to other organs (Pai et al., 2016).

1.2.2 Central nervous system tuberculosis

Invasion of the central nervous system (CNS) by infectious agents is a major healthcare concern (John et al., 2015, Robertson et al., 2018). Despite the presence of effective barriers, various pathogens such as viruses, bacteria, fungi, protozoa, and parasites can disrupt the blood-brain barrier (BBB), often with chronic implications or fatal outcomes. Clinically, the primary classification of CNS infections is based on the affected anatomical regions, notably meningitis, encephalitis, and myelitis. Viral infections of the CNS have been discussed extensively in current literature, with emphasis on disease progression, especially in immune-compromised individuals (McGavern and Kang, 2011, Eugenin et al., 2006, Pruitt, 2021, Verma et al., 2009, Hollander and Stringari, 1987). However,

bacterial infections of the CNS are potentially more threatening in terms of disease severity, particularly in developing countries where bacterial meningitis is a leading cause of severe neurological sequelae and high mortality (Lucas et al., 2016, Pelkonen et al., 2009, Zainel et al., 2021, Hsu et al., 2018). Among a few of the bacterial species involved in CNS infections are *Listeria monocytogenes, Borrelia burgdorferi, Neisseria meningitides, Streptococcus pneumoniae, Staphylococcus aureus, Haemophilus influenzae, Mycobacteria tuberculosis, Brucella abortus,* and *Brucella melitensis.* (Drevets et al., 2004, John et al., 2015, Robertson et al., 2018). Bacterial infections in the CNS have received significant attention, and considerable research is dedicated to understanding their pathogenesis, diagnosis, treatment, and prevention. Although comprehensive studies are available on *M. tuberculosis* in the CNS, there are areas where further investigation is needed. Elucidating the pathogenesis of central nervous system tuberculosis (CNS-TB), particularly exploring host-pathogen interactions, is necessary to identify potential targets for new therapies.

Pulmonary TB is the predominant form of tuberculosis, but patients can present with various clinical manifestations. Of the 7.1 million notified cases of TB in 2019, 5.9 million had pulmonary TB (84%), and extrapulmonary TB accounted for the other 16% (Fig. 1.3) (WHO, 2020). The severest extrapulmonary development is CNS-TB, with an incidence of 1% of all and 10% of extrapulmonary cases. CNS-TB has been classified as a global research priority (John et al., 2015) and can emerge in several forms namely tuberculosis meningitis (TBM), intra-cranial tuberculomas, tuberculous abscess, spinal tuberculoma, spinal meningitis, vasculopathy, encephalopathy, Pott's spine, and Pott's paraplegia (Smith, 2003, Rock et al., 2008, Garg, 1999, Cherian and Thomas, 2011). This disease is associated with high mortality and significant morbidity, disproportionately affecting young children (Lincoln et al., 1960, Girgis et al., 1998, Cruz and Starke, 2007) and immune-compromised individuals (Leeds et al., 2012, Kingkaew et al., 2009, Jones et al., 1993, Singh and Paterson, 1998). Although the neonatal Bacillus Calmette–Guérin (BCG) vaccine is presumably 73% effective at TBM prevention, several thousand children are estimated to die of TBM each year (Chiang et al., 2014). HIV co-infected individuals have an increased risk of developing TBM (Berenguer et al., 1992, Katrak et al., 2000), and those co-infected with MDR-TB have a poor prognosis with virtually 100% mortality

(Tho et al., 2012). Early diagnostic challenges, inadequate treatment outcomes (Chiang et al., 2014) and limited anti-microbial penetration of the CNS increases the risk of irreversible neurological damage and mortality (Sofia et al., 2001, Padayatchi et al., 2006).

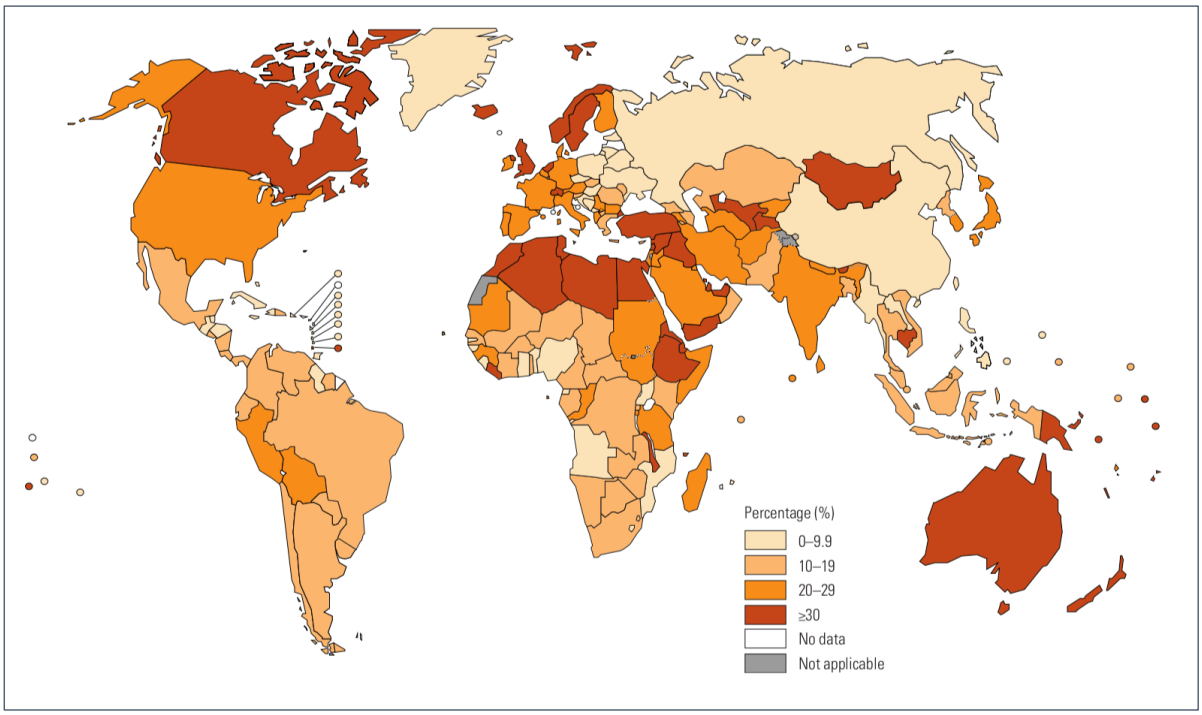


Figure 1.3 | Extrapulmonary TB cases made up 16% of total new and relapse TB cases globally in 2019. Incidence ranged from 8% in the Western Pacific to 24% in the Eastern Mediterranean regions (WHO, 2020).

Following initial infection and granuloma escape in the lungs, *M. tuberculosis* bacilli disseminate via the circulatory system (Krishnan et al., 2010, Jain et al., 2006, Nguyen and Pieters, 2005) (Fig. 1.4). The bacilli then migrate across the BBB and blood-CSF barrier via two proposed mechanisms. Animal models (Wu et al., 2000, Sánchez-Garibay et al., 2018, Rock et al., 2008, Rich, 1933) and *in vitro* studies (Jain et al., 2006) have shown that extracellular bacilli can freely traverse the vascular endothelial cells, with the *M. tuberculosis* gene Rv0931c (pknD) facilitating endothelial adhesion (Be et al., 2012, Skerry et al., 2013). The second mode-of-entry is the "Trojan Horse" mechanism whereby infected DCs, neutrophils, and macrophages enable bacilli access across the BBB (Nguyen and Pieters, 2005, Davis et al., 2019, Jain et al., 2018). Permeating *M. tuberculosis* bacilli preferentially infect microglia in the CNS (Peterson et al., 1995,

Spanos et al., 2015, Rock et al., 2005), leading to their activation and the release of an array of cytokines and chemokines which disrupt the BBB and regulate the recruitment of peripheral immune cells (Wilkinson et al., 2017, Almolda et al., 2015). Infected cells and infiltrating neutrophils, lymphocytes, and monocytes form tuberculous granulomas or "Rich's foci" around the mycobacteria in the meninges or brain parenchyma (Fig. 1.4). The eventual rupture of these foci allows bacterial dissemination into the subarachnoid space or ventricular system, resulting in inflammatory meningitis (Be et al., 2009, Rich, 1933). Most recovered patients present various neurological sequelae and systemic complications, often consequences of pathological neuroinflammation (Be et al., 2009, Rock et al., 2008).

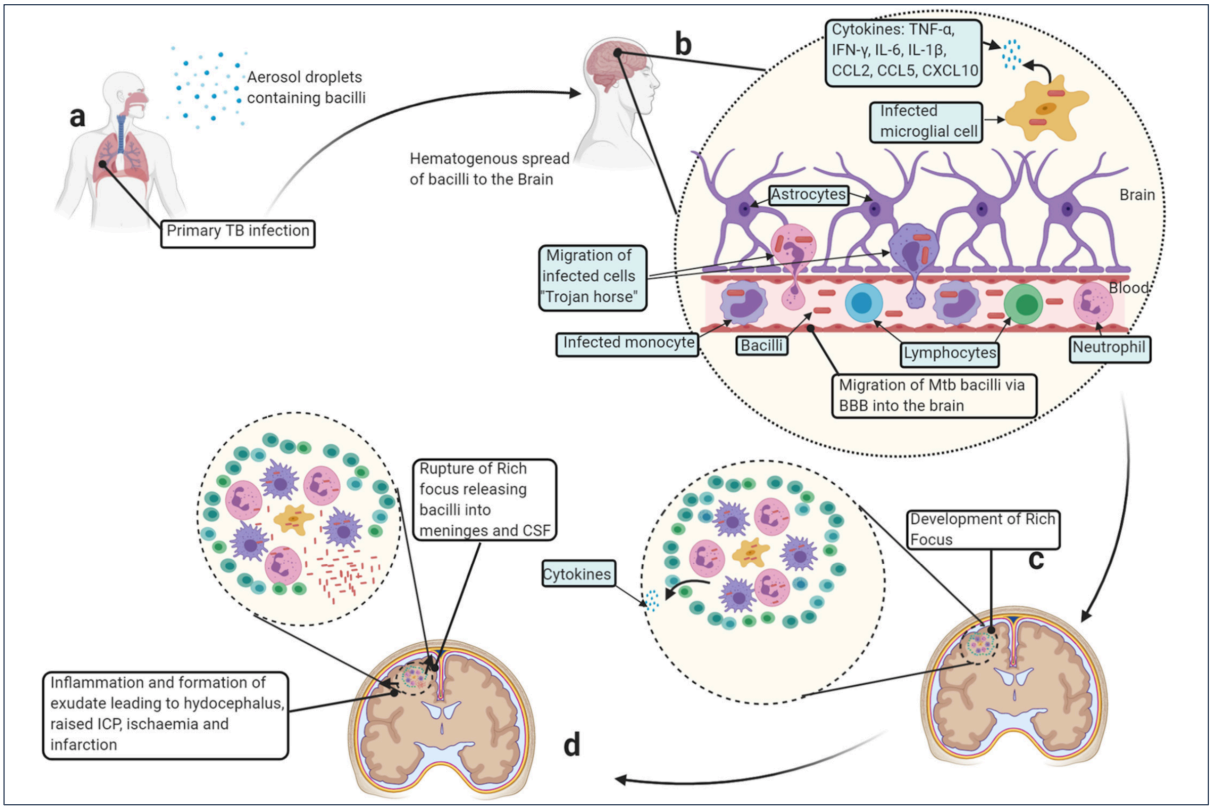


Figure 1.4 | Pathogenesis of Central Nervous System Tuberculosis. Following the establishment of a primary infection within the lungs, bacilli may be released from a collapsing granuloma allowing it to disseminate to the brain. Infected cells and extracellular bacteria traverse the BBB and infect resident brain cells which respond through the release of immune mediators such as cytokines and chemokines. These mediators disrupt the BBB and influence the infiltration of immune cells into the brain parenchyma. Tuberculous granulomas, known as "Rich foci", eventually develop, and their ensuing rupture causes bacteria to disperse into the meninges and CSF leading to meningitis and exudate build up (Manyelo et al., 2021).

1.3 Host immune response to central nervous system tuberculosis

Anatomically, the CNS is uniquely compartmentalized in disparate regions where barriers established by endothelial cells, epithelial cells, and the glial limitans effectively control access of immune cells to the CNS (Engelhardt et al., 2017). This evolutionary adaptation protecting it from damaging immune-mediated inflammation has given the perception of the CNS as an immune-privileged site. However, the mechanisms of immune privilege were redefined by the discovery of a lymphatic system within the meninges, representing a credible path for circulating immune cells to access and patrol meningeal compartments (Louveau et al., 2015). Compartmentalization of immune privilege allows areas of the CNS to be under constant surveillance, enabling resident cells to respond quickly and effectively to pathogenic challenges. Cells of the peripheral adaptive immune system are simultaneously recruited while the tight regulation of entry into the CNS parenchyma maintains tissue homeostasis. (Engelhardt et al., 2017, Galea et al., 2007).

The host inflammatory response is an integral part of TB meningitis pathology. Resident cells such as microglia, astrocytes, and potentially neurons (Peterson et al., 1995, Randall et al., 2014, Rock et al., 2005) regulate inflammation through cytokine and chemokine release during CNS-TB infection. Production of various cytokines and chemokines stimulates the expression of cell adhesion molecules on endothelial cells allowing peripheral cells to adhere and migrate into the CNS via diapedesis. Recruited neutrophils, activated macrophages, and lymphocytes amplify the response via cytokine supplementation. IL-1β, IL6, IL8, IL10, IFN-γ and TNF are some of the mediators elevated in the CSF of TB meningitis patients (Simmons et al., 2006, Mastroianni et al., 1997, Yadav et al., 2010, Marais et al., 2010, Nagesh Babu et al., 2008, Yilmaz et al., 2002, Misra et al., 2010).

TNF is critical to host control of mycobacteria (Mastroianni et al., 1997, Tobin et al., 2012, Tsenova et al., 1999, Kaplan and Freedman, 1996). Studies using mice with TNF deletions have shown that it is crucial for protective immunity against CNS-TB (Hsu et al., 2017, Francisco et al., 2015). Rabbit models have demonstrated that elevated levels of TNF in CSF corresponded with a more severe presentation of TB meningitis (Tsenova

et al., 1999). Following treatment with a TNF antagonist, thalidomide, their rabbit survival improved (Tsenova et al., 1998). Although an early clinical trial of thalidomide treatment in children with TB meningitis was discontinued due to adverse side effects (Schoeman et al., 2004), more recent trials have shown that thalidomide treatment was safe, well-tolerated, and clinically efficacious at lower doses (van Toorn et al., 2021, van Toorn et al., 2015, Schoeman et al., 2006, Schoeman et al., 2004).

Similar to TNF, IL-6 is a pleiotropic cytokine released during early stages of brain infection by microglia, astrocytes, macrophages, and endothelial cells (Isabel and Rogelio, 2014). While one study showed that IL-6 deficient mice failed to control *M. tuberculosis* infection (Ladel et al., 1997); another demonstrated that IL-6 is not essential for immunity against *M. tuberculosis* even though it is involved in IFN-γ production (Saunders et al., 2000). Moreover, studies have noted that CSF concentrations of IL-6 were independently associated with worse outcomes in TBM patients (Simmons et al., 2006). Whether this is due to its pro- or anti-inflammatory properties is unknown. The inflammatory response driven by IL-1 also promotes resistance to *M. tuberculosis* (Van Der Niet et al., 2020, Bohrer et al., 2018, Mayer-Barber et al., 2014, Mayer-Barber et al., 2011). Mice deficient in IL-1α, IL-1β or IL-1R1 demonstrated high susceptibility to *M. tuberculosis* infection (Juffermans et al., 2000, Yamada et al., 2000, Fremond et al., 2007, Mayer-Barber et al., 2011).

In addition to their role in host defence against *M. tuberculosis* infection, TNF, IL-6, and IL-1 regulate BBB function and integrity, thereby controlling cellular entry into the brain (de Vries et al., 1996, de Vries et al., 1997, Figiel and Dzwonek, 2007, Probert, 2015). The integrity of the BBB is regulated by cytokine/chemokine concentrations, and disruption of the BBB enhances inflammation that may lead to brain oedema. A balance between proinflammatory and anti-inflammatory responses is crucial for immune protection while reducing immunopathology. Furthermore, it is important to consider the role of host proteases in TB and their contribution to tissue destruction (Elkington et al., 2011a, Ong et al., 2014). Proteolytic matrix metalloproteinases (MMPs), particularly, have been implicated in the breakdown of the extracellular matrix and are associated with poor outcomes in adults with tuberculous meningitis TBM (Elkington et al., 2011b). These

MMPs can contribute to the breakdown of the protective BBB and subsequent tissue destruction in CNS-TB. It is worth noting that while chemokines and cytokines play significant roles in the immune response during TB, host proteases, such as MMPs, are key effectors of the destructive phenotype observed in CNS-TB, but their activity is amplified by the presence of cytokines and chemokines resulting in the degradation of the extracellular matrix and subsequent tissue destruction (Rohlwink et al., 2019). They form part of an elaborate network of inflammation and injury, constituting one element of the complex inflammatory process in TB.

1.4 Astrocytes

Astrocytes, from the literal Greek “star cell” are resident cells of the CNS. Originally defined as neuroglia in the 1850s by the acclaimed pathologist Rudolf Virchow (Somjen, 1988), he described this brain connective tissue as “nervenkitt’ or nerve-cement (Virchow, 1856, Kettenmann and Verkhratsky, 2008). The concept of neuroglia developed from here, but our appreciation of neuroglial attributes and disparities, particularly astrocytes, has drastically changed since then. Through the development of sophisticated cellular and molecular approaches, progressive investigations have revealed the dynamic role that astrocytes exert in maintaining CNS homeostasis, particularly in regulating synapse formation and supporting neuronal function in both healthy and injured brains (Allen and Lyons, 2018, Eroglu and Barres, 2010, Zamanian et al., 2012, Zhang et al., 2014b). There is a significant interest in the role of astrocytes following injury and disease, as they reportedly have both beneficial and detrimental effects. Improved understanding of astrocyte activity following an insult could enable manipulation of their responses to enhance their beneficial attributes and limit negative effects, ultimately leading to better disease outcomes.

1.4.1 Astrocyte function in the CNS

Astrocytes are complex cells effectively involved in a wide range of CNS functions. To concisely define the astrocyte function was a remarkable journey, with their role often described as “supportive.” Although a common practice, this simplistic descriptor does not thoroughly encompass the multitude and diversity of their purpose. Strategically

located at the interface between blood vessels and the brain parenchyma, they can influence blood-brain regulation. Their radially arranged processes provide structural support to the CNS by creating a framework onto which blood vessels and other cells anchor, while their end-feet processes create an additional barrier into the CNS known as the glia limitans. This direct network with blood vessels, neural and non-neural cells allow astrocytes to influence neuronal behaviour and facilitate their functions (Khakh, 2019) while maintaining CNS homeostasis.

As the most abundant CNS glial cell population, astrocytes play a dynamic part in regulating principal functions in the CNS. One of their fundamental roles is to provide neurons with energy metabolites via the astrocyte-neuron lactate shuttle, whereby astrocyte glycogen reserves get converted to glucose and lactose (Magistretti and Allaman, 2018). Astrocytes play an integral role in synaptogenesis and modulating synaptic activity (Allen and Eroglu, 2017). Recent evidence has supported the existence of the 'tripartite synapse' concept whereby astrocytes regulate synapse transmission through bi-directional communication with neurons (Perea et al., 2009). One of their pivotal functions is neurotransmitter buffering through uptake and regulation of glutamate released at neuronal synaptic clefts. Once taken up by astrocytes, it is converted to glutamine, by glutamine synthetase and then released into the extracellular space for neuronal uptake. Neurons are then able to convert it back to glutamate with glutaminase. Furthermore, ion balance in the extracellular fluid is maintained by their regulation of extracellular ions K^+, H^+, and H_2O (Verkhratsky and Nedergaard, 2018). Existing studies recognise the significance of astrocytes, having demonstrated neuronal degeneration and eventual death in neuron cultures lacking astrocytes or following astrocyte ablation *in vivo* (Cui et al., 2001, Schmalenbach and Müller, 1993). Thus, astrocytes are active participants in neurogenesis, and secrete neurotrophic factors necessary for neuronal survival and myelination.

Increasing evidence indicates that astrocytes regulate innate and adaptive immune responses in the CNS under pathological conditions (Jensen et al., 2013, Geyer et al., 2019, Farina et al., 2007, Dong and Benveniste, 2001), in addition to their role in the maintenance of CNS homeostasis and neuronal function (Allen and Lyons, 2018, Baldwin

and Eroglu, 2017, Halassa and Haydon, 2010). Following antigen recognition, astrocytes participate in the initiation of innate immune responses and prompt an adaptive immune response to recruit peripheral immune cells (Giovannoni and Quintana, 2020, Constantinescu et al., 2005, Aschner, 1998a, Aschner, 1998b). By acting on endothelial cells via the release of angiogenic factors, they can modulate the BBB to assist cellular infiltration (Horng et al., 2017, Argaw et al., 2012, Argaw et al., 2006). Studies have also indicated an immunosuppressive function of astrocytes where they attenuate immune activity (Cooley et al., 2014). Investigations to understand the immunological role of astrocytes in CNS disease and injury have been conducted, however, their part in CNS-TB has not been fully evaluated (Geyer et al., 2019).

1.4.2 Astrocyte activation

Under healthy conditions, astrocytes maintain homeostasis and support neuronal survival through metabolic support. However, following CNS insults such as injury and infection, astrocytes undergo a transformation process termed 'reactive astrocytosis,' during which the expression of multiple genes is altered (Liddelow et al., 2017, Zamanian et al., 2012, Clarke et al., 2018). The upregulation of glial fibrillary acidic protein (GFAP), the main cytoskeletal constituent of astrocytes, is a typical characteristic change in reactive astrocytosis and is instrumental in controlling pathogenic spread (Stenzel et al., 2004). Astrocyte reactivity is an integral component of CNS innate immunity and is generally considered to be a hallmark of disease, but this state is simply a normal physiological response to maintain neurological processes and conserve tissue homeostasis. Evidence from *in vivo* studies has demonstrated that through activation and glial scar formation, astrocytes implement beneficial functions, including neuronal protection, BBB restoration, and limitation of neuroinflammation (Pekny and Nilsson, 2005, Kang and Hébert, 2011, Anderson et al., 2014, Sofroniew and Vinters, 2010, Sofroniew, 2015a). Amidst adverse conditions, however, this reaction may be detrimental and contribute to neurological dysfunction through the loss of beneficial functions and acquisition of damaging functions such as amplifying inflammation or restricting axon growth (Sofroniew and Vinters, 2010, Sofroniew, 2015b, Silver and Miller, 2004). The astrocytic protein S100B, a calcium-binding protein, has also been used as a potential biomarker for CNS injury and diseases (Liddelow et al., 2017, Sen and Belli, 2007). S100B protein

secretion can be induced by LPS administration in rats and cultures (Guerra et al., 2011), and its physiological effect is dose dependent. At high levels, S100B is neurotoxic through its upregulation of iNOS and NO production *in vitro* and promotes reactive astrocytosis *in vivo* (Hu et al., 1997, Villarreal et al., 2014). Further investigations are necessary to clarify the extent to which astrocyte functions are altered that may offer insight into targeted therapy for various disorders.

1.4.3 Astrocytes during bacterial infection

Microglia are arguably the prime immune-effector cells of the CNS that initiate innate immune responses through antigen presentation and facilitate subsequent neuroinflammation. Microglial function is regulated by different cell types, either in a paracrine manner or through direct interaction, amongst which astrocytes play a critical role (Ransohoff and Cardona, 2010). Activation of microglia and astrocytes is a sensitive indicator of changes in the CNS microenvironment. Glial cells are able to elicit an innate immune response through recognition of highly conserved motifs, generally referred to as pathogen-associated molecular patterns (PAMPs) by different classes of pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs), (NOD)-like receptors (NLR), scavenger, complement, and mannose receptors (Liddelow et al., 2017, Jack et al., 2005, Zamanian et al., 2012, Braun et al., 2011). Bacterial products such as lipopolysaccharides (LPS) and bacterial DNA provide adequate stimuli to activate astrocytes. Interestingly, astrocytes become reactive upon stimulation and contribute to brain inflammation by releasing specific cytokines and chemokines (Table 1-1). Studies conducted on both humans and animals, involving cell lines or tissues sourced from various species are shown in Table 1-1. These studies include research using rhesus macaques (Lee et al., 2013), mice (Constantinescu et al., 2005, Chauhan et al., 2009, Chauhan et al., 2008, Cooley et al., 2014, Rasley et al., 2006, Stenzel et al., 2008b, Esen et al., 2003) and humans (Cooley et al., 2014, Rock et al., 2005, Stoner et al., 2015). Ancillary to facilitating innate immune responses, reactive astrocytes express major histocompatibility complex (MHC) class II and co-stimulatory molecules, such as CD40, that may contribute to T cell activation and integrate communication between resident CNS cells and hematopoietic cells, driving an adaptive immune response (Carpentier et al., 2005).

Under most circumstances, bacterial pathogens invade the CNS via the bloodstream. Although precise mechanisms of entry into the CNS remains contested, it is now known that intracellular and extracellular bacteria evolved different strategies to circumvent host defence systems (Drevets et al., 2004). Once CNS barriers are breached, resident cells of the CNS recognise infectious non-self-entities through a series of PRRs, which initiate a rapid immune response.

TLRs are evolutionarily conserved type I membrane glycoproteins, comprising eleven members in humans and thirteen in mice, with discrete affiliations to specific ligands. Under physiological conditions, TLRs are basally expressed in CNS areas lacking a BBB and, therefore, ideally positioned to interact with infiltrating pathogens in these areas. In human astrocytes, TLR3 is expressed constitutively at basal levels and significantly upregulated following treatment with IL-1β, IFN-β, and IFN-γ (Farina et al., 2005, Jack et al., 2005). Additionally, TLR2, TLR4, TLR5, and TLR9 expressed in resting astrocytes recognise bacterial ligands (Bowman et al., 2003, Tarassishin et al., 2014) with TLR2 recognition of bacterial peptidoglycan, lipopeptides, and lipoprotein leading to cell activation. For example, astrocyte activation combined with increased TLR2 expression was reported in the white matter of rhesus macaques infected with *B. melitensis* (Lee et al., 2013). TLR2 is also essential for the induction of cytokines and chemokines in astrocytes stimulated with *S. aureus* and peptidoglycan (Esen et al., 2004). Similar to TLR2, TLR4 signalling is necessary for protective immunity during CNS staphylococcal infection (Stenzel et al., 2008a). In response to LPS, TLR4 activation in astrocytes generates MyD88-dependent NFκB signalling and subsequent upregulation of the target genes TNF, IL-27, IL-15, MMP-9, and VCAM-1 that may cause modifications of the BBB, prompt inflammation to recruit T lymphocytes and regulate immune responses (Gorina et al., 2011). LPS causes delayed Jak1/Stat1 activation in astrocytes, which is MyD88-independent and induces the expression of the negative cytokine regulator, SOCS-1 and chemokine CXCL10 (Gorina et al., 2011). These signals create a pro-inflammatory milieu, which regulates the activity of surrounding cells, and facilitate microbial clearance. TLR2 and TLR4 are involved in recognising *M. tuberculosis* (Ferwerda et al., 2005) and clearing *Brucella* spp. infection from the lungs (Lee et al., 2013). It is, therefore, possible that their induction in astrocytes may well point to a protective role during brain infection.

Nucleotide-binding oligomerization domain (NOD)1 and NOD2 proteins are part of a TLR-related protein family that recognizes distinct motifs of intracellular pathogens. For instance, in addition to TLRs, recognition of *M. tuberculosis* by NOD2 is important for innate immunity activation (Ferwerda et al., 2005). In context of CNS infection, NOD2 receptors function as intracellular sensors to *S. aureus*, *S. pneumoniae*, *B. burgdorferi* and *N. meningitidis*, which is upregulated in astrocytes (Liu et al., 2010, Chauhan et al., 2009). Furthermore, the expression of NOD2 triggers the NFκB pathway via the adapter protein Rip2Kinase, ultimately inducing the production of IL-6 and TNF, and the expression of co-stimulatory molecules that amplify bacterially-induced immune responses in astrocytes (Sterka Jr et al., 2006).

Scavenger receptors (SRs), initially described as cell surface receptors on macrophages that bind acetylated low-density lipoproteins, are now identified as a diverse group of PRRs that recognize various ligands, including endogenous proteins and pathogens, participating in cell adhesion, phagocytosis, and activation of immune responses. SR members, SR-MARCO and SR-A, also enable innate immune responses to Gram-negative and Gram-positive bacteria (Braun et al., 2011, Godoy et al., 2012, Peiser et al., 2000, Dorrington et al., 2013). In humans, MARCO variants are associated with increased susceptibility to pulmonary tuberculosis which may be due to its regulatory role in macrophage phagocytosis (Thuong et al., 2016). In murine astrocyte cultures, *N. meningitidis* and *S. pneumoniae* induce upregulation of MARCO, which mediates the production of IL-1β in astrocytes (Braun et al., 2011). Therefore, its expression by astrocytes (Alarcón et al., 2005, Godoy et al., 2012, Braun et al., 2011) presents a compelling argument for a potential role in host defence during bacterial meningitis.

Complement forms an imperative arm of innate immunity, known for its role in pathogen recognition and killing, including bacteria (Heesterbeek et al., 2018). Complement proteins are activated and found in the cerebrospinal fluid (CSF) of patients with bacterial meningitis (Shen et al., 2017). The functional role of complement components in CNS is further supported by the decreased survival of C1q and C3 deficient mice after meningitis induction (Rupprecht et al., 2007). Notably, astrocytes can generate the majority of the

complement components modulated by various cytokines (Barnum et al., 1996). For example, LPS-activated microglia release TNF and IL-1α, and in conjunction with C1q, induce A1 reactive astrocytes with elevated levels of C3 *in vitro* and *in vivo* (Liddelow et al., 2017, Clarke et al., 2018). It is, therefore, plausible that astrocyte-dependent complement synthesis may have a significant role in regulating CNS immunity.

Table 1-1 | Astrocyte recognition of bacterial pathogens and immune mediator production

Bacteria	PRR System	Cytokines	Chemokines	Clinical Significance	Reference
Borrelia burgdorferi	TLR-1, TLR-2, TLR-5, NOD-2	IL-1β, IL-6, IL-12, IL-23, TNF, IL-10 (cultures), IL-19	COX-2, CXCL-1, CXCL-10 IL-8	Lyme Neuroborreliosis	(Lee et al., 2013, Chauhan et al., 2009, Constantinescu et al., 2005, Chauhan et al., 2008, Rasley et al., 2006, Cooley et al., 2014)
Brucella spp	TLR-2	IL-1β, IL-6, TNF	CCL2 (MCP-1), CXCL1	Neurobrucellosis	(Mosa et al., 2009, Lee et al., 2013)
Listeria monocytogenes	TLR-2, NOD-1	Unknown	Unknown	Neonatal and adult meningitis	(Mosa et al., 2009)
Mycobacterium tuberculosis	Unknown	Unknown	CXCL10	CNS Tuberculosis	(Rock et al., 2005)
Neisseria meningitidis	NOD-2, SR-MARCO, Complement-CD46	IL-6, TNF IL-10 (cultures), IL-19	Unknown	Paediatric or Infant and adult meningitis	(Chauhan et al., 2009, Braun et al., 2011, Cooley et al., 2014, Rasley et al., 2006)
Staphylococcus aureus	TLR-2, NOD-2	IL-1β, IL-6, TNF	CCL2 (MCP-1), MIP-1β, and CXCL2 (MIP-2)	Brain abscesses and meningitis	(Esen et al., 2004, Stenzel et al., 2008a, Liu et al., 2010)
Streptococcus agalactiae (Group B Streptococcus)	Unknown	IL-1β, IL-6	IL-8	Neonatal meningitis	(Stoner et al., 2015)
Streptococcus pneumoniae	TLR-2, NOD-2	IL-19	Unknown	Neonatal, paediatric and adult meningitis	(Cooley et al., 2014, Liu et al., 2010)

1.4.4 Reactive astrocytosis: effects in neuroinflammation and neuroprotection

Recent transcriptomic studies have characterized two subtypes of reactive astrocytes, namely A1 and A2, and accentuate the concept of reactive astrogliosis as a highly

heterogenous state depending on the type of insult (Liddelow et al., 2017, Clarke et al., 2018, Zamanian et al., 2012). While A2 reactive astrocytes are deemed neuroprotective through the release of neurotrophic factors that encourage CNS repair, the development of A1 reactive astrocytes is driven by LPS-activated microglia and are considered harmful by promoting neuroinflammation and neurotoxicity (Liddelow et al., 2017, Clarke et al., 2018, Zamanian et al., 2012). LPS or bacterial molecules can directly stimulate astrocytes to express various cytokines, including IL-1β, IL-6 and TNF (Tarassishin et al., 2014), as well as several chemokines, CCL2, CXCL1, CCL20 and CCL3, suggesting that astrocytes modify the chemokine framework in a pathogen-specific manner (McKimmie and Graham, 2010). Notably, direct exposure of IL-1β to human fetal astrocyte cultures can induce reactive astrogliosis and change gene expression of inflammatory mediators, among which IL-6 and CXCL5 were prominently upregulated. Elevation of neurotrophic factor genes, such as BDGF and NGF, was also induced, suggesting that IL-1β may contribute to the neuroinflammatory and neuroprotective effects of human reactive astrocytosis (Teh et al., 2017).

TNF, a potent proinflammatory cytokine, is accepted as a principal cytokine involved in antimicrobial Th1 immunity. TNF is also detected in the CSF of Lyme neuroborreliosis patients and the brains of *B. burgdorferi*-infected mice (Chauhan et al., 2008). The production of TNF together with IFN-γ, IL-12, IL-23, and NO by astrocytes supports their potential involvement in host immunity to CNS tuberculosis (McKimmie and Graham, 2010, Constantinescu et al., 2005, Tarassishin et al., 2014). Astrocytes enhance TNF and IL-12 concentrations in the brain during microbial invasion, and their increased reactivity in the presence of TNF and IFN-γ indicates their potential contribution to these pathways in CNS host defence. Furthermore, several reports suggest that reactive astrocytes may mediate neuroprotection through the release of neurotrophic factors after stimulation with TNF and LPS (Appel et al., 1997, Saha et al., 2006b); one particular is IL-6 production by astrocytes after LPS treatment that enhances neuronal survival (Li et al., 2009, Sun et al., 2017).

The upregulation of IL-6, common to many CNS bacterial infections (Bernardino et al., 2008, Stenzel et al., 2008a, Chauhan et al., 2008), is found in the CSF of meningitis

patients (Misra et al., 2010, Pinto Junior et al., 2011). Its classical pathway involves the binding of IL-6 to IL-6R and gp130, subsequently activating JAK/STAT signalling. In GFAP-IL6 transgenic mice, the over-expression of IL-6 in astrocytes was sufficient to induce reactive astrocytosis. Interestingly, the use of gp130 to block IL-6 can reduce astrocytosis in GFAP-IL6/sgp130 mice (Campbell et al., 2014). These findings suggest that chronic expression of IL-6 by astrocytes has a critical role in neuropathological effects during CNS immune responses.

Delayed microglial and astrocytic production of immunosuppressive cytokines, such as IL-10, are generated in response to *B. burgdorferi* and *N. meningitidis*, suggesting the possibility of negative feedback loops whereby mediators act to limit potentially damaging inflammation within the CNS during chronic infections (Rasley et al., 2006). Interestingly, the production of IL-10 in the mouse brain was significantly reduced following *in vivo* infection of *B. burgdorferi* or *N. meningitidis*, while the levels of both IL-6 and TNF were significantly elevated (Chauhan et al., 2008). IL-10 influences many aspects of immune responses and is an effective inhibitor of activated glia through suppression of proinflammatory cytokine response pathways (Rasley et al., 2006). Expression of IL-19 is also induced in the brains of mice as well as astrocyte cultures following bacterial challenges. Astrocyte treatment with IL-19 stimulated the upregulation of SOCS3, which inhibits cytokine synthesis. As a result, it reduced astroglial production of IL-6 and TNF following bacterial challenge (Cooley et al., 2014). Therefore, the induction and modulation of proinflammatory cytokines and immunosuppressive cytokines support the regulatory role of astrocytes to maintain a delicate balance through promoting cellular responses and limiting inflammation following CNS infection.

1.4.5 Astrocyte heterogeneity at a genomic level

To fully appreciate CNS function in healthy and injured brains an enriched understanding of astrocyte heterogeneity is needed. The term ‘astrocyte’ (astroglial cell) was first suggested by Michael von Lenhossek in 1893 (Kettenmann and Verkhratsky, 2008, von Lenhossék, 1893). During this period, anatomists and pathologists were mindful of the diversity in these non-neuronal cells and their transitions in pathology. Astrocytes were characterized into two distinct classes based on their morphology; a discovery often

attributed to Santiago Ramón y Cajal (Ramón y Cajal, 1909). These observed differences between glial cells of the white- and grey matter was however initially described by Andriezen in 1893, followed by Kölliker (Andriezen, 1893, Kölliker, 1889) and later confirmed by Cajal using the gold chloride sublimate method. The two morphological classes, notably protoplasmic and fibrous astrocytes, were further classified according to the developmental origin, location and marker protein expression. Protoplasmic astrocytes populate the grey matter and consist of a central stroma with long processes which branch out into thousands of smaller processes giving them a spongiform appearance (Khakh and Deneen, 2019). Conversely, fibrous astrocytes located in the white matter have short processes.

Considering that the adult brain is compartmentalised into multiple regions having different neuronal sub-populations, it would be plausible for regional specificity to exist within astrocytes too. In the past decade, developments in neuroscience have led to the discovery of a considerable degree of heterogeneity in astrocytes (Liddelow et al., 2017, John Lin et al., 2017). A possible explanation for the observed diversity amongst astrocytes may be the disparate lineages from which they develop. During embryonic development, the neuroepithelium gives rise to radial glial (RG) cells which act as neural progenitors for neurons and astrocytes (Malatesta et al., 2003, Noctor et al., 2001). Following neural migration along radial fibres, RG gives rise to embryonic astrocytes in the CNS during the perinatal period (Schmechel and Rakic, 1979). In addition to RG in the ventricular zone, astrocytes may also develop from glial-restricted progenitors in the postnatal subventricular zone (Schmechel and Rakic, 1979, Levison and Goldman, 1993, Cameron and Rakic, 1991, Marshall et al., 2003) or through differentiated astrocyte proliferation in the postnatal cortex (Ge et al., 2012). Variations in these glial progenitor environments elicit distinguishable patterns of transcriptional factors and gene expression in astrocytes (Sauvageot, 2002), thus influencing diversity.

Reports have described variations in astrocyte expression profiles, both locally and regionally across the CNS (Zhang and Barres, 2010, Bachoo et al., 2004, Regan et al., 2007, Doyle et al., 2008, Krencik et al., 2011). An example of such specialisation is evident with cerebellar Bergmann glia and Muller glia in the retina, each with distinct

morphologies and localized to specific regions. It is feasible that neurons release specific factors to direct the molecular profile of astrocytes, thereby regulating the local environment in the brain. A study by Farmer et al. found that neurons are able to regulate the properties of astrocytes by showing that cerebellar Bergmann glia require sonic hedgehog (Shh) signalling from Purkinje cells to retain their molecular features; and in the absence of Shh, they acquire alternative astrocyte properties (Farmer et al., 2016). Studies have also reported variations in physiological properties and molecular profiles of astrocytes isolated from different brain regions. Using RNA microarray analyses, Yeh and colleagues demonstrated discrete gene expression patterns in cultured astrocytes from day one postnatal optic nerve, neocortex, cerebellum and brainstem (Yeh et al., 2009). Regional heterogeneity in astrocyte populations was further observed in a study where distinct gene expression profiles for synaptogenic factors in the cerebral cortex, hippocampus, midbrain and cerebellum were detected, which may account for the variation in astrocyte synaptogenic potential across these regions (Buosi et al., 2018). Astroglia are therefore not homogenous, but rather a diverse group of cells with subpopulations suited to their microenvironment, constantly adapting, and changing in response to the surrounding neuronal milieu. Furthermore, local and recruited immune cells can alter the cytokine environment in a pathogen-specific manner, which in turn affects astrocyte activity. Consistent with their heterogeneous nature is the functional diversity observed in astrocytes, which needs to be considered as it may reveal compelling insight into astrocyte responses to injury and disease.

Transcriptional profiling has emerged as a powerful tool to explore gene expression and functional identities of cells under various conditions. Microarrays are increasingly used for analysing gene expression levels in a specific cell population and for comparing expression levels between different regions of the brain. Various genomic databases are now available for resident CNS cells, ranging from functional and developmental studies to transcriptomic analysis following innate immune stimulation (Cahoy et al., 2008, Lovatt et al., 2007, Bracko et al., 2012, Orre et al., 2014, Madeddu et al., 2015, Zhang et al., 2014b, Beutner et al., 2013). These genome-wide transcriptional profiles provide a resource to identify defined molecular features characteristic of astrocytic development and metabolism and can aid in advancing the understanding of CNS development,

physiology, and pathology. Liddelow et al. proposed the A1/A2 model of astrocyte activation where 2 subtypes are classified as neurotoxic and neuroprotective astrocytes, respectively (Liddelow et al., 2017). While useful, this paradigm does not consider the intricate nature of astrocyte heterogeneity or the potential of these cells to adopt several distinct activation states in different diseases. A recent meta-analysis of transcriptomic datasets investigating acute and chronic CNS injuries demonstrated different gene expression patterns observed in the two types of injury (Das et al., 2020). This study highlights the complexity of the astrocytic response to CNS injury and suggests the importance of considering the context in developing therapies targeting astrocytes. A pioneering study in tuberculosis research employed microarrays to compare the transcriptome of whole blood samples from healthy individuals and those with active tuberculosis (Berry et al., 2010). They revealed a previously underestimated neutrophil-driven type I interferon (IFN) signalling pathway in TB patients, indicating the intricacies of the immune response to tuberculosis. Their findings shed new light on the pathogenesis of tuberculosis and demonstrate the usefulness of microarray technology in investigating complex diseases. Microarray analysis can, therefore, provide valuable insights into the molecular pathways and cellular processes that are involved in astrocyte-mediated immune responses during mycobacterial infection. Moreover, the application of microarray analyses has the potential to facilitate the identification of new therapeutic targets for the treatment of neurological complications associated with tuberculosis, which may be attributed to astrocytes.

1.5 Hypothesis, Aim and Objectives

Astrocytes play a critical role in maintaining homeostasis by regulating physiological and pathological responses in the CNS. These functionally diverse cells are capable of eliciting insult-specific immune responses to maintain and restore CNS cellular function. While the understanding of astrocyte importance in response to pathogens is expanding, studies of their contribution to neuroimmune protection in the CNS during mycobacterial disease has been limited (Geyer et al., 2019, Farina et al., 2007). The hypothesis of this study is that astrocytes are key regulators of host immunity during mycobacterial infection. The principal aim of this study was to comprehensively investigate the

transcriptomic profile and multifaceted cellular immune responses of astrocytes, to accurately characterise their immunomodulatory activity and unravel their crucial role in the pathogenesis and progression of CNS-TB.

The following objectives were designed to achieve the aim (Fig. 1.5):

(i) To establish an *in vitro* model of *M. bovis* BCG and *M. tuberculosis*-infected primary murine astrocytes,

(ii) To characterise the transcriptomic profile of astrocytes during *M. bovis* BCG and *M. tuberculosis* infection *in vitro*,

(iii) To investigate potential astrocyte immune modulation during *M. bovis* BCG and *M. tuberculosis* infection by evaluating their release of cytokines and chemokines,

(iv) To evaluate the immunological profile of astrocytes during CNS-TB infection in an immune-competent mouse model by assessing their protein expression, and

(v) To determine the immunological profile of astrocytes during CNS-TB infection in immune-deficient mouse models.

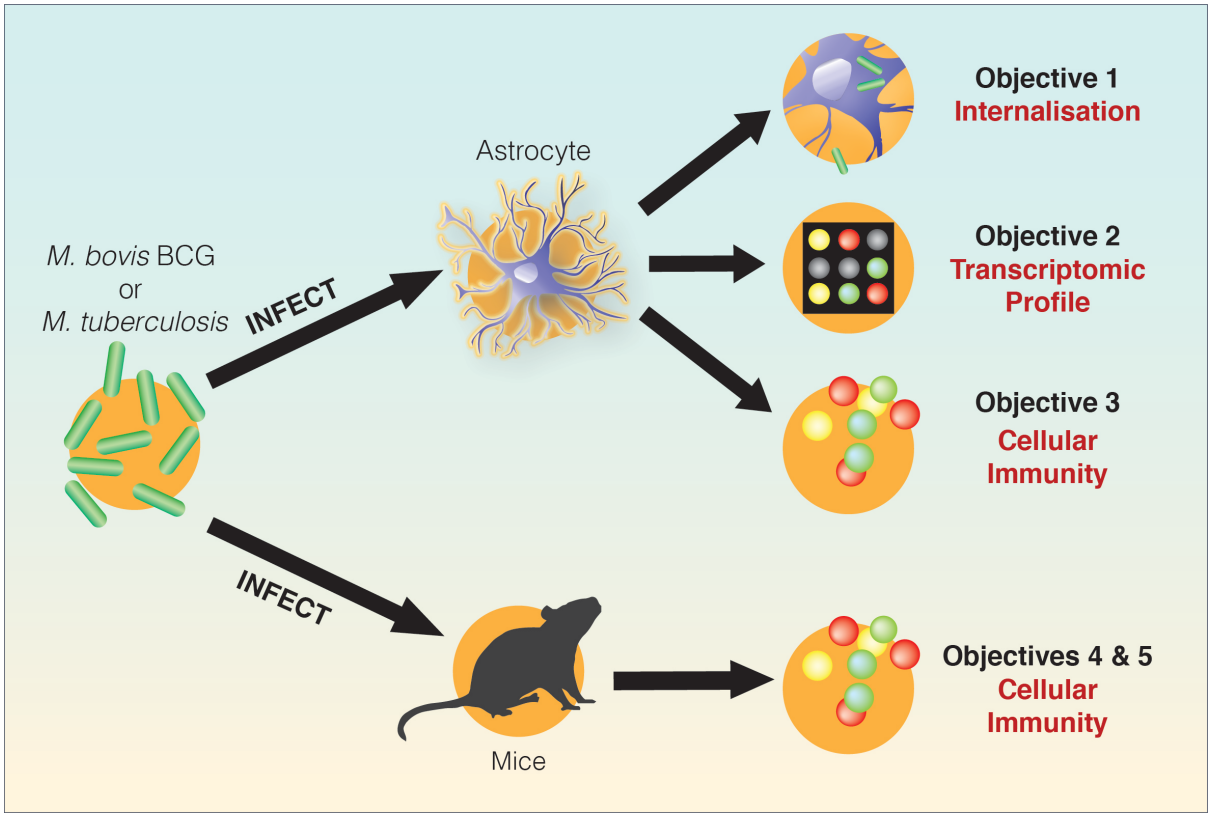


Figure 1.5 | Study objectives. The central aim of this investigation is to advance the understanding of CNS-TB by exploring the genomic profile and cellular immune responses of astrocytes to identify their immune modulatory activity during CNS-TB. Five objectives were designed to achieve this aim.

CHAPTER 2

CHAPTER 2

Methods and materials

2.1 Ethics statement

All procedures were conducted in accordance with the animal care standards of South African regulations and experimental protocols were approved by the Animal Research Ethics Committee, Faculty of Health Sciences, University of Cape Town, Cape Town, South Africa (AEC018/016, AEC018/017, AEC018/028 and AEC015/035) 021/013)

2.2 Mice

The NOD-*scid* IL2Rgamma^{null} (NSG) mouse strain was obtained from The Jackson Laboratory (Strain #:005557). Adult C57BL/6 and NSG mice were bred and maintained under specific pathogen-free (SPF) conditions within the Research Animal Facility at the University of Cape Town (South Africa). Adult mice between 8-12 weeks of age were used and housed in an individually ventilated cage (IVC) system and supplied with food and water available *ad libitum.* Experimental procedures were performed in a biosafety level-3 facility.

2.2.1 Generation of NSG-BLT mice

The NSG mouse strain (Jackson Laboratories) was used as an immune-deficient mouse model and as the recipient for human tissue due to its ability to support engraftment and proliferation of human cells (Shultz et al., 2012). Upon receipt of human foetal thymus and liver tissue (gestational age of 17–20 weeks), from Advanced Bioscience Resource (Alameda, CA), specimens were prepared as 1-2mm sections for transplantation. To recover human stem cells, remaining foetal liver was digested and CD34^{+} cells purified with a magnetic-activated cell sorter (MACS) using anti-CD3 microbeads (Miltenyi Biotec, Auburn, CA). The purity of the CD34^{+} cells was determined using flow cytometry and aliquots stored at -80°C till the day of surgery. Three days prior to the engraftment

surgery, mice were preconditioned by myeloablation with a single dose of 1.5g/kg treosulfan (medac GmbH, Hamburg, Germany) as described (Gutti et al., 2014 and Sjoo et al., 2006) via intraperitoneal injection creating a temporary deficiency in the hematopoietic system, allowing transplanted human cells to take over and establish a human immune system within the mouse. On the day of the surgery, mice were anaesthetised by intraperitoneal injection with Ketamine (100mg/kg) and Xylazine (10mg/kg). The mice were monitored throughout the procedure and the pedal withdrawal reflex method was used to ensure that the depth of anaesthesia was sufficient. Following anaesthesia administration, a pre-emptive analgesia (Buprenorphine, 0.1mg/kg) injection was given to the mice subcutaneously. The lower abdominal area was prepared for transplantation by shaving the region and making a small incision in the skin to expose the renal capsule. One fragment of the prepared human foetal thymus and liver tissue were surgically implanted in the renal subcapsular space with a cannula trocar (16G). The incision in the skin was subsequently closed with sterile skin autoclips. Immediately after tissue engraftment, $2x10^5$ human stem cells ($CD34^+$) from the digested foetal liver tissue were administered to mice intravenously. The mice were housed in a SPF flexible film isolator and allowed to recover from transplantation. Mice received acidified drinking water (pH 3.0) for two weeks after implantation and were monitored twice daily for signs of infection, wound healing, and any other adverse effects. Analgesia and antibiotic (gentamicin at a dose of 8 mg/kg) were administered accordingly. To limit the development of graft-versus-host-disease (GvHD), mice were administered with $2x10^5$ of autologous NSG bone marrow cells every 4 weeks after engraftment surgery. The humanised NSG-BLT mice were allowed to recover from transplantation for 12 weeks, allowing the human immune system to fully establish within the mice. All mice that underwent engraftment surgery survived. To evaluate the success of the human cell engraftment and the presence of a functional human immune system within the mouse, human leukocyte reconstitution was measured by harvesting mouse tail blood and staining human hematopoietic cells ($CD45^+$) with monoclonal antibodies (BD Biosciences, Inc., or BioLegend). Samples were analysed using a BD Fortessa and FlowJo software (v10.7.1). Non-humanised NSG mice were used as a control group. Flow cytometry analysis revealed the presence of human leukocytes in the humanised NSG-BLT mouse blood, indicating successful engraftment in all the mice. No $hCD45^+$ cells were detected

in the control mice (Fig. 2.1A). Kidneys were also examined for the presence of organoids. The implanted foetal tissue developed into human thymus organoids ranging from 100 to 200 mm^3 in size, which displayed a typical human thymus structure, as shown in the haematoxylin and eosin-stained histology sections (Fig. 2.1B). The organoid was easily distinguished by the presence of a clearly defined medulla and a cortex. The non-humanised NSG mice had no thymic organoid. These findings indicated successful establishment of a human immune system in the humanised NSG-BLT mice.

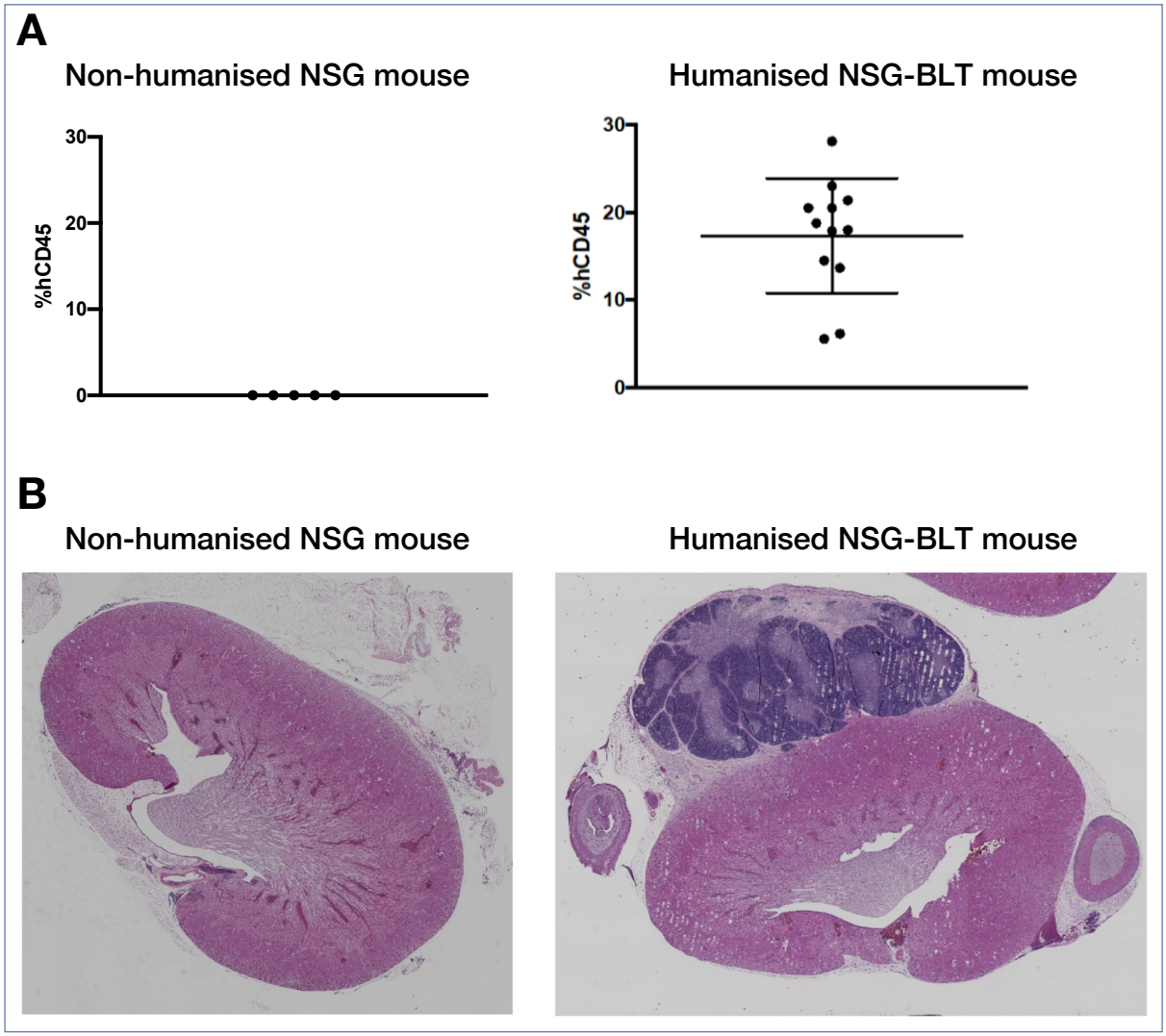


Figure 2.1 | Establishment of a human immune system in NSG-BLT mice at 12 weeks following engraftment. (A) Peripheral blood of mice were stained with human CD45 antibody and flow cytometry was used to identify the presence of human leukocytes. Reconstitution of human hematopoietic cells ($hCD45^+$) was detected in hNSG-BLT mouse blood indicating the potential for human immune responses (n=13). (B) Haematoxylin and eosin-stained section of the human thymus organoid on the kidney in non-humanised NSG and humanised NSG-BLT mice.

2.3 Isolation and Propagation of Mixed Cortical Cells

2.3.1 Poly-(L)-Lysine plate preparation

25cm^2 (T25) flasks were coated using 10ml filter sterilized 0.01% poly-L-lysine (Sigma, MO, USA) in distilled water (d.H_2O). The flasks were left overnight in a 4°C fridge and rinsed thrice with d.H_2O the following day. Prior to cortex removal the water was discarded, and plates left to dry in a Heal Force HF151 biosafety cabinet until use.

2.3.2 Cortex dissection

Mixed cortical cell isolation for astrocyte cultures was prepared using P0 to P1 C57BL/6 mice (Fig. 2.2A). The pups were sacrificed by decapitation using surgical scissors and sprayed with 70% ethanol (Fig. 2.2B). The skin and cranium were removed using sharp forceps to expose the brain (Fig. 2.2C, D). The brain (Fig. 2.2E) was then transferred to a petri dish containing HBSS (Lonza, MD, USA) (Fig. 2.2F), and the remainder of the dissecting procedure was performed under a stereomicroscope. Before the meninges could be dissected from the cortical hemispheres, the midbrain, cerebellum, and olfactory bulbs had to be removed (Fig. 2.3A-D)). The prepared cortex hemispheres were transferred into a 10ml tube containing HBSS and placed on ice (Fig. 2.3E).

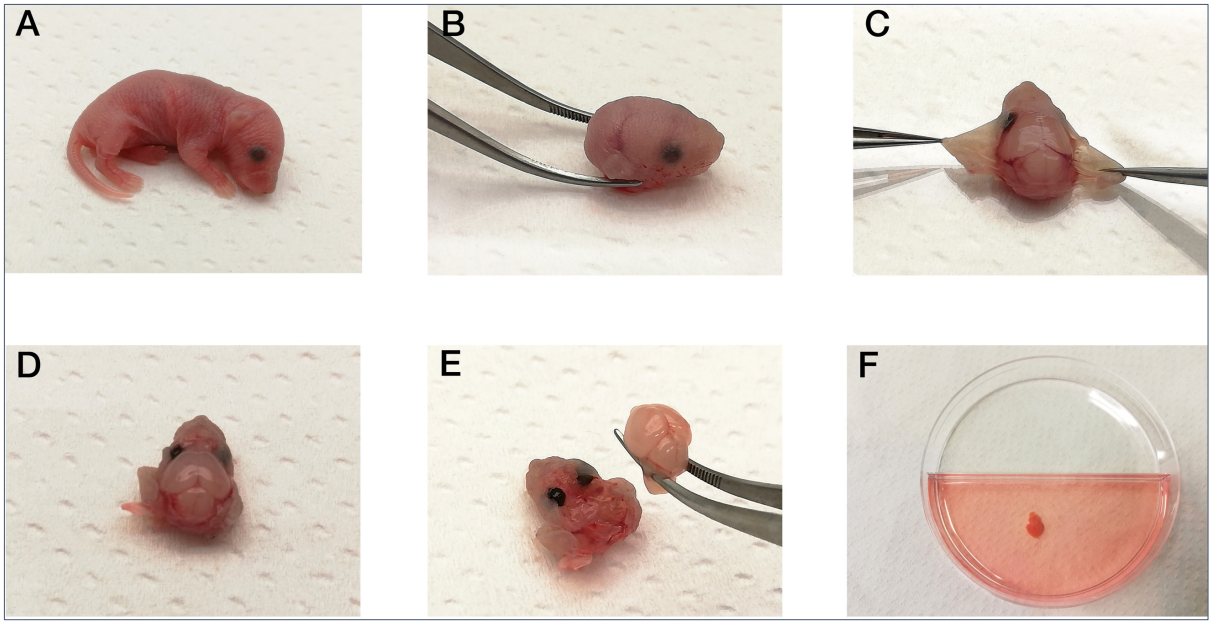


Figure 2.2 | Brain removal technique for mice pups. (A) P0 to P1 C57BL/6 mice pups were used for primary cell isolation. (B) Pups were sacrificed by decapitation and (C) the skin and (D) cranium were subsequently removed with sharp forceps to reveal the brain. (E) The brain was gently separated and removed from the skull (F) then transferred to a petri dish where it was submerged in HBSS.

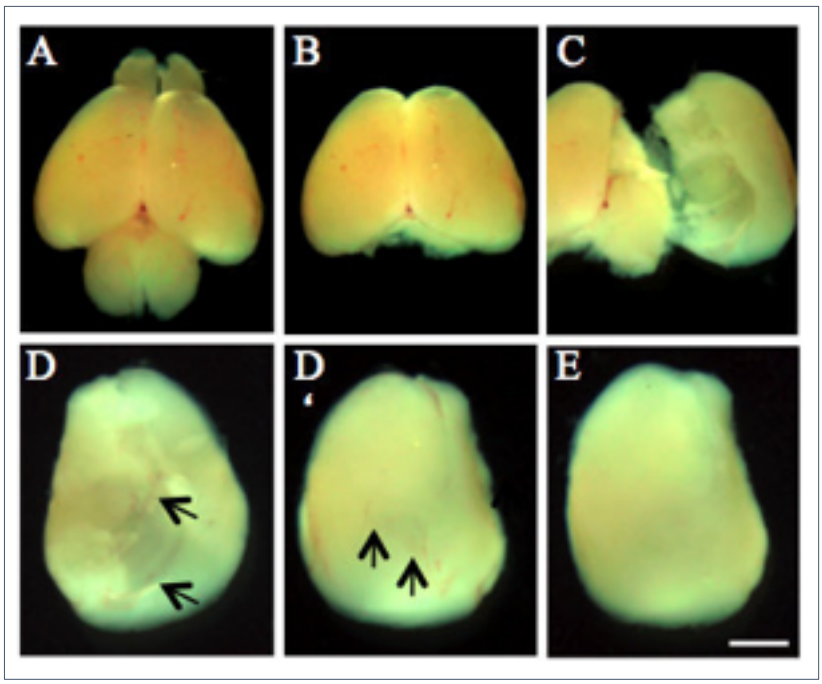


Figure 2.3 | Cortex isolation and meningeal removal. (A) The brain was submerged in HBSS and positioned dorsally. (B) The midbrain, cerebellum and olfactory bulbs were removed and the (C) two cortical hemispheres isolated. Meninges were removed on the ventral and dorsal surfaces of each cortex. (D and D') Meninges can be distinguished by the visible blood vessels as indicated by the arrows. (E) The dissected cortex hemispheres free of meninges were transferred to HBSS on ice (Schildge et al., 2013).

2.3.3 Primary cell culture isolation and maintenance

Under sterile conditions, the HBSS was removed and cortices were resuspended in 5ml Dulbecco's Modified Eagle's Medium-F12 (DMEM-F12) (Gibco, Thermo Fisher Scientific, Inc, Waltham, MA, USA) supplemented with 10% Fetal Bovine Serum (FBS) (Gibco, Thermo Fisher Scientific, Inc, Waltham, MA, USA), penicillin (100 U/ml) and streptomycin (100µg/ml) (p/s) (Gibco, Thermo Fisher Scientific, Inc, Waltham, MA, USA). The cortices were then mechanically dissociated through a 75µm mesh strainers with sterile plastic plungers to obtain a single-cell suspension and then aliquoted into T25 flasks containing glial media. Flasks were maintained at 37°C at an atmospheric environment of 5% CO_2. Partial medium was changed after 3-4 days and thereafter, complete medium was replaced every 2-3 days to remove non-adhering cells. Confluency was achieved after 21 days *in vitro*.

2.3.4 Preparation of astrocyte cultures

Astrocyte cultures were prepared from confluent mixed glial cultures by mild trypsinisation and shaking for 1-2 hours (h) and then rinsed twice with PBS (Gibco, Thermo Fisher Scientific, Inc, Waltham, MA, USA) to remove overlaying microglia. PBS was aspirated and 10 ml 10% Trypsin/EDTA in HBSS was added and flasks incubated at 37°C until all seeded glial cells were lifted. Cultures were pooled and neutralised with glial media (DMEM-F12 with 10% FBS). Cells were spun, supernatant discarded, and pellet resuspended in 1ml glial media. 5 x 10^5 astrocytes were seeded in 24-well plates, incubated overnight at 37°C and infected the following day. Culture purity of >96% astrocyte (Fig. 2.4) was confirmed by flow cytometry analysis (Fig. 2.5, page 43; method described in section 2.10, page 40).

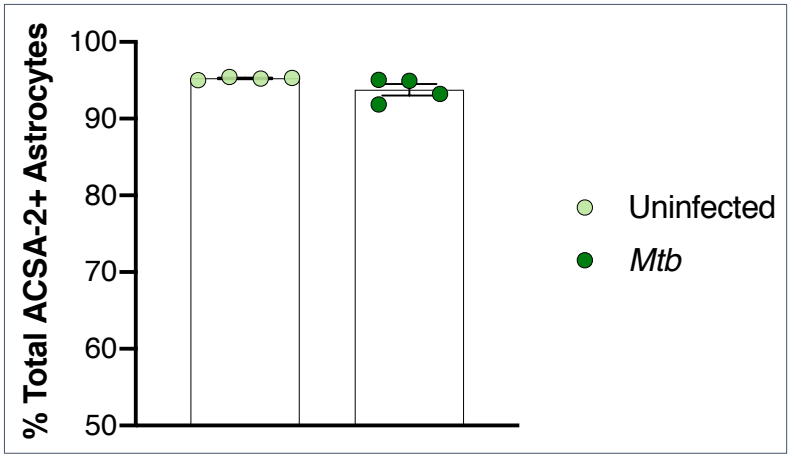


Figure 2.4 | Astrocyte culture purity. Culture purity of >96% astrocyte was confirmed by flow cytometry analysis (method described in section 2.10, page 40). Data shown is expressed as the mean ± SEM and dots represent an individual well of primary astrocytes. Experiments were performed in triplicate.

2.4 Infection Models

2.4.1 Mycobacterium

Bacillus Calmette–Guerin (BCG) and *Mycobacterium tuberculosis* strain H37Rv (Trudeau Mycobacterial Culture Collection, New York), were grown in Difco Middlebrook 7H9 broth (Becton, Dickinson and Company, Le Pont de Claix, France) containing 0.5% glycerol (Merck Chemicals Company, Germany) and enriched with 10% oleic acid-albumin-dextrose-catalase (OADC) (Becton, Dickinson and Company, France). Cultures were incubated at 37°C until log phase, then aliquoted and stored at -80°C in screw cap vials. To determine frozen stock concentrations (cfu/ml), an aliquot was thawed, passed through a 29-gauge needle 30 times to remove clumping. Thereafter 100 µl was plated in duplicate in 10-fold serial dilutions on Difco Middlebrook 7H10 agar (Appendix A) (Becton, Dickinson and Company, France) containing 10% OADC and 0.5% glycerol. Plates were semi-sealed in plastic bags and incubated for 18-21 days at 37°C and the concentration of BCG and *M. tuberculosis* determined by counting the mycobacterial colonies. BCG and *M. tuberculosis* with green fluorescent expressing protein (H37Rv-GFP, provided by Joel Ernst, New York University School of Medicine, USA) was prepared similarly with 25 µg/ml kanamycin, respectively added in broth or agar.

2.4.2 Primary cell culture and *in vitro* infection model

5 x 10^5 primary astrocytes and primary microglial cells were seeded into 24-well plates and left to adhere overnight at 37°C. The following day, frozen mycobacteria stocks were thawed and centrifuged at 10 000 rpm, 4°C for 10 minutes (mins) to remove the glycerol. The pellet was resuspended in 1ml DMEM-F12 complete media and passed through a 29-gauge needle 30 times or vigorously vortexed with 3mm glass beads to reduce clumping. Primary astrocytes and microglial cells were inoculated with *M. bovis* BCG or *M. tuberculosis* H37Rv at a multiplicity of infection (MOI) of 2:1 or 10:1 (ratios of bacilli to cells). Plates of infected cells were sealed with micropore tape and incubated at 37°C, 5% CO_2 for various experimental analysis.

2.4.3 Intra-cerebral infection model

Intra-cerebral infection procedures were performed in an Animal Biosafety Level-3 Laboratory at the University of Cape Town. C57BL/6, NSG and hNSG-BLT mice were anaesthetised by intraperitoneal injection with Ketamine (100mg/kg) (Bayer Pty Ltd, Germany) and Xylazine (10mg/kg) (Intervet, Zurich-Switzerland) and the effect of anaesthetics on the mice monitored throughout the surgery. To determine whether depth of anaesthesia was sufficient, the pedal withdrawal reflex method was utilised before proceeding with surgical procedures. Following anaesthesia administration, a pre-emptive analgesia Buprenorphine (0.1mg/kg) (Temgesic, Ricket Benkiser Healthcare SA, Pty Ltd) was administered to the mice subcutaneously, thereafter mice were placed on a stereotaxic device, nose clamped, and incisor bar swung onto place to stabilise the head. The head was shaved using veterinary clippers (Moser 1556 Trimmer, Germany) before incision and the operative site disinfected with F10 Skin-prep. By using a surgical blade and aseptic techniques, an incision of approximately 7mm in length was made with one cut between the ears to expose the skull. Thereafter, a small burr hole was drilled (Dremel 300i, Wisconsin, USA) left to the midline of the skull and anterior to the bregma exposing the duramater. A Hamilton syringe (Gastight no. 1701, Hamilton, Bonaduz, Switzerland) was lowered to a depth of 2.0 mm into the cortex and 3µl saline solution or 1 x 10^5 CFU (3µl) of *M. tuberculosis* was slowly delivered into the cortex to avoid introducing significant amount of pressure into the cortex to minimize damage. Injections into the cerebral parenchyma were based on established methodology as described in

previous publications. No adverse effects were observed with this injected volume (Francisco et al., 2015, Hsu et al., 2017, Hsu and Jacobs, 2021, Van Well et al., 2007). The needle was withdrawn and the burr hole in the skull subsequently sealed with sterile bone wax (Ethicon LLC, China). The skin was sutured in a simple interrupted pattern using monofilament suture material (5/0 Nylon, 3/8 circle reverse cutling, 13mm needle) (Ethicon LLC, China). Using antisedan (1mg/kg) the anaesthetic effect was reversed, ensuring that their body temperature returns to normal and allowing a quicker recovery rate post-surgery. Throughout the whole procedure from time of anaesthesia until recovery, the mice were kept warm with an infrared lamp, eyes lubricated when needed to prevent drying and they were continuously monitored for at least 2h post infection. For the first 72h post-surgery, the mice received analgesia (Buprenorphine) every 8-12 hourly and were monitored twice per day for the duration of the experiment. Mice that lost 20% of original weight or presented any sign of distress were humanely euthanized using 5% halothane inhalation (2-bromo-2-chloro-1,1,1-trifluoro-ethane) (Safeline Pharmaceuticals Pty Ltd, South Africa) and confirmed by cervical dislocation.

At various time points post-infection, the brain, spleen and lungs were collected separately from the naïve, infected or saline-injected control groups, and then processed for their respective analysis. Five mice were euthanised on day 1 and the brain aseptically removed to confirm the viability of the infective dose. Mouse brains, spleens and lungs were collected in 1 x PBS (pH 7.4), homogenised and plated for CFUs to determine bacterial burden on days 1-, 7-, and 14- days post-infection. To analyse the kinetic changes in the phenotype and function of astrocytes, five mouse brains per group were collected at 7- and 14- days post infection, and then dissociated into single-cell suspensions by mechanical dissociation through a 70µm nylon cell strainer (Beckton and Dickinson) and gentle trituration for flow cytometry analysis. Cells were fixed and labelled with cellular and immune activation markers (such as ACSA-2, GFAP, MHC-II, CD86, IL-10, IL-12, iNOS, IL-1β and TNF) and their expression levels were quantified using flow cytometry.

2.5 Bacterial Burden Analysis

Following *in vitro* BCG and *M. tuberculosis* infection, supernatants were collected, and cells washed with complete media. For bacterial burden enumeration, cells were lysed with 500µl ice cold water and 100µl of lysates were plated in duplicate in ten-fold serial dilutions on Difco Middlebrook 7H10 agar (DifcoTM Becton, Dickinson and Company, Sparks, MD21152, USA) supplemented with 10% OADC and 0.5% glycerol. Plates were semi-sealed in plastic bags and incubated for 18-21 days at 37°C and mycobacterial colonies determined. For *in vivo* analysis, mice were intra-cerebrally infected as described and euthanised at respective time points. Organs were harvested, homogenised in 2ml sterile 0.9% saline (0.9g NaCl in dH_2O) and ten-fold serial dilutions prepared in a solution of 0.9% NaCl/0.04% Tween 80 (v/v). Samples were similarly plated on prepared agar plates in duplicate and incubated at 37°C for 17-21 days. Mycobacterial colonies were counted, and final organ burdens calculated.

2.6 Immunocytochemistry Assay

Immunocytochemistry (ICC) was performed to characterise astrocyte infection through visualising bacterial localisation. Sterilised coverslips were placed into the wells of a 24-well plate. 5 x 10^4 astrocytes were seeded onto the coverslips and culture media added up to 500µl. Plates were incubated overnight at 37°C to allow cell adhesion. The following day cells were infected with BCG-GFP or *M. tuberculosis* H37Rv-GFP at a MOI of 10:1 and incubated at 37°C. At 2h, 24h, and 48h following infection media was aspirated from the wells, cells were washed thrice to remove any extracellular bacilli and then fixed with 4% paraformaldehyde (PFA) (Merck, Darmstadt, Germany) in 1 x PBS (pH 7.4) for 30 mins at RT. The PFA was discarded and cold methanol added for 1 minute. Thereafter, cells were washed twice with PBS for 5-10 mins on a shaker at RT. Non-specific staining was blocked using 1% Bovine Serum Albumin (BSA) (Roche, Roche Diagnostics GmbH, Mannheim, Germany) in 1 x PBS (pH 7.4) for 30 mins at RT. Cells were then incubated with primary antibody overnight at 4°C. Primary astrocytes were incubated with polyclonal antibody against GFAP (1:1000 dilution, Biolegend Clone: Poly2800, San Diego, CA, USA) or Iba1 (1:1000 dilution, Abcam, Clone: ab5076, Cambridge, UK)

diluted in 1% BSA (Roche, Roche Diagnostics GmbH, Mannheim, Germany) in PBS (pH 7.4). The astrocyte cytoskeletal marker, GFAP, was used to identify the presence of astrocytes, whereas the microglial marker Iba1 was used to identify possible microglial contamination in the cultures. The following day cells were washed twice with PBS (pH 7.4) for 5-10 mins on a shaker at RT and subsequently incubated with their respective secondary antibody. Cy3-conjugated goat anti-rabbit IgG secondary antibody (1:1000 dilution, Jackson ImmunoResearch Laboratories, PA, USA) or Cy3-conjugated rabbit anti-goat IgG secondary antibody (1:1000 dilution, Jackson ImmunoResearch Laboratories, PA, USA) were used accordingly. Antibodies were diluted in 1% BSA (Roche, Roche Diagnostics GmbH, Mannheim, Germany) prepared in PBS (pH 7.4) and samples incubated overnight at 4°C. Secondary only controls were included to exclude the possibility of non-specific antibody binding. The following day cells were washed thrice using PBS (pH 7.4) for 5-10 mins on a shaker at RT and stained for nuclei using 6-diamidino-phenylidone (DAPI) (1:1000; Sigma, MO, USA) for 10 mins in the dark at RT. DAPI binds to DNA and is a convenient nuclear counterstain. Cells were washed twice with using PBS (pH 7.4) and coverslips carefully removed from the wells and mounted in fluorescent mounting medium diaminobenzidine (Dako, CA, USA). Images were captured with a Zeiss LSM 880 Airy Scan high resolution confocal microscope (Carl Zeiss International, Germany).

2.7 Immunohistochemistry

2.7.1 Perfusion

Mice were intra-cerebrally inoculated with H37Rv-GFP as described above (Section 2.4.3., page 33). At specific time points mice were anaesthetised with a Ketamine (100mg/kg) and Xylazine (10mg/kg) cocktail and perfused with saline followed by 4% PFA in PBS. Dissected brains, lungs and spleens were post-fixed overnight in 4% PFA in PBS and cryoprotected with 15% and then 30% sucrose.

2.7.2 Staining

Following sucrose treatment, organs were embedded in Tissue-Tek OCT compound before snap-freezing in liquid nitrogen and stored at -80°C until further processing.

Frozen tissues were sectioned at 10µm using a cryostat and mounted onto slides. Tissue sections were washed with PBS (pH7.4) for 5 mins and then blocked with 1% BSA (Roche, Roche Diagnostics GmbH, Mannheim, Germany) in PBS (pH7.4) for 1h. Slices were subsequently stained with primary antibody against astrocyte specific cytoskeletal marker, GFAP (1:1000 dilution, Biolegend Clone: Poly2800, San Diego, CA, USA) or microglia Iba1 (1:1000 dilution, Abcam, Clone: ab5076, Cambridge, UK) diluted in 1% BSA (Roche, Roche Diagnostics GmbH, Mannheim, Germany) in PBS (pH7.4). Sections were incubated at 4°C in a humidified chamber overnight. The following day, slices were washed thrice with PBS (pH7.4) for 5 mins and incubated with appropriate secondary antibody diluted in 1% BSA (Roche, Roche Diagnostics GmbH, Mannheim, Germany) in 1 x PBS (pH7.4) at 4°C in a humidified chamber overnight. The Cy3-conjugated secondary antibodies (1:1000; Jackson ImmunoResearch Laboratories, PA, USA) included goat anti-rabbit IgG against astrocyte GFAP and rabbit anti-goat IgG against microglia Iba1. The following day, slices were washed thrice with PBS and thereafter counterstained for nuclear DNA visualisation with DAPI (1:1000; Sigma, MO, USA), for 15 mins at RT. After staining, samples were washed twice with PBS (pH 7.4) for 5 mins and mounted onto coverslips with a chromogenic substrate, DAKO (Dako, CA, USA). Negative control sections were processed without primary antibodies. Sections were left to dry at RT overnight and were imaged by fluorescent or confocal microscopy.

2.7.3 Histology haematoxylin and eosin staining

To validate engraftment of human tissue, humanized NSG-BLT mice were euthanised at 17 days post-infection. The left kidneys, containing the thymic organoid developed from the transplanted foetal tissue, were harvested and fixed in 10% neutral buffered formalin (Sigma Aldrich, Saint Louis, MO, USA). Tissues were embedded in paraffin wax and 2µm-thick sections cut using a Leica Sliding Microtome 2000R. Tissue sections were subsequently deparaffinized and washed thrice in 100% ethanol for 1 min, followed by two 96% ethanol washes and one 70% ethanol wash. Samples were subsequently rinsed with tap water, incubated with haematoxylin for 9 min and rinsed again with tap water. Tissues were then washed with Scott's water for 3 min and tap water for 2 min. 1% Eosin solution was subsequently used to counter stain for 2 min, followed by a final tap water wash. Finally, tissue sections were dehydrated with 70% and 90% ethanol,

followed by xylol. Prepared sections were mounted with Canada Balsam (Sigma-Aldrich, Kempton Park, South Africa). Slides were stored until image capturing.

2.8 Imaging and Software

For immunohistochemical analysis of bacilli in primary astrocyte cultures and in the brain, a Zeiss fluorescence microscope and a Zeiss LSM 880 Airy Scan high resolution confocal microscope (Carl Zeiss International, Germany) were utilised. Images were captured using Axiovision 4.8 software. A Nikon Eclipse 90i microscope with NIS-Elements AR software (Nikon Corporation, Tokyo, Japan) was used to capture images of histology slides.

2.9 Transcriptomics

2.9.1 RNA extraction

Once astrocyte cultures had reached confluency, 24-well plates were seeded with 1 x 10^5 astrocytes and incubated overnight at 37°C. The following day cells were cultured with *M. bovis* BCG or *M. tuberculosis* H37Rv at a MOI of 10:1 and incubated at 37°C. RNA was extracted using Trizol and glycogen. Briefly, after 24h of infection, the media was aspirated, and astrocytes were washed using fresh media. The cells were lysed with 800µl TRIzol and stored at -80°C. Prior to RNA extraction, samples were thawed and left to stand for 5 mins at room temperature. 200µl chloroform was subsequently added and the Eppendorf tube vigorously shaken for 15 seconds by hand. Samples were allowed to stand for 5 mins at RT before spinning for 15 mins at 13000-14000 rpm at 4°C. The aqueous phase was then carefully removed ensuring that the interphase was not disturbed. 500µl isopropanol, 120µl 5M ammonium acetate and 10µl Glycogen was added and the samples given a short vortex to mix before allowing to stand for 15-30 mins at -20°C. Thereafter, they were spun for 15min at 13000-14000 rpm at 4°C and the supernatant was removed by draining on a paper towel. 750µl 70% cold ethanol (stored at -20°C) was added and samples briefly vortexed before being spun at 14000 rpm for 10 mins at 4°C. The supernatant was once again discarded on a paper towel and left to air dry for a minimum of 15 mins to ensure all remaining ethanol was removed.

Once dry, the RNA pellet was resuspended in water (25-50µl depending on size) and left on ice for 30 mins to dissolve. Quality control and quantification of isolated RNA was performed with an Agilent 2100 Bioanalyzer (Agilent Technologies) and a NanoDrop 1000 UV-Vis spectrophotometer (Thermo Fisher Scientific). Samples were stored at -80°C until shipment to the Max Planck Institute for Infection Biology (Berlin, Germany) where microarray analysis was conducted.

2.9.2 Microarray

Microarray experiments were completed Dr Hans-Joachim Mollenkopf at the Core Facility Genomics/Microarray, Max Planck Institute for Infection Biology, Berlin, Germany. Microarrays were performed as single-color hybridisations using total RNA labelled with the Low Input Quick Amp Labelling kit (Agilent Technologies). In brief, mRNA was reverse transcribed and amplified using an oligo-dT-T7 promoter primer and labelled with cyanine 3-CTP by T7 in vitro transcription. After precipitation, purification and quantification, 0.75µg labelled cRNA was fragmented and hybridised to whole genome mouse 8 × 60K multipack microarrays according to the supplier's protocol (Agilent Technologies). After SSPE Wash (Agilent Technologies) scanning of microarrays was performed with 3µm resolution and 20-bit image depth using a G2565CA high-resolution laser microarray scanner (Agilent Technologies). Microarray image data were processed with the Image Analysis/Feature Extraction software G2567AA v.A.11.5.1.1 (Agilent Technologies) using the protocol GE1_1105_Oct12 and recommended settings. Uninfected astrocyte cultures were used as the control group for comparative analysis.

2.9.3 Analysis

All microarray data analysis was conducted in R and coding assistance was received from Dr Hans-Joachim Mollenkopf and Dr Natalie Nieuwenhuizen (Core Facility Genomics/Microarray, Max Planck Institute for Infection Biology, Berlin, Germany) as well as Dr Katie S. Lennard (Division of Computational Biology, Department of Integrative Biomedical Sciences and Institute of Infectious Disease and Molecular Medicine, Faculty of Health Sciences, University of Cape Town, South Africa). The particular figures that were generated for this thesis were selected, finalised and analysed by Sohair Geyer. Microarrays were background corrected, normalised and controlled for quality using the

R package limma (Smyth, 2005). For background correction the method 'normexp' was used on the gProcessedSignal and the gProcessedBackground data. Between-array normalisation was done using the 'quantile' method in limma. The gene expression values were averaged for each probe over all replicates of that probe on the microarray, using the 'avereps' function. Differences in gene expression were assessed using the linear model 'lmFit' and 'makeContrasts' in limma. Genes with corrected p-values of <0.05 were considered significant after Benjamini-Hochberg correction for multiple testing. All downstream analyses were conducted in R on background-corrected (expnorm) and quantile normalised gene expression data. Array quality was assessed using the R package arrayQualityMetrics (Kauffmann et al., 2008). Batch correction was performed using the ComBat function from the R package sva (Leek et al., 2012). Differential expression testing was performed with the R package limma's (Ritchie et al., 2015) lmFit function. Transcriptional module analysis was performed using the tmod (Weiner 3Rd and Domaszewska, 2016) package's functions tmodLimmaTest (with default parameters) and tmodDecideTest.

2.10 Flow Cytometry Analysis

2.10.1 Surface and intra-cellular Staining

After a 24h infection, plates were centrifuged for 10 min at 1600 rpm. The supernatants were removed, filtered twice through a 0.2µm filter and stored at -80°C for LUMINEX analysis. For *in vivo* samples, mouse brains were collected in PBS buffer (pH 7.4) and single cell suspensions were prepared using a 70µm strainer (Corning cell strainer, Merck, Darmstadt, Germany). To discriminate viable from nonviable cells, the sample pellets were stained in a v-bottom plate with 25µl Fixable Viability Stain 510 (1:2000, BD Horizon™, BD Biosciences) prepared in FACS buffer, and incubated for 15mins at RT then spun at 1600 rpm for 10mins. The pellets were surface stained with 25 µl diluted antibody cocktail (Table 2-1) of ACSA-2, MHC II and CD86 prepared in 2% FBS (Gibco, Thermo Fisher Scientific, Inc, Waltham, MA, USA) in FACS buffer solution. Samples were subsequently incubated for 40 – 50 mins in the dark at RT. Following incubation, the cells were washed with 180µl FACS buffer and centrifuged for 10 mins at 1600 rpm. The supernatant was decanted, and cells fixed overnight at 4°C with 100µl Cyto/Fix buffer

(BD Cytofix/Cytoperm Plus Fixation/Permeabilization Solution Kit). Following overnight fixation, cells were centrifuged for 10 min at 1600 rpm, and supernatant discarded. The pellet was washed with 180 µl Cyto/Perm, cells centrifuged for 10 min at 1600 rpm, and supernatant discarded. Cyto/Perm (1:10) working solutions were prepared from stock buffers according to the manufacturer guidelines. Intracellular staining was performed with 25µl diluted antibodies (Table 2-1) prepared in 2% FBS (Gibco, Thermo Fisher Scientific, Inc, Waltham, MA, USA) block solution in Cyto/Perm buffer and samples subsequently incubated for 40 – 50 mins in the dark at RT. Thereafter, cells were washed with 180µl Cyto/Perm, cells centrifuged for 10 mins at 1600 rpm, supernatant discarded and resuspended in 200µl Cyto/Perm for acquisition on the BD LSRFortessa.

Table 2-1 | Antibody panel for flow cytometry analysis of astrocytes

Antibody	Fluorophore	Clone	Dilution	Manufacturer
ACSA 2	PE	IH3-18A3	1:25	Miltenyi Biotec
GFAP	Alexa Fluor® 647	EPR1034Y	1:25	Abcam
Iba-1	Alexa Fluor® 647	EPR6136(2)	1:25	Abcam
MHC II	Alexa Fluor® 700	M5/114.15.2	1:50	eBioscience
CD 86	BV605	GL1 (RUO)	1:50	eBioscience
IL-1β	PE-Cyanine7	NJTEN3	1:25	eBioscience
IL-10	BV711	JES5-16E3	1:25	BD Horizon™
iNOS	PerCP-eFluor™ 710	CXNFT	1:25	eBioscience
TNF	APC-Cy™7	MP6-XT22	1:25	BD Pharmingen™
IL-12 (p40/p70)	V450	C15.6	1:25	BD Horizon™

2.10.2 Compensation

The compensation matrix was calculated to determine the fluorescent contribution of each fluorophore in each detector for accurate determination of primary fluorescence signals. For each flow cytometry run, compensation beads (anti-mouse Ig-κ or anti-rat Ig-κ beads; BD Biosciences) corresponding to each antibody-conjugate used in the panel were individually stained. The antibodies (at the optimal titre) were incubated with one drop of compensation beads in a 5ml polystyrene tube for 20 min in the dark at RT. After washing off the excess antibody with 2 ml FACS buffer and centrifugation at 1800 rpm for 10 min, the supernatant was decanted and the beads were resuspended in 300µl of Cell Fix buffer (BD Biosciences).

2.10.3 Acquisition and data analysis

Samples were acquired on a BD LSRFortessa flow cytometer using FACSDiva software. Flow cytometry data was analysed using FlowJo software Version 10.7.1 (Becton Dickson and Company, BD).

2.10.4 Gating strategy

The same gating approach was applied to assess *in vitro* primary astrocyte cultures and *in vivo* brain samples (Fig. 2.5). To ensure uniformity in data acquisition, a time gate was set for one channel on a laser and any fluorescence variations disregarded. Subsequently, single cells were selected (singlets), followed by live cells, FSC/SSC was used to exclude debris and finally ACSA-2^+ astrocytes were identified. FMOs were used to set the ACSA-2^+ astrocyte gate and back gating was performed to assess the distribution of the astrocyte population in that parameter, thereby ensuring that the gates were accurate. Once the astrocyte population was identified, gates were established for cytokines and other immune mediators.

2.11 Luminex Analysis

Luminex Bead Array Multiplex Immunoassay is a multi-analyte technology that allows the simultaneous detection of multiple analytes in a single sample. Once primary astrocyte cultures had reached confluency, 5 x 10^5 astrocytes were seeded in 24-well plates and left to adhere overnight at 37°C. the following day, seeded cells were infected with either *M. bovis* BCG or *M. tuberculosis* H37Rv at a MOI of 10:1. Uninfected cells were included as an experimental control to establish baseline levels of astrocyte cytokine and chemokine production. At 24h and 48h post-infection, plates were spun for 10 min at 1600 rpm and supernatants were collected and filtered twice with a 0.2µm filter. Supernatants were stored at -80°C until the experimental day. Samples were shipped on dry ice to Stellenbosch University to perform the Luminex Analysis. Samples were thawed and diluted to obtain a suitable concentration for analysis then prepared according to the manufacturer's instructions using the diluent provided in the assay kit (Merck). The Luminex® Bioplex 200 system (Bio-rad, USA) was used to detect and quantify the analytes and the Bioplex Manager 6.1 was used to analyse the levels of

analytes in the samples. Calibration and verification were performed as part of the daily quality assurance procedures.

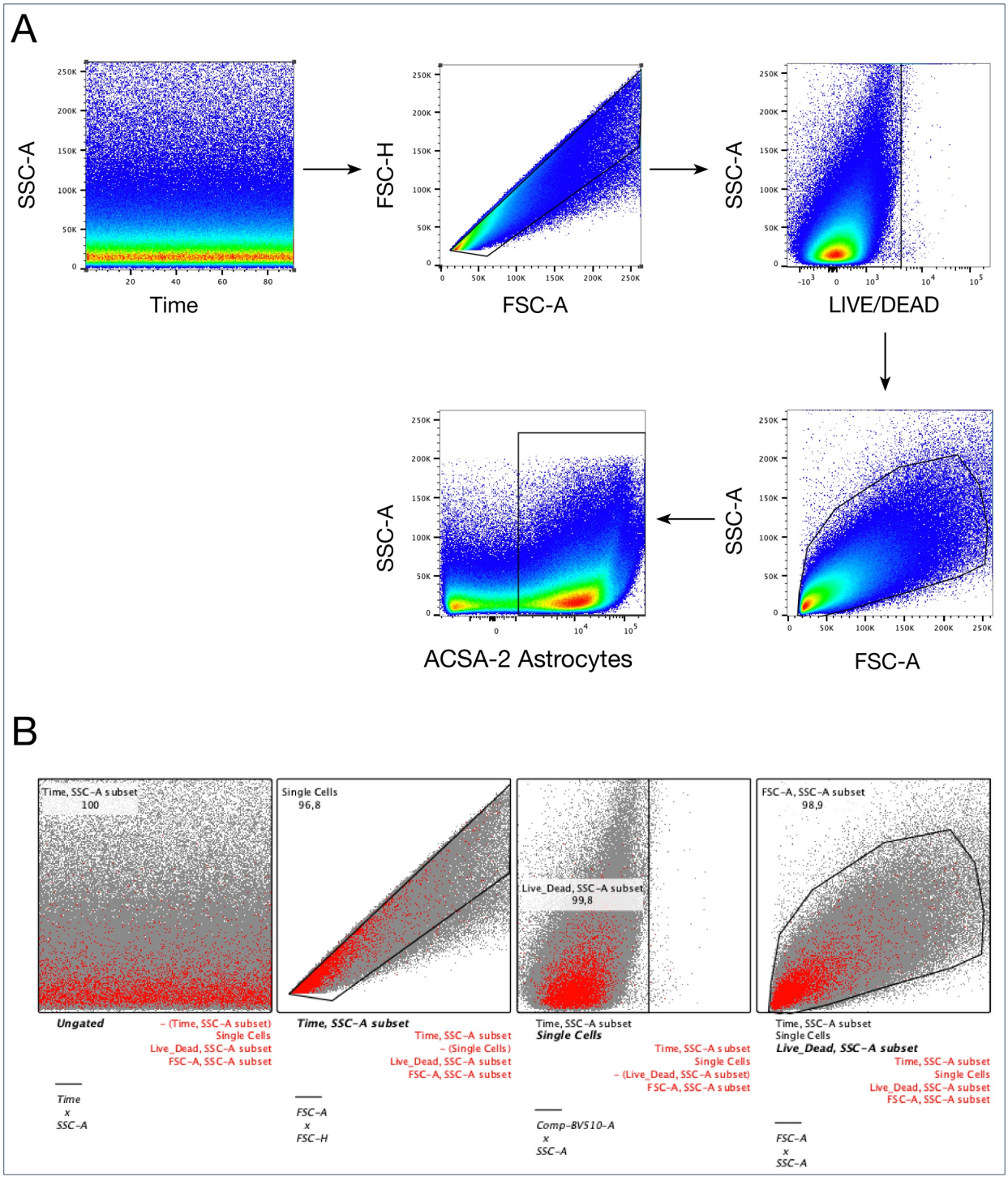


Figure 2.5 | Representative flow cytometry plots showing gating strategy for ACSA-2⁺ astrocytes. (A) Brains and primary cultures were stained with a cocktail containing the ACSA-2 antibody to identify the astrocyte population. Cells were gated further to identify the percentage of astrocytes expressing immune factors of interest. (B) Back-gating was performed to ensure accurate gating and that the entire astrocyte population was included.

2.12 Statistical Analysis

All statistical analyses were completed using GraphPad Prism software Ver. 6.0 (GraphPad Software Inc. La Jolla, CA, USA). Except where otherwise indicated, the data is represented as the mean ± SEM. Data was analysed by the students two-tailed t-test and Mann-Whitney U test or ANOVA for multiple comparisons. A “p” value of less than 0.05 was considered significant.

CHAPTER 3

CHAPTER 3

Astrocyte modulation of immune responses during BCG infection

Host immunity to mycobacteria is a complex process involving innate and adaptive mechanisms. Bovine tuberculosis is caused by the intracellular pathogen *Mycobacterium bovis* and upon infection presents as a respiratory disease comparable to *M. tuberculosis* infection in humans (Waters et al., 2011, Pollock et al., 2005). The current TB vaccine, Bacille Calmette–Guérin (BCG), is an attenuated strain of *Mycobacterium bovis*. There are various strains of the BCG vaccines and according to the BCG World Atlas 2020 update, the Danish 1331 (16.6%), Pasteur 1173P2 (9.2%), and Tokyo 172 (7.3%) strains are the most commonly used strains for BCG vaccine production worldwide (Zwerling 2011). Each BCG strain has distinct characteristics, including differences in virulence, growth rate, and immunogenicity (Asadian 2022, Brosch 2007, Behr 1999). These variations in BCG strains can result in differences in the level of protection provided against TB and can impact the interpretation of BCG vaccination efficacy studies (Brosch 2007, Davids 2006, Fine 1995). Despite it being only 75-86% effective at preventing disseminated miliary and meningeal TB in early childhood (Trunz et al., 2006, Rodrigues et al., 1993) and its variability in protection against pulmonary TB, BCG remains the most administered vaccine worldwide. Following BCG vaccination there is an epigenetic reprogramming of myeloid cells and progenitor cells in the bone marrow in a process termed trained immunity (Kong et al., 2021, Cirovic et al., 2020, Netea et al., 2016). These changes in innate immune cell phenotype results in a heightened secondary response associated with increased expression of specific cytokines such as IL-1β, TNF and IL-6 (Kleinnijenhuis et al., 2012) that offers significant protection against multiple pathogens other than *M. tuberculosis* (Kong et al., 2021, Kleinnijenhuis et al., 2012, Netea et al., 2016). This may explain why individuals vaccinated against TB generally display better antimicrobial and antiviral responses (Kong et al., 2021, Arts et al., 2018).

Analysis of astrocyte-mediated immune responses to BCG may contribute to the understanding of the cellular mechanisms shaping mycobacteria-astrocyte interactions, and how modifications of pathways induced by such interactions impact disease

pathogenesis. In this study, purified primary murine astrocytes were used for *in vitro* challenge experiments with cultured *M. bovis* BCG as the infecting agent. The objective of this study was to identify novel components of the complex astrocyte molecular pathways generated during uptake of BCG and the role these cells play in regulating host immune responses to avirulent mycobacteria.

3.1 Establishing BCG infection in primary astrocytes

Astrocytes form part of the glia limitans as a functional barrier between neural and non-neural cells restricting the entry of inflammatory cells into the CNS parenchyma from adjacent tissue (Sofroniew, 2015a). The proximity of astrocytes to non-neural cells makes them one of the first resident cells to encounter pathogens once they've traversed the barriers and gained access into the brain. Many studies have demonstrated the immunological contribution of astrocytes in cerebral infections and their role as a first line of defence against microbial insults together with microglia (Mucke and Eddleston, 1993, Gehrmann et al., 1995, Fontana et al., 1987, Streit et al., 1988, Geyer et al., 2019, Stoner et al., 2015, Braun et al., 2011, Liu et al., 2022). The interaction of BCG and microglia is well established and protective immunity in the CNS is often attributed to these cells (Lee et al., 2009, Cannas et al., 2011, Peterson et al., 1996, Mazzolla et al., 2002). Although studies have indicated that astrocytes respond to peripheral vaccination and intracerebral inoculation with BCG (Mazzolla et al., 2002, Qi et al., 2017), evidence is limited on their potential as host cells for mycobacteria and their capacity to modulate immune activity following infection.

To establish cultures for BCG infection, primary mixed cortical cells were harvested from mouse pups and cultured for 21 days until astrocytes reached confluency. The culture media used (DMEM-F12) favours survival and growth of glial cells with neurons diminishing within the first week. Microglia and oligodendrocyte progenitor cells were removed by vigorous shaking to obtain astrocyte cultures of 96% purity. Once astrocytes reached confluency, culture purity was checked using fluorescence microscopy. Cells (5 x 10^4) were seeded onto microscope slides and stained with the nuclei marker 4',6'-diamidino-2-phenylindole (DAPI) and the astrocyte lineage marker GFAP (Fig. 3.1A). The

confocal image displays GFAP signal in the red channel (Fig. 3.1A-i), DAPI in the blue channel (Fig. 3.1A-ii), a phase contrast image of the cell (Fig. 3.1A-iii) and an image of all overlapping channels (Fig. 3.1A-iv). The red, GFAP-positive signal displays the cytoskeletal filaments of primary murine astrocytes indicating the presence of astrocytes in the prepared cultures. Although the isolation method used has been reported to yield an astrocyte purity greater than 98% (Schildge et al., 2013), cultures may contain limited microglia. To examine possible culture contamination with microglial cells, the microglial marker Iba-1 was used to stain the cultures (Fig. 3.1B). Iba-1 is visualized in the red channel (Fig. 3.1B-i), DAPI-stained nuclei in the blue channel (Fig. 3.1B-ii), attached cells in the phase contrast image (Fig. 3.1B-iii) and an overlap of all channels is also displayed (Fig. 3.1B-iv). The phase contrast and DAPI-stained (blue) images exhibit a high cell density but the lack of any signal in the Iba-1 stained (red) channel illustrates the lack of microglia, confirming a suitable astrocyte culture for further analyses.

To investigate the ability of astrocytes to internalize BCG, primary astrocyte cultures were infected with recombinant GFP-expressing BCG at a multiplicity of infection (MOI) of 10:1 for 24hours (h). Previous published data (Randall et al., 2014) have shown efficient bacilli uptake by cultured microglia and were therefore included as a positive control cell type against which bacilli internalisation by astrocytes could be measured. Primary microglial cultures were infected at a MOI of 2:1. Using immunohistochemistry, astrocytes and microglia were labelled with anti-GFAP and anti-Iba-1 respectively, and bacilli uptake was analysed by confocal microscopy (Fig 3.2). Astrocyte cytoskeletal filaments were visible in the red channel, GFP^+ bacilli in the green and DAPI-stained nuclei were in the blue channel (Fig. 3.2A). An image of all three channels combined displayed GFP-labelled bacilli (green) in association with the astrocyte cytoskeletal filaments (red), however, the data does not distinguish between surface and internalised bacilli (Fig. 3.2A). A set of z-stack confocal images was therefore acquired to ascertain a three-dimensional aspect of the infected cell and to verify astrocytic internalisation of BCG bacilli. An orthogonal projection of a single z-stack was created with bacilli positioned at the intersection of a horizontal green and vertical red line representing the x- and y-planes, respectively. The x-plane is visualized in the side panel of the image and the y-plane in the top panel. Both the x and y planes demonstrate the localization of GFP^+ bacilli (green) between the $GFAP^+$

astrocyte filaments (red) indicating bacilli internalization within the astrocyte cytoskeleton (Fig. 3.2B). In some instances, yellow, fluorescent bacilli were observed within astrocytes (Fig. 3.2C). Yellow emissions are a result of the overlapping red and green, fluorescent signals characteristic of co-localisation and indicate a very close association between the bacilli and the cytoskeleton. To verify the relationship between the astrocyte structures and bacilli, an arrow was drawn through the yellow bacilli and the fluorescent intensity measured along the length of the arrow. The area of interest within which the channel intensity was measured is enclosed in a white rectangle, and the plane through which the arrow passes is represented by the graph displayed in Figure 3.2C1. The distance of the arrow length is shown on the x-axis, while the fluorescent intensity is visible on the y-axis of the graph. Unsurprisingly, emission in the blue channel remained low as no DAPI-stained nuclei were visible in the image (Fig. 3.2C, C1). Emission in the green channel started equally low with minimal background signal but eventually peaked due to the fluorescence emitted by the bacilli. Initial emission in the red channel was similarly moderate after which the fluorescent signal peaked indicating increased cytoskeleton intensity. Synchronized peak emissions within the red and green channels and the associated co-localized yellow emission typically suggests condensation of the cytoskeletal filaments around the bacilli, thus validating the internalisation of the bacilli within astrocytes. Although co-localization is indicative of internalisation it is possible for bacilli to be internalised and existing free within the astrocyte cell body (as seen in Fig. 3.2B). Microglia were previously shown to internalise mycobacteria (Randall et al., 2014), thus as a positive control for bacilli internalisation primary microglia were included (Fig. 3.2D). Z-stacked sections were acquired, and orthogonal projections created to verify the position of the bacilli in relation to the microglia. The GFP^+ bacilli (green) were clearly visible within the $Iba1^+$ microglia (red) in both the x- and y-planes. Although the kinetics of uptake may differ, the data demonstrated that astrocytes have the ability to internalise mycobacteria, similar to microglia.

To characterise the kinetics of bacilli uptake in astrocytes, colony forming units (CFUs) were enumerated in BCG-infected primary astrocytes at 2h, 24h and 48h post-infection at MOIs of 2:1 and 10:1. The data demonstrated bacilli uptake at 2h in both MOI 2:1 and MOI 10:1 infected astrocytes. Interestingly, astrocytes infected with an MOI 2:1 and MOI

10:1 demonstrated a significant decrease in bacterial burden at 24h compared to 2h ($p < 0.01$) and at 48h compared to 2h ($p < 0.05$) (Fig. 3.2 E). However, bacterial burden from 24h to 48h was consistent as no significant differences were observed ($p < 0.05$) at both MOIs.

Therefore, collectively the orthogonal view of the z-stacked section together with the yellow fluorescent co-localization image unequivocally confirms that BCG-GFP bacilli is indeed positioned within the astrocyte cytoskeleton, demonstrating that astrocytes have the ability to internalise avirulent BCG *in vitro*.

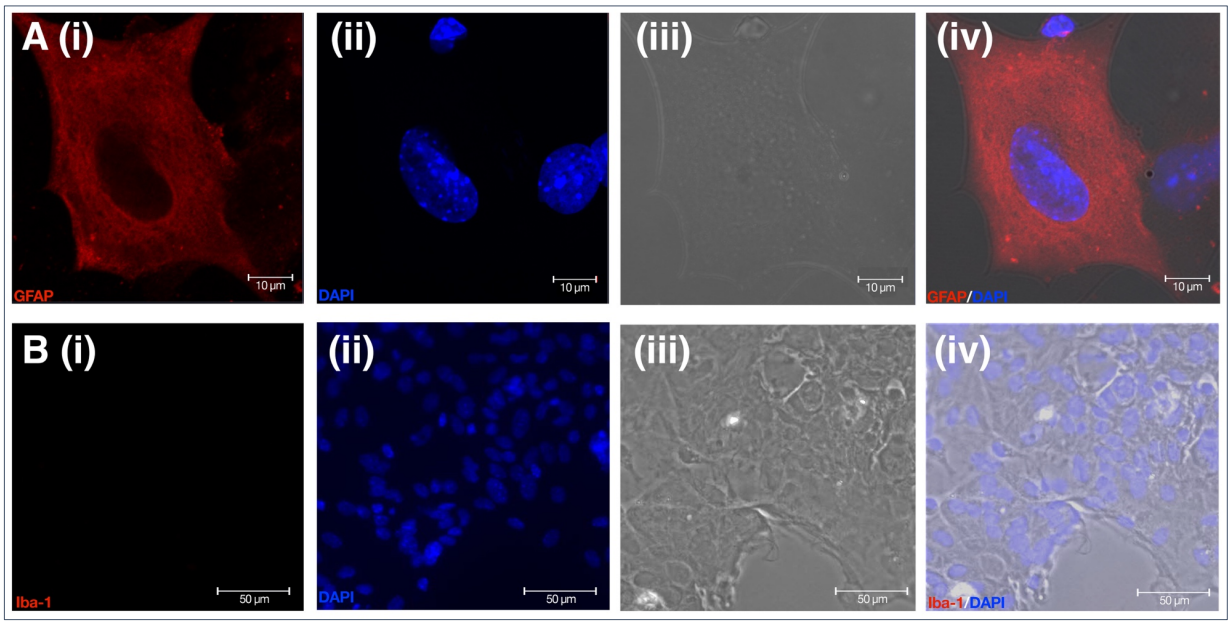


Figure 3.1 | Primary astrocyte culture. (A) Primary murine astrocytes were grown to confluency and cells were seeded onto microscope slides. After 24h cells were fluorescently stained with anti-GFAP and DAPI to confirm the presence of astrocytes. (Ai) The astrocyte lineage marker GFAP is displayed in the red channel, (Aii) nuclear marker DAPI in the blue channel, and (Aiii) a phase contrast image. (Aiv) A combined image of all overlapping channels is shown. (B) To confirm the absence of microglia, seeded cells from the astrocyte culture were stained with (Bi) the microglial marker Iba-1 in the red channel. (Bii) The DAPI marker in the blue channel and (Biii) a phase contrast image to confirm the presence of cells. (Biv) A combined image of overlapping channels illustrates the presence of cells, and the lack of a red signal demonstrates that these cells are not microglia. Images are representative of 4 wells from 1 of 3 individual experiments.

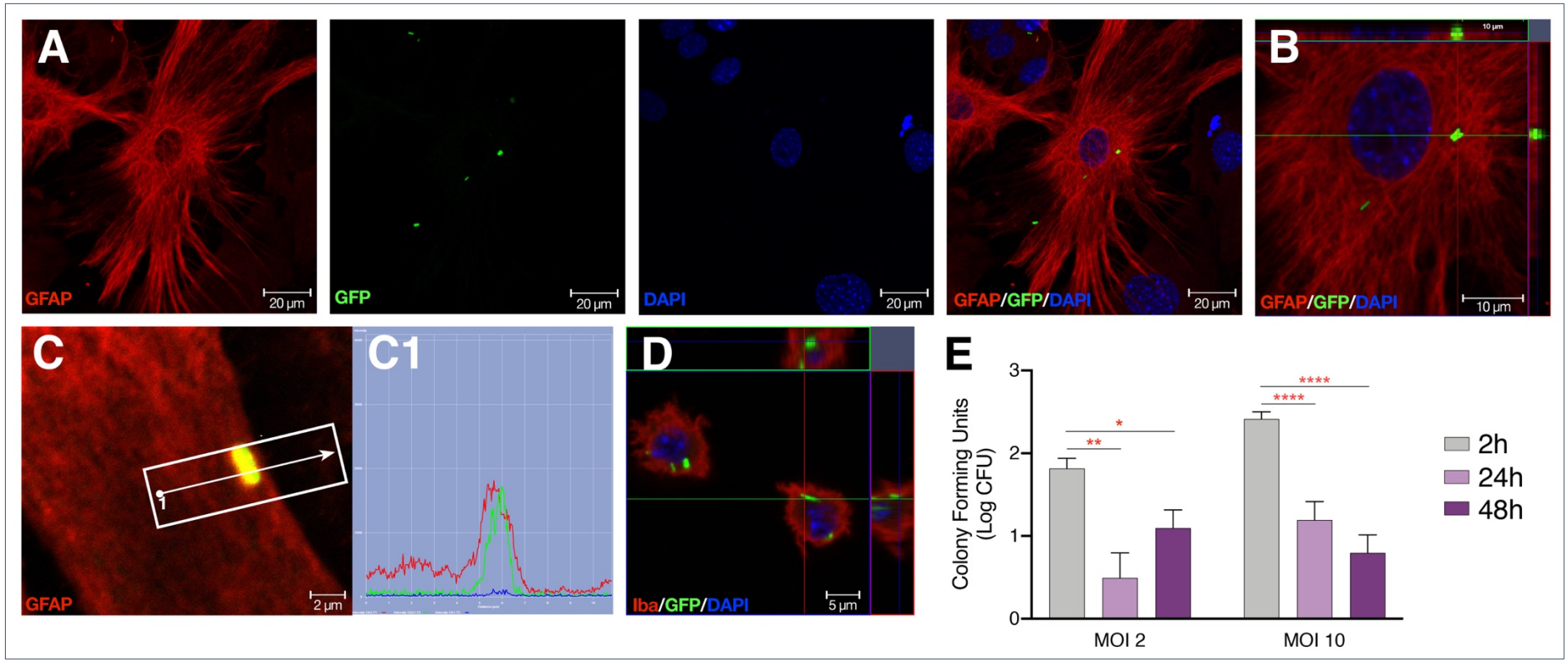


Figure 3.2 | Astrocytes internalise BCG bacilli. Following a BCG challenge, primary murine GFAP$^+$ astrocytes were analysed by immunocytochemistry at 24h and CFU numbers determined at 2h, 24h and 48h. Confocal microscopy images showing (A) GFAP-labelled astrocytes (red), GFP-bacilli (green) and DAPI-labelled cell nuclei (blue) in separate channels as well as all the channels combined. (B) Fluorescent image demonstrates astrocyte and bacilli association while the colocalization of the bacilli and cytoskeletal filaments in the orthogonal XY plane, indicate internalization. (C) Captured z-stacked image demonstrates yellow co-localisation signals of the green, fluorescent GFP-labelled bacilli and red astrocyte marker (GFAP) indicating interaction. A white arrow was drawn through the yellow bacilli as the selected path for fluorescent intensity analysis (1). This region was demarcated by a white square and is represented in C1 as a fluorescent intensity graph. (C1) Overlapping peaks of the red and green signals indicate interaction of the astrocyte cytoskeletal filaments and bacilli. (D) Internalization was comparable to Iba-1-stained microglia. (E) CFU determined from lysed cultures indicated bacilli viability at 2h following primary astrocyte infection, but viability is reduced after 24h. The results are the mean and SEM of 3 individual experiments (* $p < 0.05$).

3.2 BCG induces differential gene expression in astrocytes

There is compelling evidence that astrocytes have complicated roles in recruiting and restricting leukocytes during neurodegenerative disease, traumatic injury or infection (Owens et al., 2008, Engelhardt and Coisne, 2011, Wilson et al., 2010). Transcriptomic profiling is increasingly used to obtain comprehensive overviews of cellular components that are perturbed particularly during infection. Exploring gene transcriptional changes in astrocytes during mycobacterial challenge *in vitro* provides a model for understanding composite host-pathogen interactions. Identification of underlying cellular signalling pathways that drive host responses to mycobacterial infection can be identified and their contribution to infection control or disease progression evaluated.

To investigate the differential gene expression of reactive astrocytes induced by BCG, transcriptome changes were compared between infected and uninfected astrocyte cultures using transcriptional profiling. Purified primary astrocytes were infected with BCG (MOI 10:1) and total RNA was isolated after 24h. Each experimental group consisted of 5 samples and all experiments were conducted in triplicate. Data is representative of three independent experiments. Once RNA integrity and concentration were verified, SurePrint microarrays were utilized to detect gene expression in BCG-infected and uninfected astrocytes. Transcriptomic analysis using R software packages was then performed to determine the expression levels of mRNA. Following data normalisation and batch correction on R, further analysis of the entire microarray dataset revealed a total of 8824 differentially expressed genes (DEGs) between infected and uninfected astrocytes (See Appendix, Table S-1 for the top 30 DEGs induced in astrocytes during BCG infection.). Based on a log2FC difference of at least two, 213 genes were significantly upregulated and 35 downregulated. A heat map was created with parameters set at a fold change (FC) ≥ 2 and an adjusted p-value (adj. p) < 0.05, allowing for global visualization of gene expression changes between the two conditions (Fig. 3.3A). Gene expression values were converted to a colour scale where blue and red hues were used to represent low and high expression levels, respectively. Hierarchical clustering on the x- and y-axis was used to display groupings based on strong connections. Close associations within infected and uninfected astrocytes groups were

noteworthy, as was the distinct separation of gene expression profiles between these two groups, as indicated by the clustering on the x-axis of the heatmap. Considering the size of the dataset, a volcano plot was then constructed to visualize the overall effects of BCG infection on astrocyte gene expression (Fig. 3.3B). These scatterplots are commonly used to illustrate the relationship between effect size and statistical significance of events in two groups. The -log10 adj. p value was plotted on the y-axis as a measure of statistical significance and the log2FC was plotted on the x-axis as a measure of effect size. Thresholds were set at adj. p value < 0.01 and log2FC > 1 and < -1 represented by horizontal and vertical dashed lines, respectively. Upregulation of gene expression is denoted by a positive value on the x-axis, whereas downregulation is presented as a negative value. Each gene is displayed as a specific dot on the plot and the dot colour corresponds with minimum threshold requirements. Grey dots represent genes that do not pass any set thresholds. Green dots illustrate genes that pass the threshold for logFC, whereas blue dots depict statistically significant genes. Genes plotted in red are above the threshold for both statistical significance and fold change and may therefore be the most biologically significant genes. Top genes that passed both thresholds were labelled. The most statistically significant genes are found toward the top of the graph and outliers on the right and left of the graph represent the most differentially expressed genes. The plot displayed a clear distinction in gene expression between BCG-infected and uninfected control astrocytes. Interestingly, cytokine and chemokine transcript expression between the infected and uninfected cells were disparate, notably *Cxcl1*, *Cxcl2*, *Cxcl3*, *Ccl4*, *Tnf*, *Il1b*, and colony stimulating factor *Csf3*. Similarly, guanylate binding proteins *Gbp4* and *Gbp5* as well as *S100a8* and *Nos2* expression were altered, which is not surprising given their roles in regulating inflammatory responses (Wang et al., 2018, Tretina et al., 2019, Coleman, 2001). These findings show that astrocytes not only generate a transcriptional signature in response to BCG in culture, but also suggests potential immunoregulation. Further investigations are required to determine whether the elicited responses form a component of immune function.

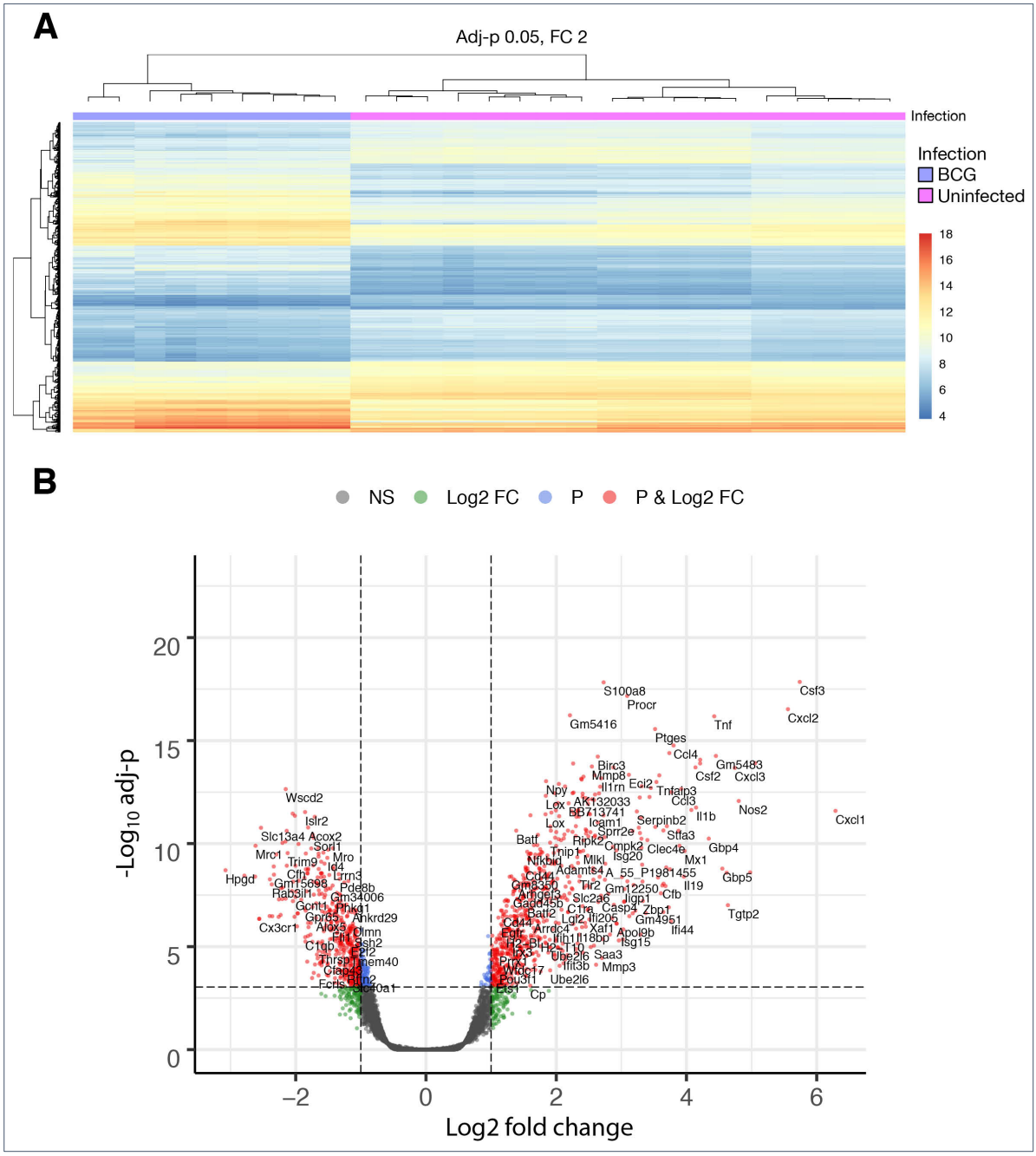


Figure 3.3 | BCG infection induces differential gene expression in astrocytes. (A) Heat map summary highlighting differential gene expression between BCG-infected primary astrocytes compared to uninfected astrocytes. Red and blue indicate high and low expression, respectively. (B) Volcano plot of DEGs in BCG-infected astrocytes. The x-axis shows the fold change (FC) in DEGs versus the adjusted p values (on a log10 scale) on the Y-axis. The horizontal dashed line indicates the threshold for statistical significance by adj. p, while the vertical dashed line is the threshold for FC. Red dots represent thresholds reached for FC and adj. p, while the green dots correspond to genes with no significant differences. Blue dots are significant however do not reach the required threshold for log2FC.

3.3 Diverse immune-related modules are enriched in astrocytes during BCG infection

Depending on their functional characteristics, genes can be classified into gene sets which generally relate to specific biological processes. The pervasive nature of modularity in biological systems allows pathways to be identified through co-expressed or co-regulated groups of genes enabling biological phenotypes to be distinguished. The fundamental principle of gene enrichment is that co-functioning genes act via related biochemical pathways that have already been defined and stored on various databases. To explore pathway enrichment in astrocytes, gene module-level analysis was conducted after a 24h BCG challenge using the transcriptional module analysis (tmod) package on R. The tmod package provides a functional analysis where data is associated to phenotypes using a gene set library created from blood transcriptional modules (Chaussabel et al., 2008, Li et al., 2014). The tmodDecideTest function was selected to distinguish whether a module is up-, down- or not regulated (Fig. 3.4). This classification is dependent on the number of differentially expressed genes within that particular module. The gene expression changes within the modules are denoted by red and blue bars for up- and down- regulated genes, respectively, while grey bars represent unchanged genes. The size of the bars illustrates effect size, and the colour opacity indicates the p-value. The significance of module enrichment between infected and uninfected astrocytes was assessed using the CERNO statistical test, a modification of Fisher's method for integrating probabilities, first described by Yamaguchi et al. (Yamaguchi et al., 2008) and module ID's utilized were defined by Li et al. (Li et al., 2014). With a threshold set at an adj. p value of 0.01 in the tmodDecideTest, 44 significantly enriched modules were identified (Fig. 3.4). A robust modification of immune-related pathways was discerned in infected astrocyte, including: "chemokine clusters (cluster I and II)" (LI.M27.0 and LI.M27.1); "chemokines and inflammatory molecules in myeloid cells" (LI.M86.0); "proinflammatory cytokines and chemokines" (LI.M29); and "enriched in activated dendritic cells/monocytes" (LI.M64). An increasing body of scientific literature has documented amplifications in the innate immune component of host defence following BCG vaccination (Jensen et al., 2015, Ota et al., 2002, Kleinnijenhuis et al., 2012). This adaptive feature of innate immunity has been termed "trained immunity".

Given that BCG vaccination boosts cytokine responses to immunological challenges, it was anticipated that modules relating to cytokine and chemokine regulation would be enriched in BCG-infected astrocytes. Further, a significant enrichment of modules apparent in viral infections such as “innate activation by cytosolic DNA sensing” (LI.M13); “antiviral IFN signature” (LI.M75); “Retinoic acid-inducible gene 1 (RIG-1) like receptor signalling” (LI.M68); and “viral sensing & immunity; IRF2 targets network (I)” (LI.M111.0). A tentative explanation for this may be the internalisation of BCG which consequently triggers intracellular receptors belonging to these modules. Moreover, these modules correlate with type I IFN responses, suggesting that IFN-related activity is incited in astrocytes during BCG infection. Consistent with this data are previous studies which showed an upregulation of type I IFN genes and interferon stimulated genes (ISGs) in the lungs and spleens of mice infected with *M. bovis* (Wang et al., 2019). Similarly, reports on *M. bovis*-infected cattle have demonstrated increased IFN-mediated gene signatures (Wang et al., 2013). Type I IFN signalling has however been shown to promote bacterial proliferation and contribute to the pathogenesis of *M. bovis* (Wang et al., 2019). It is therefore critical to investigate host responses in astrocytes, to determine how this may influence disease progression. A notable observation was that although modules involved in adaptive immunity such as “enriched in antigen presentation (I)” (LI.M71); “signalling in T cells (II)” (LI.M35.1) and “immuregulation – monocytes, T and B cells” (LI.M57) were detectable, they were not as significantly induced and therefore may not be as biologically relevant in BCG-infected astrocytes. Profiling of astrocytes during BCG infection has thus far demonstrated changes in their transcriptome signature characteristic of innate immune cells. Adjustments were apparent in modules related to pathogen recognition signalling systems as well as cytokine and chemokine pathways. Despite the tmodDecideTest presenting a clear ability of astrocytes to induce innate immune activity through these inflammatory and intracellular immune pathways, the enriched modules alone do not provide sufficient information on astrocyte gene activity. To present a more complete assessment of the impact of BCG on astrocyte function and to identify the individual genes involved, a chord diagram was constructed to look at the relationship between modules and highly expressed genes, as well as by investigating gene expression changes within modules of interest.

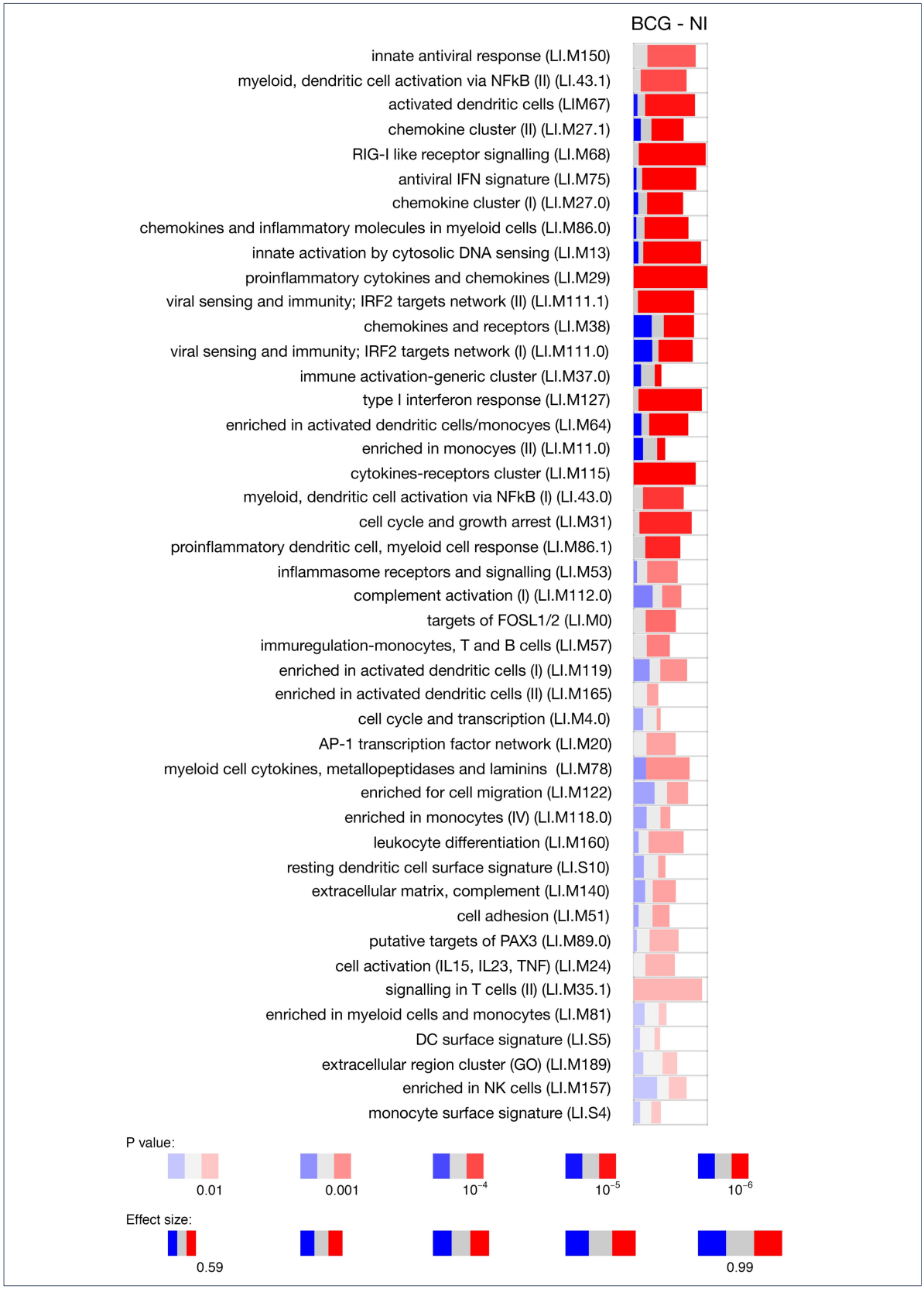


Figure 3.4 | Gene module enrichment in BCG-infected primary astrocytes. Data was generated with the tmod R package. The red and blue bars indicate gene up- and down-regulation, respectively, with bar size representing effect size and opacity corresponding to the p-value. Thresholds were set at $p < 0.01$ and effect size > 0.59.

Transcriptome module analysis provides an extensive overview of cellular activity, however, for a more extensive evaluation of cell function, gene expression changes of module members are required. To distinguish the inter-relationship between induced modules and differentially expressed genes in BCG-infected astrocytes, a chord diagram was created. Chord diagrams are graphical illustrations used to visualise connections between multiple entities for comparative purposes and to display relationships between them. The circos plot visualised 16 modules of interest with a high effect size as measured by AUC $\geq$ 0.8 and an adj. $p < 0.001$, and the differentially expressed gene transcripts within those modules (adj. $p < 0.05$, FC > 1.5) (Fig. 3.5). The right outer half of the circle displays the different modules, each one represented as a separate section in a specific colour, whereas the left half of the circle shows individual genes. Gene expression changes are indicated by the red and blue boxes representing up- and down-regulation, respectively, while the opacity corresponds to the logFC. Modules were joined to individual genes by arcs illustrating connections between these entities. Among the genes associated with the various modules were those encoding chemokines *Cxcl1*, *Cxcl2*, *Ccl7*, *Cxcl3*, *Cxcl5*, *Ccl4*, *Ccl3*, *Ccl2*, *Cxcl10*, *Ccl5*, *Cxcl12*, *Cxcl9*, and *Ccl8* as well as cytokines *Il6*, *Tnf*, *Il1a*, and *Il1b*. Many genes were shown to contribute to multiple modules, particularly *Tnf*, *Il1b*, *Rsad2*, *Ccl4*, *Cxcl10*, *Irf7*, *Ccl5*, *Nfkbia*, *Tap1*, *Ddx58* and *Ifih1* were associated with at least four modules. There were also notable crossovers with genes contributing to similar modules. Both the “proinflammatory dendritic cell, myeloid cell response” and “chemokines and inflammatory molecules in myeloid cells” modules contained the genes *Tnf*, *Il1b*, and *Ccl4*, whereas *Ccl4*, *Ccl5* and *Cxcl10* were involved in “chemokine cluster (I) and (II)”. This inflammatory framework suggests considerable astrocyte immune modulation following BCG infection. Interestingly, *Il1b*, *Ccl4*, *Cxcl10*, *Irf7*, *Ccl5*, *Nfkbia*, and *Ddx58* were all linked to “innate activation by cytosolic DNA sensing”; *Tnf*, *Cxcl10*, *Irf7*, *Nfkbia*, *Ddx58* and *Ifih1* were connected to “RIG-like receptor signalling”; and *Il1b*, *Rsad2*, *Cxcl10*, *Irf7*, *Ddx58* and *Ifih1* were associated with “antiviral interferon signature”. This is a significant finding as all three modules are directly involved in IFN responses, indicating immune functionality via IFN-related activity. A prominent gene contributing to these modules as well as four other modules on the chord diagram was *Irf7*. Given the compelling evidence of an IFN response, the involvement of *Irf7* is not surprising as it encodes interferon regulatory factor 7, an activator of type I IFN genes

(Sin et al., 2020, Honda et al., 2005, Marié et al., 1998). Of particular interest were *Cxcl10* and *Il1b* for their participation in six modules on the circos plot including those related to IFN and inflammatory responses, suggesting an appreciable influence of these factors on astrocyte immune activity during BCG infection.

The chord diagram therefore presents an elegant display of gene and module interactions with multiple genes intersecting and participating in separate modules. Induction of multiple co-regulatory mechanisms further highlights the close regulation of genes and pathways involved in immune mediation. The modules presented in this section play a principal role in immune regulation through signalling, cellular recruitment, and activation. The relevance of module relationships as well as how the genes within these modules act to modify and amplify responses require further investigation. Detailed evaluations of these genes and their functions are subsequently discussed.

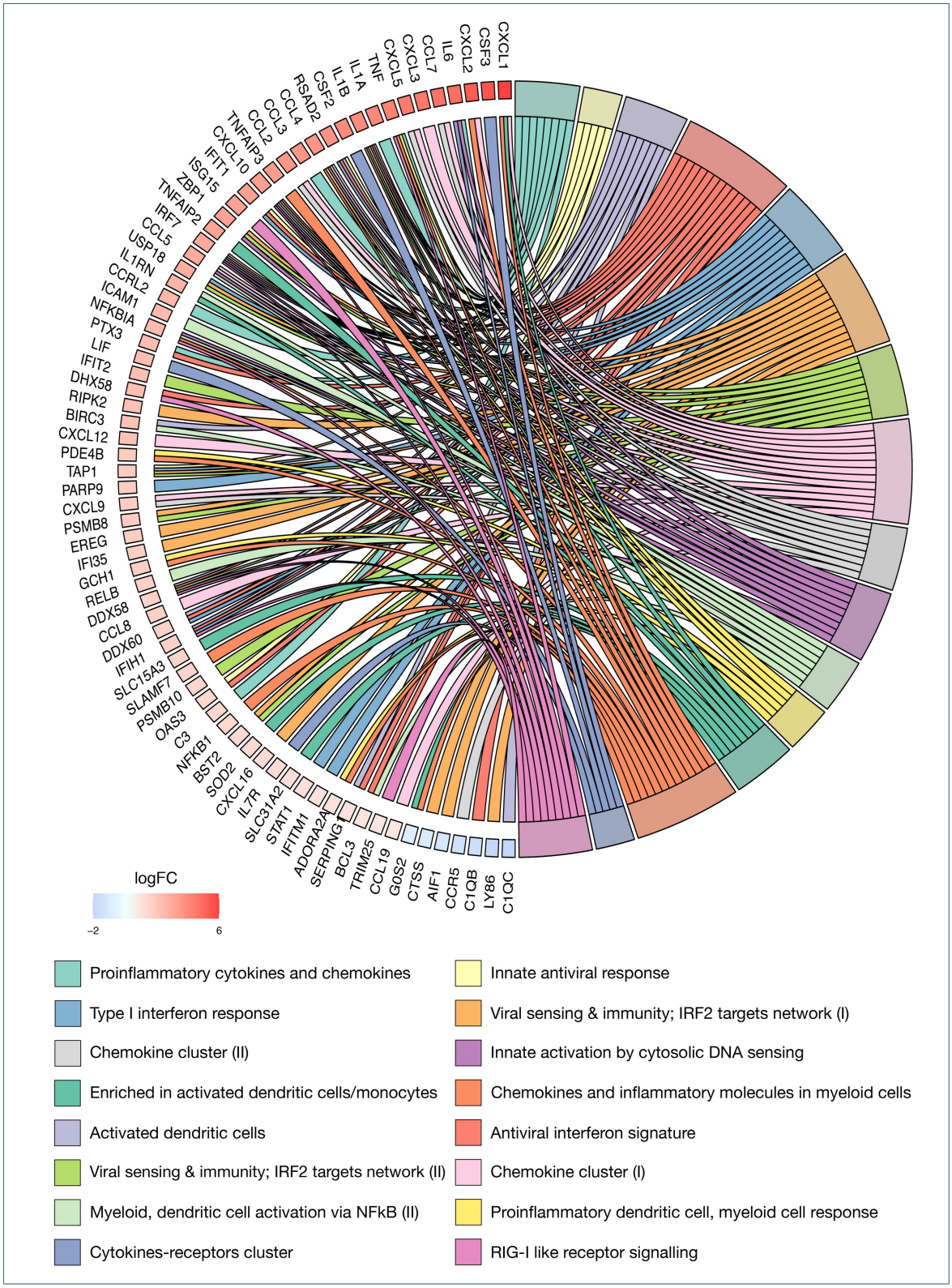


Figure 3.5 | Enrichment circos plot of selected modules. Circos plot representation of significantly enriched modules linked to associated differentially expressed gene transcripts in BCG infected astrocytes (AUC>0.7, adj. p<0.001). Each module is denoted by a unique colour band and the connection inside the circle represents an association of a particular gene to the respective modules. Red and blue outer squares represent gene up- and down-regulation, respectively, and opacity relates to logFC.

The comprehensive analysis of global astrocyte responses to BCG reflected overall changes in gene expression but characterising the complex molecular changes of these cells required considerable scrutiny. For a more thorough gene profile assessment, five modules were selected and the DEGs contributing to the enrichment of these modules was investigated. The most significantly enriched module was chosen as well as four modules with high effect size. The log2FC of DEGs were plotted in a side-by-side graph to analyse these genes within the context of their respective modules (Fig. 3.6A). The five modules of interest are denoted by a specific colour on the graph, gene IDs are displayed on the y-axis and the log2FC as a measure of gene expression is shown on the x-axis. Box and whisker plots of the significant DEGs (adj. $p < 0.05$) within each module were also generated to evaluate log2expression of uninfected (NI) and BCG-infected cells (Fig. 3.6B-F). Box plot graphs provide a clear and concise visual representation of the data by displaying several summary statistics, including the median, quartiles, and outliers, which can help to identify any patterns or trends in the data.

The most significantly enriched module in the BCG infected astrocytes, with an adj. p value of 5.4×10^{-14} and an effect size of 0.79 was the "chemokine cluster (I)" (LI.M27) module. Chemokines are chemotactic cytokines or secreted proteins that are released by a variety of cell types to direct cell migration and to facilitate inflammatory responses and immunity during infection and injury (Oppenheim et al., 1991). These small peptides (8- to- 10-kd) are classified into four subfamilies on the basis of the relative position of the conserved cysteine residues near the N-terminus of the mature protein (CXC, CC, C, and CX3C) (Domanska et al., 2011, Charo and Ransohoff, 2006). Apart from their immune-modulatory activity, studies have shown that chemokines drive neuron and neural crest migration during embryonic migration (Mayor and Theveneau, 2013, Lewellis and Knaut, 2012), they mediate vascular patterning and remodelling (Bussmann et al., 2011, Cha et al., 2012, Siekmann et al., 2009) and they are actively involved in maintaining homeostasis under basal conditions (Moser and Loetscher, 2001). Their activity in the nervous system is however not restricted to neurodevelopment as they also participate in neuroinflammation and synaptic transmission (Ramesh et al., 2013). Chemokine transcripts *Ccl2*, *Ccl4*, *Ccl5*, *Ccl7*, *Ccl8* and *Ccl19*, all showed significant (adj. $p < 0.05$) increases in expression during BCG infection (Fig. 3.6A and B). Similarly,

Cxcl1, *Cxcl2*, *Cxcl3*, *Cxcl5*, *Cxcl9*, *Cxcl10*, *Cxcl11*, and *Cxcl12* showed significant (adj. $p < 0.05$) elevations in expression whereas *Cxcl13* was significantly down regulated. One of the features of innate immunity is the rapid recruitment of immune cells to the site of infection through the release of chemokines. Responses to chemokines are mediated by a cell's expression of seven-transmembrane–domain G-protein–coupled chemokine receptors (Bonecchi et al., 2009, Premack and Schall, 1996) which are expressed on different cell types, particularly leukocytes. Some chemokine receptors are restricted to specific cells or tissues, whereas others are expressed ubiquitously (Proudfoot, 2002, Roy et al., 2014, Charo and Ransohoff, 2006, Luster, 1998). Moreover, while several of these receptors are constitutively expressed on certain cells, others are inducible and restricted to cellular activation (Proudfoot, 2002, Roy et al., 2014, Charo and Ransohoff, 2006, Luster, 1998). Chemokine receptors are remarkably redundant and able to bind to different chemokines therefore allowing a high level of regulation. The binding of chemokines to their cognate receptors elicits a series of signalling events leading to the modulation of cellular activities. Directed chemotaxis is notably associated with hematopoietic cells, but neurons, astrocytes and epithelial cells also express chemokine receptors suggesting multiple functionalities of the chemokine system (Zhang et al., 2017, De Haas et al., 2007, Choi et al., 2014, Bajetto et al., 2001, Horuk et al., 1997, Dorf et al., 2000, Flynn et al., 2003, Dwinell et al., 1999). Enrichment of this chemokine module suggests that astrocytes regulate cellular recruitment to the CNS during BCG infection through the release of chemotactic factors. Leukocyte migration to infected tissue is crucial for inflammation, emphasising the impact of chemokines on resolving infections.

Three key parameters determining the outcome of chemokine and cytokine secretion are the local concentration of these proteins, their combination with other cytokines and the stage of disease. These factors influence biological function by directing cell differentiation, migration, and polarisation. Cytokine regulation of cellular activities and inflammation is a complex process with many functioning as both proinflammatory and anti-inflammatory mediators. The "proinflammatory cytokines and chemokines" module (LI.M29) was the most enriched module with an effect size of 0.99 and an adj. p value of 4.13×10^{-11}. The data demonstrated a significant increase in *Il1a*, *Il1b*, *Tnf* and *Il6*

expression in response to BCG (Fig. 3.6A and C). This is consistent with previous studies that have showed a heightened transcription and secretion of ILβ, TNF and IL-6 in pathogen-stimulated monocytes from BCG vaccinated individuals (Kleinnijenhuis et al., 2012, Kong et al., 2021, Cirovic et al., 2020). There was an increase in *Ccl3*, an effective chemokine for lymphocytes and monocytes, as well as the chemokine receptor *Ccrl2* which regulates leukocyte recruitment and controls inflammation (Mantovani et al., 2006, Del Prete et al., 2013). Interestingly, Kong et al. observed a close association between *Ccl3* expression and *Tnf* as well as *Il1b* expression in monocytes after BCG vaccination (Kong et al., 2021). However, caution needs to be exercised when comparing transcriptional responses between different cell types and species as experimental models directly influence expression patterns. Further examination of the data revealed an amplification in *C3* expression, a central element of the complement system, as well as in *Ptx3* encoding the acute phase pentraxin protein (also known as TNF-inducible gene 14 protein). PTX3 is a key component of innate immunity expressed during infection and inflammation, and is released by numerous cell types including immune cells (Deban et al., 2011). Although a large portion of its role in innate immunity is owed to participation in complement cascade activation and amplification (Baruah et al., 2006, Garlanda et al., 2005), it is also able to limit excessive complement activation by binding to complement regulators (Braunschweig and Józsi, 2011). An additional feature of PTX3 activity is its ability to downregulate neutrophil transmigration in the brain during neuroinflammation (Rajkovic et al., 2019, Deban et al., 2010) and in this way could inhibit extreme inflammation which may be deleterious to the host. This module demonstrates the ability of astrocytes to induce a proinflammatory response concurrently with genes that specifically inhibit inflammation, indicating an astrocyte mediated regulatory process to limit potential tissue damage following BCG infection.

One of the modules strongly enriched in the BCG-infected primary astrocytes *in vitro*, was “innate activation by cytosolic DNA sensing” (LI.M13). A proinflammatory response was demonstrated within this module by the upregulation of chemokine genes *Ccl4*, *Ccl5*, and *Cxcl10* as well as the cytokine genes *Il6* and *Il1b* (Fig. 3.6A and D). *Pycard* and *Aim* expressions were however significantly reduced which was unexpected considering their roles in inflammasome assembly (Martinon et al., 2002, Borrego et al., 2022, Lugrin

and Martinon, 2018). The gene encoding Z-DNA binding protein 1 (*Zbp1*), which not only facilitates assembly and activation of the NLRP3 inflammasome (Zheng and Kanneganti, 2020) but is also a regulator of the cytosolic DNA-sensing system (Jiao et al., 2020), was shown to have a significant increase in expression. Interestingly, potential negative regulation of the inflammatory response was displayed through the elevation of *Nfkbia*, encoding an NFKB inhibitor downstream from ZBP1. Also located downstream from ZBP1 was the transcription factor, *Irf7*, which showed a significant increase in expression. IRF7 is produced in response to cytosolic viral DNA and RNA in host cells and acts as a regulator of type I IFN production (Mcnab et al., 2015). *Ddx58*, also mediates IFN production and had a significant logFC increase in the BCG-infected astrocytes compared to uninfected samples. In addition, the cytosolic PRR, RIG-1 (retinoic acid-inducible gene I), is encoded by the DDX58 gene. It was therefore unsurprising to observe a strong induction of the "RIG-1 like receptor signalling" module (LI.M68) with an overall effect size of 0.98 and an adj. p value of 8.43×10^{-9}. This is an interesting finding as cytoplasmic RIG-1 like receptors (RLRs) not only induce ISGs via IRF3 or IRF7 (Fensterl and Sen, 2015, Ikushima et al., 2013) but they are reported to be involved in mediating macrophage responses to *M. bovis* (Magee et al., 2012). In addition to *Ddx58*, the LI.M68 module showed increased transcript expression of RIG-1 regulators *Dhx58* and *Trim25* (Fig. 3.6A and E). Moreover, several genes typically induced by interferons, including *Ifih1* (interferon induced with helicase C domain 1) encoding melanoma differentiation associated factor 5 (MDA5) and *Isg15* (interferon-stimulated gene product 15), were upregulated. ISG15 is crucial for the control of *M. bovis* BCG (Bogunovic et al., 2012), while MDA5 is another RIG-1-like receptor that triggers interferon production. Notably, both of these genes play a key role in type I IFN signalling (Kim et al., 2008). Previously discussed genes also appeared in this module, notably *Tnf*, *Irf7* and *Cxcl10* as well as Nfkbia.

Since type I IFN production is the main outcome of cytosolic DNA sensors and RIG-1 receptor activation (Cavlar et al., 2012, Barber, 2011, Yoneyama et al., 2008), enrichment of the "type I IFN response" (LI.M127) module was probable. As expected, this module was one of the top four enriched modules with an effect size of 0,95, an adj. p value of 2×10^{-7}, and contained both unique genes and shared genes found in previously

analysed modules. All of the genes enriched in this module are typically induced by IFNs, particularly *Irf7*, *Tap1* (transporter associated with antigen processing-1), *Ifih1*, *Ifitm1* (Interferon-induced transmembrane protein 1), *Ifit1* (Interferon-induced protein with tetratricopeptide repeats 1), *Stat1* (Signal transducer and activator of transcription 1), *Plscr1* (Phospholipid Scramblase 1), *Usp18* (Ubiquitin specific peptidase 18), *Rsad2* (Radical S-adenosyl methionine domain-containing protein 2), *Ddx60* (DExD/H-Box Helicase 60) and *Parp9* (poly(ADP-ribose) polymerase 9) (Fig. 3.6A and F). The functional transcription factor STAT1 plays a major role in immunity (Ramana et al., 2000) and is necessary for the activation of ISGs and the induction of IRF1 and IRF7 (Remoli et al., 2002). IRF7 not only regulates the IFN-inducible protein IFIH1, but it is also the central transcription factor and principal regulator of type I IFN immunity (Honda et al., 2005). IFIH1 (Kato et al., 2006), as well as the other IFN-inducible proteins IFITM1 (Liao et al., 2019, Zhao et al., 2019), IFIT1 (Fensterl and Sen, 2015, Pichlmair et al., 2011), RSAD2 (Seo et al., 2011), PLSCR1 (Dong et al., 2004) and PARP9 (Xing 2021) all play roles in cellular antiviral states by amplifying and enhancing IFN responses. Interestingly, PARP9 is also a positive regulator of pro-inflammatory cytokine production. Of note was the enhanced expression of *Ddx60* an antiviral helicase that acts as a positive regulator of RLR signalling pathways (Miyashita et al., 2011) as well as *Usp18*, a negative regulator of IFN activity (Shaabani et al., 2018, Malakhova et al., 2006, Taft and Bogunovic, 2018, Martin-Fernandez et al., 2022). This simultaneous induction of positive and negative regulators alludes to tight regulation of the IFN pathways. It is widely accepted that transcriptional regulation of ISGs through binding of IFN to their receptors results in the biological activities associated with IFNs. The last enriched member in this module was *Tap1*, encoding a transport protein involved in transporting antigen across the endoplasmic reticulum and facilitating peptide loading onto major histocompatibility complex (MHC) class I molecules (Grandea III et al., 1995, Suh et al., 1994). These results suggest that BCG activates an innate response in astrocytes involving cytosolic DNA and RNA sensing pathways which then drive IFN-mediated responses. IFN immunity is known for its resistance to viral pathogens (Isaacs et al., 1957, Müller et al., 1994) but is similarly important for host immunity against bacteria (Du et al., 2013, Mancuso et al., 2007, Desvignes et al., 2012, Wang et al., 2019). Impaired type I IFN signalling has been shown to compromise host resistance to bacterial pathogens (Mancuso et al., 2007,

Berry et al., 2010, Bouchonnet et al., 2002, Manca et al., 2001, Ordway et al., 2007) which may be due to the associated defective production of IFN-γ, NO, and TNF (Mancuso et al., 2007). In contrast to this, studies investigating *M. tuberculosis* have shown that increased induction of type I IFNs have a detrimental effect on pulmonary TB outcome (Bouchonnet et al., 2002, Manca et al., 2001, Ordway et al., 2007) which was attributed to suppressed TNF and IL-12 production; and reduced T cell activation (Manca et al., 2005). In the CNS, type I IFN's regulate innate immunity in response to infection and injury (Khorooshi and Owens, 2010). Resident CNS cells respond to IFN-α/β stimulation (Delhaye et al., 2006, Khorooshi and Owens, 2010) with astrocytes in particular being a major source of type I IFNs (Tedeschi et al., 1986, Detje et al., 2015) and consequently contribute to immunoprotective and immunopathological responses in the CNS.

In this study, a strong type I IFN response was observed in BCG-infected astrocytes indicating a principal role in astrocyte immune activity during infection. Overall, the transcriptomic data supports that astrocytes are able elicit an immune response. as demonstrated by the induction of several immune related gene modules following BCG challenge. This may result in the release of cytokines and chemokines from astrocytes to drive inflammation, leukocyte recruitment and cell activation. Moreover, several genes demonstrated co-expression across modules suggesting complex co-regulation during host immune responses.

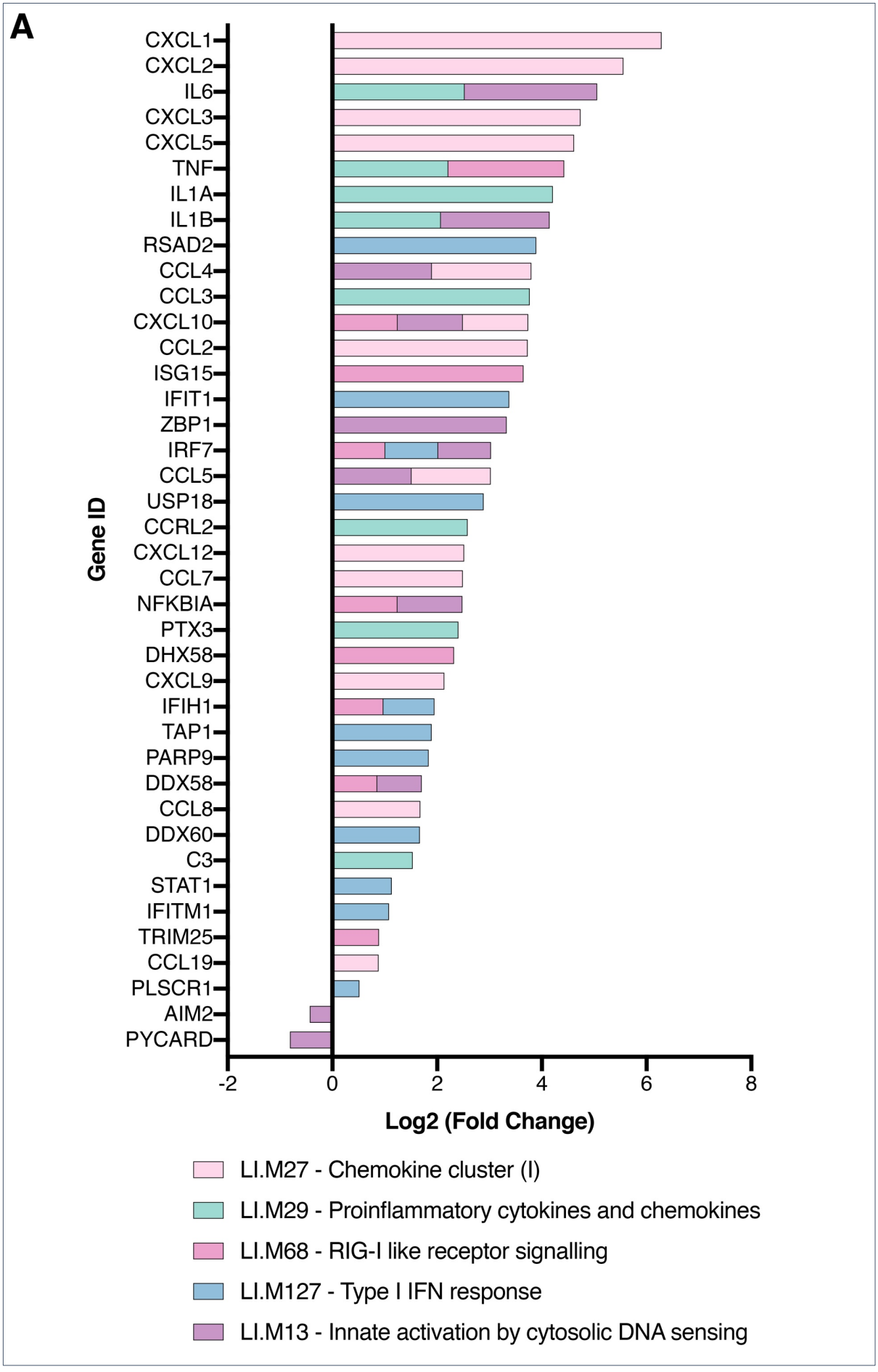
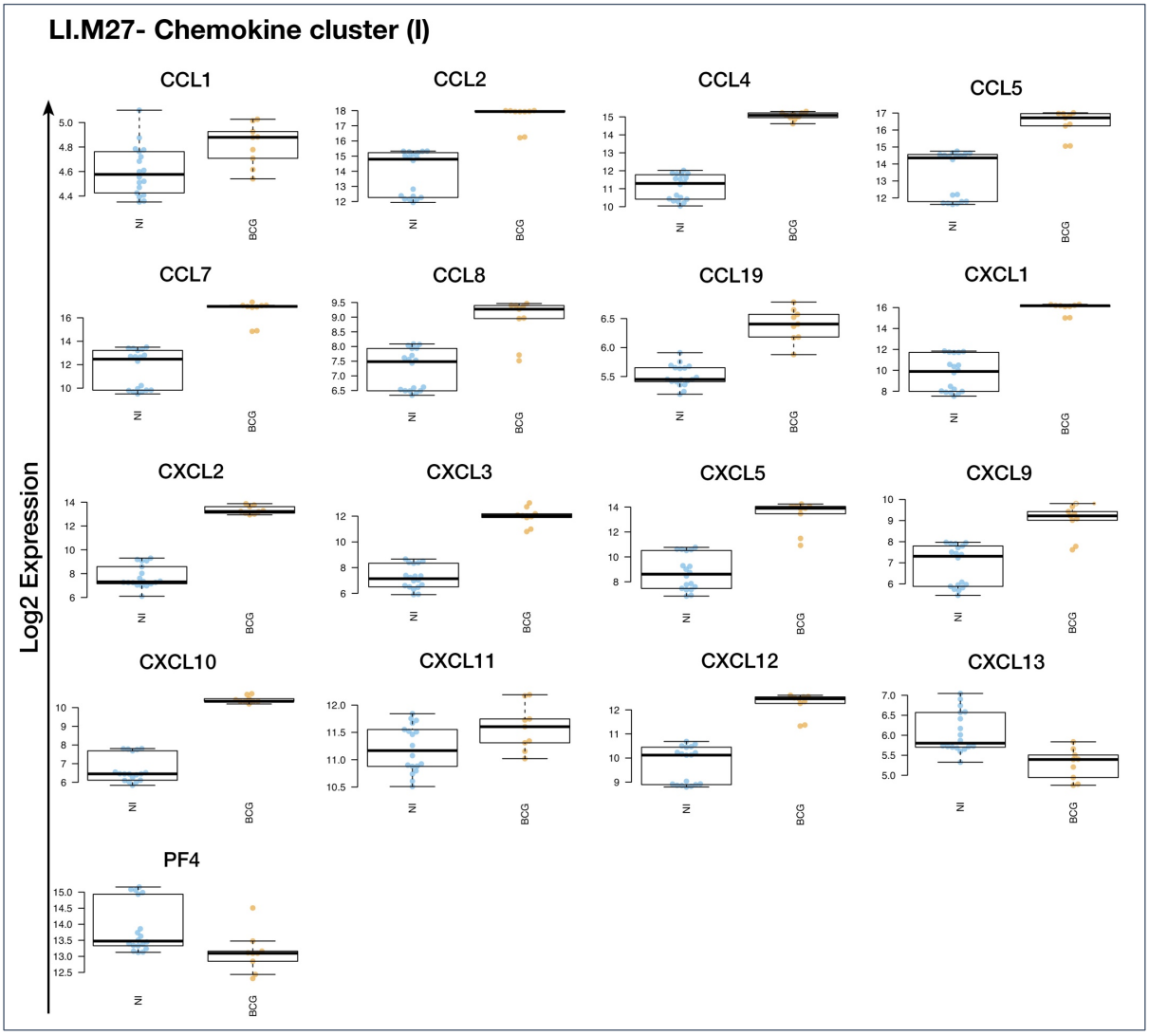


Figure 3.6 | Continued

B



C

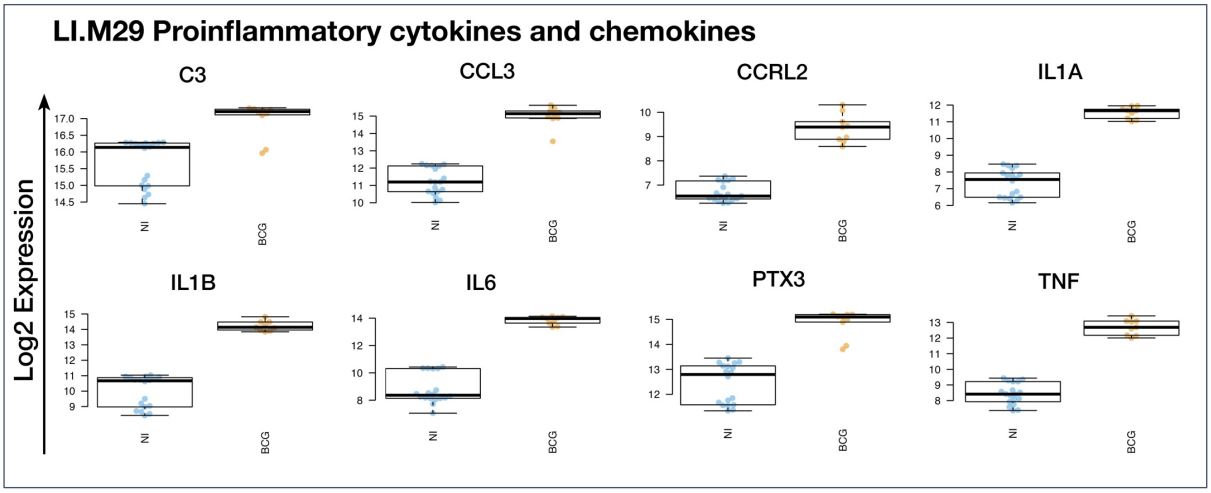
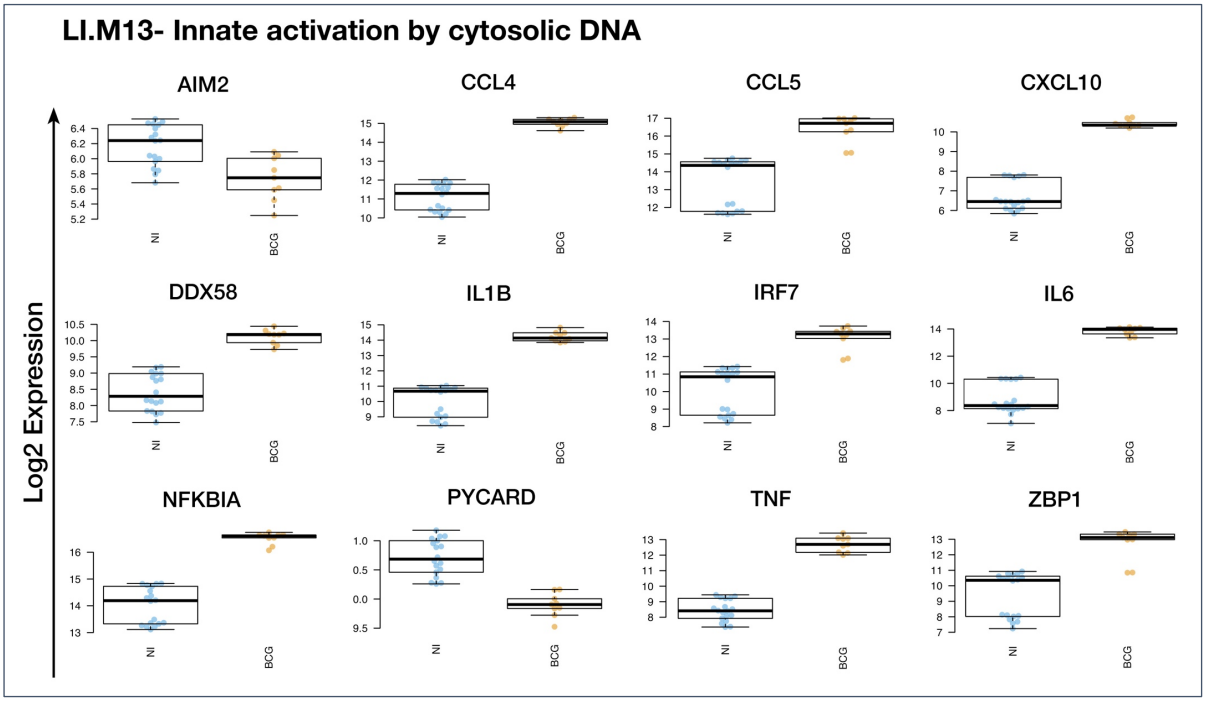


Figure 3.6 | Continued

D



E

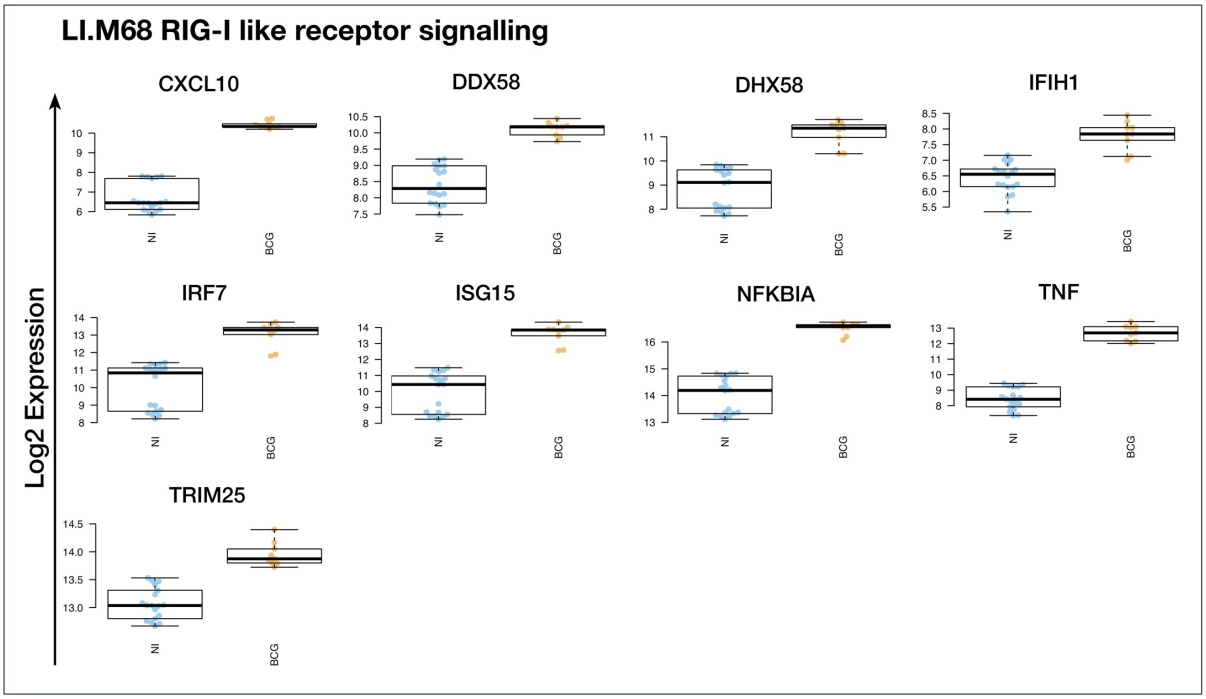


Figure 3.6 | Continued

F

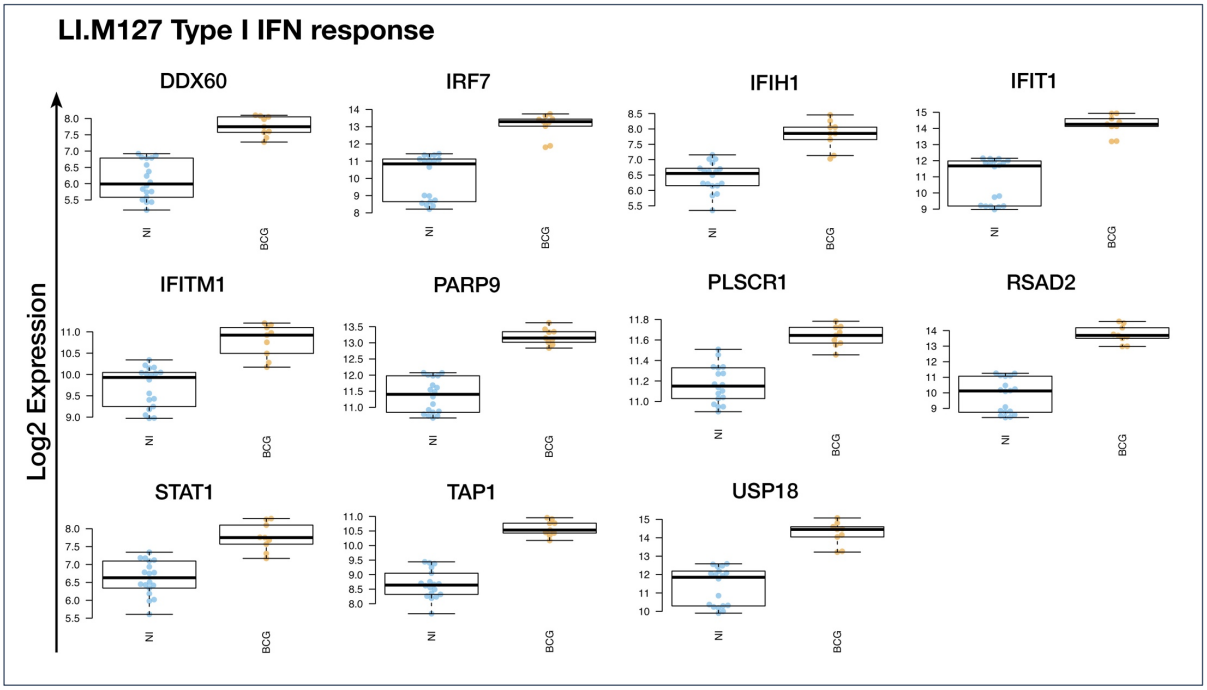


Figure 3.6 | Graph indicating Log2 fold changes of DEGs in BCG infected primary astrocytes and the modules with which they associate. (A) Five modules were selected, namely: LI.M27.0 "Chemokine cluster (I)" (Total module effect size 0.79 and adj. p value 5.4 x 10^{-14}), LI.M29 "Proinflammatory cytokines and chemokines" (Total module effect size 0.99 and adj. p value 4.13 x 10^{-11}), LI.M68 "RIG-1 like receptor signalling" (Total module effect size 0.97 and adj. p value 8.43 x 10^{-9}), LI.M127 "Type I IFN response" (Total module effect size 0.95 and adj. p value 2 x 10^{-7}), and LI.M13- "Innate activation by cytosolic DNA sensing" (Total module effect size 0.94 and adj. p value 6.87 x 10^{-11}). Positive and negative values signify gene up- and down-regulation, respectively. Modules are denoted by a colour code; gene IDs are displayed on the y-axis, and the Log2FC as a measure of gene expression is shown on the x-axis. Box plots of significant DEGs ($p < 0.05$) were constructed for modules: (B) "Chemokine cluster (I)", (C) "Proinflammatory cytokines and chemokines", (D) "RIG-1 like receptor signalling", (E) "Innate activation by cytosolic DNA sensing", and (F) "Type I IFN response". Box plots exhibit Log2 expression of BCG-infected and uninfected (NI) astrocytes.

3.4 Astrocytes enhance inflammatory factor transcripts in response to BCG

During infection or tissue injury, inflammatory responses are initiated to eliminate pathogens and to promote tissue repair (Netea et al., 2017, Kotas and Medzhitov, 2015). Such responses are characterized by the activation of both immune and non-immune cells resulting in immune function coordination through chemical signalling cascades. Inflammation is a critical process by which immune cells are mobilized to affected sites to restore homeostasis. It is a tightly co-ordinated process which usually resolves once the threat is removed, however, dysregulated or persistent acute inflammation can

progress to chronic inflammatory disease, causing further tissue damage (Kotas and Medzhitov, 2015, Chen et al., 2018, Fullerton and Gilroy, 2016). Inflammation in the brain can induce cytotoxicity, amplify neuronal excitability and enhance BBB permeability (Nelson et al., 2002, Vezzani and Granata, 2005). Although neuronal dysfunctions of some disorders may be attributed to uncontrolled inflammatory responses, multiple studies have accentuated the dichotomous role of inflammation in the CNS whereby certain mediators can be beneficial (Nguyen et al., 2002, Stoll et al., 2000). The main contributors of inflammatory mediators in the brain are microglia, astrocytes, and neurons, as well as cells of the choroid plexus and BBB. Moreover, astrocytes, neurons and microglia express chemokine receptors and can react to chemokines in their milieu.

The module enrichment data indicated a strong inflammatory response in astrocytes during BCG infection. Although box plot graphs are a useful tool for providing a quick and informative summary of the distribution and range of data, they do not provide the same level of precision and statistical analysis as exact logFC and adjusted p values. To gain a more comprehensive understanding of the specific genes upregulated in astrocytes during BCG infection, differentially expressed chemokines, cytokines, and neurotrophic factors were investigated. Precise numerical values of these genes were extracted from the data and tables containing the details of the logFC and adjusted p values were constructed. A notable increase in the expression levels of multiple cytokine and chemokine genes were identified (Tables 3-1 and 3-2). Chemokines released during inflammatory responses are associated with the mobilisation of immune cells through chemotaxis and often contribute to the pathogenesis of neuroinflammatory disease (Asensio and Campbell, 1999). *Cxcl1* had the highest upregulated fold change in gene expression with a logFC of 6.288, while *Cxcl2*, *Ccl7*, *Cxcl3*, and *Cxcl5* were ranked in the top 10 DEGs with a logFC of 5.55, 4.97, 4.74, and 4.61 respectively (Table 3-1). Under basal conditions production of CXCL1 is negligible but is enhanced during infection. This chemokine acts as a neutrophil chemoattractant and functions in microbial elimination by activating tissue release of protease and reactive oxygen species (ROS) (De Filippo et al., 2013, Jin et al., 2014, Ritzman et al., 2010). CXCL2, CXCL3 and CXCL5 are similarly known for their neutrophil recruiting activity (Sadik et al., 2011, Rouault et al., 2013, Olson and Ley, 2002, Jeyaseelan et al., 2005, Mei et al., 2010) and along with

CXCL1 are ligands for CXCR2, a G protein-coupled receptor primarily expressed by neutrophils (Gershengorn et al., 1998, Gouwy et al., 2014). The use of anti-CXCL5 during pulmonary inflammation of mice attenuated neutrophil accumulation, illustrating its contribution to neutrophil recruitment (Jeyaseelan et al., 2005).

In contrast to these CXC chemokines, CXCL10 is a T lymphocyte specific chemoattractant (Taub et al., 1993b). Primary murine astrocytes also indicated *Cxcl10* induction with a 3.7logFC increase in BCG infected cells demonstrating activity comparable to innate immune cells as CXCL10 is reportedly produced by human monocytes when infected with BCG (Samperio et al., 2004). Closely related to CXCL10 is CXCL9 (also known as Monokine induced by IFN-γ or MIG) which shares the ability to signal though CXC chemokine receptor 3 (CXCR3) present on T cells and NK cells (Egesten et al., 2007). Thus the 2.1logFC increase in *Cxcl9* further suggests that BCG uptake by astrocytes may induce recruitment of lymphocytes to infection sites. Interestingly, both CXCL9 and CXCL10 exert antimicrobial activity *in vitro* (Cole et al., 2001, Yang et al., 2003, Egesten et al., 2007) and may act as first line of defence against pathogens. In addition to CXCL9 and CXCL10, CXCL12 also controls the trafficking of T cells (Loetscher et al., 1999) and the data indicated a 2.5logFC increase in *Cxcl12* in astrocytes following BCG infection. CXC chemokines are classically associated with the immune system but some of these chemokines, such as CXCL12, have pleiotropic activity. CXCL12 is a homeostatic chemokine constitutively expressed by astrocytes under normal physiological conditions and is essential for neural migration during cerebellar development (Huising et al., 2003, Ma et al., 1998, Lewellis and Knaut, 2012). It is actively involved in vascular patterning (Cha et al., 2012), angiogenesis (Siekmann et al., 2009) and acts as an attractant for microglia as well as a stimulant for Purkinje cells in the cerebellum (Huising et al., 2003). Although chemokines have a plethora of activities that extend beyond immune function, most CXC chemokines are involved in neutrophil recruitment and activation, and some in T cell and NK activation (Zlotnik and Yoshie, 2000, Méndez-Samperio, 2008). Conversely, the CC chemokines generally mobilise monocytes, eosinophils, basophils, and T cells to the affected tissue. Astrocyte induction of *Ccl2*, *Ccl3*, *Ccl4*, *Ccl5*, *Ccl7*, *Ccl8* and *Ccl17* was observed with logFC changes of 3.7, 3.8, 3.8, 3.0, 5.0, 1.7 and 2.2 respectively (Table 3-1). It is well documented that CC

chemokines are crucial to host immune responses in tuberculosis. Previous studies on human monocytes have shown their ability to produce CCL2, CCL3, CCL4 and CCL5 during BCG infection (Méndez-Samperio et al., 2010, Mendez-Samperio et al., 2003) whereas dendritic cells increased production of CCL2 and CCL5 as well as the cytokines IL-12, IL-6 and TNF (Zhang et al., 2013). Enhanced chemokine expression therefore suggests astrocytes can recruit leukocytes to the brain during BCG infection thus influencing both innate and adaptive immunity. Others have reported that chemokines play a role in BBB permeability which would allow effector cells to cross the BBB and enter the brain parenchyma following cellular recruitment (Glabinski and Ransohoff, 1999, Man et al., 2007, Zozulya et al., 2007, Kuang et al., 2009). Elevated expression of CXCL9, CXCL10 and CCL5 in the CNS was shown to correlate with leukocyte influx in multiple sclerosis (Sørensen et al., 1999). Mice infected with mouse hepatitis virus showed a considerable decrease of infiltrating T lymphocytes following treatment with rabbit antisera for CXCL9 or CXCL10 (Liu et al., 2001). CXCL10 plays a prominent role in T cell recruitment and BBB integrity. Mice deficient of the CXCL10 receptor, CXCR3, displayed reduced T cell trafficking to the CNS during Dengue virus (Hsieh et al., 2006) and West Nile virus infection (Zhang et al., 2008). A separate study on mice infected with attenuated rabies virus displayed an upregulation of CXCL10, and to a lesser extent CCL3, which corresponded with enhanced BBB permeability (Kuang et al., 2009). CCL3 also modulates T lymphocyte and dendritic cell transmigration across brain endothelial cells in Alzheimer's disease and multiple sclerosis, respectively (Zozulya et al., 2007, Man et al., 2007). Apparently CCL3 is unable to cross the BBB, but may indirectly influence transmigration of these cells by binding to BBB endothelia (Banks and Kastin, 1996). This is consistent with another report that demonstrated interaction of CCL3 and its CCR5 receptor stimulated endothelial tight junction opening in the BBB (Man et al., 2007). In a co-culture system, astrocyte-derived CCL2 was demonstrated to mediate monocyte and lymphocyte transmigration across a BBB model (Weiss et al., 1998). Therefore, given the importance of astrocytes to BBB functionality, chemokine induction due to BCG infection may compromise BBB integrity and facilitate cell transmigration.

In addition to chemokines, cytokines reportedly contribute to BBB permeability and leukocyte entry into the CNS. Most notably is IL-1β which acts directly on endothelial

cells through tight junction protein modifications (Blamire et al., 2000, Laflamme et al., 1999, Wang et al., 2014) or indirectly through the induction of vascular endothelial growth factor (VEGF) from astrocytes (Argaw et al., 2006). Increased expression of *Il1b* was observed with a 4.15logFC providing further evidence of astrocyte involvement of BBB disruption during BCG infection (Table 3-2). This cytokine is also established for its activity in T cell recruitment. A significant upregulation of other genes encoding various Th1-type cytokines was evident, particularly *Tnf*, *Il1a,* and *Il12b* with logFCs of 4.43, 4.21, and 1.80, respectively (Table 3-2). Interestingly *Il6*, encoding the pleiotropic cytokine was the most differentially expressed cytokine with a 5.06logFC increase. A regulator of inflammation was also induced namely, *Il19*, which had a 3.69logFC increase in expression. *Ifnb1* also showed a notable 3.65logFC increase.

Cytokines and chemokines are involved in regulating homeostasis of the nervous system under physiological conditions (Becher et al., 2017). During pathogenic challenges they act to restore homeostasis by eliminating pathogens through mediating the recruitment of circulating leukocytes and assisting cellular transmigration across the BBB via tight junction disruption (Rochfort and Cummins, 2015, Librizzi et al., 2012, Tsao et al., 2001, Annunziata et al., 2002, Roberto et al., 2017). Amplified transcript expression of these mediators in BCG-infected astrocytes therefore demonstrates the chemotactic potential of these cells and their ability to mediate the transmigratory processes of peripheral leukocytes across the BBB and into the brain parenchyma during infection. To discern whether the amplified cytokine and chemokine expression observed were biologically relevant, its corresponding protein concentrations had to be measured.

Table 3-1 | Chemokines induced by astrocytes during BCG infection

Gene Symbol	Gene Title	LogFC	Adj. P Val	Biological Function/Mechanism	Reference
Ccl2 (Mcp1)	Chemokine (C-C motif) ligand 2	3.7	7.4e-08	Chemotactic activity for monocytes and basophils. T cell activation, migration and cytokine production	(Deshmane et al., 2009, Proost et al., 1996, Méndez-Samperio, 2008)
Ccl3 (Mip1a)	Chemokine (C-C motif) ligand 3	3.8	1.2e-12	Chemotactic for neutrophils and inflammatory function. T cell activation, migration and cytokine production	(Taub et al., 1993a, Schall et al., 1993, Singh et al., 2021, Méndez-Samperio, 2008)
Ccl4 (Mip1b)	Chemokine (C-C motif) ligand 4	3.8	3.5e-14	Chemotactic and inflammatory function. T cell activation and migration	(Taub et al., 1993a, Schall et al., 1993)
Ccl5 (Rantes)	Chemokine (C-C motif) ligand 5	3.0	1.2e-06	Chemoattractant for monocytes, T cells, dendritic cells and eosinophils	(Schall et al., 1990, Alam et al., 1993, Sozzani et al., 1995, Méndez-Samperio, 2008)
Ccl7 Mcp3	Chemokine (C-C motif) ligand 7	5.0	4.6e-09	Chemoattractant for monocytes, T cells, eosinophils, basophils, dendritic cells, and neutrophils	(Proost et al., 1996, Allavena et al., 1994, Noso et al., 1994, Alam et al., 1994, Sozzani et al., 1995, Menten et al., 2002)
Ccl8 Mcp2	Chemokine (C-C motif) ligand 8	1.7	2.4e-06	Chemotactic factor attracting monocytes, lymphocytes, NK cells, basophils and eosinophils	(Noso et al., 1994, Proost et al., 1996, Alam et al., 1994)
Ccl17 (Tarc)	Chemokine (C-C motif) ligand 17	2.2	2.2e-12	T cell chemoattractant. Mediates inflammation	(Imai et al., 1997, Achuthan et al., 2016)
Cxcl1 (Kc/Mgsa-a)	Chemokine (C-X-C motif) ligand 1	6.3	2.4e-11	Neutrophil chemoattractant and microbial clearance	(De Filippo et al., 2013, Jin et al., 2014, Ritzman et al., 2010, Méndez-Samperio, 2008)
Cxcl2 (Mip2a)	Chemokine (C-X-C motif) ligand 2	5.6	6.2e-15	Neutrophil chemoattractant. (Immune regulatory and inflammatory processes. Hematoregulatory)	(Sadik et al., 2011, Rouault et al., 2013)

Cxcl3 (Gro3)	Chemokine (C-X-C motif) ligand 3	4.7	3.3e-13	Role in inflammation and chemoattractant for neutrophils	(Olson and Ley, 2002)
Cxcl5 (Ena78)	Chemokine (C-X-C motif) ligand 5	4.6	3.9e-09	Neutrophil chemoattractant	(Wuyts et al., 1999, Mei et al., 2010, Jeyaseelan et al., 2005)
Cxcl9 (Mig)	Chemokine (C-X-C motif) ligand 9	2.1	3.0e-06	Chemoattractant for T cells	(Sørensen et al., 1999, Liu et al., 2001)
Cxcl10	Chemokine (C-X-C motif) ligand 10	3.7	5.3e-14	Monocyte and T cell chemoattractant. T cell adhesion	(Taub et al., 1993b, Samperio et al., 2004, Tokunaga et al., 2018)
Cxcl12 (Sdf1)	chemokine (C-X-C motif) ligand 12	2.5	1.4e-09	Homeostatic chemokine. Chemoattractant for T cells and monocytes. Vascular patterning, angiogenesis and neural migration during embryogenesis	(Loetscher et al., 1999, Huising et al., 2003, Cha et al., 2012, Siekmann et al., 2009, Lewellis and Knaut, 2012)
Cxcl16	chemokine (C-X-C motif) ligand 16	1.3	6.9e-11	T cell chemoattractant. Cell adhesion	(Matloubian et al., 2000, Shimaoka et al., 2004, Nakayama et al., 2003)
Cx3cl1	chemokine (C-X3-C motif) ligand 1	1.6	2.0e-09	Chemotactic for T cells and monocytes. Promotes leukocyte adhesion to endothelial cells. Involved in neuron-glia crosstalk. Regulates cytokines.	(Fong et al., 1998, Haskell et al., 1999, Luo et al., 2019, Laing et al., 2010, Schwarz et al., 2010)

Table 3-2 | Cytokines induced by astrocytes during BCG infection

Gene Symbol	Gene Title	LogFC	Adj. P Val	Biological Function/Mechanism	Reference
Csf2	Granulocyte macrophage colony stimulating factor 2	4.1	2.2e-13	Survival, proliferation and differentiation of myeloid cells, Controls dendritic and T cell function.	(Guthridge et al., 1998, Mellman and Steinman, 2001, Barouch et al., 2002, Hansen et al., 2008)
Il1a	Interleukin 1 alpha	4.2	1.3e-13	Immune response, inflammatory processes, transcription factor.	(Malik and Kanneganti, 2018, Di Paolo and

					Shayakhmetov, 2016, Dinarello, 2018, Werman et al., 2004)
Il1b	Interleukin 1 beta	4.1	6.5e-12	Inflammatory response mediator. Involved in cell proliferation, differentiation and apoptosis. Induces neutrophil influx and T cell activation, cytokine production.	(Lopez-Castejon and Brough, 2011, Kaneko et al., 1999, Dinarello, 2018, Dinarello and van der Meer, 2013)
Il6	Interleukin 6	5.1	2.8e-13	Leukocyte recruitment, induces chemokine expression, Th17 differentiation	(Hurst et al., 2001, Korn et al., 2009, Romano et al., 1997, Penkowa et al., 1999)
Il12b	Interleukin 12b	1.8	4.1e-09	P40 subunit of IL12 and IL23. Induces Th1 development	(Watford et al., 2004, Trinchieri, 2003)
Il15	Interleukin 15	0.8	1.4e-04	Development, maintenance and proliferation of memory CD8 T cells, NK and iNKT cells	(Stonier et al., 2008, Castillo and Schluns, 2012, Mortier et al., 2009, Mishra et al., 2014)
Il19	Interleukin 19	4.0	3.6e-09	Activates Th2 responses and limits proinflammatory mediator production.	(Liao et al., 2004, Gallagher et al., 2004)
Il21	Interleukin 21	1.4	2.3e-09	Promotes T cell and NK cell activation	(Kasaian et al., 2002, Parrish-Novak et al., 2000)
Il23a	Interleukin 23, alpha subunit p19	0.9	1.4e-02	Promotes Th17 cells	(Lim et al., 2020, McGeachy et al., 2009, McGeachy and Cua, 2007)
Il34	Interleukin 34	0.9	1.8e-05	Promotes proliferation, differentiation and survival of monocytes and macrophages. Promotes the release of proinflammatory macrophages	(Wei et al., 2010, Lin et al., 2008, Foucher et al., 2013)
Ifnb1	Interferon beta 1, fibroblast	3.6	3.1e-11	Facilitates adaptive and humoral immune response. Enhances the activation and capacity of dendritic cells to stimulate T cells. Resolves inflammation.	(Mcnab et al., 2015, Le Bon et al., 2003, Montoya et al., 2002, Kumaran Satyanarayanan et al., 2019, Stetson and Medzhitov, 2006)

Lif	Leukaemia inhibitory factor	2.3	1.1e-12	Modulates inflammation and T-cell lineage maturation.	(Metcalfe, 2011, Zhang et al., 2021, Janssens et al., 2015)
Tgfb1	Transforming growth factor, beta 1	0.4	9.6e-04	Regulates T cell proliferation, differentiation, and survival. Initiates and resolves inflammation	(Blobe et al., 2000, Gorelik and Flavell, 2002, Cerwenka and Swain, 1999)
Tnf	Tumor necrosis factor	4.4	6.2e-15	Multifunctional proinflammatory cytokine. Induces cell survival and proliferation. Modulates Tregs.	(Brenner et al., 2015, Aggarwal et al., 2012, Tracey and Cerami, 1993, Jung et al., 2019)

3.5 Astrocytes induce the expression of factors with neurotrophic potential during BCG infection *in vitro*

Astrocytes are a major source of neurotrophic factors, crucial for the maintenance, survival, development, and function of neurons in the brain. During CNS insults, astrocytes actively participate in inflammatory regulation through the production of pro- and anti-inflammatory cytokines and chemokines as well as growth factors. A prominent feature of damage and disease in the CNS is reactive astrogliosis, depicted as morphological alterations, such as an increase in cell size and branching, as well as changes in gene expression that regulate the release of various cytokines, chemokines, and growth factors (Hyvärinen et al., 2019, Meeuwsen et al., 2003, Choi et al., 2014, Aloisi et al., 1992). Compelling evidence showed that astrogliosis can modulate neural activity through the regulation of extracellular ion concentrations and neurotransmitter uptake as well as support neuron survival with neurotrophic factor release. Additionally, astrocytes can promote the formation of the blood-brain barrier and aid in tissue repair and regeneration in response to injury or disease (Faulkner, 2004, Sofroniew and Vinters, 2010, Sofroniew, 2009, Sofroniew, 2015b, Pekny and Nilsson, 2005, Kang and Hébert, 2011, Sofroniew, 2005). The dynamic behaviour of astrocytes is context dependent but the fundamental aspect of the process is not defined in mycobacterial infection, requiring investigation to adequately understand their contribution to disease progression and resolution.

Neurotrophin activity during neuroinflammation is well established (Davis and Pennypacker, 2018, Lima Giacobbo et al., 2019, Hu et al., 2020, Yin et al., 2020). Glial cell line derived neurotrophic factor (GDNF) is not only an important regulator of neuronal development, but its neuroprotective activity against inflammation-induced damage has been described following their release from activated astrocytes (Marco et al., 2002, Chen et al., 2014, Duarte Azevedo et al., 2020, Bresjanac and Antauer, 2000). GDNF can also influence endothelial cells by enhancing barrier function of the tight junctions thereby directly reducing BBB permeability (Igarashi et al., 1999). *Gdnf* as well as *Artn* (Artemin, belonging to the GDNF family of ligands) exhibited increased expression in BCG infected astrocytes in comparison to the uninfected controls with logFC increases of 1.5 and 1.1, respectively (Table 3-3). The production of certain pleiotropic chemokines and cytokines reportedly have beneficial effects in the CNS including growth, survival and differentiation (Liberto et al., 2004, Spranger et al., 1990, Lindholm et al., 1990), with some triggering the release of neurotrophic factors too (Spranger et al., 1990, Morganti-Kossman et al., 1997). Although chemokines and cytokines are mainly produced by astrocytes and microglia in the CNS, neurons do express receptors and can therefore be influenced by their presence. An increase in chemokine and cytokine gene transcripts which offer neuronal support was observed in astrocytes, notably, *Ccl2*, *Cx3cl1*, *Cxcl16*, *Il6*, *Il19*, interferon beta 1 (*Ifnb1*) and the IL-6 class cytokine-leukaemia inhibitory factor (*Lif1*) (Table 3-3). The cytokine inhibitor- suppressor of cytokine signalling 1 (*Socs1*) was also upregulated. IL-6 and transforming growth factor beta 1 (TGF-β) are pleiotropic cytokines and their production is associated with increased neuronal survival and enhanced neuron growth factor (NGF) release (Morganti-Kossman et al., 1997, Lindholm et al., 1990, Loddick et al., 1998, Hirota et al., 1996, Kumar et al., 2010, Fujita et al., 2009, Sun et al., 2017, Longhi et al., 2011). NGF is essential for the development, differentiation, and maintenance of neurons (Levi-Montalcini, 1987, Aloe and Calzà, 2003, Aloe et al., 2015). Although NGF was not enhanced in the dataset, nerve growth factor receptor (*Ngfr*) was upregulated in astrocytes suggesting that amplification sensitivity to NGF that will facilitate a greater physiological response. *In vitro* and *in vivo* studies have demonstrated that IL-1 and TNF are capable of inducing astrocyte proliferation (Barna et al., 1990, Brosnan et al., 1989, Giulian and Lachman, 1985, Giulian et al., 1988) and can also act as indirect neurotrophic factors through their stimulation of

fibroblast growth factors (FGFs) or NGF secretion (Yoshida et al., 1992, Gadient et al., 1990, Čarman-Kržan et al., 1991). Furthermore, TNF was shown to play a role as a neurotrophic factor by improving neuronal survival in an animal seizure model (Jankowsky and Patterson, 2001). Although IL-1, IL-6, TNF and TGF-β are typically involved in inflammation, they may have beneficial neuroprotective effects in the CNS during BCG infection too.

Like cytokines, growth factors are chemical messengers that bind to cell surface receptors, and through intracellular signalling cascades, regulate cell proliferation, differentiation, and survival (Landreth, 1999, Garcia et al., 2016, Oliveira et al., 2013). In addition to *Gdnf*, various other growth factors were induced, including insulin-like growth factor binding protein (*Igf2bp2*) vascular endothelial growth factors (*Vegfa* and *Vegfc*), fibroblast growth factors (*Fgf-2, -7, -9*,), neuregulin 1 (*Nrg1*), and platelet derived growth factor B (*Pdgfb*). Interestingly, FGF2 and FGF9 are glia activating factors (Kang et al., 2014, Kanda et al., 2000). Regulation of growth factors in astrocytes are necessary to prevent a chronic state of activation, thus it was unsurprising that certain growth factors exhibited a decrease in expression, specifically, *Fgf-1* and *Igf2bp3*.

This data suggests that in response to BCG infection, astrocytes upregulate the gene expression of various factors which have neuroprotective and neurorestorative functions. Many of the factors secreted by astrocytes, including IL-6, LIF, TGF-β and IL-1, can behave in an autocrine manner directly influencing the proliferation, migration and adhesion of cells (Merrill and Benveniste, 1996) or in a paracrine manner affect nearby neurons (Srinivasan et al., 2004, Huang et al., 2011, Park et al., 2018, Oh et al., 2010, Loddick et al., 1998, Hirota et al., 1996, Li et al., 2017, Cekanaviciute et al., 2014, Linnerbauer and Rothhammer, 2020). Although the functional role of these cytokines during injury or infection are still controversial, over expression may be deleterious while low levels of production may be beneficial to the host. The increased expression of neuroprotective factors also suggests astrocyte-mediated neuronal recovery but whether this activity is sufficient to prevent inflammation-induced neurotoxicity is unlikely.

Table 3-3 | Differentially expressed factors with neurotrophic potential induced in astrocytes during BCG infection

Gene Symbol	Gene Title	LogFC	Adj. P Val	Biological Function/Mechanism	Reference
Artn	Artemin	1.1	5.5e-10	Glial cell line derived neurotrophic factor. Survival, differentiation, and maintenance of neurons	(Wong et al., 2015, Airaksinen and Saarma, 2002, Honma et al., 2002, Baloh et al., 1998, Zihlmann et al., 2005, Zhu et al., 2020, Ilieva et al., 2019)
Ccl2 (Mcp1)	Chemokine (C-C motif) ligand 2	3.7	7.4e-08	Neurological repair and survival. Inhibits glutamate-induced neuronal apoptosis.	(Andres et al., 2011, Réaux-Le Goazigo et al., 2013, Fang et al., 2018, Eugenin et al., 2003)
Cx3cl1	Chemokine (C-X3-C motif) ligand 1 (Fractalkine)	1.6	2.0e-09	Neuroprotection. Promotes neuron survival and reduces excitotoxicity.	(Deiva et al., 2004, Limatola et al., 2005, Meucci et al., 1998, Tong et al., 2000)
Cxcl16	Chemokine (C-C motif) ligand 16	1.3	6.9e-11	Neuromodulation. Acts on astrocytes to release neuroprotective soluble factors. Neuroprotective against glutamate-excitotoxicity.	(Rosito et al., 2012, Trettel et al., 2020, Rosito et al., 2014, Di Castro et al., 2016)
Fgf1	Fibroblast growth factor 1	-1.1	1.4e-07	Cell proliferation and differentiation. Neuroprotection	(Russell et al., 2006, Everall et al., 2001, Ghazavi et al., 2017, Boilly et al., 2000)
Fgf2	Fibroblast growth factor 2	0.6	1.5e-04	Glia activating factor. Involved in neurogenesis, axonal growth, neuroprotection and regeneration.	(Kang et al., 2014, Noda et al., 2014, Belluardo et al., 2000, Jin et al., 2005, Li et al., 2010)
Fgf7	Fibroblast growth factor 7	0.7	6.0e-06	Neuron survival. Inhibitory synapse formation.	(Lee et al., 2012, Dabrowski et al., 2015, Klimaschewski and Claus, 2021, Terauchi et al., 2010)

Fgf9	Fibroblast growth factor 9	0.4	8.9e-05	Glia activating factor. Promotes neuron survival and protection.	(Kang et al., 2014, Kanda et al., 2000, Kinkl et al., 2003)
Gdnf	Glial cell line derived neurotrophic factor	1.5	2.1e-08	Survival of dopaminergic, noradrenergic and motor neurons. Inhibits neuroinflammation	(Lin et al., 1993, Arenas et al., 1995, Henderson et al., 1994, Rocha et al., 2012)
Ifnb1	Interferon beta 1	3.6	3.1e-11	Neuroprotective	(Liu et al., 2002, Marsh et al., 2009, Longhi et al., 2011, Khorooshi et al., 2015)
Igf2bp2	Insulin-like growth factor binding protein-2	1.3	6.1e-10	Neuroprotection. Enhances biological and neuroprotective action of IGF-1.	(Yao et al., 1995, Breese et al., 1996, Logan et al., 1994)
Igf2bp3	Insulin-like growth factor binding protein 3	-0.2	2.5e-02	Blocks IGF-1, a neurotrophic and neuroprotective peptide	(Carro et al., 2003, Logan et al., 1994, Walter et al., 1997, Titone et al., 2019)
Il6	Interleukin 6	5.1	2.8e-13	Induces neurotrophin expression. Promotion of neuronal survival and regeneration.	(Erta et al., 2012, Tanaka et al., 2014, März et al., 1999, Kushima et al., 1992, Quarta et al., 2014, Bowen et al., 2011, Hama et al., 1991, Hama et al., 1989)
Il19	Interleukin 19	3.95	3.6e-09	Anti-inflammatory signalling. Neuroprotective.	(Cooley et al., 2014, Gallagher et al., 2004, Xie et al., 2016)
Lif	Leukaemia inhibitory factor	2.3	1.1e-12	Neurotrophin, neuroprotection and axon growth	(Janssens et al., 2015, Marriott et al., 2008, Butzkueven et al., 2002, Davis et al., 2019, Ajmo Jr et al., 2006)

Ngfr	Nerve growth factor receptor	0.5	1.2e-02	Development, differentiation and maintenance of neurons.	(Levi-Montalcini, 1987, Aloe and Calzà, 2003, Aloe et al., 2015, Balzamino et al., 2015)
Nrg1	Neuregulin 1	0.9	8.3e-09	Role in neural development, neuroprotection, axonal regulation and synaptic plasticity.	(Li et al., 2007, Noll et al., 2019, Xu et al., 2005, Nave and Salzer, 2006, Mei and Xiong, 2008)
Pdgfb	Platelet derived growth factor, B	1.1	7.3e-10	Neuroprotection and angiogenesis	(Krupinski et al., 1997, Arimura et al., 2012, Cheng and Mattson, 1995, Risau, 1997, Osborne et al., 2016)
Socs1	Suppressor of cytokine signalling 1	1.6	1.1e-10	Neuroprotective. Suppressors of cytokine signalling-Inhibits neuroinflammation.	(Xiao-Lei et al., 2018, Wang et al., 2021, Dragone et al., 2014, Park et al., 2003)
Tgfb1	Transforming growth factor, beta 1	0.4	9.6e-04	Regulate neuronal survival. Increase the neuroprotective action of neurotrophic factors	(Krieglstein et al., 2002, Buisson et al., 1998)
Tnf	Tumor necrosis factor	4.4	6.2e-15	Improve neuronal survival. Astrocyte proliferation.	(Jankowsky and Patterson, 2001, Barna et al., 1990)
Vegfa	Vascular endothelial growth factor A	0.8	1.8e-03	Neuroprotection, neurogenesis and angiogenesis	(Nishijima et al., 2007, Foxton et al., 2013, Sun et al., 2003)
Vegfc	Vascular endothelial growth factor C	1.5	1.1e-11	Neuroprotection and angiogenesis	(Piltonen et al., 2011, Le Bras et al., 2006)

3.6 Specific chemokines and cytokines are produced by astrocytes in response to BCG infection

Following the recognition of mycobacterial PAMPs via host PRRs, signalling pathways are induced resulting in the release of proinflammatory cytokines. Transcriptomic analysis

is a useful tool to obtain a global image of cellular activity, by providing insight into differential gene expression and possible pathway inductions. Due to transcriptional regulation and post-transcriptional modifications within cells, gene transcription alone cannot be used as an indicator of cellular functionality; hence protein expression needs to be measured as a functional assessment of cellular responses. To assess whether the gene expression changes observed in the transcriptomic studies were biologically relevant, a Luminex multiplex assay was selected to quantitate secreted proteins in astrocyte supernatants following BCG infection *in vitro*. As experimental conditions for Luminex analysis, an MOI 10 was selected for infection as well as a 48h time point, and astrocyte sample numbers were increased to 5 x 10^5 cells. Following infection, supernatants of infected and uninfected astrocytes were collected at the specified time points. For each experiment, the sample supernatants of each group were pooled, and the analytes measured. Each experimental group therefore consisted of five individual samples that were combined. As a result, every data point plotted on the graph represented a single experiment with five individual samples (Fig. 3.7). Based on the transcriptomic data, the analytes selected for further analysis were chemokines CXCL5, CXCL1, CXCL2, CXCL10, CCL5, CCL3 and CCL2; as well as cytokines TNF, IL-1β, IL-10 and IL-6 (Fig. 3.7). There was no detectable amount of IL-1β in the uninfected astrocyte samples, but it was induced following BCG infection. Low, but detectable, concentrations of TNF, IL-10 and IL-6 were measured in uninfected samples, which increased significantly during infection ($p < 0.05$). The results align with past findings that BCG exposure triggers TNF, IL-10, IL-1β and IL-6. Previous research on mice and clinically relevant studies on human blood monocytes and PBMCs showed increased levels of these cytokines following BCG stimulation (Huygen et al., 1992, Suzuki et al., 1993, Arts et al., 2015, Kleinnijenhuis et al., 2012). Additionally, a study found that low birth weight infants showed increased levels of IL-1β, TNF, IL-6, and IFN-γ following heterologous bacterial stimulation four months post-vaccination (Jensen et al., 2015). Therefore, although the data presented in this study is from astrocytes rather than typical immune-modulating leukocytes, the signatures are consistent with previously published reports.

Primary murine astrocytes treated with IL-1β, stimulated expression of CCL2, CXCL2 and CCL20 chemokines (Wang and Knaut, 2014) thereby inducing the recruitment of peripheral immune cells. Chemokines are essential for TB immunity, particularly in directing cellular traffic during granuloma formation (Scott Algood et al., 2005) and astrocyte derived chemokines may direct and regulate pathogenesis. In uninfected astrocytes, CXCL10 was expressed at low levels whereas CCL3, a chemoattractant involved in T cell activation, migration, and cytokine production (Taub et al., 1993a, Schall et al., 1993, Singh et al., 2021) was undetectable. Following BCG infection, both chemokine analytes displayed enhanced production compared to the uninfected controls. BCG vaccination in infants induces CXCL10 and CCL3 expression, regulated by type I IFN in monocytes (Lalor et al., 2010, Samperio et al., 2004). The strong type I IFN response observed in the study suggests a potential role in regulating CXCL10 activity in astrocytes. BCG infection also increased secretion of CXCL5, CXCL1, CXCL2, CCL5 (Chemoattractant for monocytes, T cells, dendritic cells, and eosinophils), and CCL2 (Chemotactic activity for monocytes and basophils and a mediator of T cell activation, migration, and cytokine production), in astrocytes compared to uninfected controls. The concentrations of CXCL1 and CXCL2 at 48h post-infection (Fig. 3.7) were found to be unexpectedly elevated compared to the other analytes in the multiplex assay, exceeding the quantifiable range of the absorbance values. Estimate values from the highest standard concentrations were therefore used for these 2 analytes. The maximum detectable concentration on the standard curve indicates the highest measurable level of that protein. As the actual concentrations were found to be greater than the standard values, it can be assumed that there was a difference in protein production compared to the control group.

The Luminex data therefore indicates the synthesis of biologically active proteins from RNA transcripts. The contribution of astrocytes to the cytokine and chemokine milieu provides evidence of astrocytes to induce an inflammatory response, signal cellular recruitment and potentially enhance BBB permeability during BCG infection. In an *in vivo* setting, resident brain cells and infiltrating immune cells also elevate specific cytokines or chemokines during infection, and they are likely to compensate if astrocytes fail to produce them. A network of glial and immune cells influences the repertoire of immune

factors, and the overall effect is tightly linked to the type of stimulus, time after the insult, the concentration of cytokines and chemokines as well as their interactions with other cytokines and chemokines.

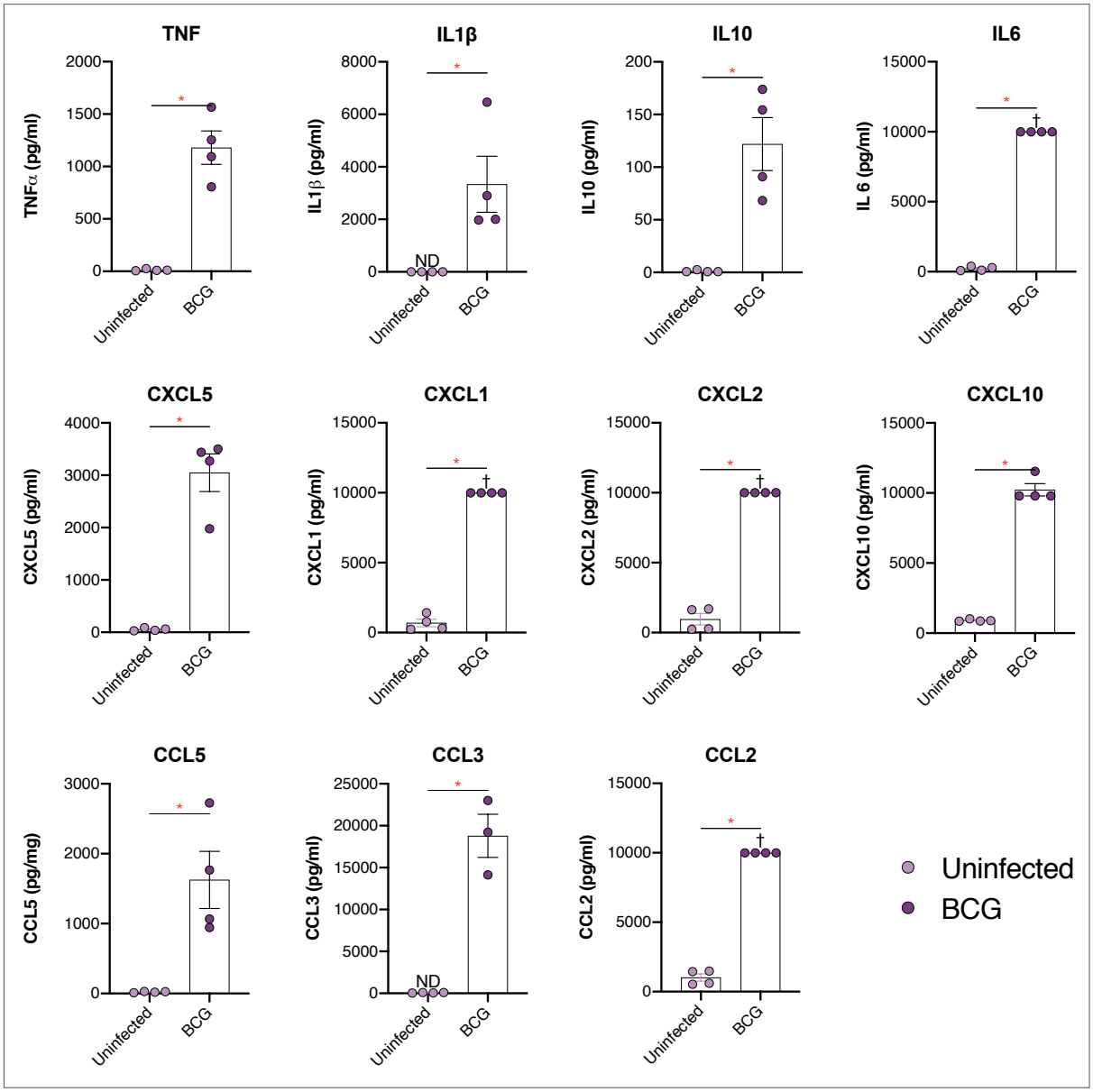


Figure 3.7 | Astrocytes induce an immune response during BCG infection. Primary astrocytes were infected with BCG at MOI 10 or uninfected, and supernatants collected at 48h for Luminex analysis. Production of TNF, IL-1β, IL-10, IL-6, CXCL5, CXCL1, CXCL2, CXCL10, CCL5, CCL3 and CCL2 was measured. In individual experiments, each group was composed of five samples that were pooled for analysis. As a result, each data point on the graph represents the pooled value of five samples of the group. Data shown is expressed as the mean ± SEM and is representative of three to four experiments (n=4 or 5 per group). Statistical significance was determined (* $p < 0.05$; ND = not detected).

CHAPTER 4

CHAPTER 4

Astrocyte modulation of immune responses during *Mycobacterium tuberculosis* infection

Although microglia have been classified as the main hosts for *M. tuberculosis* in the CNS, other resident cells, particularly astrocytes, are also infected by *M. tuberculosis* (Rock et al., 2005). Despite the expanding appreciation of astrocyte immune regulation in response to pathogens, a paucity of evidence on their activity during *M. tuberculosis* infection remains (Geyer et al., 2019, Farina et al., 2007). *M. tuberculosis* is a more virulent pathogen than *M. bovis* BCG and has developed methods of persisting in host cells by overcoming immune defences (Flynn and Chan, 2003, Divangahi et al., 2009). The development of active disease is largely due to the ability of the bacilli to evade or suppress protective cellular immune responses. Multiple factors determine disease outcome, one of the most important being the strength of the immune response elicited by the host. To determine whether astrocytes are host cells for *M. tuberculosis* and whether mycobacterial virulence has an impact on the astrocyte response profile, a clinical laboratory mycobacterial strain *M. tuberculosis* H37Rv was used to challenge astrocytes and their responses were evaluated. This chapter therefore aims to advance the understanding of CNS-TB by exploring the profile of reactive astrocytes and their contribution to host defences during *M. tuberculosis* infection. This is the first comprehensive analysis of astrocytes during *M. tuberculosis* infection, providing new insights into the essential immunological role that these resident CNS cells play.

4.1 Primary murine astrocytes internalise *M. tuberculosis*

Primary astrocyte cultures were purified from cultures of mixed glia derived from mouse pups' cortices. Once primary astrocytes had reached confluency, 5 x 10^4 cells were seeded onto microscope slides and left to adhere overnight. For this part of the study a recombinant GFP expressing *M. tuberculosis* strain, H37Rv-GFP, was used to infect astrocytes at a MOI of 2:1 and 10:1 for 24h. Synonymous to the BCG study, samples were stained with the nuclei marker DAPI and the astrocyte lineage marker anti-GFAP.

Fluorescent confocal microscopy images captured GFAP$^+$-astrocyte cytoskeletal filaments in the red channel, GFP$^+$-bacilli in the green channel, DAPI-stained nuclei in the blue channel and an overlap of all three channels (Fig. 4.1A). A z-stacked image was created in which an association between the astrocyte cytoskeletal filaments and *M. tuberculosis* bacilli were observed. This association however does not determine the position of the bacilli nor whether it is extracellular or internalised. To confirm the location of the bacilli, an orthogonal projection of a single z-stacked image in the XY plane was generated as a three-dimensional visual representation of the cell (Fig. 4.1B). Two lines representing the X (green) and Y (red) planes were constructed to intersect at selected bacilli to assess its location. The X- and Y-planes were visualised in the right (red line) and top (green line) panels, respectively. Images revealed the presence of a GFP$^+$-bacillus (green) between the astrocyte cytoskeletal filaments (red) in both planes, illustrating internalisation of the *M. tuberculosis* bacillus within the astrocyte filaments (Fig. 4.1B). A characteristic indicative of bacillus-cytoskeletal interaction is the co-localisation of fluorescent signals which appear yellow as a result of the combined red and green fluorescence. Yellow bacilli were observed in the captured image, and to confirm co-localisation of the GFAP$^+$-astrocyte cytoskeleton and GFP$^+$-bacilli, an area of the image, denoted by the white rectangle, was selected and fluorescent intensity analysed (Fig. 4.1C-1). Fluorescent peaks on the graph represent areas within the white rectangle where the fluorescent signal is enhanced. The green-fluorescent peaks indicate the presence of bacilli, while the red-fluorescent peaks reflect an increase in astrocyte cytoskeletal filaments. Interestingly, the green and red peaks were synchronised providing evidence of co-localised signals from the bacilli and astrocyte, suggesting tight interaction. Given that the bacilli were undoubtedly internalised, it is plausible that the astrocyte cytoskeletal filaments assembled around the bacilli and resulted in the intensified fluorescent signal observed in the red channel.

To determine whether *M. tuberculosis* bacilli can replicate following their uptake in primary astrocytes, cells were challenged with *M. tuberculosis* at a MOI of 2:1 and 10:1, lysed at 2h and 24h post-infection and bacterial colonies quantified. In both MOI of 2:1 and 10:1 CFU enumeration revealed a significant increase in bacterial burden at 24h ($p < 0.05$) compared to 2h post-infection (Fig. 4.1C), demonstrating bacilli replication in

astrocytes over the experimental period. These findings provide conclusive evidence that *M. tuberculosis* is capable of directly infecting astrocytes, consistent with previous reports on the ability of *M. tuberculosis* to infect resident CNS cells (Rock et al., 2005, Randall et al., 2014). More importantly, it demonstrates that astrocytes act as suitable host cells that permits bacilli replication.

4.2 *M. tuberculosis* induces differential gene expression in astrocytes

Gene profiling analysis has emerged as a powerful tool to fully characterise the molecular changes that occur within distinct cell types during development or in response to injury and disease (Zamanian et al., 2012, Doyle et al., 2008). Previous gene expression studies on *M. tuberculosis* have mainly focused on responses in macrophages (Volpe et al., 2006, Giacomini et al., 2001, Ragno et al., 2001, Wang et al., 2003) and resident pulmonary cells (Maertzdorf et al., 2018). Moreover, reports on cellular responses in the brain with respect to gene expression and its relationship to CNS disease are not only limited but are completely lacking in CNS-TB. Within the CNS, gene expression studies on astrocytes are focused on developmental characterisations, heterogeneity, reactive astrogliosis, and their contribution to neuroinflammation and neuroprotection (Cahoy et al., 2008, Cuevas-Diaz Duran et al., 2019, Zhang et al., 2014b, Liddelow et al., 2017, Batiuk et al., 2020, Zamanian et al., 2012, Hasel et al., 2021, Teh et al., 2017, Zhang et al., 2016). Astrocyte responses to *M. tuberculosis* has received very little attention in the literature and gene expression profiling in these cells are noticeably unexplored in the context of CNS-TB. Analysis of astrocyte transcriptional activity could assist with their characterisation by clarifying their cellular phenotype during infection and determining their possible contribution to host immune responses during CNS-TB. In the previous chapter, astrocytes were shown to induce an immune response to BCG, but to determine whether transcription is modulated by bacterial virulence further investigations explored gene expression differences between uninfected and *M. tuberculosis*-infected astrocytes. Astrocytes were infected with *M. tuberculosis* H37Rv for 24h and total RNA was subsequently extracted. Each experimental group consisted of 5 samples and all experiments were conducted in triplicate. Data is representative of three independent experiments.

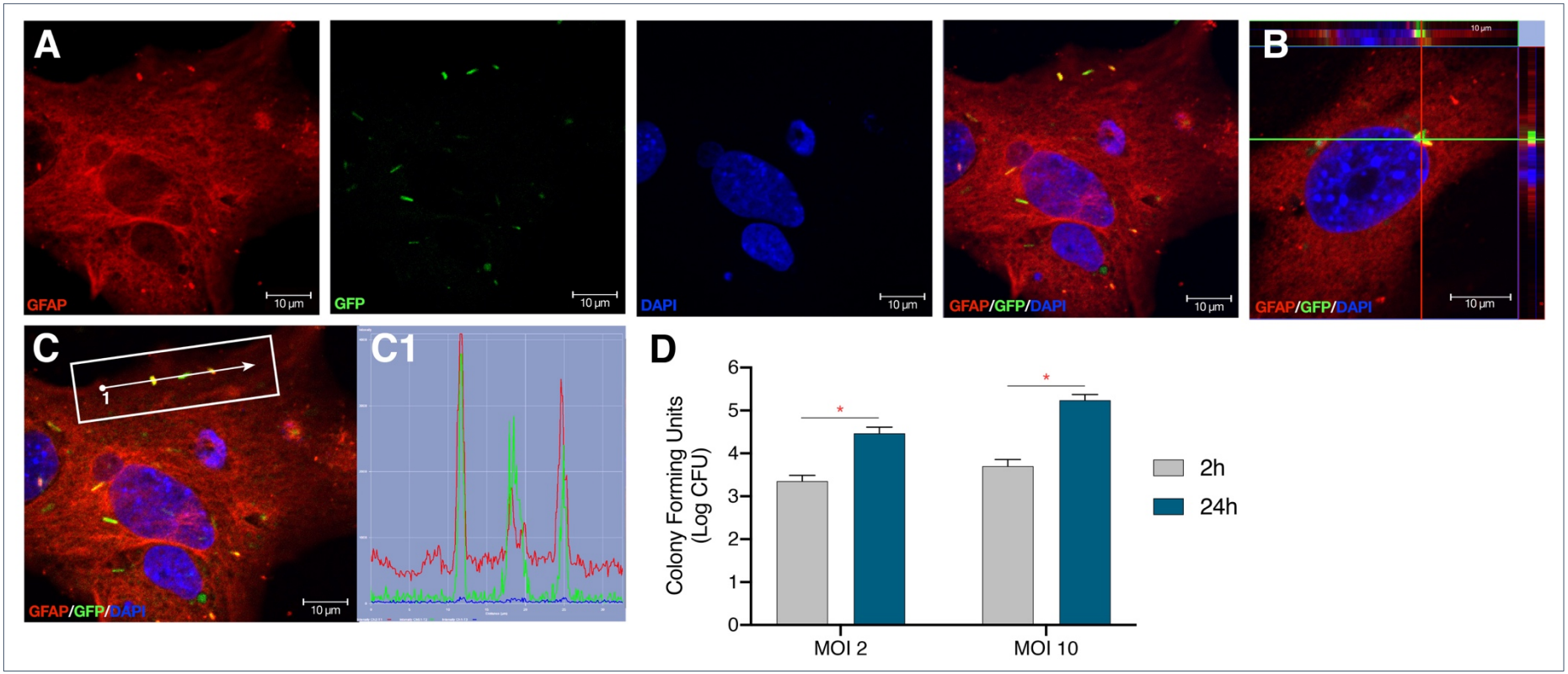


Figure 4.1 | Astrocytes internalise *M. tuberculosis* bacilli. Following a 24h *M. tuberculosis* challenge, primary murine GFAP$^+$ astrocytes were analysed by immunocytochemistry and CFU numbers determined. Confocal microscopy images showing (A) GFAP-labelled astrocytes (red), GFP-bacilli (green), and DAPI-labelled cell nuclei (blue) in separate and combined channels. (B) Fluorescent image of combined channels demonstrates astrocyte and bacilli association while the colocalization of the bacilli and cytoskeletal filaments in the orthogonal XY plane, indicate internalisation. (C) Captured z-stacked image demonstrates yellow co-localisation signals of the green, fluorescent GFP-labelled bacilli and red astrocyte marker (GFAP) indicating interaction. (C1) Simultaneous peaks of the red and green signals indicate interaction of the astrocyte cytoskeletal filaments and bacilli. Images are representative of 4 wells from 1 of 3 individual experiments. (D) CFU determined from lysed cultures indicated bacilli viability and replication over 24h following primary astrocyte infection. The results are the mean and SEM of three experiments (* $p < 0.05$).

SurePrint microarrays were used to evaluate differential gene expression of infected and uninfected cultured primary astrocytes. Analysis of the microarray data revealed 7,620 significant differentially expressed genes (DEGs) (adj. p value < 0.05) in *M. tuberculosis* infected compared to uninfected astrocyte samples. A two-fold log change is commonly used in literature as the arbitrary cut-off value to define significantly different levels of gene expression. Using this parameter as the threshold, 91 genes were established as significantly upregulated and 28 downregulated. Following data normalisation, a heat map was constructed with parameters set at fold change (FC) ≥ 2 and an adj. p value < 0.05, allowing for global visualisation of gene expression changes between the two conditions (Fig. 4.2A). Data values were transformed to a colour scale where the magnitude of gene expression was presented as red and blue hues representing higher and lower levels of expression, respectively. Closer examination of the heat map revealed clustering of the uninfected and *M. tuberculosis* groups, as indicated by the hierarchical clusters on the x-axis which orders data by similarity. To efficiently visualise genes with large fold changes between infected and uninfected astrocytes, a volcano plot was created (Fig. 4.2B). The log10 adj. p values were plotted on the y-axis with the threshold for statistical significance denoted by the horizontal dashed line (adj. p < 0.01). Effect size as measured by log2FC was plotted on the x-axis, and vertical dashed lines represent the cut-off values set at log2FC > 1 and < -1. Positive values on the x-axis indicate upregulation, whereas negative values are indicative of a downregulation in genes in the *M. tuberculosis*-infected astrocytes compared to the uninfected control. Each event is annotated as a dot on the plot and represents a specific gene, and each dot is further depicted as a colour to categorise the event according to the thresholds they meet (grey, green, blue, or red). Grey dots represent genes that do not pass any of the set thresholds, whereas green, blue, and red dots represent genes that pass defined thresholds. Genes that pass the threshold for logFC are denoted in green, statistically significant genes are denoted in blue, and genes that are both statistically significant and pass the fold change cut-off values are depicted in red. Top genes that passed both thresholds were labelled. It is worth noting that amongst the visible outliers there was a pronounced appearance of upregulated chemokines, particularly *Cxcl1*, *Cxcl2* and *Cxcl5*; the guanylate binding proteins: *Gbp4*, *Gbp5* and *Gbp11*; as well as the reactive free radical, *Nos2*. Interestingly,

expression of the chemokine receptor *Cx3cr1* gene was significantly downregulated along with complement factor, *C1q*.

One measure of pathogen virulence is the ability to reside within a cell which is usually achieved through the perturbation of host cell function. The capacity of *M. tuberculosis* to replicate within astrocytes indicates that they can evade host responses and reside within these cells. *M. bovis* BCG, in contrast, demonstrated lack of bacilli replication possibly because of its reduced virulence. Distinct inflammatory signalling pathways may be initiated by these two pathogens contributing to their disparate virulence. Comparative studies have shown that several differences exist in gene expression profiles between virulent and attenuated mycobacteria that are important determinants of survival and persistence in host cells. For example, genes involved in secretion systems and lipid metabolism are identified as virulence determinants for mycobacteria (Ates et al., 2018, Aguilo et al., 2017, Augenstreich et al., 2017, Quigley et al., 2017, Roberts et al., 2004, Braunstein et al., 2003, Bottai et al., 2012, Pym et al., 2002, Aguilar-Ayala et al., 2017, Lovewell et al., 2016, Mendum et al., 2015) and are distinctly different between BCG and *M. tuberculosis*. Astrocyte responses to BCG and *M. tuberculosis* are likely to have a degree of variation due to virulence differences. With the aid of a principal component analysis (PCA), a comparative analysis was done to distinguish the relationship between *M. tuberculosis*- and *M. bovis* BCG-infected astrocytes. By performing this dimensionality-reduction method, data sets with multiple variables could be transformed into a smaller set of variables while maintaining variation between samples. In addition, this data transformation technique separates samples according to their variation enabling the identification of trends in complex biological data. A PCA plot for uninfected, *M. tuberculosis*-infected and BCG-infected astrocytes was created. The results displayed in Figure 4.2C illustrate how samples with similar expression profiles cluster together. Three clusters were immediately apparent with BCG (orange), *M. tuberculosis* (green) and uninfected (blue) astrocytes forming distinct groups. The BCG and *M. tuberculosis* samples clustered closer together, indicating a closer relationship to each other than to the uninfected astrocyte group. Given the similarities between BCG and *M. tuberculosis* as mycobacterial pathogens they are likely to activate and induce similar responses in astrocytes, and as a result cluster more closely. Uninfected astrocytes in

comparison would be unreactive with a very different gene expression profile and hence would appear as a more distant cluster. Another useful tool which was employed to assess the relationship between BCG- and *M. tuberculosis*-induced responses in astrocytes was a Venn diagram. The transcriptomic data was used to generate a Venn diagram with a threshold of adj. $p < 0.05$ and $FC > 1.5$ for differentially expressed genes. There were 583 differentially expressed genes common to both BCG and *M. tuberculosis*; interestingly BCG-infected astrocytes had 841 distinctly expressed genes while *M. tuberculosis*-infected astrocytes had 105 unique differentially expressed genes. These results demonstrate that *M. tuberculosis* is capable of inducing gene expression in astrocytes but do so in a manner quite distinct from astrocytes infected with BCG. This raises several questions, the most important ones being which genes are differentially expressed, how do these genes impact astrocyte function and does this involve immune mediation? Identifying astrocyte responses to *M. tuberculosis* may help elucidate their contribution to disease progression or alternatively, host defence and pathogen clearance.

4.3 RNA expression analysis of astrocytes elucidates immune pathway enrichment during *M. tuberculosis* infection

Expression patterns in cells need to be identified to acquire insight into the functional relevance of specific genes. Observed cellular phenotypes are not a product of expression changes of one gene but rather a result of the interrelationships of several differentially expressed genes. It is therefore necessary to analyse modules or pathways to assess the effective impact of expression changes of connected genes and clarify cellular function. The transcriptomic data on *M. tuberculosis*-infected astrocytes was explored by conducting gene enrichment analysis with the transcriptional module analysis (tmod) package. The tmodDecideTest function was used to determine gene module enrichment following *M. tuberculosis* infection. Red, blue, and grey bars represent genes that are up-, down- or not-regulated within their respective modules. The opacity of the bars displays the p-value, while bar size depicts the relative effect size. After a 24h *M. tuberculosis* infection the gene expression analysis plot showed adjustments in 35 modules ($p < 0.001$ and effect size > 0.61) with compelling

modifications of immunity-related modules in the infected compared to uninfected astrocytes (Fig. 4.3). Of the 35 enriched modules, a range of pathways related to the inflammatory response were induced, namely: “proinflammatory cytokines and chemokines”; “activated dendritic cells”; “chemokine cluster (I and II)”; “chemokines and inflammatory molecules in myeloid cells” and “cytokine receptor cluster”. Interestingly many intracellular mechanisms were significantly upregulated, notably “viral sensing and immunity”, “IRF2 targets network (I and II)”; “innate activation by cytosolic DNA sensing”; and “RIG-1 like receptor signalling” pathways. The plot also showed strong pathway enrichment for “type I IFN response” and “antiviral IFN signature” modules. While most modules associated with B cell activities showed little significance, the data displayed a decrease in expression levels of genes within “antigen presentation (II and III)” and “leukocyte activation and migration modules”, suggesting the regulation of adaptive immune responses by infected astrocytes. There were further indications of immune regulation with simultaneous downregulation of genes within the “complement and other receptors in DCs (dendritic cells)” module.

These findings demonstrate robust enrichment of IFN-related modules, as well as cytokine- and chemokine-associated modules in *M. tuberculosis* infected astrocytes. Pathway and modular analyses of peripheral blood cells have identified gene signatures indicative of active TB disease which are consistent with these results. Accumulating evidence on blood transcript signatures has shown that although IFN-inducible genes dominate (Berry et al., 2010, Maertzdorf et al., 2011, Ottenhoff et al., 2012), host responses in TB patients are more heterogeneous than previously considered with studies demonstrating substantial variability in gene expression patterns across cohorts and between individuals (Domaszewska et al., 2021, Joosten et al., 2013, Cliff et al., 2015). In addition to IFN-inducible genes, the upregulation of myeloid and inflammatory gene transcripts and the downregulation of transcripts encoding B- and T-lymphocyte function (Cliff et al., 2015, Berry et al., 2010) were previously shown to be correlates of TB. Numerous molecular mechanisms therefore direct the TB response. Immune module enrichment in the presented data elucidates the ability of *M. tuberculosis*-infected astrocytes to elicit immune responses that correspond to TB transcriptional signatures.

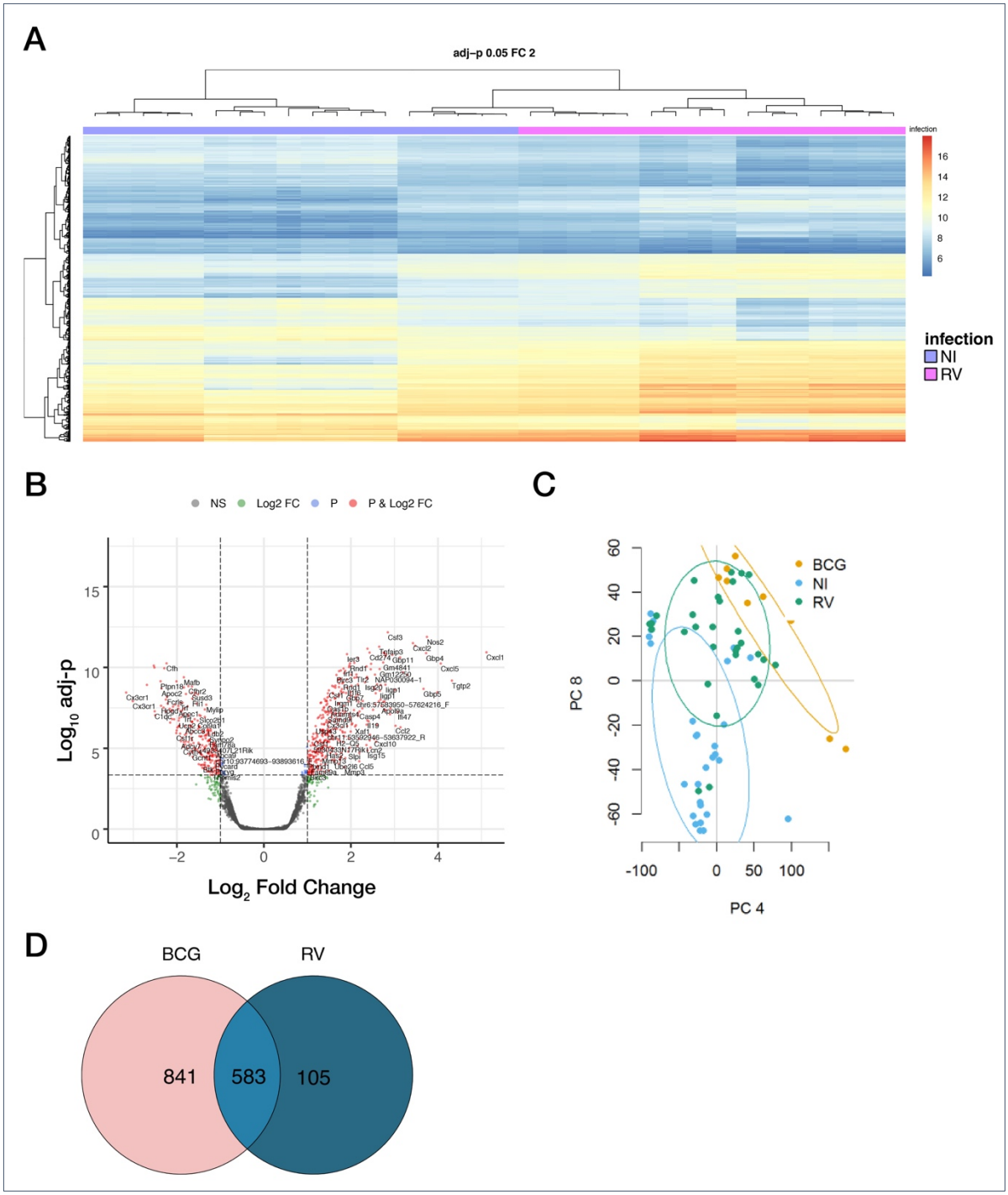


Figure 4.2 | *M. tuberculosis* infection induces differential gene expression in astrocytes. (A) Heat map summary highlighting differential gene expression between *M. tuberculosis*-infected primary astrocytes (RV) compared to uninfected astrocytes (NI). Red and blue indicate the level of gene expression with red representing higher expression. (B) Volcano plot of DEGs in *M. tuberculosis*-infected astrocytes. The x-axis shows the fold change in DEGs versus the adjusted p values (on a log10 scale) on the Y-axis. The horizontal dashed line indicates the threshold for statistical significance, while the vertical dashed lines are the thresholds for effect size. Grey dots are not significant, blue dots represent statistical significance, green dots indicate effect size and red dots are genes that have reached thresholds for both adj. p and effect size. (C) PCA plot shows distinct groupings of uninfected (blue), BCG-infected (orange) and *M. tuberculosis*-infected (green) astrocytes. (D) Venn diagram of the comparison of differentially expressed genes in BCG- and *M. tuberculosis*-infected astrocytes, and the overlap between the two groups. Threshold adj. $p < 0.05$ and $FC > 1.5$.

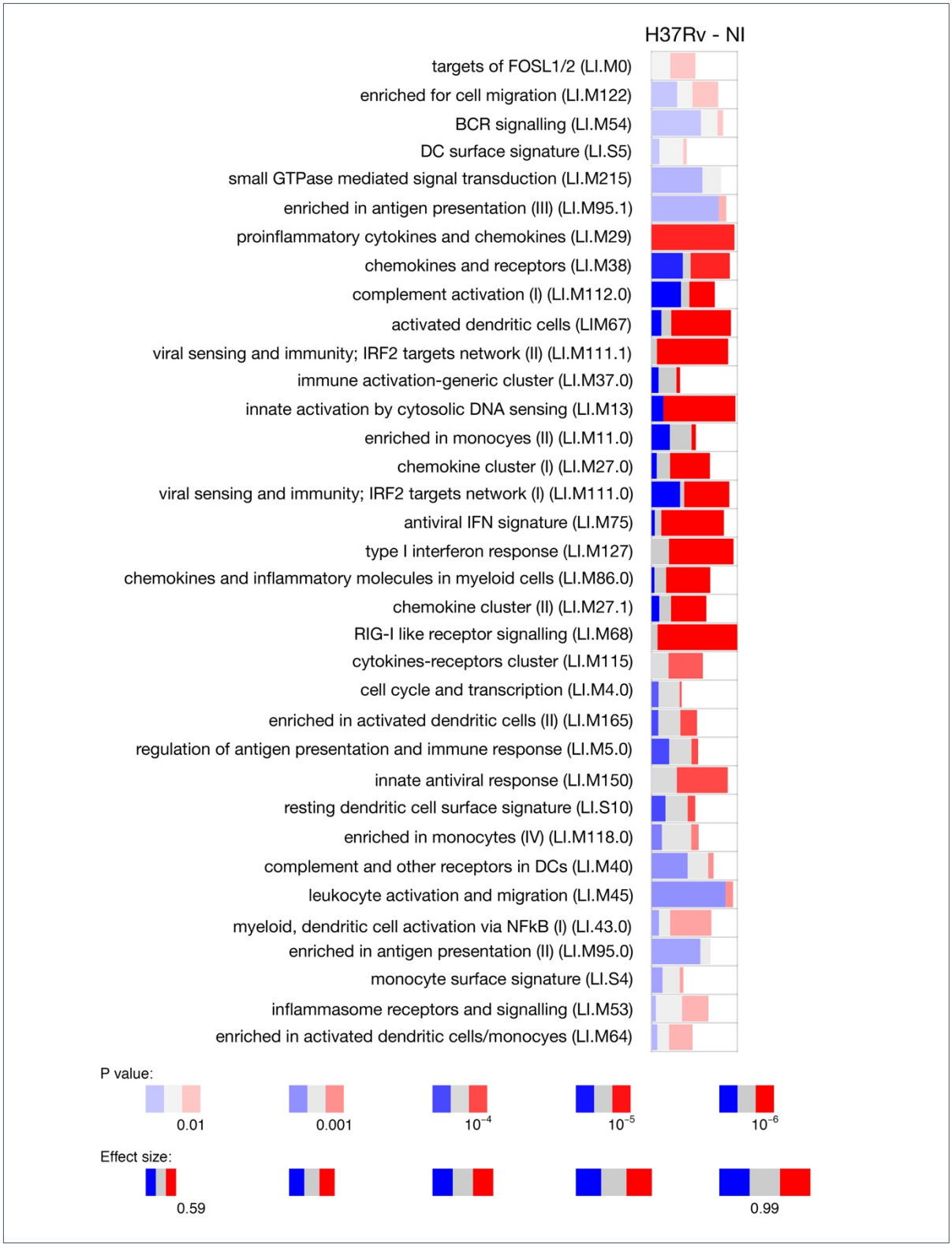


Figure 4.3 | Gene module enrichment in *M. tuberculosis*-infected primary astrocytes. The red and blue bars indicate gene up- and down-regulation, respectively, with bar size representing effect size and colour opacity corresponding to the p-value. Thresholds were set at $p < 0.001$ and effect size > 0.61. Figure generated with tmod R package.

To distinguish the inter-relationship between the differentially expressed genes and modules a chord diagram was created. The circos plot visualised significant differentially expressed genes (adj. $p < 0.05$, $FC > 1.5$) in sixteen modules enriched in *M. tuberculosis* infected astrocytes (Fig. 4.4). For comparative purposes the same modules as BCG were selected. Chemokine expression was strongly associated with the various modules, as seen earlier in the BCG samples; more specifically, the cytokine gene transcripts *Il6*, *Tnf*, and *Il1a*; and the chemokines *Cxcl1*, *Cxcl5*, *Ccl7*, *Cxcl3*, *Ccl2*, *Cxcl10*, *Ccl5*, *Cxcl9*, *Ccl3*, *Ccl4*, *Cxcl12* and *Ccl8*. Cytokines are essential immunomodulatory proteins produced by several innate immune cells such as macrophages, natural killer cells, dendritic cells, as well as adaptive T and B cells. Chemokines are a superfamily of chemotactic cytokines responsible for regulating inflammation and directing cell movement. Several genes contributed to multiple modules, particularly *Rsad2*, *Cxcl10*, *Irf7*, *Ccl5*, *Tnf*, *Nfkbia*, *Ccl4*, *Ifih1*, *Tap1* and *Ddx58* which were associated with at least four modules. In view of this it is reasonable to surmise that these factors are fundamental to host immune regulation during *M. tuberculosis* infection. Further inspection revealed multiple shared modules between them. *Cxcl10*, *Irf7*, *Ddx58 Ccl5*, *Tnf*, *Ccl4*, and *Nfkbia* for instance, all contribute to the “innate activation by cytosolic DNA sensing” module; *Rsad2*, *Cxcl10*, *Irf7*, *Ifih1*, and *Ddx58* are associated with the “antiviral IFN signature” module; *Cxcl10*, *Irf7*, *Ifih1*, and *Ddx58* are involved in the “RIG-1 like receptor signalling” module; and *Rsad2*, *Irf7*, and *Tap1* participate in the “innate antiviral response” module. Assessment of these genes revealed that their corresponding modules are largely related and collectively appear to form an integral network in IFN-related immunity. *Ddx58* for example connects to “innate activation by cytosolic DNA sensing”, “RIG-1 like receptor signalling”, “activated dendritic cells”, “antiviral IFN signature” and “viral sensing and immunity; Irf2 targets network (II)” modules which are all involved in IFN responses. Although there are variations in the modules there are visible overlaps of gene activity presumably due to related gene functions.

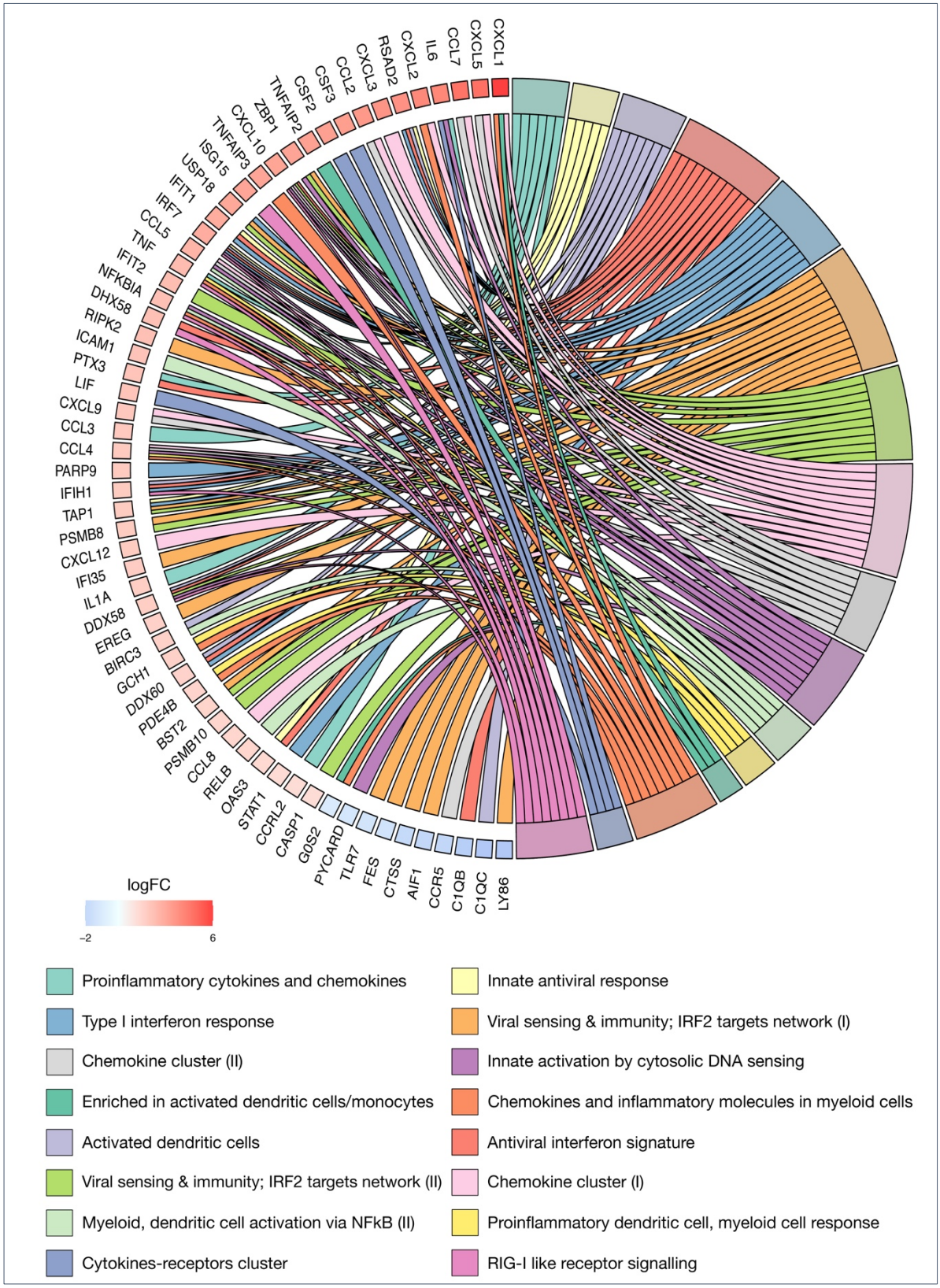


Figure 4.4 | Enrichment plot of selected modules. Circos plot representation of significantly enriched modules linked to associated differentially expressed genes in *M. tuberculosis* infected astrocytes (AUC > 0.7, adj. $p < 0.01$). Each module is denoted by a unique colour band and the connection inside the circle represents an association of a particular gene to the respective modules. Red and blue outer squares represent gene up- and down-regulation, respectively, and opacity relates to logFC.

The most significantly enriched module ($p < 0.01$) as well as the top 4 modules with the highest effect size were selected for further analysis (Fig. 4.5A). A side-by-side bar graph was generated to examine the modules in more detail and to analyse the contribution of specific genes to module enrichment. In addition, box and whisker plots were constructed for detailed analysis of the genes (Fig. 4.5B-F). The most significantly enriched module in the *M. tuberculosis*-infected astrocytes, with an adj. p of 5.94×10^{-14} and an effect size of 0.81, was the "chemokine cluster (I)" (LI.M27) module. *Ccl2*, *Ccl4*, *Ccl5*, *Ccl7*, *Ccl8* and *Ccl19*, all showed significant augmentation in this module during *M. tuberculosis* infection. Similarly, *Cxcl1*, *Cxcl2*, *Cxcl3*, *Cxcl5*, *Cxcl9*, *Cxcl10*, *Cxcl11*, and *Cxcl12* showed significant elevation in expression while *Cxcl13* and *Pf4* (also known as *Cxcl4*) were significantly downregulated. Interestingly, CXCL13 directs B lymphocytes (Ansel et al., 2002, Förster et al., 1996, Bekele Feyissa et al., 2021) whereas PF4 is a kinocidin which has a dual function as a chemokine and a microbicidal effector protein involved in antibody responses (Yeaman et al., 2007, Krauel et al., 2011, Tang et al., 2002, Yeaman, 2014). The downregulation of *Cxcl13* and *Pf4* highlights the complexity of the immune response, indicating potential modulation of B cell recruitment and antibody responses. Enhancement of this module and the upregulation of CCL and CXCL chemokines suggest that astrocytes may play a defining role in regulating cellular recruitment to the CNS during *M. tuberculosis* infection.

The module "Proinflammatory cytokines and chemokines" module (LI.M29) was also substantially enriched with an effect size of 0.96 and an adj. p of 4.47×10^{-6} (Fig. 4.5C). It is well established that IL-1α, IL-1β, TNF and IL-6 play pivotal roles in host defence against *M. tuberculosis* (Van Crevel et al., 2002, Flynn and Chan, 2001, Domingo-Gonzalez et al., 2016, Davis et al., 2021, Cooper et al., 2011, Bourigault et al., 2013), and the results in this study show significant increased expression of these genes in *M. tuberculosis*-infected astrocytes. Transcripts for chemokine *Ccl3*, and the receptor *Ccrl2* were elevated; both genes have been linked to leukocyte recruitment (Schioppa et al., 2020, Hilda et al., 2020), while *Ccl3* also contributes to *M. tuberculosis* growth inhibition (Saukkonen et al., 2002). Complement pathway associated genes *C3*, and *Ptx3* were also heightened in this module during infection. The essential component of the complement system, C3, serves as a convergence point for the classical, lectin, and

alternative pathways of complement activation (Carroll, 2004, Walport, 2001). PTX3 plays a role in pathogen recognition by binding to pathogens and mediating immune responses, enhancing phagocytosis and increasing opsonisation (Reading et al., 2008, D'Angelo et al., 2009, Garlanda et al., 2002, Deban et al., 2009, Bottazzi et al., 2009, Bozza et al., 2006). In addition, it also activates and regulates the complement pathway by binding to the classical complement pathway component C1q thereby limiting inflammatory damage (Deban et al., 2008). Reduced *C1q* expression shown in *M. tuberculosis*-infected samples could therefore be attributed to heightened *Ptx3* expression. Thus, through PTX3 activity, astrocytes may regulate inflammatory responses by amplifying certain pathways while inhibiting others, thereby providing immunological and neuroprotective support. This module highlights the ability of astrocytes to modulate proinflammatory responses following infection with *M. tuberculosis*.

The "RIG-1 like receptor signalling" module (LI.M68) was the most enriched module with an effect size of 0.98 and adj. p of 1.18×10^{-8} (Fig. 4.4B). RIG-1 is a cytosolic PRR responsible for the type 1 IFN response in the innate immune system (Choi et al., 2009, Rehwinkel and Gack, 2020). *Ddx58* which encodes RIG-1, as well as its regulators *Dhx58* and *Trim25*, all had increased transcript expression. Other inflammatory regulating genes such as *Ifih1*, *Isg15*, *Irf7*, and *Nfkbia* showed significant upregulation, as did *Tnf* and *Cxcl10*. ISG15 mediates a crucial cellular process known as ISGylation which facilitates immune signalling pathways and is a key component in host responses to *M. tuberculosis* (Wang et al., 2022, Bogunovic et al., 2012).

Interestingly, RIG-1 like modules and their associated receptors were not the only nucleic acid related modules upregulated in infected cells. The next module significantly enriched in *M. tuberculosis*-infected primary astrocytes *in vitro* was "innate activation by cytosolic DNA sensing" (LI.M13; Fig. 4.5E). This is in line with a recent study which showed that *M. tuberculosis*-RNA released during infection can trigger host DNA and RNA sensors promoting IFNβ production (Cheng and Schorey, 2018). In macrophages the ESX-1 secretion system is important for cytoplasmic exposure of extracellular mycobacterial DNA to host cytosolic receptors through phagosomal membrane permeabilization

(Manzanillo et al., 2012, van der Wel et al., 2007). Cytosolic DNA receptor activation stimulates IFNβ production via the Sting/Tbk1/Irf3 axis (Manzanillo et al., 2012), possibly promoting the elevated type I IFN signature associated with active TB disease (Berry et al., 2010, Novikov et al., 2011, Watson et al., 2015). The ESX-1 system is also necessary for type I IFN production in murine and human macrophages during *M. tuberculosis* infection (Novikov et al., 2011, Stanley et al., 2007) and may similarly be involved in astrocyte responses. Within this module a mediator of IFN activity *Ddx58*, and an IFN inducible protein *Cxcl10*, also showed a significant upregulation in transcript expression. The module member, Z-DNA binding protein 1 (ZBP1, also known as DNA-dependent activator of IFN-regulatory factors (DAI) and DLM-1) is similarly involved in mediating a type I IFN responses after binding to foreign DNA (Takaoka et al., 2007). *Zbp1* and its downstream factors, *Irf7* and *Nfkbia*, were shown to have significant logFC increases in *M. tuberculosis*-infected compared to uninfected astrocyte samples. ZBP1 contributes to NLRP3 inflammasome activation and subsequent IL-1β secretion in response to influenza A virus (Kuriakose et al., 2016) which is noteworthy given that *Il1b* was significantly upregulated in this module. Interestingly, transcript expression of the NLRP3 adaptor protein, *Pycard* (ASC), as well as absent in melanoma 2 (*Aim2*), a receptor involved in AIM2 inflammasome activation, were both significantly reduced. IL-1β induction as part of NLRP3 inflammasome activation and AIM2 reportedly have important roles in regulating human and mouse macrophage responses during infection (Mishra et al., 2010, Wong and Jacobs Jr, 2011, Saiga et al., 2012, Wassermann et al., 2015). Contradictions regarding inflammasome contribution to *M. tuberculosis* induced immunity, however, exists. Some studies have found the NLRP3 inflammasome to be essential for immunity to *M. tuberculosis* (Wawrocki and Druszczynska, 2017, Van de Veerdonk et al., 2011, Briken et al., 2013, Wassermann et al., 2015), while others suggest that inflammasome-independent IL-1β production is the main contributor to protective immunity in *M. tuberculosis* (Saiga et al., 2012, Mayer-Barber et al., 2010, Cooper et al., 2011, Walter et al., 2010, Mcelvania Tekippe et al., 2010, Dorhoi et al., 2012). This could be due to *M. tuberculosis* inhibition of inflammasome activation or that the inflammasome function is not as crucial as previously thought. A study on *Pycard*$^{-/-}$ mice showed an abrupt decline in survival during chronic *M. tuberculosis* infection due to decreased granuloma formation (Mcelvania Tekippe et al., 2010). Although PYCARD

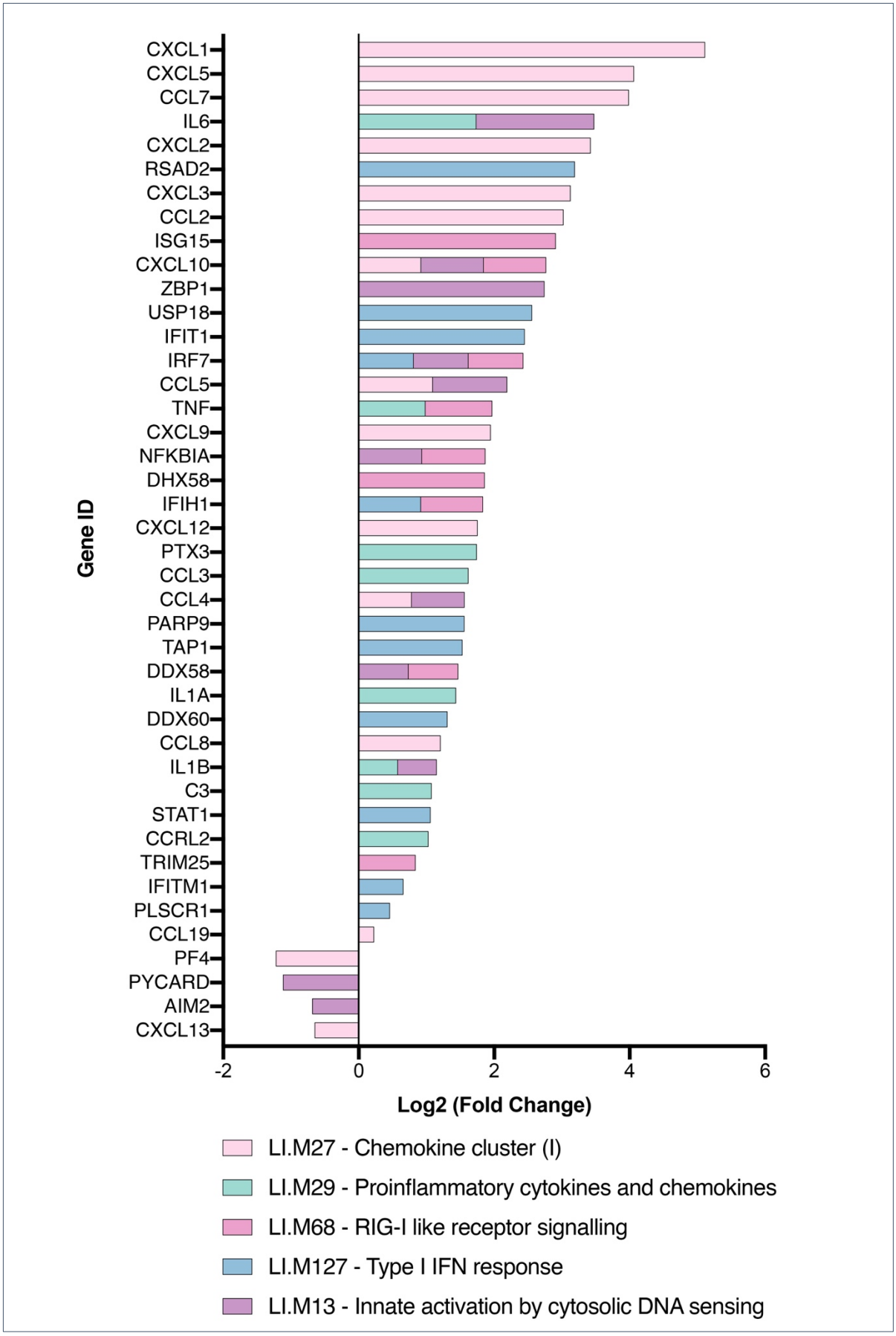
plays a pivotal role in inflammasome assembly, it also offers host protection during *M. tuberculosis* infection in an NLRP3 independent manner (Mcelvania Tekippe et al., 2010, Mayer-Barber et al., 2010). AIM2 is a cytosolic immune sensor that recognises microbial DNA and is important for host defence during *M. tuberculosis* infection (Saiga et al., 2012), but possibly induces IL-1 production through inflammasome-independent pathways too. Despite the downregulation of *Aim2* and *Pycard*, *Il1b* expression was increased and could possibly be inflammasome-independent. In addition to *Il1b*, chemokine transcripts *Ccl4*, *Ccl5*, and *Cxcl10* as well as the cytokine transcript *Il6* showed elevated expression. This is notable as DNA sensing pathways are an essential component of the innate immune system, and can induce inflammatory gene expression (Barber, 2011). IL-6 is crucial for immune cell recruitment and *M. tuberculosis* control (Ladel et al., 1997). An unexpected observation was the absence of *Il18* in this module as it is produced in conjunction with *Il1b* and did display increased expression (Table 4-2). This could be explained by their distinct regulatory mechanisms despite both cytokines being produced as precursor pro-forms and cleaved by the protease caspase-1 (Zhu and Kanneganti, 2017, Marshall et al., 1999, Puren et al., 1999). These transcriptional changes present a complex system of nucleic acid-sensing innate immune signalling pathways that are activated in astrocytes during *M. tuberculosis* infection. Activation of these pathways results in the synthesis of pro-inflammatory cytokines, chemokines and IFNs that drive inflammatory and cell-mediated immune responses. Based on these findings, it is likely that RNA- and DNA- sensing pathways contribute to the inflammatory signature seen in *M. tuberculosis*–infected astrocytes, particularly those related to IFN responses. Reductions in certain genes such as those involved in the inflammasome-related pathways may be due to astrocyte-induced regulation of inflammatory responses as a protective mechanism, or bacterial inhibition of innate immune responses. Further research is needed to fully understand the functional significance of these observations and their implications for the pathogenesis of TB in the CNS.

Corresponding to the “RIG-1 like receptor signalling” module and “innate activation cytosolic DNA sensing” module which induce a downstream IFN response, enrichment of the “type I IFN response” module (LI.M127; Fig. 4.5F) was also observed. Similarly,

increased transcript expression of *Ifih1* and positive regulator *Irf7* were noted. *Stat1*, the transcription activator downstream of IFN receptors also showed enhanced transcript expression. *In vitro* studies have previously demonstrated activation of *Stat1* and *Irf7* after *M. tuberculosis* infection of human macrophages, DCs and monocytes (Wu et al., 2012, Remoli et al., 2002, Ameixa and Friedland, 2001). *Tap1*, the antigen transporting protein involved in loading peptide onto MHC I molecules (Grandea III et al., 1995, Suh et al., 1994), demonstrated the most significant expressional change with an adj. p of 1.51 x 10^{-11}. Increased expression was detected in *Ifitm1*, *Ifit1*, *Rsad2*, *Parp9* and *Plscr1* in infected astrocytes. An upregulation of these genes was observed in the previous chapter exploring BCG-infected astrocytes and their functions discussed (see Chapter 3, Section 3.3, page 65). *Ddx60* which is involved in promoting RIG-1-like receptor mediated signalling (Miyashita et al., 2011) was also induced but was not associated with the "RIG-1 -like receptor mediated signalling" module (LI.M68). Analogous to BCG-infected astrocytes, the negative regulator of IFN activity, *Usp18*, also displayed elevated transcript expression following *M. tuberculosis* infection. These results highlight the prominent type I IFN signature in *M. tuberculosis*-infected astrocytes and the common transcriptional signatures to BCG-infected astrocytes provide strong evidence that virulence may not be a significant influence on type I IFN expression. Moreover, simultaneous transcription of positive and negative regulators alludes to a tightly controlled type I IFN pathway.

Analysis of the gene expression profiles of astrocytes infected with *M. tuberculosis* revealed a significant enrichment of type I IFN-related signalling modules, highlighting the involvement of IFN activity in the astrocyte-mediated immune response. The upregulation of key genes involved in IFN signalling, including *Ddx58*, *Dhx58*, *Trim25*, and *Irf7*, suggests that *M. tuberculosis* infection in astrocytes induces the production of IFNs. Secretion of type I IFNs leads to the induction of IFN stimulated genes such as CXCL10 and ISG15 that upregulate the effector function of immune cells to coordinate inflammatory responses and resolve infections. However, the system is more complex than initially thought as IFN stimulated genes can be activated by factors besides IFN signalling, such as IRF7, NFκB, or IL-1 signalling. Some of these factors, including IRF7, can also be induced by IFN, potentially resulting in multiple pathways to trigger a single

IFN stimulated gene. While the upregulation of type I IFN response pathways in astrocytes during *M. tuberculosis* infection is consistent with previous research (Berry et al., 2010, Manzanillo et al., 2012, Watson et al., 2015), the implications of this response in the context of CNS-TB pathogenesis remain unclear. Investigating astrocyte immune function against *M. tuberculosis* in the CNS may enable underlying mechanisms of host defence to be identified. Understanding the balance of immune-regulatory factors produced by astrocytes and how they can generate both protective and pathological consequences is critical to appreciate CNS-TB pathogenesis and for developing effective, novel therapies.



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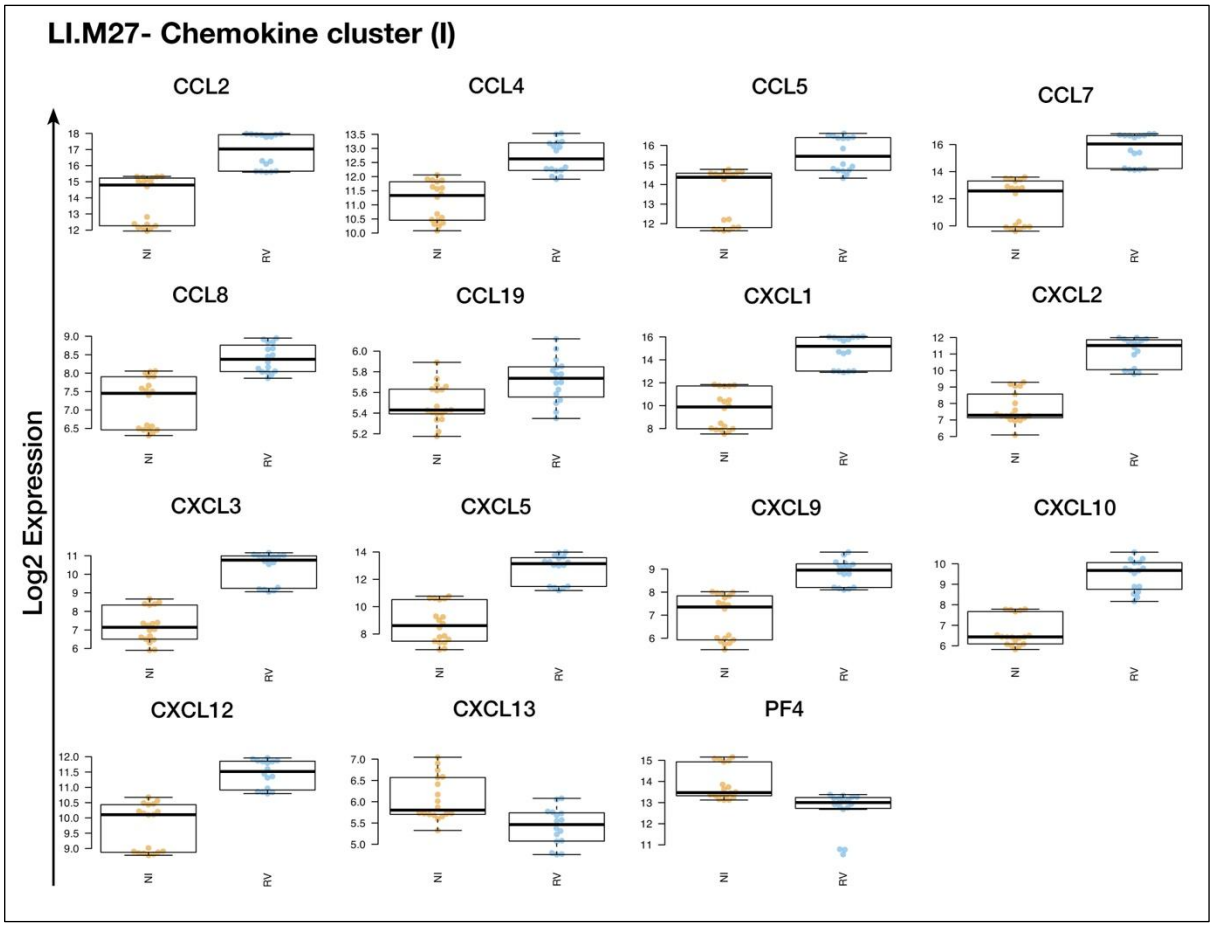

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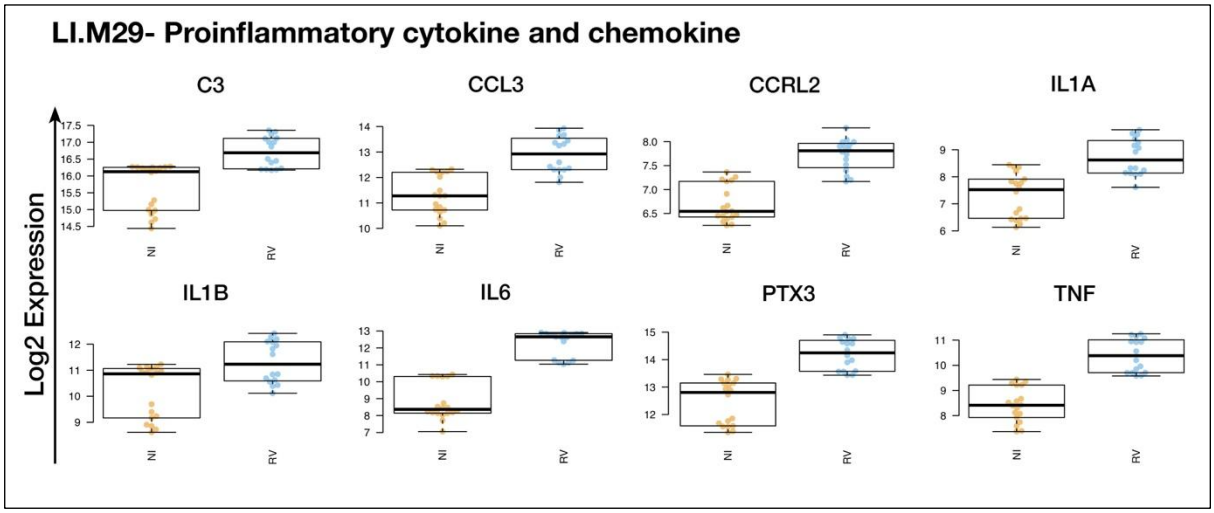

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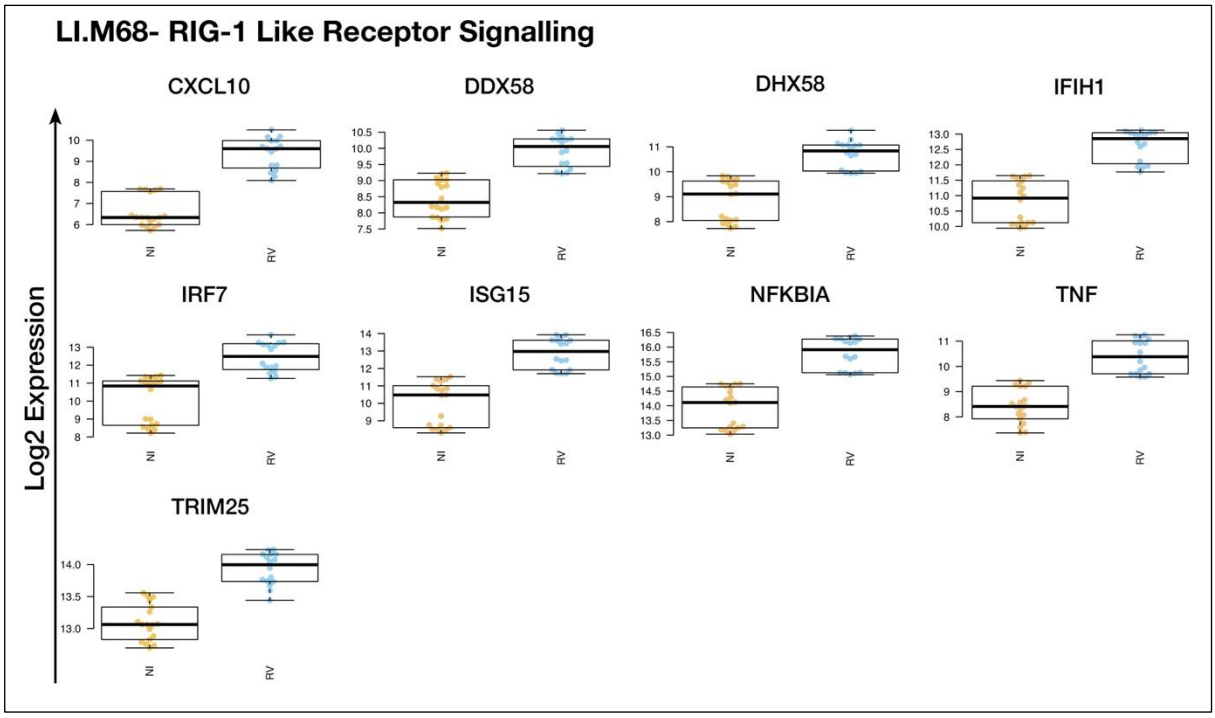

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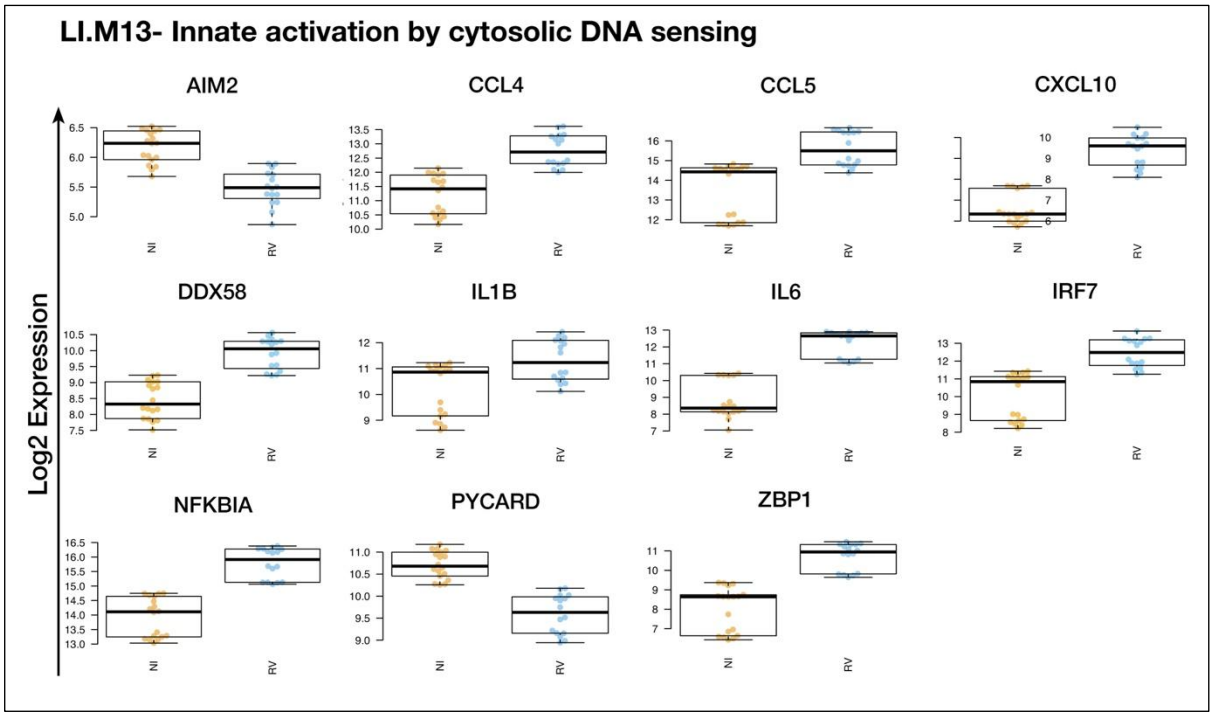

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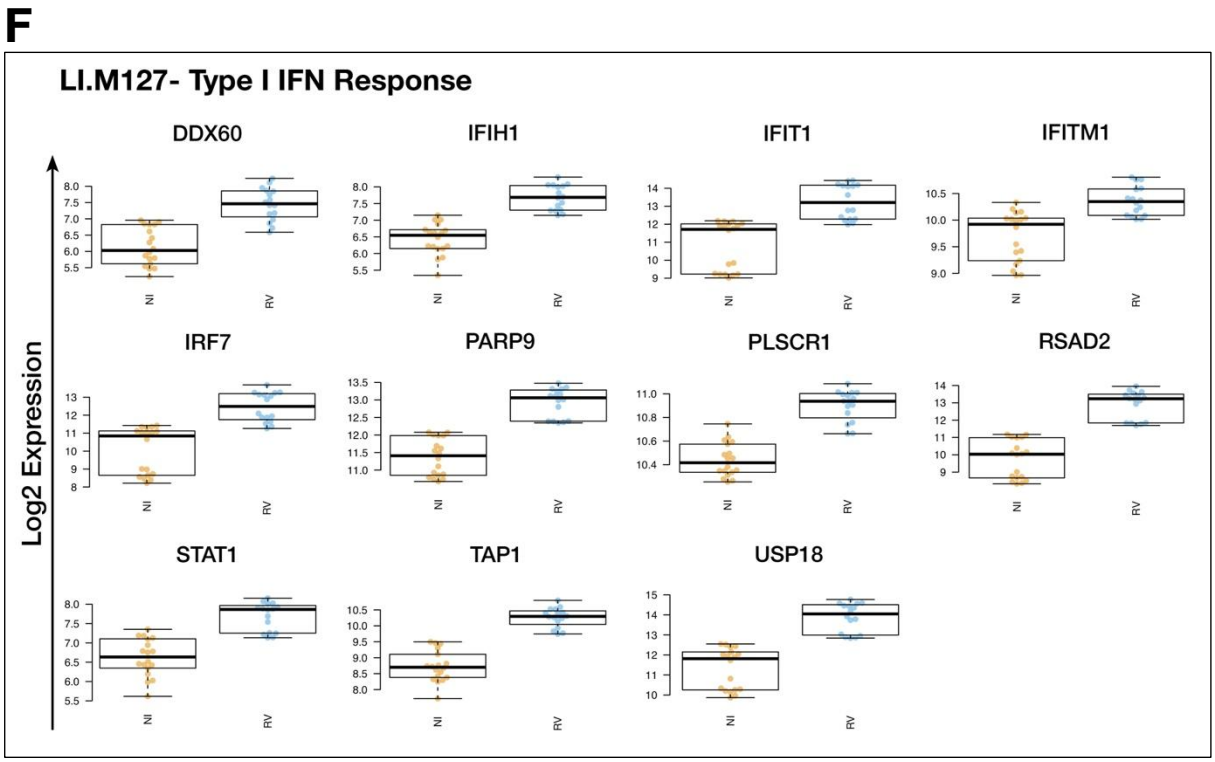


Figure 4.5 | Gene expression within modules upregulated in astrocytes during *M. tuberculosis* infection. (A) Graph indicating Log2 fold changes of DEGs in M. tuberculosis infected astrocytes and the modules with which they associate. The most significantly enriched module "LI.M27 Chemokine cluster (I)" (Total module effect size 0.81 and adj. p value 5.94 x 10^{-14}) was selected as well the four most enriched modules, namely "LI.M68 RIG-1 like receptor signalling" (Total module effect size: 0.98 and adj. p value: 1.18 x 10^{-8}), "LI.M13- Innate activation by cytosolic DNA sensing" (Total module effect size 0.97 and adj. p value 1.46 x 10^{-9}), "LI.M29 Proinflammatory cytokines and chemokines" (Total module effect size 0.97 and adj. p value 4.47 x 10^{-6}), and "LI.M29 Proinflammatory cytokines and chemokines" (Total module effect size 0.97 and adj. p value 4.47 x 10^{-6}). Positive and negative values signify gene up- and down-regulation, respectively. Modules are denoted by a colour code; gene IDs are displayed on the y-axis; and the Log2FC as a measure of gene expression is shown on the x-axis. Box plots of significant DEGs ($p < 0.05$) were constructed for modules: (B) "Chemokine cluster (I)", (C) "Proinflammatory cytokines and chemokines", (D) "RIG-1 like receptor signalling", (E) "Innate activation by cytosolic DNA sensing", and (F) "Type I IFN response". Plotted are Log2 expression levels of M. tuberculosis-infected and uninfected (NI) astrocytes. Box plots exhibit median with first and third quartile, whiskers extend to the outermost data point.

4.4 Astrocytes express genes with neuroinflammatory and neuroprotective function in response to *M. tuberculosis*

Differential expression of individual genes was assessed, particularly those that coded for chemokines and cytokines as their transcripts were visibly enhanced in the enrichment data. To date, the only published chemokine expressed in astrocytes in response to *M. tuberculosis* is CXCL10 (Rock et al., 2008). The data reported here corroborated this earlier report as it showed an increase in *Cxcl10* expression. The

findings reported here are novel and significantly expands on the chemokine range astrocytes can produce in response to mycobacterial infection.

Cxcl1 had the highest upregulated fold change in gene expression with a logFC of 5.11, whilst *Cxcl5*, *Ccl7*, *Cxcl2*, *Cxcl3* and *Ccl2* were ranked in the top twenty DEGs with a logFC of 4.06, 3.99, 3.43, 3.13 and 3.02 respectively (Table 4-1). These findings demonstrate the potential of astrocytes to generate chemotactic signals to induce cellular recruitment following *M. tuberculosis* infection. In addition to chemokines, *M. tuberculosis* is documented to induce cytokine expression in various cell types. These include but are not limited to TNF, ILs and IFNs. *Tnf* a notable Th1-cytokine crucial for *M. tuberculosis* control in CNS-TB (Francisco et al., 2015, Hsu et al., 2017, Tsenova et al., 1999) displayed a 1.97 logFC increase in *M. tuberculosis* infected astrocytes compared to control cells (Table 4-2). Equally relevant to host control of *M. tuberculosis*, are ILs especially IL-1α and IL-1β which mediate a number of cellular immune activities in response to infection (Bourigault et al., 2013). A 1.44, 1.15 and 0.38 logFC was observed for *IL1a*, *IL1b*, and *IL12b* (encoding the IL12p40 subunit) expression, respectively, suggesting their possible participation in astrocyte mediated innate host responses. Interestingly *IL6*, illustrated a logFC increase of 3.48 (Table 4-2), making it the most differentially expressed cytokine transcript. Juxtaposing this was the unexpected absence of modifications in *Il10* expression, a cytokine typically recognized for its importance in regulating immune responses during *M. tuberculosis* infection (Redford et al., 2011). Another regulator of inflammation was identified, namely *Il19*, which had a 2.60 logFC increase in expression.

IFNs are cytokines typically produced by human and mouse macrophages, neutrophils, and DCs (Donovan et al., 2017, Remoli et al., 2002, Stanley et al., 2007, Novikov et al., 2011, Berry et al., 2010). Multiple IFN-related signalling pathways were elevated during *M. tuberculosis* infection which is in line with current dogma (Desvignes et al., 2012, Moreira-Teixeira et al., 2018, Berry et al., 2010). A 2.11 logFC upregulation of *Ifnb1* was detected in infected astrocytes supporting the type I IFN signature described earlier (Table 4-2). The prominent expression of genes encoding the guanylate-binding proteins (GBPs), particularly *Gbp4*, *Gbp5*, *Gbp6*, and *Gbp11* (See Appendix, Fig. S-2),

was another interesting finding given that GBPs are a group of IFN-inducible GTPases involved in cell-autonomous immunity and host defence against intracellular pathogens (Pilla-Moffett et al., 2016, Man et al., 2017). Type I IFNs and associated immune response networks elicit both protective and pathological outcomes during *M. tuberculosis* infection (Moreira-Teixeira et al., 2018). Whether the type I IFN signature observed in astrocytes is associated with TB disease pathogenesis is yet to be determined and requires further investigation.

Interestingly, *M. tuberculosis* infected astrocytes induced various complement component transcripts, particularly *C1ra*, *C1rb*, *C1s1*, *C1s2*, and *C3* (Table 4-3), known to play important roles in inflammation and host defence. Complement system signalling was shown to be associated with active TB (Cliff et al., 2013, Cai et al., 2014). In a genomic study by Zamanian et. al., upregulation of the complement factors *C1r*, *C1s*, *C3* and *C4* were reported in astrocytes following LPS treatment (Zamanian et al., 2012). They linked these factors to an A1 (inflammatory) astrocyte phenotype, a state postulated to aggravate neurodegeneration through synapse loss (Zamanian et al., 2012). Astrocytes may contribute to a regulated inflammatory response that leads to mycobacterial resolution and as part of this process secrete neurotrophic factors such as neurotrophins and growth factors that facilitate neuronal survival (Pöyhönen et al., 2019, Jean et al., 2008, Emsley and Hagg, 2003, Gómez-Casati et al., 2010, Béjot et al., 2011, Vignoli et al., 2016, Albrecht et al., 2002, Liu et al., 2017). There was a notable upregulation in astrocyte gene expression of various neurotrophic, anti-inflammatory and growth factors which are known to minimise neuronal death and ultimately limit tissue damage (Table 4-4). Increased gene expression of the neuropoietic cytokines *Lif* (Leukaemia inhibitory factor) and *Il6* was detected and together with *Ptx3* are proposed as being protective (Suzuki et al., 2009, Suzuki et al., 2005, Davis and Pennypacker, 2018, Jung et al., 2011, Rajkovic et al., 2018). *Gdnf* (glial derived neurotrophic factor) and *Artn* (artemin), belonging to the GDNF family of ligands, also displayed increased expression. Several growth factors were induced, particularly: *Igfbp* (insulin-like growth factor binding protein), *Vegf* (vascular endothelial growth factor), *Pdgfb* (platelet derived growth factor), *Ngfr* (nerve growth factor receptor), *Nrg1* (neuregulin 1), and *Nrg2* (neuregulin2). Functions of these factors were discussed in the previous chapter. These

factors all play a role in the maintenance and development of neurons as well as neuroprotection (Table 4-4). GDNF and ARTN, modulate neuroinflammation and promote the survival of neurons (Eugenin et al., 2003, Madrigal et al., 2009, Kotliarova and Sidorova, 2021, Duarte Azevedo et al., 2020, Chen et al., 2015, Rocha et al., 2012). IGFBP enhances the neuroprotective effects of IGF-1 (Yao et al., 1995, Breese et al., 1996, Logan et al., 1994), while VEGF and PDGFB promote neuroprotection and angiogenesis (Krupinski et al., 1997, Arimura et al., 2012, Cheng and Mattson, 1995, Risau, 1997, Osborne et al., 2016, Nishijima et al., 2007, Foxton et al., 2013, Sun et al., 2003). Ngfr is involved in the development, differentiation, and maintenance of neurons, Nrg1 and Nrg2 play roles in neural development, synaptic plasticity, axonal regulation, and neuroprotection (Levi-Montalcini, 1987, Aloe and Calzà, 2003, Aloe et al., 2015, Balzamino et al., 2015, Li et al., 2007, Noll et al., 2019, Nave and Salzer, 2006, Longart et al., 2004, Nakano et al., 2016). Thus, based on this expression profile it is evident that *M. tuberculosis*-infected astrocytes perform neuroprotective functions which can in part be attributed to their production of neurotrophic and growth factors.

These results demonstrated that *M. tuberculosis* infection of astrocytes leads to the upregulation of specific chemokines, cytokines, and type I IFN genes. The range of chemokines induced in astrocytes in response to mycobacterial infection, notably *Cxcl1*, *Cxcl2*, *Cxcl3, Cxcl5*, *Ccl2* and *Ccl7,* and suggests the potential for astrocytes to generate chemotactic signals for cellular recruitment. Additionally, complement component transcripts were induced in infected astrocytes, indicating the involvement of the complement system in inflammation and host defence. Through the expression of *Il1* and *Tnf,* astrocytes may contribute to a regulated inflammatory response that leads to mycobacterial resolution and as part of this process secrete neurotrophic factors such as neurotrophins and growth factors to facilitate neuronal survival. These neuroprotective factors may play a crucial role in mitigating the damage caused by neuroinflammation during CNS-TB infection. Overall, the findings provide insight into the complex immune response in the central nervous system during *M. tuberculosis* infection and highlights the potential therapeutic targets for mitigating neurodegeneration. Further investigation is needed to determine the exact role of these immune response networks in astrocytes during TB pathogenesis.

Table 4-1 | Chemokines induced by astrocytes during *M. tuberculosis* infection

Gene Symbol	Gene Title	LogFC	Adj. P Val	Function in Tuberculosis	Reference
Ccl2 (Mcp1)	Chemokine (C-C motif) ligand 2	3.02	1.16e-07	Granuloma formation. T cell and monocyte recruitment.	(Hasan et al., 2009, Saunders and Britton, 2007, Penido et al., 2003, Hilda et al., 2020)
Ccl3 (Mip1a)	Chemokine (C-C motif) ligand 3	1.62	4.24e-07	Inhibits *M. tuberculosis* growth. Monocyte recruitment.	(Saukkonen et al., 2002, Hilda et al., 2020)
Ccl4 (Mip1b)	Chemokine (C-C motif) ligand 4	1.57	6.39e-08	Inhibits *M. tuberculosis* growth	(Saukkonen et al., 2002)
Ccl5 (Rantes)	Chemokine (C-C motif) ligand 5	2.19	6.02e-06	Inhibits *M. tuberculosis* growth. Granuloma formation	(Saukkonen et al., 2002)
Ccl7 (Mcp3)	Chemokine (C-C motif) ligand 7	3.99	3.74e-09	Chemotactic activity for macrophages	(Scott, 2002, Menten et al., 2002)
Ccl8 (Mcp2)	Chemokine (C-C motif) ligand 8	1.21	6.40e-07	Chemotactic factor for T cells.	(Liu et al., 2013)
Ccl17 (Tarc)	Chemokine (C-C motif) ligand 17	1.52	2.43e-10	Chemotactic activity for eosinophils and DCs. Polarisation of Tregs and Th2 cells	(Lasco et al., 2004, Feng et al., 2011)
Ccl19	Chemokine (C-C motif) ligand 19	0.23	6.6e-03	Triggers DC accumulation in the draining lymph node. Granuloma formation. T cell priming and activation.	(Torres-Bacete et al., 2015, Wolf et al., 2007, Khader et al., 2009)
Cxcl1 (Kc/ Mgsa-a)	Chemokine (C-X-C motif) ligand 1	5.11	7.64e-11	Regulates production of antimicrobial peptides (beta-defensins). Inflammatory role and triggers iNOS production. NLRP3 Inflammasome activation.	(Boro and Balaji, 2017, Boro et al., 2016)
Cxcl2 (Mip2a)	Chemokine (C-X-C motif) ligand 2	3.43	9.18e-12	Regulates production of antimicrobial peptides (beta-defensins). Inflammatory role and triggers iNOS production. NLRP3 Inflammasome activation.	(Boro and Balaji, 2017, Boro et al., 2016)
Cxcl3 (Gro3)	Chemokine (C-X-C motif) ligand 3	3.13	2.31e-11	Regulates lipid metabolism. Role in inflammation and chemoattractant for neutrophils	(Chen et al., 2022, de Oliveira et al., 2016)
Cxcl5 (Ena78)	Chemokine (C-X-C motif) ligand 5	4.06	1.31e-10	Granulocyte recruitment.	(Nouailles et al., 2014, Gopalakrishnan et al., 2019)

Cxcl9 (MIG)	Chemokine (C-X-C motif) ligand 9	1.35	4.53e-08	Granuloma formation. Chemoattractant for T cells and NK cells	(Seiler et al., 2003, Lande et al., 2003)
Cxcl10	Chemokine (C-X-C motif) ligand 10	2.77	9.18e-12	Inhibition of mycobacterial replication. Chemoattractant for T cells and NK cells	(Palucci et al., 2019, Lande et al., 2003)
CXCL12 (SDF1)	Chemokine (C-X-C motif) ligand 12	1.29	5.85e-07	Granuloma formation. Chemoattractant for T cells and neutrophils	(Rocca et al., 2013, Slight and Khader, 2013, Rawat et al., 2018)
Cxcl16	Chemokine (C-X-C motif) ligand 16	0.71	3.15e-07	Chemoattractant for T cells	(Lee et al., 2011)
Cx_3cl1	Chemokine (C-X3-C motif) ligand 1	1.44	1.18e-10	Monocyte recruitment.	(Hingley-Wilson et al., 2014)

Table 4-2 | Cytokines induced by astrocytes during *M. tuberculosis* infection

Gene Symbol	Gene Title	LogFC	Adj. P Val	Function in Tuberculosis	Reference
Il1a	Interleukin 1 alpha	1.44	4.94e-06	Mediates immune responses, cellular recruitment	(Silvério et al., 2021, Cooper et al., 2011, Domingo-Gonzalez et al., 2016, O'Garra et al., 2013)
Il1b	Interleukin 1 beta	1.15	0.001	Inflammatory response mediator. Cellular recruitment. Critical for host resistance to *M. tuberculosis*	(Silvério et al., 2021, Cooper et al., 2011, Domingo-Gonzalez et al., 2016, O'Garra et al., 2013, Mayer-Barber et al., 2010, Bourigault et al., 2013, Juffermans et al., 2000, Fremond et al., 2007)
Il6	Interleukin 6	3.48	1.13e-11	Essential for protective immunity against *M. tuberculosis.* Early T cell differentiation and recruitment.	(Boni et al., 2022, Dienz and Rincon, 2009, Ladel et al., 1997, Leal et al., 1999, Hilda et al., 2020)
Il12b	Interleukin 12b	0.38	0.006	Essential for protective immunity against *M. tuberculosis.* Th1 priming	(Robert and Miossec, 2021, Orme and Basaraba,

				and activation. Controlling bacterial growth	2014, Jo et al., 2003, Cooper et al., 1997, Leal et al., 1999)
Il18	Interleukin 18	0.45	0.0002	Essential for protective immunity against *M. tuberculosis.* Th1 cell activation. Involved in IFN-γ production	(Schneider et al., 2010, Wawrocki et al., 2020, Sugawara et al., 1999)
Il19	Interleukin 19	2.60	4.65e-08	Modulates T cell function	(Kumar et al., 2018)
IL34	Interleukin 34	1.14	1.24e-09	Regulates Tregs. Monocyte and macrophage proliferation and differentiation.	(Bézie et al., 2015, Wei et al., 2010, Lin et al., 2008, Foucher et al., 2013)
Ifnb1	Interferon beta 1, fibroblast	2.11	1.28e-08	Regulates IL1β production	(Novikov et al., 2011, Mayer-Barber et al., 2011, Sousa et al., 2020)
Lif	Leukaemia inhibitory factor	1.73	6.75e-12	Elevated in TB, possibly modulates inflammatory responses and T-cell lineage maturation.	(La Manna et al., 2018, Metcalfe, 2011, Zhang et al., 2021)
Tnf	Tumour necrosis factor	1.97	1.37e-09	Essential for protective immunity against *M. tuberculosis.* Macrophage activation and granuloma formation. Essential for protective immunity against *M. tuberculosis*. Influence chemokine expression.	(Flynn et al., 1995, Bean et al., 1999, Flynn and Chan, 2001, Jo et al., 2003, Orme, 1993, O'Garra et al., 2013, Chakravarty et al., 2008, Kaneko et al., 1999, Jacobs et al., 2007)

Table 4-3 | Complement factors induced by astrocytes during *M. tuberculosis* infection

Gene Symbol	Gene Title	LogFC	Adj. P Val	Function in Tuberculosis	Reference
C1ra	Complement component 1r	1.53	1.24e-09	Classical pathway of the complement system by forming the C1 complex. Component of innate immunity.	(Lu and Kishore, 2017, Mortensen et al., 2017)
C1rb	Complement component 1r	1.36	3.98e-10	Classical pathway of the complement system by forming the C1 complex.	(Lu and Kishore, 2017,

				Component of innate immunity.	Mortensen et al., 2017)
C1s1	Complement component 1s	0.92	9.35e-08	Associates with C1r and C1q, to form C1 complex. Component of innate immunity.	(Lu and Kishore, 2017, Mortensen et al., 2017)
C1s2	Complement component 1s	0.92	3.04e-08	Associates with C1r and C1q, to form C1 complex. Component of innate immunity.	(Lu and Kishore, 2017, Mortensen et al., 2017, Jagatia and Tsolaki, 2021)
C3	Complement component 3	1.08	1.30e-05	Component of innate immunity. Opsonises *M. tuberculosis*	(Jagatia and Tsolaki, 2021, Ferguson et al., 2004, Schlesinger et al., 1990)

Table 4-4 | Factors with neurotrophic properties induced by astrocytes during *M. tuberculosis* infection

Gene Symbol	Gene Title	LogFC	Adj. P Val	Function in the CNS	Reference
Artn	Artemin	0.97	1.04e-09	Glial cell line derived neurotrophic factor. Survival and growth of neurons. Modulates neuroinflammation.	(Eugenin et al., 2003, Madrigal et al., 2009, Kotliarova and Sidorova, 2021, Duarte Azevedo et al., 2020)
Ccl2 (Mcp1)	Chemokine (C-C motif) ligand 2	3.02	1.16e-07	Neurotransmission and neuronal cell survival. Astrocyte survival. Inhibits glutamate-induced neuronal apoptosis.	(Madrigal et al., 2009, Eugenin et al., 2003, Edman et al., 2008, Bruno et al., 2000, Guyon et al., 2009, Brunet et al., 2001, Réaux-Le Goazigo et al., 2013)
Cx3cl1	Chemokine (C-X3-C motif) ligand 1	1.44	1.18e-10	Neuroprotection. Promotes neuron survival and reduces excitotoxicity.	Deiva et al., 2004, Limatola et al., 2005, Meucci et al., 1998, Tong et al., 2000)
Cxcl16	Chemokine (C-X-C motif) ligand 16	0.71	3.15e-07	Acts on astrocytes to release neuroprotective soluble factors. Neuroprotective against glutamate-excitotoxicity.	(Rosito et al., 2012, Trettel et al., 2020, Rosito et al., 2014)
Fgf1	Fibroblast growth factor 1			Neuroprotection	(Russell et al., 2006, Everall et al., 2001, Ghazavi et al., 2017)

Fgf2	Fibroblast growth factor 2			Glia activating factor. Involved in neurogenesis, axonal growth, neuroprotection and regeneration.	(Kang et al., 2014, Noda et al., 2014, Belluardo et al., 2000, Jin et al., 2005, Li et al., 2010)
Fgf7	Fibroblast growth factor 7			Neuron survival. Inhibitory synapse formation.	(Lee et al., 2012, Dabrowski et al., 2015, Klimaschewski and Claus, 2021, Terauchi et al., 2010)
Fgf9	Fibroblast growth factor 9			Glia activating factor. Promotes neuron survival and protection.	(Kang et al., 2014, Kanda et al., 2000, Kinkl et al., 2003)
Gdnf	Glial cell line derived neurotrophic factor	0.94	5.40e-06	Inhibits microglial activation and modulates inflammation. Neuroprotective during inflammation.	(Kotliarova and Sidorova, 2021, Duarte Azevedo et al., 2020, Chen et al., 2015, Rocha et al., 2012)
Ifnb1	Interferon beta 1	2.11	1.28e-08	Neuroprotective	(Liu et al., 2002, Marsh et al., 2009, Longhi et al., 2011, Khorooshi et al., 2015)
Igf2bp2	Insulin-like growth factor binding protein-2	0.90	5.42e-08	Neuroprotection. Enhances biological and neuroprotective action of IGF-1.	(Yao et al., 1995, Breese et al., 1996, Logan et al., 1994)
Igf2bp3	Insulin-like growth factor binding protein 3	-0.38	3.44e-05	Blocks IGF-1, a neurotrophic and neuroprotective peptide	(Carro et al., 2003, Logan et al., 1994, Walter et al., 1997, Titone et al., 2019)
Il6	Interleukin 6	3.48	1.13e-11	Promotion of neuronal survival and regeneration. Enhances NGF release.	(Loddick et al., 1998, Hirota et al., 1996, Kumar et al., 2010, Fujita et al., 2009, Sun et al., 2017, Longhi et al., 2011, Morganti-Kossman et al., 1997)
Il19	Interleukin 19	2.38	1.45e-08	Anti-inflammatory signalling and neuroprotection.	(Xie et al., 2016, Gallagher et al., 2004, Cooley et al., 2014)
Lif	Leukaemia inhibitory factor	1.73	6.75e-12	Neurotrophin, neuroprotection and axon growth	(Janssens et al., 2015, Marriott et al., 2008, Butzkueven et al., 2002, Davis et al.,

					2019, Ajmo Jr et al., 2006)
Ngfr	Nerve growth factor receptor	0.41	0.01	Development, differentiation and maintenance of neurons	(Levi-Montalcini, 1987, Aloe and Calzà, 2003, Aloe et al., 2015, Balzamino et al., 2015)
Nrg1	Neuregulin 1	0.57	1.01e-07	Neural development, neuroprotection, axonal regulation and synaptic plasticity.	(Li et al., 2007, Noll et al., 2019, Xu et al., 2005, Nave and Salzer, 2006, Mei and Xiong, 2008)
Nrg2	Neuregulin 2	-0.31	3.75e-05	Neural development, synaptic plasticity and neuroprotection.	(Longart et al., 2004, Nakano et al., 2016)
Pdgfb	Platelet derived growth factor, B	0.96	5.05e-10	Neuroprotection and angiogenesis	(Krupinski et al., 1997, Arimura et al., 2012, Cheng and Mattson, 1995, Risau, 1997, Osborne et al., 2016)
Ptx3	Pentraxin 3	1.74	3.15e-08	Neuron survival and enhancing neurogenesis. BBB integrity	(Zhou et al., 2020, Shindo et al., 2016, Rajkovic et al., 2018)
Socs1	Suppressor of cytokine signalling 1	1.00	3.62e-07	Neuroprotective. Suppressors of cytokine signalling-Inhibits neuroinflammation	(Xiao-Lei et al., 2018, Wang et al., 2021, Dragone et al., 2014, Park et al., 2003)
Tnf	Tumour necrosis factor	1.97	1.37e-09	Improve neuronal survival. Astrocyte proliferation.	(Jankowsky and Patterson, 2001, Barna et al., 1990)
Vegfa	Vascular endothelial growth factor A	0.37	0.01	Neuroprotection, neurogenesis and angiogenesis	(Nishijima et al., 2007, Foxton et al., 2013, Sun et al., 2003)
Vegfc	Vascular endothelial growth factor C	1.10	4.50e-10	Neuroprotection and angiogenesis	(Piltonen et al., 2011, Le Bras et al., 2006)

4.5 KEGG pathway mapping demonstrates signalling pathway enrichment in astrocytes during *M. tuberculosis* infection

The biological functioning of any cell is dependent on global gene expression and the interaction of various molecules. With functional analyses, pathways of differentially

expressed genes can be explored and predictions of biological outcomes made. To gain insight on the molecular interactions and resultant pathway perturbations in astrocytes during *M. tuberculosis* infection, pathway topology analysis was performed using SPIA (Signalling Pathway Impact Analysis) (Tarca et al., 2009) and mapped onto the Kyoto Encyclopaedia of Genes and Genomes (KEGG) reference pathways (Ogata et al., 1999). The net accumulated perturbation of a pathway (tA) was determined by calculating the sum of all perturbations for every gene in each pathway. If the accumulated tA value was positive, the pathway was considered as activated; whereas if the value was negative the pathway was considered as inhibited. A total of sixty-nine KEGG pathways showed significant perturbation in astrocytes at 24h during *M. tuberculosis* infection (adj. $p < 0.05$). The top twenty most significant pathways are shown in Figure 4.6 (See Appendix, Table S-3 for details on each pathway). The x-axis represents the net accumulated pathway perturbation, and the y-axis indicates the reference pathways. Dot size depicts the number of differentially expressed genes within the individual pathways and the dot colour is an indication of the adjusted p value. The "cytokine-cytokine receptor" pathways displayed the most significant and greatest pathway perturbation, while the "chemokine signalling pathway" showed the second highest perturbation. Interestingly the "osteoclast differentiation" pathway displayed the second most significant perturbation. Although this pathway is primarily involved in bone metabolism and bone remodelling, it can interact with immune factors and signalling pathways, particularly those involved in the regulation of inflammation. For example, several pro-inflammatory cytokines, such as TNF, IL-1 and IL-6, that activate the immune response are also involved in the osteoclast differentiation pathway (Amarasekara et al., 2018, Wei et al., 2005, Gao et al., 1998, Kitaura et al., 2020). For further analysis the activated "tuberculosis" pathway was selected (Fig. 4.7). This pathway showed 71 differentially expressed genes, a total perturbation of 21.18 and an adj. p value of $1{,}11 \times 10^{-6}$. Various pathogen recognition receptor (PRR) genes such as Mincle, Dectin-1 and TLRs (toll like receptors) were differentially expressed. TLRs are a particular group of PRRs known to play a critical role in the activation of immune responses during *M. tuberculosis* infection. Two of the most recognized TLRs in TB innate immunity are TLR2 (Reiling et al., 2002, Stenger and Modlin, 2002) and TLR4 (Abel et al., 2002, Quesniaux et al., 2004). Although *Tlr4* gene expression was stable, differential expression of *Tlr2* and the subsequent activation of

the Myd88 pathway were observed. Furthermore, notable activation was observed in the pathways linked to tuberculosis, which were among the top twenty pathways identified (Fig. 4.6). These included the "toll-like receptor signalling pathway", "NOD-like receptor signalling pathway", "MAPK signalling pathway", "NF-kappa B signalling pathway", and the "apoptosis" pathway. Downstream from these pathways was the apparent differential expression of several inflammatory cytokines, including *Tnf*, *Il6*, *Il12*, *Il18*, *Il1* and *Ifnb*. Activation of viral, parasite and bacterial pathways was unsurprising and likely correspond to the upregulation of common intracellular genes. An interesting finding was the differential gene expression of the host defence peptide, cathelicidin. These peptides are usually found in innate immune cells and function as antimicrobial and immunomodulatory agents (Van Harten et al., 2018). It is evident that during *M. tuberculosis* infection, various PRRs are enriched in astrocytes, and the activation of the conserved tuberculosis pathway demonstrates that astrocytes induce a higher order biological function for host protection against tuberculosis.

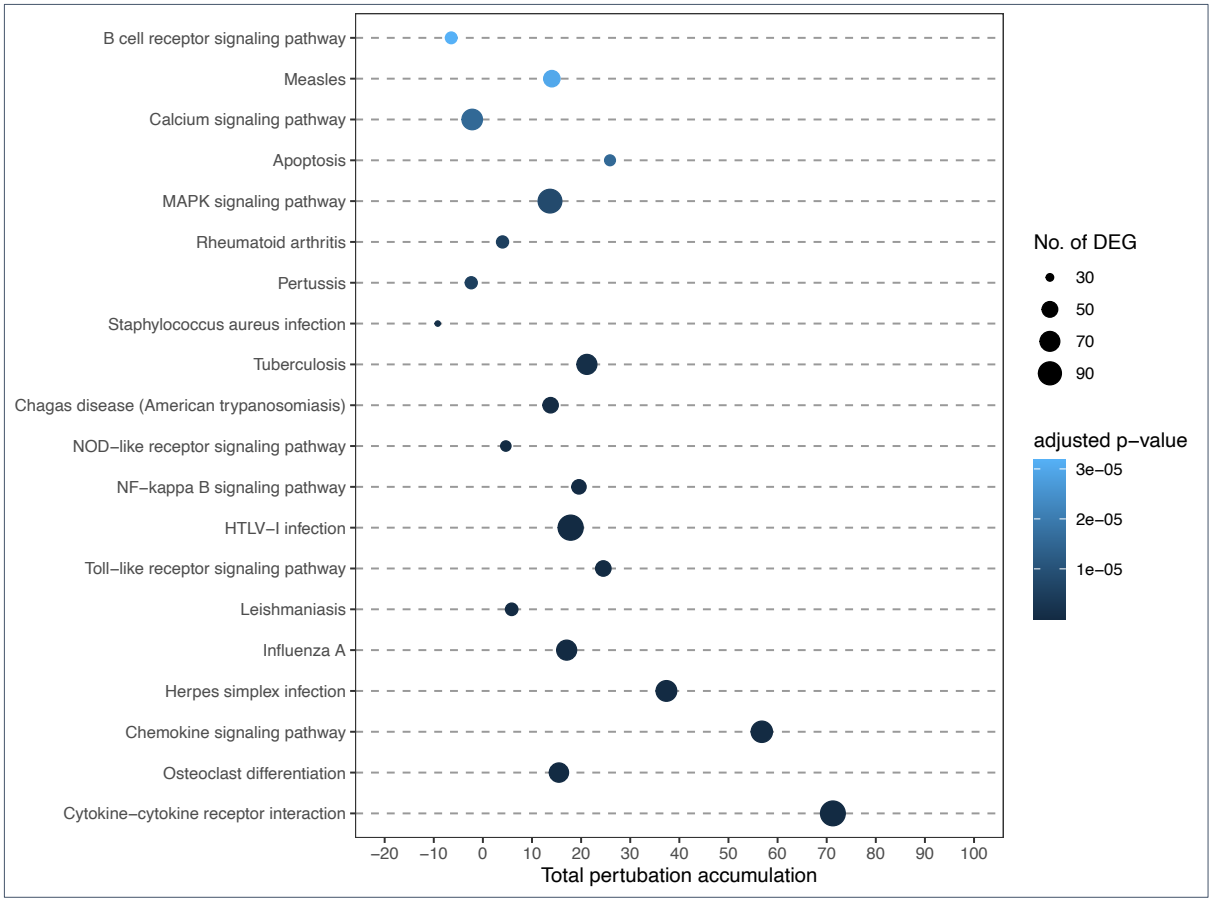


Figure 4.6 | Top 20 KEGG most significantly enriched pathways in astrocytes during *M. tuberculosis* infection. Pathways are displayed on the y-axis. The x-axis indicates the total pathway perturbation (tA), the size of the dot represents the number of differentially expressed genes within the reference pathway, and the colour represents the adjusted p value.

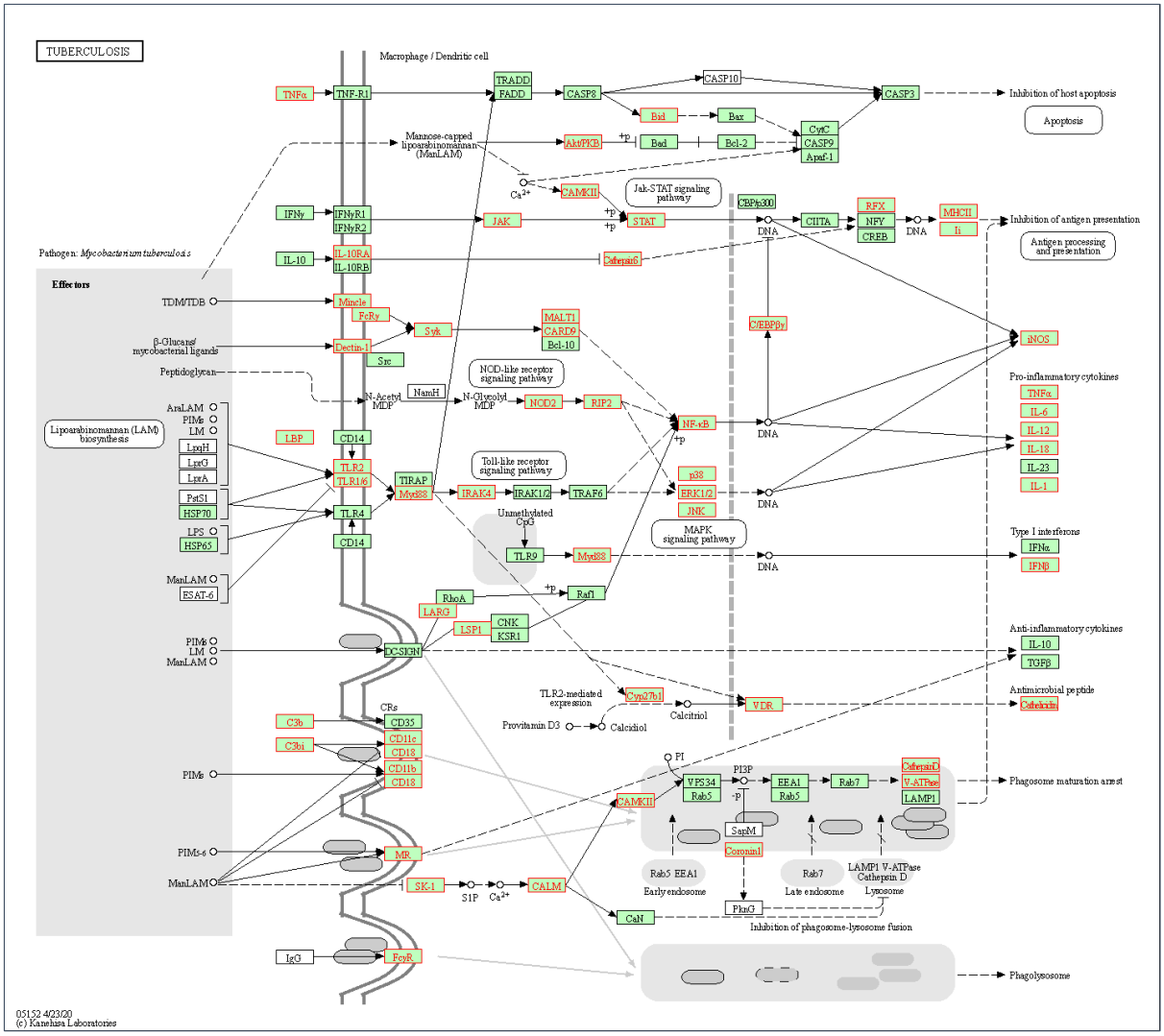


Figure 4.7 | Tuberculosis pathway diagram of astrocytes during *M. tuberculosis* infection. Red indicates differentially expressed genes, black indicates stable gene expression (adj. $p < 0.05$).

4.6 Astrocytes contribute to the cytokine and chemokine protein milieu in response to *M. tuberculosis* infection *in vitro*

Although transcriptomic data is a useful tool to analyse cellular behaviour, the observed signature does not always correlate with secreted protein (Brion et al., 2020, Carlyle et al., 2017, Franks et al., 2017). Thus, as validation of the transcriptomic data studies were performed to quantify specific proteins that were secreted by astrocytes in response to *M. tuberculosis* infection. Proteins of interest were selected based on the transcriptome database and Luminex analysis was performed on the supernatant of uninfected and *M.*

tuberculosis-infected astrocyte cultures. Analytes were selected based on their immune activity and importance to host responses during *M. tuberculosis* infection.

An MOI 10 was selected for infection, 24h and 48h were chosen as time points, and astrocyte sample numbers were increased to 5 x 10^5 cells as experimental conditions for analysis. Following infection, supernatants of infected and uninfected groups were collected at 24h and 48h and analytes measured (Figure 4.8). For the purpose of analysis, each group was composed of five individual samples that were then pooled together for each separate experiment. This means that each data point on the graph represents the average of five individual samples from a single experiment. CXCL10 was the only chemokine that showed a significant ($p<0.05$) upregulation at 24h in *M. tuberculosis*-infected compared to uninfected samples. Cytokine levels of TNF, IL-1β, IL-10, IL-6, as well the chemokines CXCL5, CXCL1, CXCL2, CCL5, CCL3, and CCL2 were not significantly higher than controls at 24h post-infection, but there was a visible trend toward significance. All analytes showed a significant increase at 48h in *M. tuberculosis* infected cells compared to the uninfected control cells. No differences were observed in the control cells at 24h and 48h. The purpose of this analysis was to determine if *M. tuberculosis* caused a significant increase in protein secretion from astrocytes compared to the control group. However, concentrations of CXCL2 at 24h post-infection as well as CXCL1 and CXCL2 at 48h post-infection were unexpectedly higher than the other analytes of the multiplex assay and beyond the quantifiable range of the absorbance values. The concentrations of CXCL2 at 24h and CXCL1 and CXCL2 at 48h post-infection (Fig. 4.8) were estimate values from the highest standard concentrations. The highest detectable point on the standard curve represents the highest measurable concentration of that specific protein. Therefore, since the actual concentrations were higher than the standard values, it would be reasonable to use these values as an indication of a change in protein production compared to the control group.

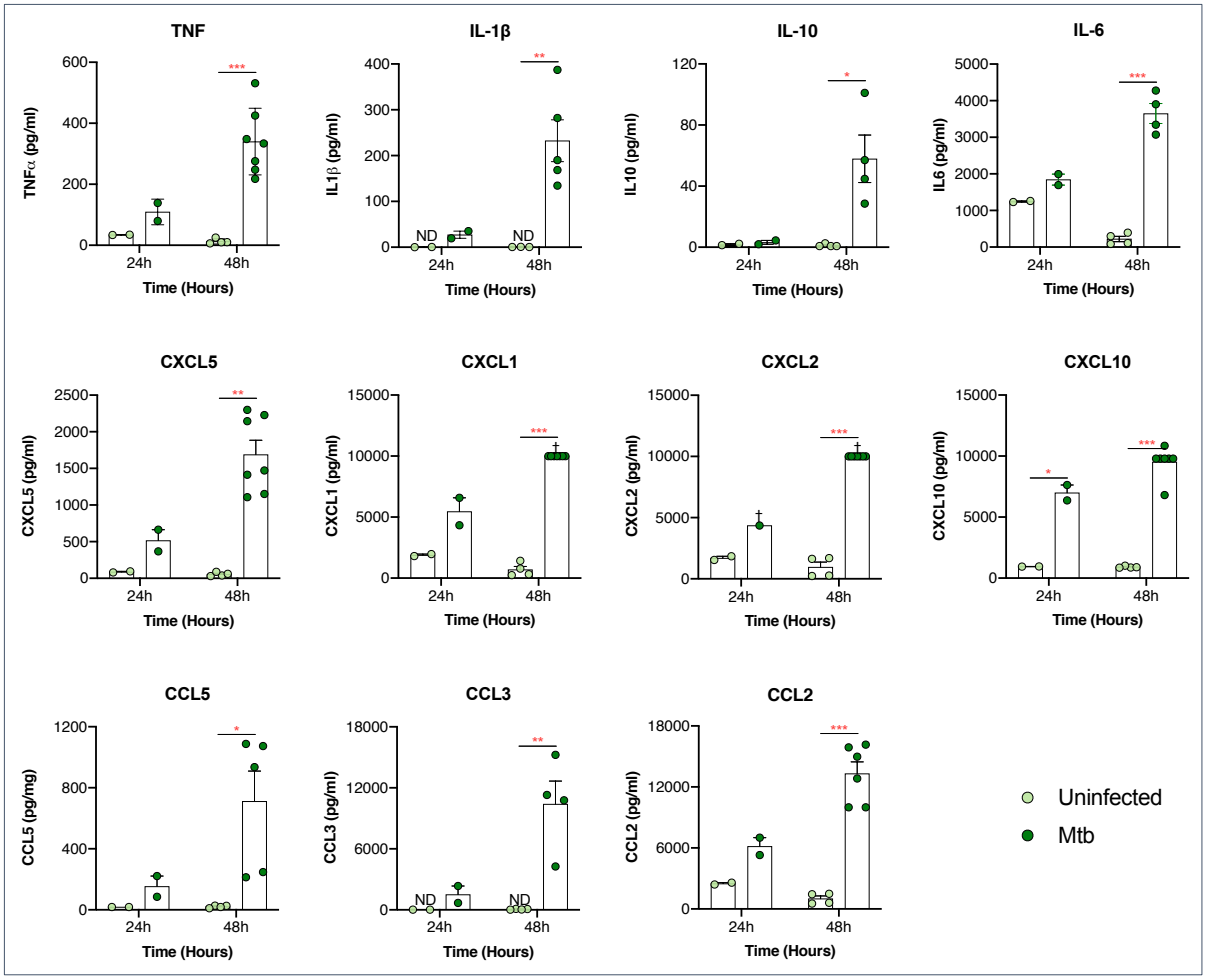


Figure 4.8 | Astrocytes induce an immune response during *M. tuberculosis* infection. Primary astrocytes were infected with *M. tuberculosis*-H37Rv at MOI 10 for 24h and 48h or left uninfected. Supernatants of control and infected groups were collected and analysed for TNF, IL-1β, IL-10, IL-6, CXCL5, CXCL1, CXCL2, CXCL10, CCL5, CCL3, and CCL2. Analytes were measured by Luminex assay. In individual experiments, each group was composed of five samples that were pooled for analysis. As a result, each data point on the graph represents the pooled value of five samples of the group. Data shown is expressed as the mean ± SEM and is representative of two to seven experiments). Statistical significance was determined (* $p < 0.05$, **$p < 0.01$, *** $p < 0.001$; ND = not detected). †: The highest observed concentration in the standard curve was used as an estimated value for sample concentrations that exceeded the readable range.

Intracellular cytokine staining and flow cytometry analysis are powerful techniques used to evaluate intracellular cytokine expression at a single cell level (Lovelace and Maecker, 2011). This method is widely performed in the fields of immunology and infectious disease for the simultaneous analysis of multiple cytokine signals within specific cells. To confirm cellular cytokine expression in astrocytes, intracellular cytokine staining was employed. This method is advantageous over multi-analyte analysis as flow cytometry enables the analysis of individual cells, permitting the percentage of astrocytes expressing a particular cytokine to be quantified. In addition, multiple parameters can be

analysed. Cell viability and surface marker expression can be measured which is not possible when investigating protein secretions. Furthermore, the quantifiable range is less constrained, and the sensitivity of flow cytometry analysis facilitates the detection of low levels of protein expression which can be particularly advantageous when analysing samples with low protein abundance. Cells were infected with *M. tuberculosis* for 24h, then processed and stained with specific monoclonal antibodies targeted towards selected proteins, and expression was quantified by flow cytometry analysis (Fig. 4.9). A live/dead marker was used to distinguish live cells and primary astrocytes were identified using the ACSA-2 lineage marker. The percentage of ACSA-2$^+$ astrocytes expressing the proteins of interest were then assessed. The cytokines IL-1β, TNF, and IL-12 were selected as each play a critical role in the activation and regulation of immune responses during CNS-TB (Van Crevel et al., 2002, Flynn and Chan, 2001, Domingo-Gonzalez et al., 2016, Davis et al., 2021, Cooper et al., 2011, Bourigault et al., 2013), while iNOS was selected as it is involved in the production of nitric oxide (NO) that has potent antimicrobial effects against *M. tuberculosis* (Landes et al., 2015, Ehrt et al., 2001). In contrast, a notable anti-inflammatory cytokine IL-10, was chosen as it is beneficial in controlling inflammation and limiting tissue damage during CNS infection (Burmeister and Marriott, 2018, Lobo-Silva et al., 2016). The APC surface markers, MHCII and CD86, were selected as markers crucial for antigen presentation and T cell activation (Nikcevich et al., 1997, Girvin et al., 2002).

There was a significant upregulation in the percentage of astrocytes expressing the proinflammatory cytokines IL-1β, TNF, and IL-12 in *M. tuberculosis* infected samples compared to the uninfected control samples ($p < 0.05$) (Fig. 4.9). In addition, the percentage of astrocytes producing iNOS was also markedly increased by *M. tuberculosis* infection. In contrast to the pro-inflammatory cytokines there was an increase in the percentage of astrocytes expressing the anti-inflammatory cytokine, IL-10. This contrasted with the transcriptional data which did not show an increase in *Il10* expression. This discrepancy could be attributed to mRNA instability which is an important post-transcriptional regulatory mechanism to regulate cytokine production (Anderson, 2008, Mino and Takeuchi, 2013). IL-10 mRNA contains multiple copies of the destabilising motifs in the 3' untranslated region (UTR) which targets mRNA for

degradation (Powell et al., 2000, Brown et al., 1996, Kishore et al., 1999, Németh et al., 2005, Saraiva and O'Garra, 2010). Secondly, gene transcription and translation are multi-step, sequential processes where RNA and protein represent different steps and don't necessarily correlate (Wang, 2008, Perl et al., 2017, Guo et al., 2008). It is therefore possible that the IL-10 mRNA was already degraded at the investigated time points, hence its expression was not reflected in the transcriptome. Reports in human and mouse models have demonstrated that increased IL-10 production in response to *M. tuberculosis* is associated with susceptibility to TB (Redford et al., 2011); whereas IL-10 ablation resulted in a reduced pulmonary bacterial burden and improved survival due to restored Th1 function (Jacobs et al., 2000, Beamer et al., 2008). Heightened production of MHC II and CD86 producing astrocytes was observed following *M. tuberculosis* infection. The production of functional proteins by astrocytes supported the transcriptomic data, indicating their involvement in regulating immune activity during *M. tuberculosis* infection. Overall, the findings suggest that astrocytes induce a higher order biological function for host protection against tuberculosis, and the observed signature can be used as a basis for further research on host-pathogen interactions.

This chapter presents compelling evidence that primary murine astrocytes are susceptible to direct infection by *M. tuberculosis* and can support its replication. Notably, the transcriptomic analysis revealed a robust enrichment of IFN-related modules, whereas the "chemokine cluster (I)" and "proinflammatory cytokines and chemokines" modules were found to be the most significantly enriched in *M. tuberculosis*-infected astrocytes. These modules highlight the ability of astrocytes to regulate inflammatory responses, thereby providing immunological support. Further exploration of the molecular interactions and pathway perturbations in astrocytes during *M. tuberculosis* infection displayed significant activation of cytokine-cytokine receptor and tuberculosis pathways. Astrocytes upregulated the expression of various chemokines, cytokines, and complement component transcripts, which likely induce cellular recruitment and activate immune responses. Additional protein analysis confirmed the increased production of pro-inflammatory cytokines IL-1β, TNF, and IL-12, and iNOS, as well as the anti-inflammatory cytokine IL-10, in *M. tuberculosis*-infected astrocytes compared to uninfected controls. Moreover, heightened production of MHC II and CD86-producing

astrocytes was observed following *M. tuberculosis* infection, suggesting their involvement in adaptive immunity. Increases in the expression of neuroprotective factors by astrocytes supports their role in limiting inflammation-induced tissue damage and supporting neuronal function. These findings highlight the importance of astrocytes in shaping immune responses against *M. tuberculosis*, provide insights into their complex activity in the CNS during *M. tuberculosis* infection, and offer potential therapeutic targets for mitigating neurodegeneration.

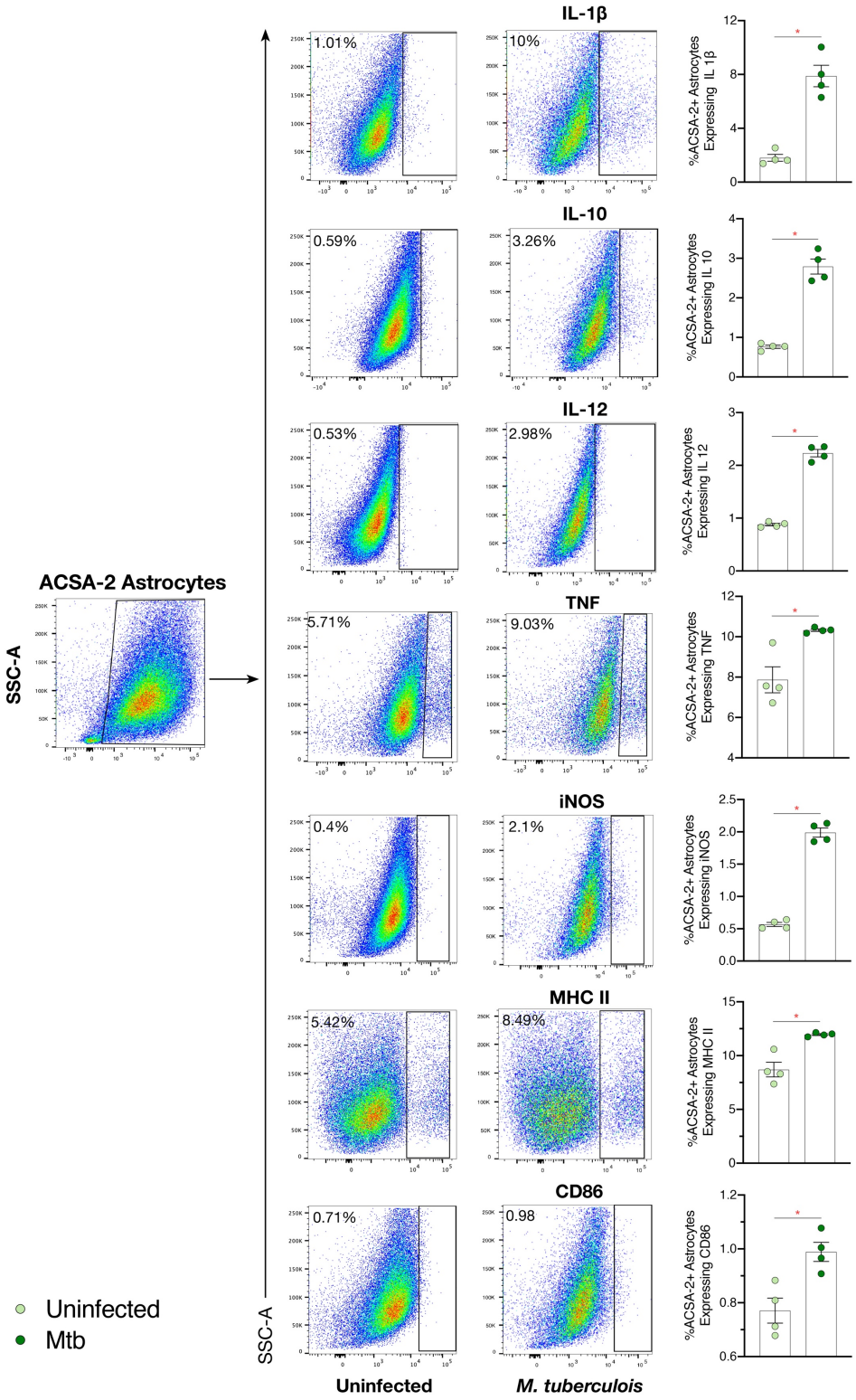


Figure 4.9 | Expression of immune mediators during *M. tuberculosis* infection of primary astrocytes. Primary astrocytes were infected with *M. tuberculosis*-H37Rv at MOI 10 for 24h. Cells were stained for flow cytometry analysis. The first plot represents the gating strategy for astrocytes using the ACSA-2 lineage marker. Further analysis was conducted on the astrocyte population by gating for various immune factors. Flow cytometry analyses were quantified and expressed as the percentage of ACSA-2^{+}-astrocytes expressing IL-1β, IL-10, IL-12, TNF, iNOS, MHC-II and CD-86. Data shown is the mean ± SEM (n=4). Significant differences were determined between uninfected and M. tuberculosis infected groups using the Mann Whitney test (* $p < 0.05$).

CHAPTER 5

CHAPTER 5

Investigating astrocyte immune modulation during cerebral *Mycobacterium tuberculosis* infection in mice

Diagnosis and therapy of CNS-TB continues to be a challenge with inadequate knowledge of disease pathogenesis. Previous studies have explored microglia and neuronal responses to *M. tuberculosis* challenge (Randall et al., 2014, Spanos et al., 2015, Rock et al., 2005), but reports on astrocyte responses during CNS-TB infection are limited and their influence on disease progression has not been addressed. Despite compartmentalization of the brain, synergy between the nervous and immune systems enable resident cells to coordinate immune responses and facilitate pathogen clearance (Godinho-Silva et al., 2019, Sternberg, 2006). Primary astrocyte cultures provide a controlled environment for the assessment of cellular processes and functions during *M. bovis* BCG and *M. tuberculosis* infection. *In vitro* models are therefore important investigative tools but are unable to fully recapitulate host-pathogen interactions of the brain. They not only lack the complex physiological functions and systemic interactions present in humans and other mammals but are homogenous by nature and therefore not a true representation of the heterogenous astrocyte populations that exist in whole organisms.

An advanced understanding of the complexity of astrocytic molecular interactions could, however, be achieved in an animal model where all systems are intact and functional. Animal models are essential to biological research and have been indispensable tools for investigating the pathophysiology of CNS-TB (Bolin et al., 1997, Tucker et al., 2016, Van Well et al., 2007, Mazzolla et al., 2002, Tsenova et al., 2002, Tsenova et al., 1998). Several animal models have been described for studying CNS-TB, that includes guinea pigs, mice, rabbits and pigs (Bolin et al., 1997, Tucker et al., 2016, Van Well et al., 2007, Mazzolla et al., 2002, Tsenova et al., 2002, Tsenova et al., 1998). Although the rabbit presents as the most appropriate model for CNS-TB to investigate brain pathology, immunological tools for studies on rabbits are limited thereby hindering in depth immunological evaluation. In comparison, gene expression and protein secretion profiling

tools are readily available for mice making them more suitable candidates for the purpose of this study. Despite C57BL/6 mice not developing necrotic lesions such as Kramnik and Nos2$^{-/-}$ mice (Kramnik et al., 2000, Irwin et al., 2015, Gengenbacher et al., 2017, Poh et al., 2022a), C57BL/6 mice are resistant to TB and known to generate Th1 cells needed to activate mycobacteriostatic function in macrophages to control *M. tuberculosis* growth. Murine models are well established and have been extremely useful in identifying key immune mechanisms during disease, particularly for investigations on CNS-TB infection (Hsu and Jacobs, 2021, Hsu et al., 2017, Francisco et al., 2015, Van Well et al., 2007), however, current literature is devoid of its application to examine astrocyte responses to *M. tuberculosis in vivo*. Therefore, to investigate the role of astrocytes in host-pathogen relationships immunocompetent C57BL/6 mice, immunodeficient non-obese diabetic (NOD) SCID/IL2Rγ^{null} (NSG) mice and humanised NSG-BLT mice were used in *M. tuberculosis* challenge studies.

5.1 Intracerebral *M. tuberculosis* infection in C57BL/6 mice

The transcriptional and translational data obtained from *M. tuberculosis* challenged studies in culture have indicated that astrocytes are potentially important modulators of host immune responses during neuroinfection. To investigate and validate these findings under physiological homeostasis conditions, astrocyte responses were analysed in C57BL/6 mice. C57BL/6 mice are considered resistant to CNS-TB infection making them appropriate candidates to evaluate the immunological contribution of astrocytes during infection (Van Well et al., 2007). The efficiency of cerebral *M. tuberculosis* infection was evaluated by first establishing a viable infection in immune competent C57BL/6 mice. Intracerebral infection was performed by inoculating mice with 1 x 10^5 CFU *M. tuberculosis* H37Rv-GFP or alternatively with sterile saline as a sham control. Mice were monitored daily for 28 days, and body weights recorded. The physiological condition of infected mice was clinically comparable to the saline inoculated sham mice with all mice surviving the clinical procedure, no visible health deterioration, and no loss in body weight observed for the duration of the experiment (Fig. 5.1A). The initial decline in body weight is likely due to the surgical procedure as this was observed in both sham challenged and

infected mice, consistent with prior reports (Kirby et al., 2012, Weiergräber et al., 2005, Francisco et al., 2015).

To assess disease progression, brains were homogenised at 1-, 7- and 14-days following infection and CFU enumeration used to evaluate the bacterial burden. (Fig. 5.1B). Mycobacterial replication was observed at day 7 post infection and similar mycobacterial levels at day 14 indicated bacterial control. Bacterial burden in the spleens and lungs were assessed as a measure of mycobacterial dissemination. Splenic colonies were visible at day 1, with a significant increase in growth at day 7 which remained constant at day 14 (Fig. 5.1C). Although pulmonary bacilli were observed as early as one day post infection, the bacterial burden was relatively unchanged at day 7, and 14 (Fig. 5.1D). CFU presence in the spleen and lungs after 24h possibly occurred due to hematogenous dissemination via damaged vasculature caused by the inoculation procedure. Mice did not display any abnormal physical or behavioural signs despite disease development in the *M. tuberculosis*-infected mice. These findings supported previous studies demonstrating the long-term survival of C57BL/6 mice following cerebral *M. tuberculosis* and saline inoculation (Olin et al., 2008, Van Well et al., 2007).

To further characterise disease development in the brain, mice were sacrificed at 14 days post-infection, brains were perfused and tissue immediately isolated and snap frozen. Brains were sectioned, then labelled with an astrocyte specific fluorescent-conjugated antibody to GFAP (red), to identify resident astrocytes. The nuclear marker DAPI was visible in the blue channel and GFP-labelled bacilli in the green channel (Fig. 5.1E). Captured confocal images displayed a well-defined network of astrocytes in the red channel, while overlapping channels revealed the green bacilli to be positioned within the parenchymal tissue amongst the astrocytes (Fig. 5.1E), thus substantiating the presence of *M. tuberculosis* bacilli in the brain. Despite examination of images at high magnification, no bacilli were detected within astrocytes, probably due to the lower uptake frequency by astrocytes compared to microglia and the initial relatively low bacilli to astrocyte ratio on inoculation.

The data indicates that intracerebral inoculation of *M. tuberculosis* establishes a viable infection in C57BL/6 mice, without morbidity or mortality. Having demonstrated deposition of *M. tuberculosis* in the mouse brain, further studies focused on investigating cellular responses to bacilli challenge.

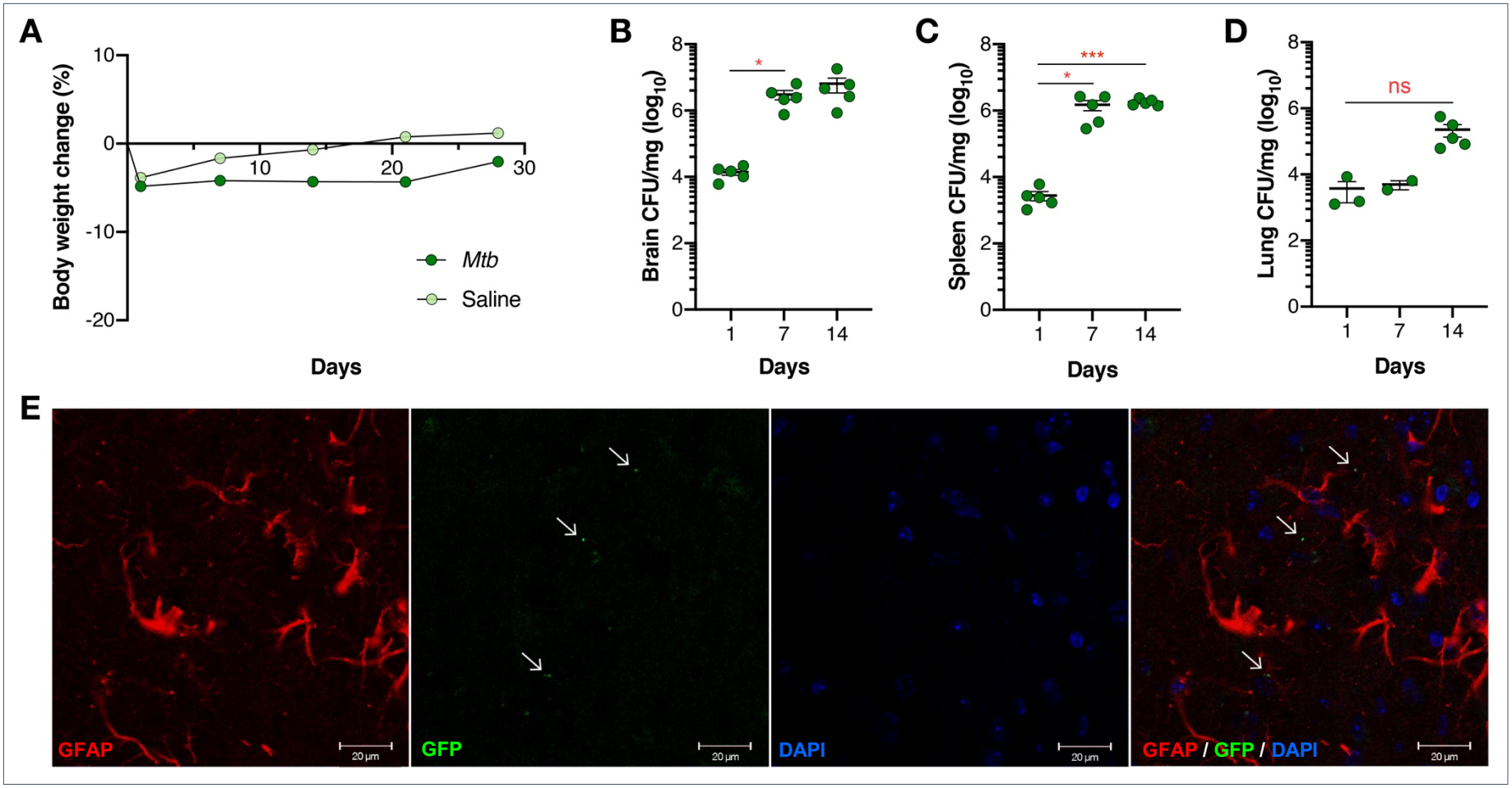


Figure 5.1 | Intracerebral *M. tuberculosis* infection in C57BL/6 mice. (A) Body weight average was consistent in infected mice and was comparable to the sham control mice. (B) Bacterial burden was confirmed in the brain at day 1 and disseminated to the (C) spleen and (D) lungs. Brain and spleen bacterial burdens increase at day 7, whereas the lungs showed no significant increase. (E) Confocal microscopy fluorescent images of astrocytes and GFP-labelled bacilli in the cerebral cortex at day 14 post infection. Fixed brains were sectioned and labelled with a fluorescent-conjugated antibody. Astrocyte cytoskeletal filaments were labelled with GFAP antibody (red), and cell nuclei were labelled with DAPI (blue). *M. tuberculosis* H37Rv-GFP bacilli (green) were visible in the brain parenchyma (indicated by the white arrows). Data is representative of two independent experiments, n=5 mice. Scale bars: 20µm. Analysis was performed by Student t-test (*$p < 0.05$; ns= not significant).

5.2 Astrocytes induce an immune response in C57BL/6 mice during CNS-*M. tuberculosis* infection

As a proof-of-concept study, an intracerebral inoculation was performed with 1 x 10^5 CFU *M. tuberculosis* H37Rv and a sterile saline substitute to determine whether astrocytes generate a response to *M. tuberculosis* challenge. The purpose of the sham challenged group was to affirm that observed responses were due to bacilli infection as opposed to the invasive surgical procedure, whereas uninfected, naïve mice were included as experimental controls to establish basal astrocyte activity. Mice were humanely euthanised at 7- and 14-days post-infection and whole brains were processed for flow cytometric analysis. Samples were immunostained with the monoclonal ACSA-2 lineage marker for astrocytes as well as for the immune markers IL-1β, IL-10, iNOS, TNF, MHC II and CD86, data acquisition was via BD Fortessa, and analyses done using the FlowJo software program. Seventy percent (70%) of total live cells were ACSA-2^+-astrocytes which were similar in all the groups at day 7 and day 14 post-infection (Fig. 5.2A), indicating that infection does not alter the overall astrocyte population number. Analysis of immune active astrocytes in the ACSA-2^+-cell population revealed a significant ($p < 0.05$) increase in the percentage of astrocytes expressing IL-1β in the *M. tuberculosis* infected mice compared to naïve and saline control groups at both days 7 and 14 (Fig. 5.2B). The percentage of ACSA-2^+ astrocytes expressing IL-10 showed significant ($p < 0.05$) elevations at both day 7 and 14 in the infected compared to naïve mice. Moreover, the percentage of ACSA-2^+ astrocytes expressing IL-10 in infected mice were significantly ($p < 0.01$) higher to the saline sham group at day 14 but not day 7 (Fig. 5.2C). This suggests that the initial observed elevation of IL-10 producing astrocytes in the first week may be injury induced, while the subsequent increase is driven by infection. In contrast to IL-1β, IL-10 attenuates local inflammation (Lobo-Silva et al., 2016) and mediates neuroprotection (Chen et al., 2016, Lim et al., 2013). INOS expression by astrocytes peaked at day 7 and was significantly higher in infected mice compared to both naïve and saline control mice ($p < 0.05$), however no significant differences were observed at day 14 (Fig. 5.2D). MHC II expression in astrocytes were enhanced in infected samples compared to the saline group at day 14 ($p < 0.05$) but not at day 7 (Fig. 5.2F), suggesting its potential to prime T cells and contribute to adaptive responses. No

significant ($p < 0.05$) changes were observed in astrocytes expressing CD86 and TNF in *M. tuberculosis* challenged mice compared to the controls (Fig. 5.2E and G).

The presented results demonstrate a constitutive low level of cytokine expression by astrocytes in the healthy brain, which is unsurprising as IL-1β, IL-10 and TNF, in particular, are imperative for synaptic physiology and plasticity (Maggio and Vlachos, 2018, Lenz et al., 2021, Rizzo et al., 2018, Levin and Godukhin, 2017). During infection astrocytes expression of IL-1β, IL-10, iNOS and MHC II increased, in response to *M. tuberculosis,* a novel finding that supports active participation of astrocytes to regulate immune responses *in vivo*.

5.3 Immunodeficient NSG mice and humanised NSG-BLT mice are susceptible to intracerebral *M. tuberculosis* infection

An important objective of these animal studies was to confirm the *in vitro* observations that *M. tuberculosis* bacilli can infect astrocytes under physiological conditions. Infection studies using C57BL/6 mice failed to show internalised bacilli within astrocytes, potentially due to an initial strong immune response leading to control of bacilli. It was hypothesised that NSG mice would be less successful at limiting bacilli replication due to their defective immune system. NSG mice exhibit multiple defects in immunological function, including defective cytokine and complement signalling, deficiencies in T cells and B cells and natural killer cells, as well as maturation defects in macrophages and DCs (Shultz et al., 1995, Pearson et al., 2003, Kataoka et al., 1983, Serreze et al., 1993, Shultz et al., 2005). Thus, it was postulated that the potential for unrestricted bacilli growth would increase the number of bacilli available for cellular uptake and hence improve the likelihood of astrocyte internalisation. More importantly, the use of NSG mice allows for investigation of infection under conditions of immune restriction. Most individuals who are infected with *M. tuberculosis* bacilli mount a successful immune response to tuberculosis that does not result in clinical disease. Nonetheless, children and immunocompromised individuals are at higher risk of developing disseminated TB, particularly CNS-TB, due to their weakened immune systems (Khan et al., 2019, Qian et al., 2018). Therefore, as an additional aim, this study investigated the role of astrocytes

in NSG mice to simulate clinical outcomes under conditions of immune suppression. Further, the lack of a functional immune system in NSG mice support better engraftment of human-derived immune cells to create humanised mice (Lan et al., 2006, Brainard et al., 2009, Karpel et al., 2015, Gillgrass et al., 2021). The NSG mouse is a well-established recipient typically used for the engraftment of human cells and tissue to study specific immune responses implicated in disease pathogenesis (Rongvaux et al., 2013, Ito et al., 2002). In recent years the development of humanised mice has facilitated TB studies by emulating human immune responses and the corresponding pathology following infection (Calderon et al., 2013, Arrey et al., 2019). Several humanised mouse models exist (Shultz et al., 2012) from amongst which the bone marrow, liver, thymus (or BLT) mice generated by surgically implanting human fetal liver and thymic tissue under the renal capsule of NSG mice is considered the most advanced to study human immune responses (Covassin et al., 2013, Lan et al., 2006, Melkus et al., 2006, Brainard et al., 2009). The thymic fragments develop into human thymic organoids allowing the maturation of human T cells (Lan et al., 2006, Melkus et al., 2006), and together with hematopoietic stem cell injection, a functional human immune system develops. The inclusion of humanised mice in this study provided an opportunity to investigate whether a reconstituted human immune system can rescue the potential susceptibility expected in NSG infected mice. On a cellular level it offers a tool to investigate how interactions with circulating human immune cells modify astrocyte function. Communication between astrocytes and immune cells in physiological and pathological processes has been established (Han et al., 2021, Sanmarco et al., 2021, Greenhalgh et al., 2020, Geyer et al., 2019). Although peripheral immune cells are usually restricted from entering the parenchyma, during disease and injury the repertoire of soluble molecules changes and circulating cells can infiltrate via a disrupted BBB (Engelhardt et al., 2017). The release of soluble factors from neutrophils, monocytes, macrophages (Andjelkovic et al., 2002, Haan et al., 2015, Harris et al., 2007, Frik et al., 2018), and T cells (Filiano et al., 2017, Filiano et al., 2016, Ito et al., 2019) can directly impact astrocyte function and affect disease progression (Greenhalgh et al., 2020).

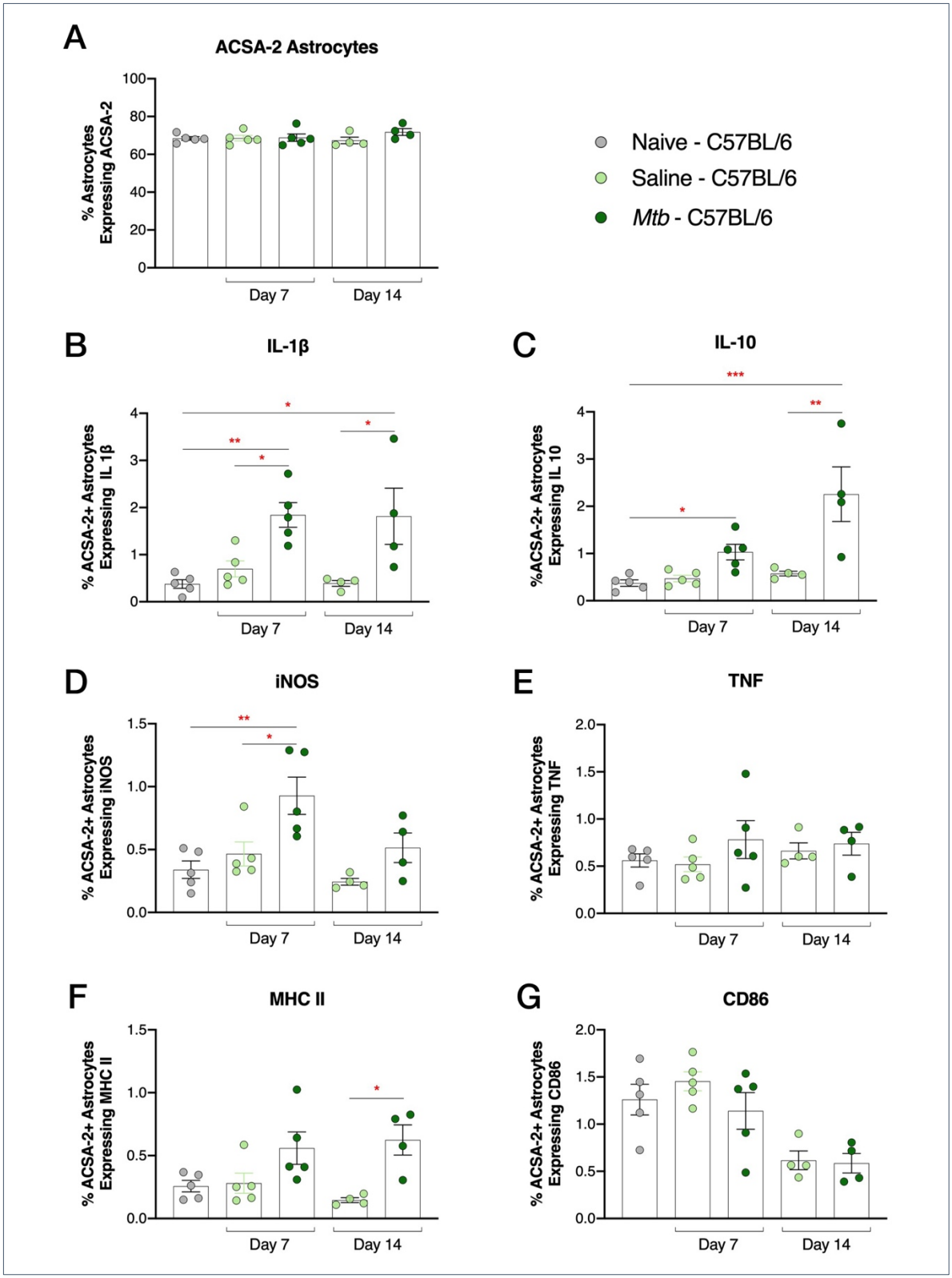


Figure 5.2 | Astrocyte expression of immune mediators during CNS *M. tuberculosis* infection. C57BL/6 mice were left untouched (naïve), intracerebrally inoculated with saline or intracerebrally infected with a dose of 1 x 10^5 CFU of M. tuberculosis (H37Rv). At 7- and 14-days post infection brains were isolated and samples prepared for flow cytometry analysis. (A) The population of ACSA-2^+ astrocytes in the brain were identified and the percentage of cells that produce (B) IL-1β, (C) IL-10, (D) iNOS, (E) TNF, (F) MHC II, and (G) CD86 were assessed. Data is representative of 1 of 2 independent experiments. Results are mean ± SEM of n=5 mice/group. (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

Non-humanised NSG mice and successfully engrafted humanised NSG-BLT (hNSG-BLT mice) (hCD45^{+}>10%) were intracerebrally inoculated with a dose of 1 x 10^{5} CFU of *M. tuberculosis* H37Rv-GFP or sterile saline and their ability to control infection was assessed. Saline challenged NSG and hNSG-BLT mice did not display any clinical deterioration both physically or behaviourally. All mice were alert and active, exhibited healthy coats, showed no indication of pain or distress, and maintained a constant average body weight for the experimental period (Table 5-1, Fig. 5.3A). These observations confirm that the invasive intracerebral procedure had a limited effect on animal health. In contrast, clinical regression was apparent in *M. tuberculosis* infected NSG mice which were distressed and displayed a rapid loss of body weight (average = 19%) over the experimental period (Table 5-1, Fig 5.3A). Disease progression in *M. tuberculosis* infected NSG mice was characterised by adverse physical effects, such as dull ruffled fur, with some mice showing orbital tightening, facial bulging, and rotated ears. Although the mice were actively mobile and behavioural manifestations were limited with no obvious convulsions, circling or head tilting; signs of ataxia were noted in some animals suggesting neurological damage in the cerebellum (Table 5-1). Following euthanasia, brains were inspected, and most animals presented with oedema and unresolved burr hole wounds, but no suppuration was visible. In comparison, *M. tuberculosis* infected hNSG-BLT mice exhibited clinical regression and were visibly more distressed and under-conditioned (Table 5-1). Most of the hNSG-BLT's displayed ruffled fur due to piloerection and lack of grooming, body hunching, orbital tightening, facial bulging, while also presenting substantial behavioural and clinical neurological manifestations. The behavioural effects were enhanced compared to the NSGs, with more mice displaying ataxia and some displaying otitis indicative of an ear infection as well as convulsions, and limb dragging. In addition, their brain pathology was more advanced with all animals exhibiting oedema, unrepaired burr hole wounds and suppuration. Deterioration of the hNSG-BLT mice was rapid with a 30% decline in body weight over the duration of the experiment and mice eventually succumbing to infection (Fig. 5.3A). NSG mice were more susceptible to infection than the previously observed C57BL/6 mice which was expected given their considerable immunodeficiency in comparison to the wild type mice. More surprisingly was the susceptibility of hNSG-BLT

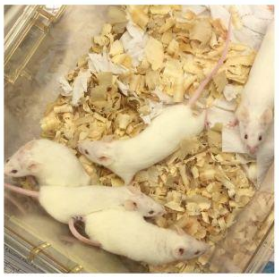
as it was hypothesised that the reconstituted human immune system would be sufficient to rescue mice.

Uncontrolled bacilli replication can disrupt normal cellular function thereby causing severe tissue damage which could result in physical and behavioural clinical manifestations. To assess the bacterial burden in non-humanised NSG and hNSG-BLT mice, brains, spleens, and lungs were harvested and CFUs measured at day 17 post infection (Fig. 5.3B). Bacterial burdens in the brains of NSG and hNSG-BLT mice were exceptionally high, 100-fold greater than that observed earlier in the C57BL/6 mice (Fig. 5.1B), indicating uncontrolled bacilli replication (Fig. 5.3B). The NSG and hNSG-BLT mice displayed comparable CFU's in the spleens and lungs ($p < 0.05$) (Fig. 5.3B) illustrating bacilli dissemination from the brain to distant organs. The data therefore demonstrate that hNSG-BLT mice were as susceptible as non-humanised NSG mice to cerebral *M. tuberculosis* infection but that clinical deterioration in the hNSG-BLT mice was noticeably enhanced. Reconstitution of immune cells therefore augmented *M. tuberculosis* pathology in humanised mice rather than rescued bacterial susceptibility.

To further analyse disease progression in the brain, NSG mice were infected with *M. tuberculosis* (H37Rv-GFP), humanely euthanised at 21 days post infection, and brains fixed and frozen. Brain tissue was sectioned and incubated with a fluorescent-conjugated GFAP antibody to label astrocyte cytoskeletal filaments (red). Localisation of the GFP-expressing bacilli (green) in the cerebral tissue was assessed by fluorescent imaging. Immunohistochemistry was used to inspect the infection site or the saline injury site of NSG mouse brains. Saline challenged control mice had uniform GFAP-positive astrocytes, undisturbed DAPI-stained nuclei, and no visible GFP-expressing bacilli (Fig. 5.4A). In clear contrast *M. tuberculosis* infected mice displayed a focal area of green bacilli within a defined boundary (Fig. 5.4B). Supporting dissemination to distant organs, the image confirms rapid uncontrolled bacilli replication at the site of inoculation. Within the bacilli focal region, astrocytes were visibly disrupted, while peripheral integrity of the surrounding astrocyte network was maintained. The apparent border could be an attempt by resident cells to contain bacterial dispersal and prevent neuronal injury.

Astrocyte uptake of *M. tuberculosis* was not established in C57BL/6 mice, which was potentially due to infection control by resident microglia and infiltrating peripheral cells. In contrast, NSG mice were less successful at limiting infection, an unsurprising outcome given their defective immune system. The elevated bacterial burden increases the number of available bacilli thereby improving the likelihood of astrocyte internalisation. Based on bacilli internalisation observed in culture, it was hypothesised that astrocytes could internalise *M. tuberculosis* bacilli in NSG mice. Orthogonal projections created from confocal z-stack images were used to ascertain the localisation of bacilli in relation to astrocytes (Fig. 5.5). These projections illustrate a 3-dimensional rendition of a z-stack section by displaying the x-y and z-planes. A green horizontal line represents the x-plane (side panel); and the red vertical line represents the y-plane (top panel). Fluorescent bacilli (green) positioned at the intersection of the red and green lines were shown to be positioned within the astrocyte cytoskeletal filaments (red) in both the x- and y-planes, confirming that *M. tuberculosis* was able to infect astrocytes *in vivo*. Despite the internalisation of the bacilli within the astrocyte, there was no strong association between the cell's cytoskeletal filaments and the bacilli as no yellow colocalization signal was observed. This data revealed successful intracerebral *M. tuberculosis* infection of NSG mice, and for the first time established that astrocytes are host cells for *M. tuberculosis in vivo.*

Table 5-1 | Clinical parameters of NSG and hNSG-BLT mice following intracerebral *M. tuberculosis* infection and saline inoculation

Clinical Manifestations		NSG		hNSG-BLT	
		Saline	*M. tuberculosis*	Saline	*M. tuberculosis*
Physical	Body Condition	 Well-conditioned	 Under conditioned	 Well-conditioned	 Emaciated
	Ears rotated back	-	+	-	+++
	Hunched	-	+	-	+++
	Facial bulge (Nose/Cheek)	-	+	-	++
	Orbital tightening	-	+	-	++
	Piloerection	-	++	-	+++
	Dull fur	-	+++	-	+++
Behavioural	Activity	Active	Active	Active	Restless
	Otitis (Head tilt/ Circling)	-	-	-	+
	Ataxia	-	+	-	++
	Abnormal gait/ dragging limbs	-	-	-	+
	Convulsions	-	-	-	+
Brain Pathology	Oedema	-	++	-	+++
	Burr hole wound	-	+++	+++	+++
	Purulent wound	-	-	-	+++

Not present (-), present in some animals (+), present in most animals (++), present in all animals (+++).

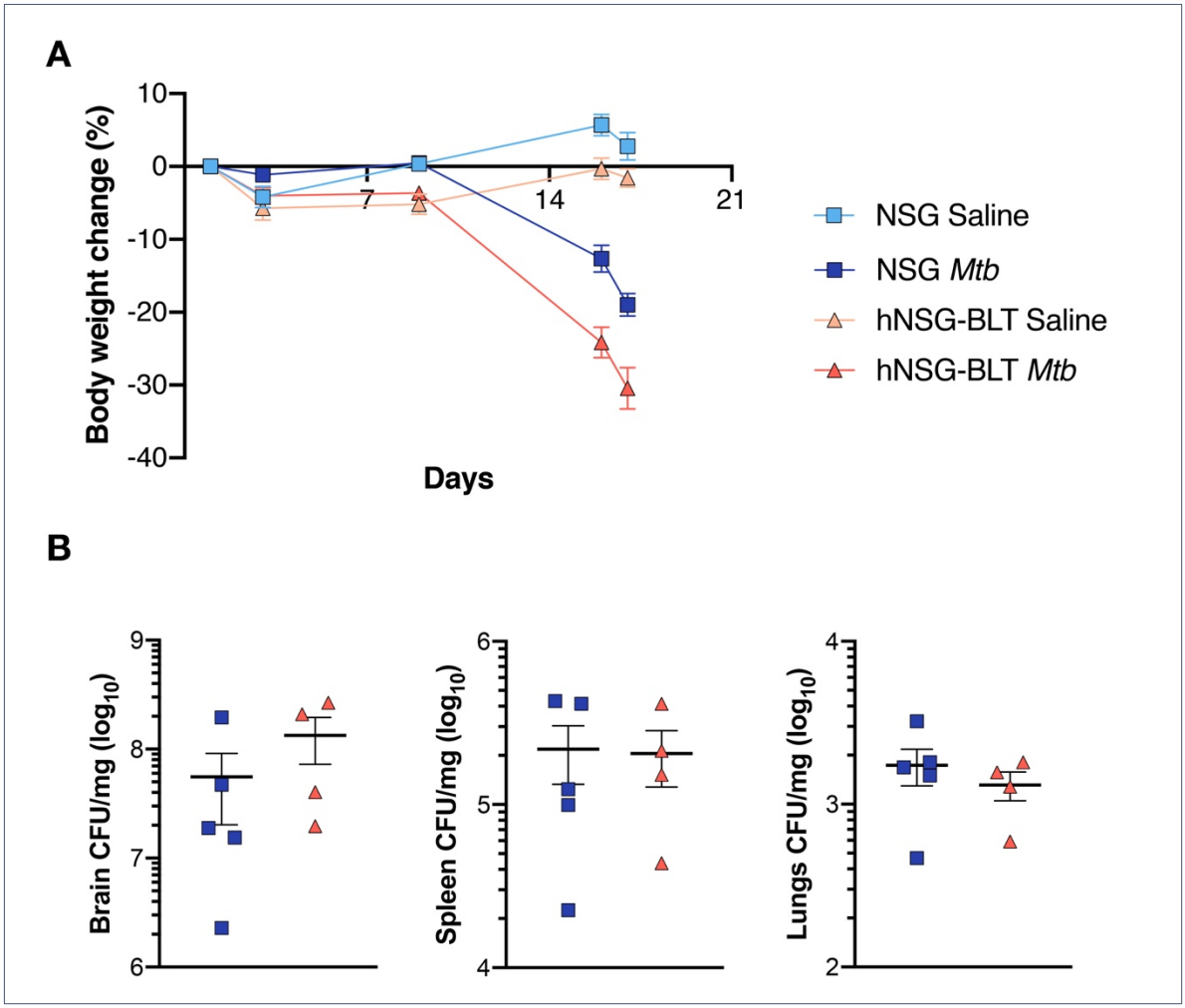


Figure 5.3 | *M. tuberculosis* infection of non-humanised NSG and humanised NSG-BLT mice. (A) Body weight change (%) in mice inoculated with sterile saline or 1 x 10^5 CFU of *M. tuberculosis* H37Rv over the duration of the experiment. NSG and hNSG-BLT sham challenged mice maintained healthy body weights and survived the experimental period. In contrast, *M. tuberculosis* infected NSG and hNSG-BLT mice lost 19% and >30% of their body weight over the experimental period, respectively. (B-D) Colony forming unit counts in the brain, spleen, and lung of NSG and hNSG-BLT mice at day 17 following infection indicate dissemination from the brain to distant organs. Bacilli replication between the two strains was comparable with no significant differences observed in the (B) brain, (C) spleen or (D) lungs ($p < 0.05$). Data shown is the mean ± SEM, n=4/5 mice per group.

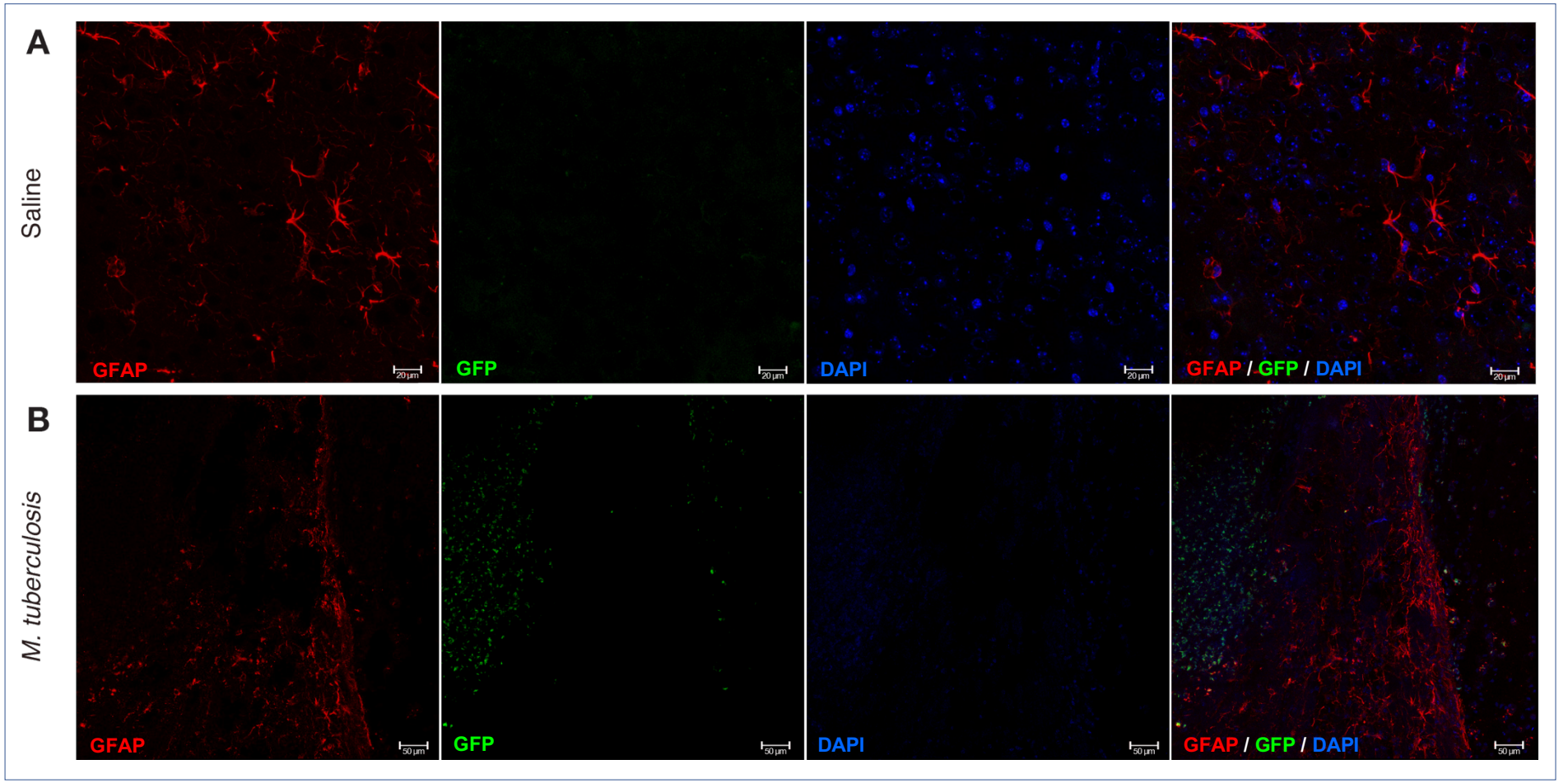


Figure 5.4 | Cerebral tissue of NSG mice at day 21 post *M. tuberculosis* infection. Brain sections of the injection site of (A) sterile saline and (B) *M. tuberculosis*-H37Rv-GFP infected mice show GFAP-positive astrocytes (red) and DAPI-labelled nuclei (blue). Infected brains display visible GFP-expressing *M. tuberculosis* bacilli (green). The astrocyte framework is disintegrated in the areas predominated by bacilli. Images are representative of brain sections from 5 mice from 1 of 2 independent experiments. Scale bars: 20µm - 50µm.

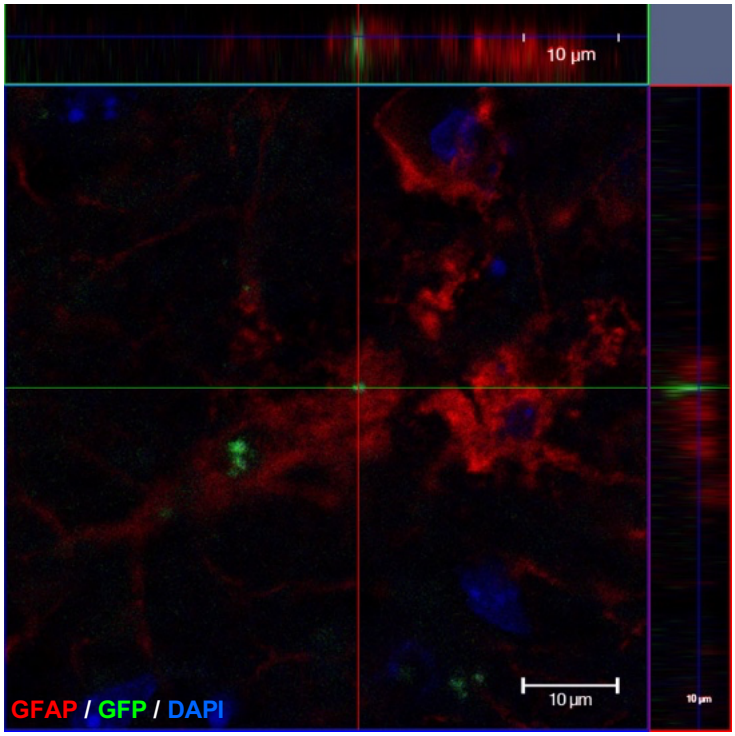


Figure 5.5 | Confocal microscopy of intracellular *M. tuberculosis* in astrocytes *in vivo*. NSG mice were intracerebrally infected with *M. tuberculosis* H37Rv-GFP. At 21 days post infection brains were fixed and sectioned. Astrocyte cytoskeletal filaments were labelled with a fluorescent-conjugated GFAP antibody (red) and cell nuclei stained with DAPI (blue). The displayed orthogonal projection is a 3-dimensional representation of a z-stack section. Although there is no yellow co-localization signal, *M. tuberculosis* bacilli (green) are visible within the astrocyte structures (red) in both x- and y-planes. Images are representative of brain sections from 5 mice, from 1 of 2 independent experiments. Scale bar: 10µm.

Considering the physical and behavioural clinical manifestations, the uncontrolled mycobacterial replication, and the susceptibility of NSG and hNSG-BLT mice to *M. tuberculosis* infection, it was apparent that they were unable to elicit efficient immune responses to control disease. The contribution of astrocytes to the pathophysiology observed in this model was, however, unknown and required further investigation. Astrocyte activity during *M. tuberculosis* infection in NSG and hNSG-BLT mice was therefore explored by intracerebrally inoculating them with either 1 x 10^5 CFU of *M. tuberculosis* H37Rv or sterile saline. Given the lack of effective immune control by NSG and hNSG mice, activity of astrocytes was investigated to determine whether astrocyte function was impaired and likely contributed to immune failure during *M. tuberculosis* challenge. On day 17 post infection, mice were euthanised, brains isolated, and samples analysed by flow cytometry. Astrocyte responses were determined by gating on ACSA-2 positive astrocytes and the relative percentage of cells that expressed IL-1β, IL-10, IL-

12, TNF, iNOS, MHC-II and CD86 were measured (Fig. 5.6). The percentage of astrocytes in NSG mice remained constant between saline and *M. tuberculosis*-infected mice encompassing 77% of total live cells (Fig. 5.6A). Interestingly, less than 0.5% of astrocytes expressed any of the selected markers, and there were no significant differences observed in the percentage of astrocytes expressing these markers in the *M. tuberculosis*-infected compared to the saline-inoculated sham control mice ($p < 0.05$) (Fig. 5.6B-H). Thus, the lack of peripheral immune cells and hence potential interaction with astrocytes may have impaired its functionality. To determine whether reconstituting immunity with human immune cells was capable of restoring astrocyte immune function, astrocyte responses in hNSG-BLT mice were evaluated. Saline inoculated hNSG-BLT mice displayed comparable levels to both saline and *M. tuberculosis* infected NSG mice, with no significant difference between the groups (Fig. 5.6B-H). Although not significant, there appeared to be an increase in astrocyte responses in mice supplemented with peripheral human immune cells following *M. tuberculosis* challenge. A trend toward significance was observed in ACSA-2^+ astrocytes expressing IL-1β, IL-10, TNF, iNOS, and MHC-II in *M. tuberculosis* infected hNSG mice compared to the sham challenged hNSG control mice (Fig. 5.6B, C, E, F, G). This trend was, however, not observed for IL-12 and CD86 expression (Fig. 5.6D, H).

The data therefore shows that although astrocytes are host cells for *M. tuberculosis*, they are unable to mount a response in NSG mice during infection. Since neuroimmune interactions are imperative for effective brain function (Herz et al., 2017) and protection against cerebral infection, it is plausible that the absence of an operative immune system impacts astrocyte activity. Interestingly, supplementation with human immune cells improved astrocyte activity suggesting that the neuroimmune interactions are important for proper astrocyte function during CNS-TB. One could speculate that the level of reconstitution or cellular recruitment to the brain was not sufficient to induce a robust response from astrocytes. These findings raise questions about the functional competency of astrocytes in immunocompromised individuals during CNS-TB infection if immunodeficiency directly influences astrocyte responses in a murine model of *M. tuberculosis.*

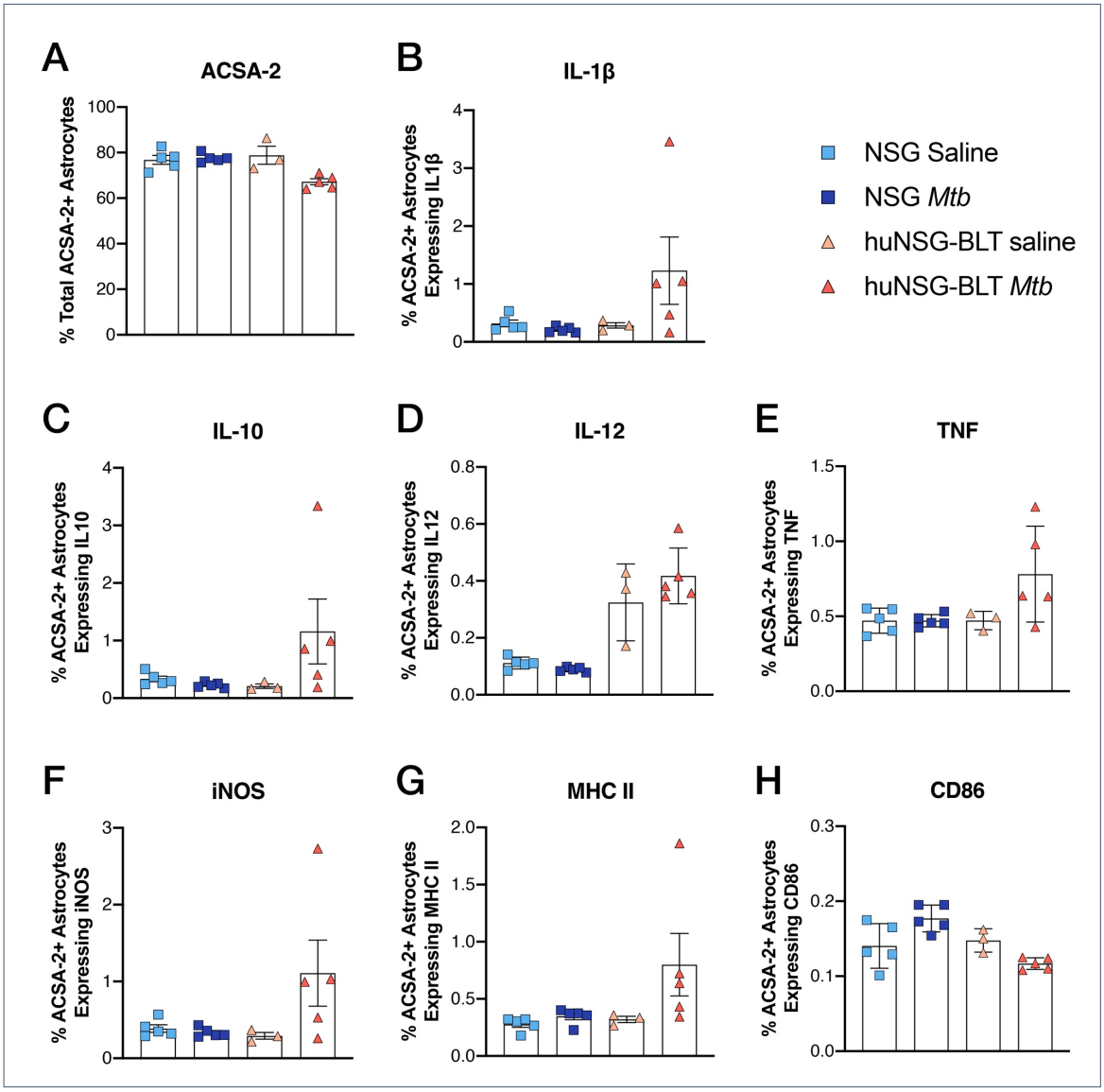


Figure 5.6 | Astrocyte expression of immune mediators during CNS *M. tuberculosis* infection in non-humanised NSG and humanised NSG-BLT mice. Following intracerebral inoculation with sterile saline or a dose of 1 x 10^5 CFU of *M. tuberculosis* H37Rv, brains were isolated, and samples prepared for flow cytometry analysis at day 17. The population of (A) ACSA-2^+ astrocytes in the brain was identified and the percentage of these cells that produce (B) IL-1β, (C) IL-10, (D) IL-12, (E) TNF, (F) iNOS, (G) MHC II and (H) CD86 were assessed. Results are from two independent experiments and are represented as the mean ± SEM of n=3-5 mice/group.

CHAPTER 6

CHAPTER 6

Discussion

Host immune responses in the CNS are highly regulated and involve the complex interaction of resident neuroglia, innate and adaptive immune cells. Astrocytes are among the first cells to encounter pathogens and reportedly contribute to innate immunity and host protection (Farina et al., 2007, Geyer et al., 2019). The contribution of astrocytes to host immunity is, however, often overlooked, yet they display several immunological functions and can respond to inflammatory stimuli (Liddelow et al., 2017, Perriot et al., 2018, Ronco et al., 2014). The primary aim of this project was to explore astrocyte immune modulation during mycobacterial infection. The initial objective of this study was to establish and optimise *in vitro* models of *M. bovis* BCG and *M. tuberculosis*- infected primary murine astrocytes as tools to characterise astrocyte activity during mycobacterial infection. Multiple reports have described bacterial infection of astrocytes (Geyer et al., 2019, Alkuwaity et al., 2012, Chauhan et al., 2009, Phulwani et al., 2006, Rasley et al., 2006, Seele et al., 2016, Sterka Jr et al., 2006, Stevens et al., 2009, Stoner et al., 2015, Cooley et al., 2014), however, studies on astrocyte activity during mycobacterial infection have been limited in scope (Geyer et al., 2019, Rock et al., 2005). This specific investigation is novel in concept and the findings present the characterisation of the astrocyte transcriptional profile and protein secretion during mycobacterial infection (summarised in Fig. 6.1). As an exploratory study it attempts to identify astrocyte specific correlates and caveats to immune function during mycobacterial infection.

Thus far, no research to date has explored *M. bovis* BCG infection in astrocytes, and only a single report referred to astrocytes as target cells for *M. tuberculosis in vitro* (Rock et al., 2005). Studies have identified microglia as the primary resident CNS cell type infected by *M. tuberculosis* (Rock et al., 2005, Peterson et al., 1995, Curto et al., 2004), and to a lesser degree, neurons (Randall et al., 2014) and astrocytes (Rock et al., 2005). A report by Rock et al., demonstrated that following a 24h *M. tuberculosis* infection, astrocytes displayed 15% cell-associated bacilli in comparison to the 76% of microglia that were infected. The current study confirms the findings of Rock et al. with the use of

confocal imaging. Internalisation of mycobacteria was confirmed by z-stack imaging, demonstrating that primary murine astrocytes can internalise both non-virulent and virulent mycobacterial strains within 24h of *in vitro* infection. The C57BL/6 mouse was then selected as an animal model of cerebral infection for further analysis due to its immune competency and established resistance to *M. tuberculosis* infection (Van Well et al., 2007). Wild type C57BL/6 mice were intracerebrally inoculated with *M. tuberculosis* or a saline solution then euthanised at various time points for analysis. Confocal imaging revealed the presence of bacilli in the brain parenchyma; however, no bacilli were visible within astrocytes. This was unsurprising given the higher affinity of *M. tuberculosis* for microglia in comparison to other CNS cell types (Rock et al., 2005, Randall et al., 2014). In contrast to this, NSG mice displayed internalisation of bacilli within astrocytes. The increased likelihood of astrocyte infection in the NSG mice could be attributed to the higher bacterial load in the brain due to uncontrolled bacterial replication. This data corroborates previous reports of mycobacterial internalisation by primary astrocytes (Rock et al., 2005) and shows novel observations of astrocyte infection *in vivo*.

Following uptake, *M. tuberculosis* bacilli were able to replicate in primary astrocytes confirming its suitability as a cellular niche for *M. tuberculosis*. In contrast to this, *M. bovis* BCG bacilli replication in astrocytes was restricted, a distinction which may be attributed to the virulence factors of bacteria. The observed difference in the replication dynamics between *M. tuberculosis* and BCG could be attributed to their inherent virulence and the host response they elicit (Fremond et al., 2004, Malone et al., 2018, Kozak et al., 2011). *M. tuberculosis*, being highly virulent, is more adept at overcoming immune surveillance mechanisms and successfully replicating within host cells, including astrocytes. This ability to evade immune responses and replicate effectively could contribute to the consistent increase in *M. tuberculosis* colony-forming units (CFU) over time. In contrast, BCG is less virulent due to its attenuated nature (Kozak et al., 2011). As a result, the host immune response, including the response of astrocytes, may be more effective in limiting BCG replication. Astrocytes, known for their role in immune surveillance and defense, may exhibit more efficient mechanisms to restrict BCG replication, leading to a reduction in BCG CFU over time. Notwithstanding BCG bacilli's replicative restriction within astrocytes, its potential to stimulate astrocyte expression of key pro- and anti-

inflammatory cytokines remained unhindered. Consistent with this is a recent study which demonstrated that no differences in cytokine production occurred in BMDM cultures after stimulation with both viable, replicating BCG and non-replicating, γ-irradiated BCG (Bickett et al., 2020). Collectively, the findings indicate that cell surface expressed molecules are sufficient to stimulate antigenic responses irrespective of mycobacterial viability and virulence.

The effects of mycobacteria on gene expression and protein induction were analysed as a measure of astrocyte immune regulatory potential. Evaluating transcriptional changes and protein secretion in astrocytes during *in vitro* mycobacterial challenge provide a framework for understanding intricate host-pathogen interactions and the cellular responses that shape host immunity to mycobacterial infection. Transcriptomic studies are increasingly utilised for the comprehensive analysis of gene expression patterns to characterise host-mycobacteria interactions in various contexts (Wu et al., 2012, Schnappinger et al., 2006). In this study using primary murine astrocytes, host transcriptome responses were profiled during *M. bovis* BCG and *M. tuberculosis* infection *in vitro*. Evaluation of the most predominant differentially expressed genes in astrocytes revealed upregulation of genes involved in immune responses, following infection with avirulent and virulent mycobacterial strains. A notable observation was the number of differentially expressed genes common to both *M. tuberculosis* and BCG which included genes associated with inflammation and cell recruitment. Genes commonly expressed by *M. bovis* BCG and *M. tuberculosis* were not the only important aspect of this study worth noting. As invaluable was the distinct number of differentially expressed genes observed only in response to BCG as opposed to *M. tuberculosis* which is likely due to their diverse virulent properties and may impact bacterial clearance and host defence. Interestingly, there is a correlation between the number of genes expressed and ability to control mycobacterial replication. Astrocytes displayed greater gene induction during BCG infection which had restricted bacterial replication compared to replicative *M. tuberculosis* infection. This data together suggests that astrocytes upregulate genes which may facilitate BCG bacilli clearance, whereas *M. tuberculosis* may inhibit these factors and permit bacilli persistence and replication.

Cytokines are commonly expressed in TB patients and play pivotal roles in host protection against mycobacteria by initiating and coordinating innate and adaptive immune responses (Van Crevel et al., 2002, Flynn and Chan, 2001, Domingo-Gonzalez et al., 2016, Davis et al., 2021, Cooper et al., 2011, Bourigault et al., 2013). Astrocytes can express a range of cytokines in response to neurotrauma and infection e.g. mechanical injury induce the release of IL-1α, TNF , IL-6 and IFN-γ (Lau and Yu, 2001), whereas stimulation with bacterial molecules or LPS induced IL-1β, TNF, and IL-6 (Tarassishin et al., 2014). Interestingly, astrocytes in this study demonstrated a significant increase in *Il6*, *Tnf*, *Il1a* and *Il1b,* which were among the genes expressing the highest fold change in expression in response to both *M. bovis* BCG and *M. tuberculosis*. Corresponding to the enhanced mRNA transcripts in *M. bovis* BCG and *M. tuberculosis*-infected astrocytes, the data presented in this study displayed an elevation in the production of IL-1β, TNF, and IL-6 proteins following infection with both pathogens. There has been substantial progress in understanding the importance of cytokines to control *M. tuberculosis* infection using gene specific deleted murine strains in challenge studies. Immunity to mycobacteria is regulated through the cellular release of several cytokines; amongst these are TNF, IL-6, the IL-1 family of cytokines, IL-12, IL-10, TGF-β, IFN-γ and VEGF (Kleinnijenhuis et al., 2011, Dlugovitzky et al., 1997, Sousa-Vasconcelos et al., 2015, Morosini et al., 2003, Helguera-Repetto et al., 2014, Sahiratmadja et al., 2007, Vankayalapati et al., 2003, Dubois-Colas et al., 2014, Davis et al., 2021, Domingo-Gonzalez et al., 2016). Astrocyte production of many of these factors suggests their facilitation of early innate immune protection during mycobacterial infection.

IL-1β and TNF, contribute to neuroinflammation by acting on microglia, and infiltrating immune cells (Meeuwsen et al., 2003). During neuroinflammation, microglia induce a reactive astrocyte phenotype through their release of IL-1β and TNF. Reactive astrocytes are characterized by morphological transformations and the activation of signalling pathways (Hyvärinen et al., 2019). Both IL-1β and TNF are major signalling molecules in the CNS (Meeuwsen et al., 2003, Benveniste and Benos, 1995), and are strongly induced following astrocyte activation (Meeuwsen et al., 2003, Choi et al., 2014, Aloisi et al., 1992). Astrocyte reactivity following IL-1β and TNF treatment has been shown *in vitro*

(Hyvärinen et al., 2019, Choi et al., 2014, Roybon et al., 2013, Rivieccio et al., 2005, Croitoru-Lamoury et al., 2003, Satoh, 2014, Satoh and Kim, 1995) and *in vivo* (Carrillo-de Sauvage et al., 2015, Clausen et al., 2008, Shinozaki et al., 2017) resulting in NFκB activation, a key transcription factor known to be activated in reactive astrocytes during neuroinflammation (Colombo and Farina, 2016). Proinflammatory cytokines, chemokines and adhesion molecules which facilitate leukocyte recruitment (Hyvärinen et al., 2019) are subsequently induced. Given that astrocytes can produce IL-1β and TNF during mycobacterial infection, this suggests a positive autoregulatory feedback loop for the expression of IL-1β and TNF. Although being considered "non-professional" immune cells, the overall cytokine profile in astrocytes correlated with the typical profile seen in specialized immune cells during mycobacterial infection.

Several studies have convincingly demonstrated the importance of TNF, IL-6 and IL-1β primarily produced by monocytes or macrophages in immune regulation during pulmonary tuberculosis (Maertzdorf et al., 2018, Bourigault et al., 2013, Mayer-Barber et al., 2010, Domingo-Gonzalez et al., 2016). Polymorphisms in the IL-6, TNF, and IL-1β genes are associated with increased susceptibility to TB (Wu et al., 2019, Meenakshi et al., 2013, Zhou et al., 2017, Zheng et al., 2018, Mao et al., 2015); while elevated levels of IFN-γ, TNF, IL-1β, and IL-6, as seen in active disease, correlates with severity (Boni et al., 2022, Kumar et al., 2019, Joshi et al., 2015, Lee et al., 2003, Ruhwald et al., 2009). Reports investigating brain homogenates from mice intracerebrally inoculated with BCG or *M. tuberculosis*, exhibited increased TNF, IL-6, IL- β and IFN-γ production (Hsu et al., 2017, Francisco et al., 2015, Mazzolla et al., 2002, Van Well et al., 2007, Palin et al., 2004). Interestingly, unlike *M. tuberculosis*, elevation of these cytokines in response to BCG following intracerebral injection fails to initiate an adaptive immune response (Matyszak and Perry, 1998). Microglia are the primary source of TNF, IL-6, IL- β and IFN-γ in the CNS during CNS-TB, and the production of these cytokines are critical for the host's response to the infection. Astrocytes which also express these cytokines may therefore assume a similar role in CNS-TB. Their location as part of the BBB may ideally suit them as dictators of early anti-mycobacterial CNS immune responses. Several studies have been conducted on CNS-TB, but a full characterisation of the cytokine and chemokine responses by astrocytes is lacking. Thus, the transcriptional functional profile

of astrocytes generated in this study provides novel insight of their immune regulatory potential during mycobacterial infection.

Constitutive levels of certain cytokines are necessary for optimal cellular function in a healthy brain. IL-1β is an example of such a cytokine, acting as an endogenous CNS molecule that coordinates neuroendocrine function in the brain. Additionally, it is a key inflammatory mediator that increases in the brain during CNS-TB (Francisco et al., 2015, Misra et al., 2010, Donald et al., 1995), particularly important for host protection during the early phase of *M. tuberculosis* infection (Francisco et al., 2015, Juffermans et al., 2000, Mayer-Barber et al., 2010, Fremond et al., 2007, Yamada et al., 2000, Bourigault et al., 2013). Analyses in this study revealed low levels of IL-1β expression in unchallenged astrocytes, as would be expected, and a considerable elevation in IL-1β production during mycobacterial infection both *in vitro* and *in vivo*. Increased cytokine production because of injury from the surgical procedure could be excluded as the saline inoculated mice showed no significant difference in levels compared to the naive mice. Thus, any significant changes in astrocyte expression observed in the *M. tuberculosis*-infected mice *in vivo* could be attributed to infection rather than injury. Interestingly, although production of IL-1β was elevated in both BCG- and *M. tuberculosis*- infected astrocytes *in vitro*, the BCG-infected samples displayed a much higher secretion of IL-1β in comparison to the *M. tuberculosis*-infected astrocytes. This suggests that more virulent mycobacterial strains may have greater inhibitory action, thereby limiting cytokine production. Supporting this is a study by Mishra et al. which demonstrated the ability of *M. tuberculosis* to reduce pro-IL-1β processing via iNOS-mediated inhibition of the NLRP3 inflammasome (Mishra et al., 2013). *M. tuberculosis* infection, however, did not completely diminish IL-1β expression, possibly due to production through inflammasome independent pathways. Furthermore, although IL-1β is commonly enhanced in various cells in response to BCG, the IL-1 pathway is presumably redundant in BCG infection. A pulmonary TB study showed that the absence of IL-1R1, IL-1α/β and neutralisation of mediators in the IL-1 pathway had no effect on mouse susceptibility to BCG infection (Bourigault et al., 2013). In contrast, delivery of anti-IL-1α/β antibodies was lethal to *M. tuberculosis*-infected mice (Guler et al., 2005) and mice deficient in IL-1R1 or IL-1α/β displayed increased susceptibility, disrupted granuloma formation and reduced IFN-γ

production following *M. tuberculosis* H37Rv infection (Bourigault et al., 2013, Mayer-Barber et al., 2011, Yamada et al., 2000, Juffermans et al., 2000, Fremond et al., 2007). In addition, in the absence of MyD88, a cytosolic adapter protein critical for IL-1β secretion via the canonical inflammasome pathway, mice rapidly succumbed to *M. tuberculosis* infection (Nicolle et al., 2004); whereas BCG-infected mice demonstrated controlled infection despite increased mycobacterial burden in the lungs (Bourigault et al., 2013, Nicolle et al., 2004). These studies suggest that during pulmonary TB pathogen virulence is a factor in this pathway, and it is reasonable to surmise that similar outcomes can be expected in the CNS. The findings of this study indicate that while astrocytes have the capacity to participate in host immunity against *M. tuberculosis* infection by producing IL-1β, the expression level of this cytokine may be influenced by the virulence factors of the pathogen.

One of the roles of IL-1β is the induction of iNOS (Landes et al., 2015, Balligand et al., 1994, Adams et al., 2002), an enzyme commonly upregulated in TB patients (Choi et al., 2002, Landes et al., 2015, Schön et al., 2004). Astrocyte expression of iNOS was elevated in *M. tuberculosis* infected primary cultures and in C57BL/6 mice, suggesting that astrocytes play a role in mycobacterial growth inhibition. iNOS is the catalyst for nitric oxide (NO) production which has versatile biological activity including immune regulation (Bogdan, 2015, Xue et al., 2018), and is central to controlling intracellular pathogens such as *M. tuberculosis* (Landes et al., 2015, MacMicking et al., 1997, Ehrt et al., 2001, Nathan and Shiloh, 2000, Rockett et al., 1998). CNS-TB models of iNOS deficient mice display severe neuropathology and the development of an immunological phenotype typically observed in tuberculosis meningitis patients (Poh et al., 2022a, Olin et al., 2008) but not seen in wild type mice. In murine models, astrocytes and microglia may both contribute to iNOS production during infection, however, astrocytes may be the principal source in human CNS-TB. Although there is evidence of iNOS production in murine (Simmons and Murphy, 1992, Galea et al., 1992) and human fetal astrocytes (Lee et al., 1993a, Lee et al., 1993b) species differences exist with respect to iNOS expression in microglia. Murine microglia are capable of producing iNOS but does not occur in human microglia (Lee et al., 1993b). The cellular disparity between species could be a contributing factor to the higher susceptibility seen in humans compared to wild type

mice. The findings of this study are therefore consistent with previous reports that astrocytes can induce iNOS expression, and it adds to the dogma by demonstrating that they enhance iNOS production during *M. tuberculosis* infection thereby improving antimicrobial activity in mice. Although microglia may contribute indirectly by stimulating astrocyte production of iNOS through microglial IL-1β (Kim et al., 2006, Lee et al., 1993a, Hu et al., 1995), this study showed that direct infection of astrocytes elevated iNOS production indicating that microglial stimulation is not essential for astrocyte activity *in vitro*.

IL-1β released by astrocytes and resident brain cells during CNS-TB play multiple roles in fighting infection, but despite the importance of IL-1β in defence against *M. tuberculosis*, levels of this cytokine requires strict regulation as excessive production promotes neutrophil influx resulting in inflammation and immunopathology (Mishra et al., 2013, Zhang et al., 2014a), whereas reduced IL-1β leads to uncontrolled bacterial replication (Mishra et al., 2017) as demonstrated by iNOS-driven NLRP3 inflammasome inhibition (Mishra et al., 2013). Taken together, the elevated expression of IL-1β and iNOS by astrocytes during mycobacterial infection suggests their participation in IL-1β modulation to maintain tissue integrity in the CNS, and their involvement in microbial clearance to resolve infection. IL-1β needs tight regulation to provide an optimal concentration to be effective, and disruption of this meticulous balance may be a contributing factor to the pathogenesis of CNS-TB. To establish the exact mechanism of astrocyte IL-1β production during *M. tuberculosis* infection, requires neutralising studies targeting the IL-1β pathway. Compellingly, IL-1 mediated signalling pathways are known to exert a protective effect against CNS-TB, and it is highly probable that the production of IL-1β by astrocytes in conjunction with resident and recruited cell types (Rock et al., 2005, Randall et al., 2014), actively contribute to host defence during CNS-TB. Notably, in situations of immune suppression, diminished production of IL-1β by astrocytes could potentially heighten susceptibility to CNS-TB infection.

The critical role of TNF in the control of mycobacterial infections has been extensively reported (Hsu et al., 2017, Flynn et al., 1995, Segueni et al., 2016, Francisco et al., 2015). These include the role of TNF in granuloma formation and maintenance (Kindler et al.,

1989, Fallahi-Sichani et al., 2011, Cilfone et al., 2013, Fallahi-Sichani et al., 2010, Warsinske et al., 2017), macrophage activation, facilitation of chemokine responses and contribution to immune cell proliferation and differentiation (Ray et al., 2009, Flynn et al., 2011, Scott Algood et al., 2005, Serbina and Flynn, 1999, Faustman and Davis, 2013, Clay et al., 2008, Kaplan and Freedman, 1996). Reductions in TNF production are reportedly linked to decreased IL-1β expression and diminished protection against CNS-TB (Francisco et al., 2015). TNF is therefore crucial to induce a protective neuroimmune response against mycobacteria in the CNS (Hsu et al., 2017, Francisco et al., 2015), however, the contribution of astrocyte-derived TNF mediated responses is not well defined. Thus far it is thought that microglia are the primary contributors of TNF although T cells also act as a major source driving a neuroimmune response (Lee et al., 2009, Spanos et al., 2015, Curto et al., 2004, Rock et al., 2005, Lee et al., 2008). Neurons, however, are unlikely to contribute to TNF mediated immunity against *M. tuberculosis* (Francisco et al., 2015). This study has shown that astrocytes do generate TNF in response to *M. bovis* BCG and *M. tuberculosis* infection and may therefore contribute to TNF-mediated host protection. Given that transmembrane TNF expressed on astrocytes plays a role in mediating inflammatory responses (Akassoglou et al., 1997), astrocyte-derived TNF can potentially regulate neuroinflammatory responses and contribute to infection control. Overexpression of TNF in the CNS is, however, associated with neuroinflammation, demyelination and inhibition of neurite outgrowth and synapse formation (Neumann et al., 2002). Astrocyte specific TNF may therefore play a critical role in ensuring host protection; however, excessive levels can result in tissue injury, highlighting the need for careful regulation.

In conjunction with IL-1 and TNF production, was the upregulation of IL-6 gene expression and protein secretion during mycobacterial infection. Induction of IL-6 by astrocytes suggests a critical role in coordinating and regulating immune responses to TB infection in the CNS as IL-6 signals leads to microglia and T cell migration to sites of infection (Boni et al., 2022, Ladel et al., 1997, Leal et al., 1999, Hilda et al., 2020, Dienz and Rincon, 2009). IL-6 also activates immune cells and increases the production of other cytokines and antibodies, further enhancing the immune response and improving bacterial clearance. Notably, IL-6 is elevated in the cerebrospinal fluid of tuberculosis

meningitis patients (Misra et al., 2010, Simmons et al., 2006, Rohlwink et al., 2017) and is essential for host resistance against murine *M. tuberculosis* infection (Dienz and Rincon, 2009, Saunders et al., 2000). Interestingly, IL-6 is upregulated in the presence of IFN-γ (Sun et al., 2017), commonly elevated during *M. tuberculosis* infection. Thus, one of the mechanisms through which astrocytes may increase IL-6 expression in the brain is following IFN-γ stimulation from microglia and infiltrating immune cells.

Deficiency of IL-6 is associated with an increased bacterial burden and altered Th1 responses (Saunders et al., 2000), yet elevated levels correlate with TB progression and susceptibility (Vivekanandan et al., 2023, Rohlwink et al., 2017). Uncontrolled inflammation can lead to tissue damage in the brain and spinal cord and may contribute to the development of neurological symptoms. However, IL-6 plays a role in regulating inflammation and promoting tissue repair in the CNS during infection (Erta et al., 2012, Rothaug et al., 2016, Tanaka et al., 2014). This is important because uncontrolled inflammation can cause harm to healthy tissue in the CNS and impair its function. Induction of IL-6 by astrocytes suggests an additional role in CNS neuroprotection as this pleiotropic cytokine is associated with neuron survival and regeneration (Loddick et al., 1998, Hirota et al., 1996, Kumar et al., 2010, Fujita et al., 2009, Sun et al., 2017). Astrocyte-derived IL-6, therefore, plays a crucial role in coordinating and regulating the immune response to TB infection in the CNS. By enhancing the immune response and limiting inflammation, regulated IL-6 production helps to minimize tissue damage and preserve CNS function.

It has been postulated that *M. tuberculosis* induces the elevation of IL-6 to inhibit type I IFN signalling, thereby allowing disease progression. In the CNS, type I IFNs regulate innate immunity in response to infection and injury (Khorooshi and Owens, 2010). Resident CNS cells respond to IFN-α/β stimulation (Delhaye et al., 2006, Khorooshi and Owens, 2010) and astrocytes in particular represent a major source of type I IFNs (Tedeschi et al., 1986, Detje et al., 2015). The IFN system is an important contributor to immune modulation, a crucial first line of defence against viruses (Isaacs et al., 1957, Müller et al., 1994) and critical for innate host responses against bacterial infections (Du et al., 2013, Mancuso et al., 2007, Moreira-Teixeira et al., 2018). Impaired type I IFN

signalling compromises host resistance to bacterial pathogens which may be due to the associated defective production of IFN-γ, NO, and TNF (Mancuso et al., 2007). Although reports have demonstrated the intrinsic contribution of type I IFN to host immune cell activation, others have shown that it can promote bacterial virulence and exacerbate disease (Antonelli et al., 2010, Rayamajhi et al., 2010, Moreira-Teixeira et al., 2018, Moreira-Teixeira et al., 2020). The findings of this study demonstrated that astrocytes induce type I IFN modules, as well as intracellular DNA-sensing and RIG-1 pathways which lead to expression of interferon stimulated genes and type I IFN production (Cavlar et al., 2012, Barber, 2011, Yoneyama et al., 2008). A tentative explanation for activation of these pathways may be internalisation of the mycobacteria which consequently triggers the intracellular receptors belonging to these modules.

Contrasting outcomes have been reported for type I IFNs in tuberculosis. Earlier studies have indicated that type I IFN signalling promotes TB pathogenesis (Bouchonnet et al., 2002, Manca et al., 2001, Ordway et al., 2007, Berry et al., 2010, Mayer-Barber et al., 2014, Mayer-Barber et al., 2011, Kimmey et al., 2017, Dorhoi et al., 2014), or may be protective (Wang et al., 2019, Desvignes et al., 2012, Mancuso et al., 2007, Du et al., 2013, Moreira-Teixeira et al., 2016). Neutralisation of IFN-IFNR signalling improved disease outcome while treatment with type I IFN resulted in disease susceptibility. In contrast, others have found that mice lacking IFNAR displayed a reduced ability to limit early bacterial growth during *M. tuberculosis* infection (Desvignes et al., 2012, Cooper et al., 2000). Type I IFN treatment was beneficial (Giosue et al., 1998) for use in patients with IFN receptor deficiencies (Ward et al., 2007, Desvignes et al., 2012, Moreira-Teixeira et al., 2016) and was proposed for use against multi-drug resistant strains (Palmero et al., 1999). Disparity in these studies indicate that the type I IFN system is complex, and differences could be ascribed to the timing of type I IFN signalling, concentrations of the type I IFNs, the cell types and differences in IFNAR subunit expression. During the early phase of infection, type I and II IFNs work synergistically by activating parallel pro-inflammatory pathways to mount an effective immune response (Desvignes et al., 2012). When adaptive immune responses are initiated, IFN-γ dominates and type I IFNs assume an anti-inflammatory regulatory role (Desvignes et al., 2012). The adverse effects of type I IFN during TB may be related to their reduction in T cell activation and suppression of

TNF and IL-12 production (Manca et al., 2005) or inhibition of IL-1α and IL-1β (Mayer-Barber et al., 2011, Novikov et al., 2011, Guarda et al., 2011) during late infection. In addition, *M. tuberculosis* may disrupt the balance of type I and II IFNs, thereby promoting an anti-inflammatory state and exacerbating disease.

By limiting the production of other cytokines, type I IFNs can prevent damage associated with excessive cytokine production, a beneficial effect, especially in the CNS (Mcnab et al., 2015, Blank and Prinz, 2017). In multiple sclerosis IFNβ, suppresses leukocyte-mediated inflammation (Boivin et al., 2015, Kavrochorianou et al., 2016, Fogarty et al., 2016, Sormani and Bruzzi, 2015). In a recent study on EAE mice, while recombinant IFN-β treatment of EAE mice generated IL-10, expression in myeloid cells was associated with suppressive effects observed in IFN treatment (Rasouli et al., 2021). In addition, under basal conditions, IFN-β is required for neuronal homeostasis and survival (Ejlerskov et al., 2015, Blank and Prinz, 2017) as mice deficient of IFNβ displayed symptoms of neurodegenerative disease (Ejlerskov et al., 2015). Thus, the regulation and production of type I IFN has important consequences in the CNS, and whether their activity is associated with homeostatic, pathological, or protective functions may be affected by various factors such as cell source, stimulus, expression levels, cytokine milieu, target cells and their receptor expression. It is therefore critical to assimilate host responses in astrocytes, to determine how they contribute to immunoprotective and immunopathological responses to appreciate their influence on disease progression in the CNS. The activity of type I IFNs is complex and multifaceted and the role of astrocyte-derived IFN-β during CNS-TB may best be determined using an astrocyte-specific IFN deficient mouse model which may potentially reveal modes of intervention during CNS-TB infection.

One of the features of innate immunity and a prominent aspect of activated astrocytes is the recruitment of immune cells to the site of infection through the release of chemokines. Chemokine production creates a gradient for the mobilisation of perivascular leukocytes, specifically T cells, to the infected tissue sites. Responses to chemokines are determined by the surface expression of seven-transmembrane–domain G-protein–coupled chemokine receptors (Bonecchi et al., 2009, Premack and Schall, 1996). Chemokine

receptors are remarkably redundant; the binding of chemokines to their cognate receptors elicits a series of signalling events leading to the modulation and tight regulation of cellular activities. Directed chemotaxis is notably associated with hematopoietic cells, but neurons, astrocytes and epithelial cells also express chemokine receptors suggesting multiple functionality of the chemokine system (Luster, 1998). Although 4 chemokine subfamilies exist, the CC and CXC clusters are the major groups involved in the recruitment of monocytes and neutrophils, respectively. To date, the only chemokine known to be produced by astrocytes in response to *M. tuberculosis* is CXCL10, as reported by Rock and colleagues (Rock et al., 2005). In this study the findings of increased CXCL10 expression in BCG- and *M. tuberculosis*-infected astrocytes were in line with the findings of Rock et al. Production of CXCL10 by astrocytes suggests that they play a role in attracting and activating monocytes and T cells to the site of infection (Taub et al., 1993b, Samperio et al., 2004, Tokunaga et al., 2018). Chemokines help direct the movement of immune cells to the infected tissue and determine the types of immune cells that are recruited. During BCG and *M. tuberculosis* infection, astrocytes increased the expression and production of several chemokines, including CXCL1, CXCL2, CXCL3 as well as the expression of *CXCL5*, all of which are ligands for receptors expressed on neutrophils, hence astrocytes may dictate neutrophil recruitment and transendothelial migration.

Neutrophils are the first responders to the infected area and their migration is orchestrated by the spatiotemporal production of chemoattractants. Quiescent circulating neutrophils recruited to the CNS are then activated by chemokines (Capucetti et al., 2020, Poh et al., 2022a) and in some instances chemokines such as CXCL2 may facilitate their transendothelial migration (Capucetti et al., 2020). Two major chemokine receptors expressed by neutrophils are CXCR1 and CXCR2 (Bachelerie et al., 2014, Coelho et al., 2008, Hu et al., 2011). Interestingly, under inflammatory conditions, the chemokine receptor repertoire on neutrophils is adjusted. CXCR2 expressed by circulating neutrophils is downregulated after extravasation and the inflammatory CC chemokine receptors CCR1, CCR2, and CCR5 are enhanced (Bonecchi et al., 2022). Remarkably, the ligands for these receptors were enhanced in BCG- and *M. tuberculosis*-infected astrocytes, particularly CCL2, CCL3, and CCL5. Given that

neutrophil receptors are modified upon entry into the brain parenchyma, astrocyte production of chemokines that target these receptors may modulate neutrophil activity. TB disease severity was associated with a neutrophil-derived type I IFN signature (Berry et al., 2010, Ahmed et al., 2021, Moreira-Teixeira et al., 2018, Donovan et al., 2017), thus neutrophil recruitment by astrocytes may aid in disease progression. The micro-environmental cues created by astrocytes during mycobacterial infection could therefore be important for neutrophil activity and immune regulation.

Astrocytes are a prominent source of CCL2 which are elevated in the CNS during inflammatory and traumatic conditions (Semple et al., 2010a, Berman et al., 1996, Glabinski et al., 1996, Delgado et al., 2013). Studies in which primary murine astrocytes were treated with IL-1β or TNF lead to increased expression of CCL2 (Oh et al., 1999, Meeuwsen et al., 2003, Wang et al., 2014, Hurwitz et al., 1995, Hyvärinen et al., 2019), CCL5 (Oh et al., 1999, Meeuwsen et al., 2003, Barnes et al., 1996, Hyvärinen et al., 2019), CCL20 (Wang et al., 2014), CXCL2 (Wang et al., 2014, Meeuwsen et al., 2003), CXCL8 (Meeuwsen et al., 2003, Oh et al., 1999, Hyvärinen et al., 2019), CXCL10 (Oh et al., 1999, Hyvärinen et al., 2019), and IL-6 (Hyvärinen et al., 2019, van Kralingen et al., 2013, Sawada et al., 1992) as well as neurotrophic factors such as BDNF (Meeuwsen et al., 2003, Saha et al., 2006a). Similarly, treatment of human astrocytes with both IL-1β and TNF lead to CCL2 induction (Meeuwsen et al., 2003, Barna et al., 1994). Production of CCL2 by astrocytes suggests its capacity to mediate the migration and infiltration of macrophages, as well as microglial activation (Rollins, 1996, Bell et al., 1996, Semple et al., 2010b, Rollins, 1991). In addition, elevated astrocyte CCL2 alludes to an active contribution to neurotransmission and neuronal cell survival (Madrigal et al., 2009, Eugenin et al., 2003, Edman et al., 2008, Bruno et al., 2000, Guyon et al., 2009) during *M. tuberculosis* infection. CCL2 deficiency *in vitro* and *in vivo* is associated with a modified cytokine profile in the brain following LPS stimulation or traumatic brain injury, respectively (Semple et al., 2010a). In their studies, the authors showed that astrocyte cultures displayed heightened levels of IL-6, TNF, CXCL1 and CCL3; whereas mice showed an exacerbation in IL-1α, IL-1β, IL-6, G-CSF, IL-12, CCL3 and CXCL1, and reductions in CCL5, IFN-γ and IL-2 (Semple et al., 2010a). Contrary to this, others reported a reduction in the production of IL-6 and IL-1β in CCL2-deficient mouse brains

following intrastriatal injection of LPS (Rankine et al., 2006) as well as in an ischaemic stroke model (Hughes et al., 2002). The observed discrepancies may well be due to the different challenge models, nonetheless it is clear that CCL2 disruption results in changes in the cytokine milieu following injury or infection.

In the inflamed CNS, CCL2 may bind to the CCR2 receptors expressed on astrocytes, thereby modulating their cytokine expression (Andjelkovic et al., 2002, Banisadr et al., 2002). Stimulation of murine astrocytes with CCL2 activates the phosphatidylinositol 3-OH kinase (PI 3-kinase)-Akt pathway and the downstream transcription factor, NFκB (Quinones et al., 2008). One of the functions of this pathway, is the inhibition of pro-apoptotic factors (Brunet et al., 2001) thus promoting astrocyte survival. CCL2 produced by astrocytes may play an intricate role during mycobacterial infection. Astrocyte-derived CCL2 can mediate immune activity by facilitating leukocyte recruitment and infiltration into the brain, and in addition can activate microglia. Through a positive feedback loop CCL2 can act on astrocytes to modulate cytokine production and support their survival, while maintaining neuronal function and survival. The production of chemokines by astrocytes is therefore essential for neuronal maintenance and coordinating inflammatory response. It, however, requires tight regulation to avoid deleterious tissue injury because of uncontrolled inflammation.

This is the first comprehensive report to present the participation of astrocytes to host immunity against CNS-TB by highlighting their regulation of early innate responses through their production of cytokines and chemokines, resulting in immune cell recruitment and activation. Cytokines can influence various aspects of the immune response in the CNS and depending on the context, they can elicit both beneficial and harmful consequences. Several cytokines were previously shown to be involved in neuroprotection by promoting the survival and growth of neurons, inhibiting apoptosis, and regulating neuroinflammation (Mousa and Bakhiet, 2013). Astrocytes can therefore possibly modulate neuroprotection in the CNS by regulating cytokine expression.

Although mainly acknowledged for its prominent role in inflammatory responses, IL-1β has pleiotropic effects in the CNS. Underappreciated functions of IL-1β are its role as a

neuromodulator in the healthy brain and its contribution to protection, while tissue remodelling and repair during injury or disease (Hewett et al., 2012) provides neuroprotection via astrocyte IL-1R1-receptor signalling (Todd et al., 2019). Another important indicator alluding to astrocyte duality and regulatory abilities was the increase in the anti-inflammatory mediator, IL-10, following *M. tuberculosis* infection *in vitro* and *in vivo*. Notably, astrocyte expression of IL-10 was elevated at day 7 and 14 following intracerebral *M. tuberculosis* infection. The increase displayed at 7 days was likely due to injury as the saline sham group was not significantly different to the infected group ($p < 0.05$), however, the upregulation observed at 14 days was significantly elevated compared to the control groups suggesting that this was driven by infection as opposed to injury. The production of IL-10 by astrocytes suggests that they may attempt to alleviate inflammation as a protective mechanism against inflammation-driven neuronal injury (Zhou et al., 2009a, Zhou et al., 2009b, Ledeboer et al., 2002, Balasingam and Yong, 1996, Lobo-Silva et al., 2016). Overexpression of IL-10 is however detrimental as it mitigates local inflammation by attenuating macrophage function and the Th1-type immune response (Jacobs et al., 2000, Murray et al., 1997, Bogdan and Nathan, 1993, Gong et al., 1996). Disrupted Th1 cytokine signalling results in higher bacterial burdens and increased susceptibility to *M. tuberculosis* infection (Flynn et al., 1995, Cooper et al., 1997, Kamijo et al., 1993, Cooper et al., 1995, Lyadova and Panteleev, 2015). Interestingly, mice deficient in IL-10 show improved bacterial clearance (Jacobs et al., 2000, Redford et al., 2010, Murray and Young, 1999), indicating that IL-10 is not necessary for protection against mycobacterial infection, and in fact, its absence can have a beneficial effect (Redford et al., 2010).

Regulation of the immune response by IL-10 involves balancing pathogen clearance and immunopathology, and by mediating cytokine expression, astrocytes can modulate neuroprotection in the CNS. Aside from their regulatory function, astrocytes attempt to preserve neuron integrity by releasing neurotrophins such as GDNF, Artn, and growth factors. Astrocytes are a principal source of neurotrophic factors in the CNS; an increase in the expression of a variety of these factors in response to BCG and *M. tuberculosis* infection suggests a significant impact on neuronal survival and promoting tissue repair during CNS-TB (Wheeler and Quintana, 2019, Chung et al., 2015, Molofsky et al., 2014,

Allen et al., 2012, Christopherson et al., 2005). A study by Chen et. al. demonstrated reduced neuroprotection following LPS challenge when astrocyte GDNF was neutralized in supernatants (Chen et al., 2015). Although GDNF and Artn are known to promote neuron survival, increasing studies show that GDNF family ligands also modulate neuroinflammation by acting on glial cells (Kotliarova and Sidorova, 2021, Duarte Azevedo et al., 2020). The pronounced production of neurotrophic and growth factors in conjunction with pleiotropic cytokine activity in astrocytes suggests their unequivocal activity to maintain the functioning of the neural network, regulation of the immune response, and promotion of tissue repair during CNS-TB. Astrocytes therefore play a dual role in the CNS and can contribute to a protective or pathological state.

An important mechanism by which astrocytes coordinate immune responses and prevent excessive inflammation is by modulating leukocyte infiltration. Leukocyte influx is necessary for an effective host immune response and pathogen clearance, however, uncontrolled infiltration in CNS-TB, can exacerbate inflammation and result in neuronal damage. By reducing inflammation during CNS-TB, the neurological complications associated with this disease can be minimized. Entry of circulating leukocytes into the CNS parenchyma requires active recruitment across the highly selective structural and biochemical barriers surrounding it. The BBB is a semi-permeable boundary between blood and the brain and is critical for sustaining a suitable neuronal environment and maintaining CNS homeostasis (Daneman and Prat, 2015, Kadry et al., 2020, Chow and Gu, 2015, Archie et al., 2021, Abbott et al., 2010, Bernacki et al., 2008). This barrier is formed by specialized endothelial cells that are supported by a basement membrane and surrounded by astrocytes and pericytes (Abbott, 2002, Abbott et al., 2010, Furtado et al., 2018, Abbott, 2013, Abbott et al., 2006, Abbott and Romero, 1996). The BBB is tightly regulated (Daneman and Prat, 2015, Kadry et al., 2020, Chow and Gu, 2015, Archie et al., 2021, Abbott et al., 2010, Bernacki et al., 2008) through endothelial cell interactions with astrocytes and pericytes. Molecules released from astrocytes and surrounding cells actively downregulate endothelial tight junction proteins allowing the opening of the BBB, thereby regulating the navigation of ions, cells, and molecules into the perivascular space.

Although astrocytes play a crucial role in maintaining BBB integrity (Abbott et al., 2006, Abbott, 2002, Daneman and Prat, 2015, Yao et al., 2014), they are also associated with BBB disruption during disease (Argaw et al., 2012, Horng et al., 2017, Bell et al., 1996). A compromised BBB facilitates neurotoxin, pathogen, and cellular entry into the brain and is a critical component for many neuropathological conditions (Zhao et al., 2015). CNS-TB is characterized by excessive inflammation and injury to the CNS tissue (Nelson and Zunt, 2011, Poh et al., 2022b). A main contributing factor to the immunopathology observed in CNS-TB is the disruption of the BBB through injury or cellular mediator release. This is associated with the accumulation of leukocytes in the perivascular space where they come into direct contact with the glia limitans which are formed by astrocyte end-foot processes. Navigation across the glia limitans also requires active recruitment and this process is facilitated by the release of molecules from various cell types including astrocytes (Horng et al., 2017, Argaw et al., 2012, Argaw et al., 2006). Thus, together the BBB and the glia limitans, create a coordinated double barrier guarding entry into the CNS parenchyma.

Astrocytes are in a critical position to modify the barriers and subsequently regulate cellular influx into the CNS parenchyma in a context-specific manner (Jin et al., 2012). Data from the *M. bovis* BCG and *M. tuberculosis* infections established that astrocytes create an inflammatory milieu by secreting various cytokines and chemokines in response to infection, some of which reportedly contribute to BBB permeability and leukocyte entry into the CNS. It is therefore possible that the release of such molecules by astrocytes directly influences leukocyte infiltration into the brain parenchyma during infection. Although it is known that cytokines and chemokines can disrupt the barriers by altering tight junction protein expression (Capaldo and Nusrat, 2009, Pan et al., 2011, Rochfort et al., 2014), their precise role in allowing leukocytes to penetrate the CNS during *M. tuberculosis* infection is not fully understood. One of the most notable BBB-altering cytokines is IL-1β which emerged as a principal cytokine released by astrocytes during mycobacterial infection. IL-1β acts directly on endothelial cells through tight junction protein modifications (Blamire et al., 2000, Laflamme et al., 1999, Wang et al., 2014) or by stimulating endothelial production of the glycoproteins VCAM-1, ICAM-1, and E-selectin which then allow leukocyte adhesion, permitting easier migration into the

parenchyma (Hofman et al., 1986, Dinarello, 1991). Given that astrocytes were shown to release IL-1β, it would be reasonable to surmise that IL-1β activity is one of the mechanisms through which they modulate leukocyte transmigration.

An important aspect to consider is, not only the production of IL-1β by astrocytes but also the capacity of astrocytes to respond to this cytokine. IL-1β was shown to stimulate the induction of vascular endothelial growth factor (VEGF) from astrocytes (Argaw et al., 2012, Argaw et al., 2006, Argaw et al., 2009), an effective angiogenic factor that is associated with BBB permeability (Yancopoulos et al., 2000, Su et al., 2006, Proescholdt et al., 2002). Access into the CNS parenchyma is partly limited via complex tight junctions, and it has been suggested that through astrocyte regulation of tight junction protein expression they can adjust BBB permeability (Horng et al., 2017, Gu et al., 2012, Jiang et al., 2014, Yang et al., 2007, Liu et al., 2016, Kong et al., 2015). Interestingly, tight junctions are usually downregulated under inflammatory conditions, disrupting the BBB. Sonic the hedgehog (SHH) is a protein involved in upregulating tight junctions (Alvarez et al., 2011), promoting endothelial BBB repair (Alvarez et al., 2011, Alvarez et al., 2013), and sustaining BBB integrity. IL-1β can disrupt astrocyte function by downregulating SHH expression, therefore disturbing endothelial cell tight junctions and affecting the BBB (Wang et al., 2014). Interestingly, during inflammation astrocytes are able to induce tight junction bonds at the glia limitans, providing an additional boundary for immune cell or molecule entry (Horng et al., 2017). Although IL-1β results in astrocyte disruption of the BBB, it reciprocally increases barrier function at the glia limitans. Astrocytes exposed to IL-1β *in vitro* and *in vivo* induce expression of the tight junction proteins claudin 1 (CLDN1), claudin 4 (CLDN4), and junctional adhesion molecule A (JAM-A) (Horng et al., 2017, Tsukita and Furuse, 2000, Severson and Parkos, 2009) in the glia limitans. Upregulation of these proteins by astrocytes not only seals the glia limitans thereby controlling access, but they shape immune responses by increasing BBB permeability causing leukocytes to accumulate in the perivascular space where they will encounter CNS antigen (Argaw et al., 2012, Argaw et al., 2009, Proescholdt et al., 2002).

Although IL-1β is a considerable contributor to BBB permeability, which can be detrimental, the neutralization of IL-1β is not a favourable therapeutic option as IL-1β also augments the beneficial effects of astrocytes on the BBB (Herx and Yong, 2001). Furthermore, despite its redundancy in BCG infection, IL-1β plays a significant protective function during *M. tuberculosis* infection (Francisco et al., 2015, Juffermans et al., 2000, Mayer-Barber et al., 2010, Fremond et al., 2007, Yamada et al., 2000, Bourigault et al., 2013). A plausible alternative, however, would be targeting SHH or VEGF as a therapy to reduce cellular infiltration, or potentially upregulating the expression of tight junction proteins, essentially protecting the CNS from neuroinflammatory damage. Managing VEGF expression could reduce TB meningitis complications as it is notably induced in tuberculous meningitis and is associated with BBB disruption and brain oedema (van der Flier et al., 2004, Yang et al., 2023, Zucchi et al., 2013). Interestingly, VEGF production is not only modulated by IL-1 but TNF too (Ryuto et al., 1996).

TNF is considerably expressed in neuropathological states (Dinarello, 1991, Hofman et al., 1986, Decourt et al., 2017, Sairanen et al., 2001, Badoer, 2010, Gong et al., 1998) and is associated with BBB breakdown, blood-CSF disruption, and neutrophil recruitment (Chen et al., 2019, Hussain et al., 2021, Zeni et al., 2007, Sayed et al., 2010, Egashira et al., 2013, Ferrari et al., 2004). Astrocyte expression of TNF in response to *M. bovis* BCG and *M. tuberculosis* suggests an influence on BBB permeability allowing the infiltration of circulating immune cells. Proteolytic enzymes released by infiltrating neutrophils contribute to BBB breakdown through disintegration of the basement membrane and loss of tight junctions allowing leukocyte entry (Klein et al., 2017). Moreover, TNF modulates microvascular permeability and facilitates leukocyte infiltration via the induction of VCAM-1, ICAM-1, and E-selectin on endothelial cells (Brilha et al., 2017). Adhesion molecules, such as ICAM-1 and/or VCAM-1, facilitate astrocyte-lymphocyte interaction which are upregulated upon astrocyte exposure to IFN-γ, TNF, IL-1β or TLR ligands such as LPS (Ransohoff et al., 2003, Gimenez et al., 2004). This increased immune cell infiltration elevates localised TNF concentrations which could then contribute to pathogenesis.

Il-6 secretion by astrocytes is implicated in cerebral endothelial cell permeability, by reducing endothelial tight junction expression (Rochfort et al., 2014). Notably, tight junction integrity is reportedly compromised during bacterial meningitis (Brilha et al., 2017, Schubert-Unkmeir et al., 2010, Leppert et al., 2001) partly attributed to elevated IL-1 and IL-6 production. IL-6 is one of the major cytokines produced by astrocytes, particularly during CNS injury and inflammation (Van Wagoner et al., 1999, Almolda et al., 2015, Aloisi et al., 1992, Benveniste, 1998, Gruol and Nelson, 1997, Erta et al., 2012). Studies suggest that IL-6 together with IL-1β and TNF, are the main cytokines detected in the CSF of patients with multiple sclerosis, stroke, and meningitis (Zhao and Schwartz, 1998, Konsman, 2022) and promote the pathophysiology observed during these inflammatory disorders (Van Wagoner et al., 1999, Eng et al., 1996, Benveniste, 1992, Konsman, 2022). Thus, the production of these cytokines by astrocytes during mycobacterial infection alludes to their participation in modulating BBB permeability and immune infiltration, while possibly contributing to the neuropathology associated with bacterial meningitis.

M. bovis BCG and *M. tuberculosis*-infected astrocytes also increases expression of multiple chemokines known to participate in BBB permeability (Glabinski and Ransohoff, 1999, Man et al., 2007, Zozulya et al., 2007, Kuang et al., 2009), particularly CCL2, CCL3, CCL5, CCL7, CXCL1, CXCL2, CXCL3, CXCL5, and CXCL10. Heightened expression of astrocyte derived CXCL1 and CCL2 following mycobacterial infection suggests a crucial role in transendothelial migration and BBB function as demonstrated in previous studies (Weiss et al., 1998, Michael et al., 2020). Abrogation of CCL2-CCR2 signalling and CXCL1-CXCR2 signalling was associated with diminished monocyte and neutrophil recruitment, respectively, and a reduction in BBB permeability (Michael et al., 2020). Elevated expression of CXCL10, and CCL5 in BCG and *M. tuberculosis* infected astrocytes indicates that they potentially contribute to leukocyte infiltration as seen in neurodegenerative disorders (Sørensen et al., 1999) and viral infections (Liu et al., 2001). CXCL10, particularly, plays a prominent role in T cell recruitment and BBB integrity and could therefore contribute to adaptive immunity against CNS-TB, however, its participation in BBB disruption may cause tissue damage. Elevated expression of CCL3 by astrocytes further indicates their contribution in BBB modulation. CCL3 enhances

BBB permeability during viral infections (Kuang et al., 2009) and modulates T lymphocyte and dendritic cell transmigration across brain endothelial cells in neurodegenerative diseases (Zozulya et al., 2007, Man et al., 2007). Apparently, CCL3 is unable to cross the BBB, but may indirectly influence transmigration of these cells by binding to BBB endothelia (Banks and Kastin, 1996). Consistent with this postulate it was reported that the interaction of CCL3 and its CCR5 receptor stimulated endothelial tight junction opening in the BBB (Man et al., 2007). Thus, the release of specific chemokines by astrocytes can regulate cellular recruitment into the parenchyma by altering BBB permeability.

Dysregulation of astrocyte activity by pathogens may, however, cause uncontrolled cellular influx. There is undoubtedly a capacity of mycobacteria to affect the BBB as mice intracerebrally infected with *M. bovis* BCG and *M. tuberculosis* H37Rv resulted in excessive inflammatory cell infiltration into the brain parenchyma (Mazzolla et al., 2002, Van Well et al., 2007). Various aspects of BBB physiology are influenced by astrocytes and because their responses are signal specific, the mechanisms through which they alter BBB permeability during mycobacterial infection are likely to be strain dependent. It is reasonable to presume that following BCG or *M. tuberculosis* infection, astrocytes disrupt the BBB and recruit circulating leukocytes into the brain parenchyma through their production of IL-1β, IL-6, TNF, and various chemokines, but may also contribute to BBB maintenance through their production of IFN-β thereby performing a regulatory function. Many studies investigating BBB regulation focus on the inflammatory cytokines, often overlooking the contribution of IFN-β. Remarkably, IFN-β can stabilize BBB integrity by enhancing tight junction formation on endothelial cells and by impeding IL-1β expression thereby indirectly preventing barrier interference (Kraus et al., 2004, Daniels et al., 2014). By counteracting the effects of the inflammatory cytokines on the BBB, astrocyte derived IFN-β may protect the CNS from excessive inflammation.

Although a reports suggest that astrocytes are not essential for BBB maintenance *in vivo*, (Kubotera et al., 2019) the notion that astrocytes are essential to BBB integrity is still widely accepted. Discrepancies in the literature may be due to differences in the extent of astrocyte loss, the duration of loss, or a consequence of the regions ablated. One

study investigating laser ablation of astrocyte end-feet concluded that these processes were not essential for BBB function (Kubotera et al., 2019). However, only the end-feet were ablated and not the entire cell. In this case astrocytes were able to extend their processes to cover exposed areas (Mills et al., 2022) thereby preventing the loss of BBB integrity. Moreover, the inability to repair the barrier following astrocyte ablation provides further evidence of their essential, nonredundant role in maintaining the BBB (Heithoff et al., 2021). Ablation of reactive astrocytes results in elevated leukocyte infiltration and neurodegeneration suggesting that activated astrocytes play a crucial role in modulating inflammation following injury (Faulkner, 2004, Bush et al., 1999). The array of factors released by astrocytes during BCG and *M. tuberculosis* infection reflects their pivotal role in the modulation of BBB endothelial cells whereby barriers are either opened or preserved in a signal-specific manner. In response to pathological conditions astrocytes can control the extent of inflammation, however, dysregulation or loss of astrocyte function during disease could result in a disrupted BBB allowing the uncontrolled influx of leukocytes.

While the selected choice for pathogen challenge occurred via intracerebral infection the natural route of bacilli dissemination would be through hematogenous spread from the lungs to the brain. In principle, pulmonary infection would precede CNS-TB in a normal clinical setting. As such, studies have shown that prior to bacilli dissemination, mediators released from the lungs could possibly influence CNS activity. Thus, infection at distant sites can result in the loss of brain barrier function and influence the inflammatory responses in the brain. The interaction between the nervous and immune systems were previously underappreciated, but recent studies (Dantzer, 2018, Sanmarco et al., 2021, Han et al., 2021, Greenhalgh et al., 2020) have suggested that although distinct, these systems are remarkably closely associated and dysfunction in their communication pathways is often a contributor to numerous pathological conditions.

Communication between the CNS and peripheral immune system through a lymphatic system within the meninges indicates that the CNS is under constant immune surveillance (Louveau et al., 2015). Patrolling APCs within this system would proceed to the cervical lymph nodes after an encounter with pathogens that invaded the CNS, and

prime naïve T cells for maturation. Expression of MHC II molecules is critical to initiate immune responses by presenting processed antigen to $CD4^+$ T-helper cells via the T cell receptor (TCR). While both glial cell types, astrocytes and microglia, induce MHC-II and co-stimulatory signals under inflammatory conditions to activate T cells (Nikcevich et al., 1997, Constantinescu et al., 2005), the role of astrocytes in presenting antigen is contentious. Interestingly, in this study astrocyte expression of MHC II was increased in wild type mice suggestive of a functional role in adaptive immunity. IFN-γ is efficient at inducing MHC II expression on microglia, but its effects on astrocytes may be delayed (Esen et al., 2004). Studies indicate that IFN-γ and TNF-treated astrocytes upregulate the expression of MHC II, CD80 (B7-1) and CD86 (B7-2), for antigen presentation and proficiently activate naïve Th1 or Th2 $CD4^+$ T cells (Nikcevich et al., 1997, Girvin et al., 2002). Given that microglia express TNF during *M. tuberculosis* infection, it is possible that they induce astrocyte expression of MHC-II and co-stimulatory factors (Curto et al., 2004) resulting in T cell activation. Others showed that IFN-γ -activated astrocytes are more efficient at stimulating Th2 rather than Th1 cells, which leads to IL-4 expression (Aloisi et al., 1998).

Notably production of IL-12 by astrocytes during *M. tuberculosis* infection indicates their potential to polarise cells towards Th1 responses. APCs and phagocytic cells produce IL-12, which is important for T-cell priming and the development of Th1 type immune responses. IL-23, a member of the IL-12 family, is also necessary for the establishment of T-cell mediated inflammation and involved in both Th1 and Th17 responses (Constantinescu et al., 2005). Under inflammatory conditions, astrocytes express IL-12 and IL-23, and present antigen to encephalitogenic T-cells in an IL-12/IL-23 dependent manner (Constantinescu et al., 2005). Together with IL-12, IFN-γ drives T-cell maturation towards a Th1 phenotype. This study also displayed the production of IL-10 in BCG and *M. tuberculosis* infected astrocytes. Astrocyte expression of IL-10 may reduce Th1 cytokine expression and induce naïve T cells to differentiate into Th2 cells (Meeuwsen et al., 2003, Bsibsi et al., 2006). Co-expression of IL-10 and IL-12 by astrocytes in response to BCG and *M. tuberculosis* is indicative of a regulatory role in the Th1/Th2 balance of the CNS. Although T cell-astrocyte interaction *in vivo* is not well documented in current

literature, it is a noteworthy association with important implications in the control of neuroinflammation.

Distinct from the Th1 or Th2 lineage is the Th17 effector $CD4^+$ T cells, also activated by IL-23, but in the presence of TGF-β and IL-6 (Mangan et al., 2006, Bettelli et al., 2006). Microglial production of TGF-β (Lehrmann et al., 1998, Hernandez Pando et al., 2010) and astrocyte production of IL-6 during mycobacterial infection therefore suggests a potential role in the progression of Th17 cell expansion (Ma et al., 2010, Weaver and Hatton, 2009, Veldhoen et al., 2006, Mangan et al., 2006). TGF-β produced by Th17 T-cells and microglia is predominantly associated with immune-suppression via Th1 and Th2 inhibition as well as the development of T-regulatory cells (Bettelli et al., 2006). However, it is now apparent that TGF-β is crucial for Th17 progression. TGF-β signalling in an inflammatory climate aids IL-23 recognition by upregulating IL-23R expression, thus promoting Th17 development (Mangan et al., 2006). Interestingly, by transferring Th17 supernatants to astrocyte cultures, Th17 effector molecules could directly influence astrocytic function and phenotype (Prajeeth et al., 2017). IL-17, in particular, can induce IL-6 production by astrocytes that triggers a positive-feedback loop of IL-6 to promote Th17 cell differentiation. The correlative and inimical action of astrocytic cytokines regulates Th1 and Th17 polarization, indirectly coordinating the cytokine reservoir, and ultimately influencing the adaptive immune response. In this way astrocytes help equip the CNS to combat permeating pathogens through expansion of the relevant T cells. An in-depth investigation on the interactions between astrocytes and T cells would uncover the direct contribution of astrocytes to T cell polarisation and their effector functions during CNS-TB.

It is known that diverse cell types contribute to the intricate balance of cytokines required for effective mycobacterial control in the CNS (Randall et al., 2014, Francisco et al., 2015, Rock et al., 2005, Davis et al., 2019). The absence of a fully functional immune system disturbs this balance and susceptibility is elevated under conditions of either uncontrolled *M. tuberculosis* proliferation or inflammatory-related tissue damage. Children and patients living with HIV or immunodeficiency commonly display exacerbated disease and are most at risk of developing extrapulmonary TB such as CNS-TB (Török, 2015, Qian

et al., 2018, Khan et al., 2019). Given the importance of effective communication between the CNS and the peripheral immune system for protection against cerebral tuberculosis, astrocyte function was evaluated in highly immunodeficient NSG mice to determine their immune contribution in the absence of functional peripheral immune cells; whereas hNSG-BLT were included to determine whether supplementation with human immune cells was able to restore immune function in NSG mice.

Injury can be classified as pathogenic or non-pathogenic, but both require effective immune responses. Although the mechanical injury caused by the surgical procedure is traumatic, both the saline challenged NSG and hNSG-BLT mice survived without clinical manifestations indicating that the cerebral surgical procedure did not impact the experimental results. In contrast, *M. tuberculosis* infected mice displayed behavioural and physical deterioration compared to the saline controls. NSG mice lack a functional immune system due to compound mutations that cause defects in cells of myeloid origin. The regression of NSG mice in comparison to C57BL/6 mice following intracerebral *M. tuberculosis* inoculation was, therefore, anticipated due to their dysfunctional immune system. Surprisingly, hNSG-BLT mice were as susceptible as NSG mice to infection despite successful engraftment, suggesting that reconstitution with human immune cells were unable to rescue the mice. In fact, the hNSG-BLT mice arguably displayed more substantial clinical impairments. These observations are consistent with previous studies indicating impaired bacterial control in humanised mice following intravenous *M. tuberculosis* infection (Heuts et al., 2013). Given that allogeneic bone marrow transplants are commonly associated with the development of xenogeneic graft-versus-host disease (GvHD)-like syndrome (Covassin et al., 2013), it is plausible that this could have contributed to the increased susceptibility of *M. tuberculosis* infected humanised BLT mice. Previous studies using humanised mice models have reported this condition following engraftment (Greenblatt et al., 2012, Lockridge et al., 2013, Gillgrass et al., 2021). Considering their ability to stimulate cytokine production, bacterial infection can also aggravate the progression of GvHD (Fuji et al., 2014). Thus, infection with *M. tuberculosis* may have contributed to early development of the condition.

Despite the excessive bacterial load, astrocyte cytokine responses were not significantly elevated in infected NSG mice as the production of IL-1β, IL-10, IL-12, TNF, iNOS, MHC II and CD86 was unchanged in comparison to the sham control mice. Interestingly, the phagocytic capability of astrocytes was unhindered and to date, this is the first report demonstrating internalisation of *M. tuberculosis* by astrocytes in an animal model. Nonetheless, the lack of astrocytic functionality in response to *M. tuberculosis* infection in NSG mice was unexpected and the reasons for this unresponsiveness unknown. Astrocytes may lack inherent full functionality due to flawed metabolic pathways because of genetic defects associated with NSG mice. Alternatively, cooperation with other cell types such as microglia were suboptimal to induce full functionality i*n vivo*. For example, the regular interactions between astrocytes and microglia may, however, be disrupted in NSG mice which exhibit a SIRP-1α mutation and defective IL-2Rγ protein in myeloid cells which likely affects microglia. Interestingly, while the bacterial burden in hNSG-BLT mice was comparable to NSG mice, *M. tuberculosis* infected hNSG-BLT mice was responsive to infection and displayed notable increases in IL-1β, IL-10, IL-12, TNF, iNOS and MHC II production and expression, trending toward significance when compared to infected NSG mice. The differences may reflect cellular cooperation between peripheral immune cells and astrocytes in hNSG-BLT mice during *M. tuberculosis* infection. Astrocytes are evidently key inflammatory mediators during early CNS-TB infection, but the findings in this study argues strongly for the presence of a fully functional peripheral immune system; for effective cooperation between the neural and immune networks to generate specific immune responses against *M. tuberculosis*.

Clinical management of CNS-TB is challenging as a result of inadequate diagnostics and inaccessibility. Identifying biomarkers that can be used to diagnose CNS-TB is important for early and effective treatment. Despite advances in the development of biomarker-based tests, most tests focus on pulmonary TB in adults. Studies exploring alternative approaches for the identification of novel diagnostics, including protein, transcriptional, microRNA, and metabolomic biomarkers are ongoing (Manyelo et al., 2021). Additional research, however, is required to enrich the depth of knowledge on disease immunopathogenesis associated with CNS-TB for improved diagnostic tools and host-directed therapies. This study provided an extensive selection of transcriptional

biomarkers which can potentially be used as diagnostic tools. Although elevated CSF cytokine levels are common among TBM patients, there are conflicting reports in the literature regarding the association between cytokines and the severity of meningitis and disease outcome in CNS-TB patients. Several cytokines were observed to be upregulated in TBM patients, including TNF, IL-10, IFN-γ, IL1-β, IL-6, and IL-8 (Kwon et al., 2019, Van Laarhoven et al., 2019, Misra et al., 2010, Mastroianni et al., 1997, Donald et al., 1995). While some studies indicated direct correlations between cytokine expression such as TNF, IL-1β, IL-2, IL-6, TNF and IFN-γ (Tsenova et al., 1999, Sharma et al., 2017) and disease progression; others have shown no link (Misra et al., 2010, Donald and Van Toorn, 2016). Novel biomarkers may therefore be useful if used in conjunction with cytokine markers. PTX3 for example, is a known astrogliosis-associated marker *in vivo* (Zamanian et al., 2012) and is a viable candidate as a prognostic biomarker. It can be measured in the CSF of patients as levels are higher in bacterial meningitis compared to aseptic meningoencephalitis (Zatta et al., 2020). In addition, elevated levels are associated with severity of infection, particularly in sepsis (Liu et al., 2014, Hu et al., 2018, Caironi et al., 2017).

Consistent with the type I IFN signature observed in this study, particular interferon related genes detected early in pulmonary TB have been identified as transcriptional biomarkers which correlate with progression to active disease and disease severity (Berry et al., 2010). Human blood transcriptional profiles display a prominent type I IFN-inducible gene signature including IFITs, GBPs, IRF1, and STAT1 (Moreira-Teixeira et al., 2018, Scriba et al., 2017, Zak et al., 2016). Interestingly, in this study, BCG-infected astrocytes displayed an upregulation in *Ifit1*, *Ifitm1*, *Stat1* and *Irf1*. Astrocytes infected with *M. tuberculosis*, similarly, exhibited an increase in *Ifit1*, *Ifitm1*, *Stat1* and *Irf1* as well as *Gbps*. In addition, both strains induced the production of positive and negative regulators, suggesting tight regulation of the IFN pathways. Given that a type I IFN signature precedes up-regulation of myeloid inflammation, understanding IFN signalling pathways in a clinical setting can improve the development of diagnostic biomarkers, possibly identifying patients at the highest risk of progression to CNS-TB.

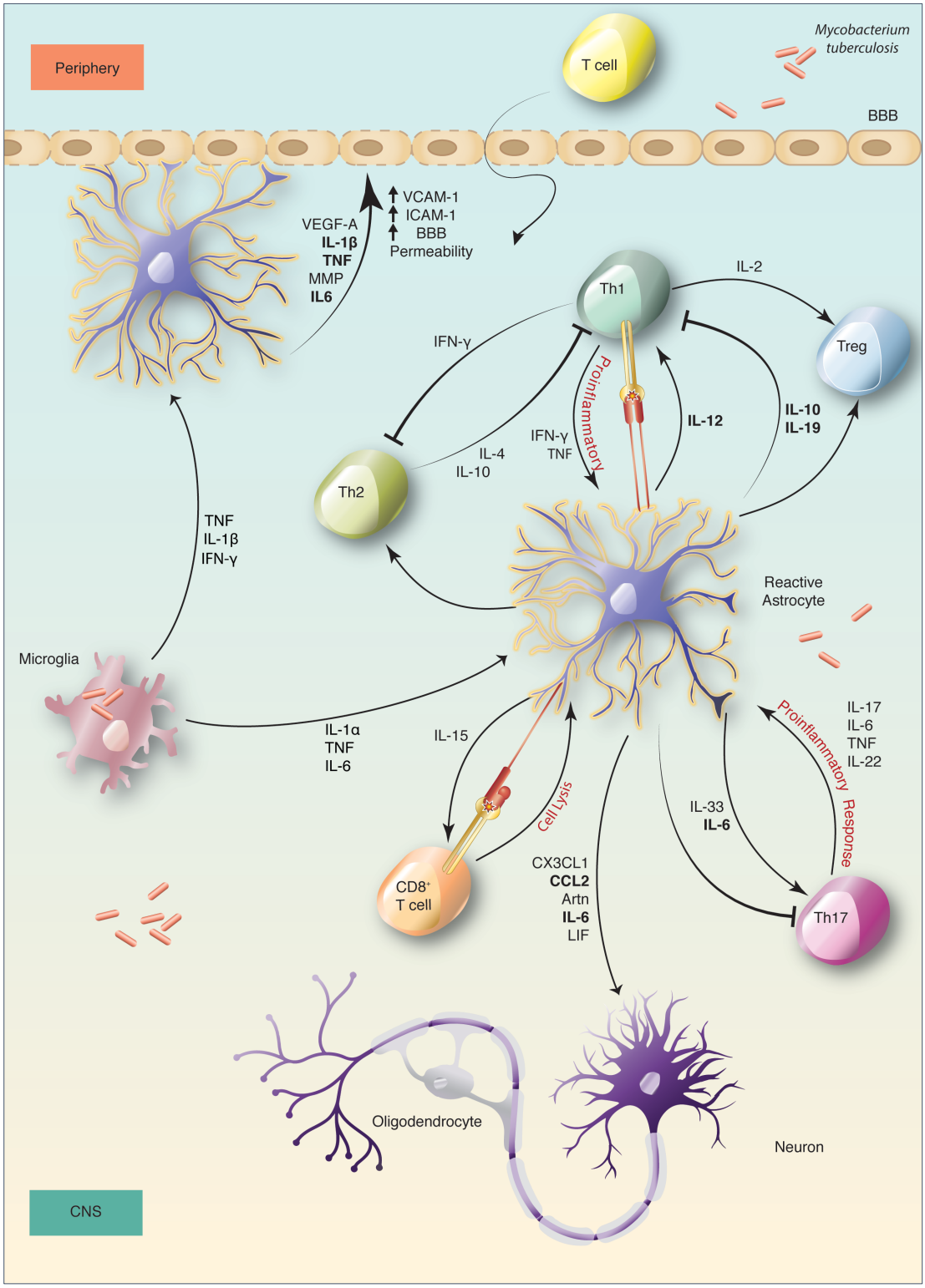


Figure 6.1 | Graphical summary of astrocyte activation and immune regulation during *M. tuberculosis* infection. Activation of microglia and astrocytes is a sensitive indication of changes following CNS infection. This leads to secretion of cytokines and chemokines by reactive astrocytes that changes the BBB permeability and promotes the recruitment of peripheral immune cells, such as T cells. Recruited T cells then infiltrate into the CNS parenchyma across the disrupted BBB and after entering, are restimulated by reactive astrocytes. T cell polarization is dependent on the cytokine milieu created by astrocytes and other resident cells. Neurotrophic factors are also released by astrocytes during M. tuberculosis infection and may influence neuronal survival and function. All factors depicted in bold were shown to be produced by astrocytes during M. tuberculosis infection (Adapted from Geyer et al. 2019).

STUDY LIMITATIONS

Primary astrocytes and *in vivo* mouse models are valuable tools for investigating the biology of astrocytes and their responses to mycobacteria. However, there are several limitations to consider when interpreting the results of these studies. Transcriptomic analysis, which examines the expression of genes at the RNA levels, is an increasingly popular method for investigating cellular and molecular processes. One of the greatest assumptions made when analysing transcriptomic data is that LogFC values assume a linear relationship between the expression levels of genes, but gene expression changes may not always be linear. Module and pathway analyses were therefore investigated to gain a more complete understanding of the data. In addition, the cut-off values for log change may result in missed gene expression changes or false positive results if these cut-offs are too stringent or lenient, respectively. To account for this limitation statistical significance was evaluated in conjunction with LogFC. A further important consideration is that transcriptomic studies capture a "snapshot" of gene expression at a specific time point but do not reflect the dynamic nature of gene expression over time. Changes in gene expression may occur rapidly in response to environmental cues, making it difficult to capture in a transcriptomic study. Interestingly although the data presented in this study is from astrocytes rather than typical immune-modulating leukocytes, the signatures observed are consistent with previously published reports. However, caution needs to be exercised when comparing transcriptional responses between different cell types and across species as selected experimental models directly influence expression patterns. Transcriptomic studies should therefore be interpreted prudently and ideally substantiated with further analyses. Protein quantification is often used for this purpose of validation, but an important caveat to consider is that protein concentrations do not necessarily correlate with mRNA expression levels due to different half-lives and post-transcriptional modulation (Brion et al., 2020, Carlyle et al., 2017, Franks et al., 2017).

The RNA extracted for these transcriptomic investigations was obtained from primary astrocyte cultures. *In vitro* astrocyte cultures have limitations and do not accurately reflect the complex *in vivo* environment of the CNS, including the impact of other cell types, and the immune milieu created by an *in vivo* system. Comparing this data to previously published results, one must be cognisant of differences associated with treatment times,

cytokine dosing, maturity of cells and, most importantly, the source of astrocytes as important variables that can confound direct comparisons. Such differences can impact behaviour and function of astrocytes and limit the ability to translate the results of *in vitro* studies to *in vivo* settings. In addition, homogenous astrocyte monocultures do not adequately display the complex heterogeneous astrocyte populations *in vivo.* Therefore, while *in vitro* astrocyte analyses are practical tools for investigating the biology of astrocytes, they should be interpreted judiciously and validated using *in vivo* models to ensure that the results are relevant to the *in vivo* environment and CNS-TB pathology.

In vivo mouse models are commonly used to study astrocytes in the CNS, but they also have several limitations with respect to clinical interpretation and application. In this study, an artificial infection method was used by intracerebrally inoculating mice with *M. tuberculosis*, which does not represent the natural progression of the disease. Mouse models also do not accurately represent human-specific pathology of CNS-TB and may not display the complex human immune response to this disease. In addition, mouse astrocytes may have behavioural, genetic, and functional differences from human astrocytes (Degl'Innocenti and Dell'Anno, 2023), particularly in the context of CNS-TB. While this study focuses on murine astrocytes, interpreting results for the human context necessitates recognizing potential divergences. Although murine insights offer valuable understanding of general astrocyte activity during infections, caution and further investigation are needed for direct translation to human astrocyte behavior. To comprehend astrocyte responses and their relevance to human biology, it's pivotal to complement murine research with human-specific studies. These investigations reveal species-specific differences, enriching our grasp of astrocyte behavior and its implications for human diseases.

Although various models exist, C57BL/6 mice was selected in this context as they are valuable for studying protective immune mechanisms. More susceptible strains such as BALB/c or Kramnik mice might provide insights into disease exacerbation. Given that different mouse strains possess distinct immunological profiles that influence their response to TB infection, this can affect the interpretation of findings and its relevance to human TB.

The incorporation of humanized mouse model in this context, was to replicate human disease conditions and recapitulate the human immune response in a murine setting. While acknowledging the complexities of the humanized mouse model and that the astrocytes present within our humanized mouse model are of non-human origin, the findings from this study strongly support the existence of a fully functional peripheral immune system. This functional immune system is pivotal in establishing effective neuro-immune interactions that result in targeted immune responses against *M. tuberculosis*. Therefore, these results highlight the presence of a robust immune system rather than a compromised one within this humanized mouse model. Moreover, the data generated by this study serves as a fundamental framework for the future development of a preclinical model.

The contribution of astrocytes to innate immunity and host protection is well documented (Geyer et al., 2019, Farina et al., 2007), nevertheless, they are non-professional immune cells and comparing their activity to prominent immune cells should be done with caution. Lastly, although the focus of this study was on astrocyte activity, it would be important to conduct further investigations into other cell types such as neutrophils and leukocytes and how they influence astrocyte responses to obtain a more complete understanding of the immune response during infection.

CONCLUSION

The threat of mortality and substantial neurological complications associated with CNS bacterial infection, specifically neurotuberculosis, is significant, especially in developing countries, due to the limitations of diagnosis and treatment. This highlights the urgent need to develop novel approaches for addressing CNS-TB. It is, therefore, important to study how brain cells respond to mycobacteria to gain a deeper understanding of their role in disease progression. Identification of novel regulatory mechanisms can lead to the development of targeted interventions that improve treatment outcomes. This innovative investigation explored astrocyte contributions to host immune responses during CNS-TB infection by examining their regulation of CNS inflammation in the presence of mycobacterial challenges. Astrocytes, as glial cells in the CNS, play a progressive role in maintaining the structural and functional integrity of the CNS while supporting neuronal

function and participating in host protection during infection of the CNS. These cells exist as distinct populations with complex morphological identities and functional modifications suited to their micro-environment, and astrocyte reactivity likely exists on a spectrum as their responses depend on the type of injury or pathogen and their proximity to the insult.

The initial hypothesis of this study was that astrocytes are crucial regulators of host immunity during mycobacterial infection, and the principal aim was to elucidate the genomic profile and cellular immune responses of astrocytes to characterise their immunomodulatory potential during CNS-TB infection. The novel findings presented here collectively demonstrate the sophistication and complexity of astrocyte activity and behaviour during host immunity. For the first time, astrocytes showed internalisation of *M. bovis* BCG and *M. tuberculosis* bacilli, demonstrating that astrocytes are target cells for non-virulent and virulent mycobacterial strains. Extensive transcriptomic analysis of astrocytes revealed elevated expression of multiple pathways in infected cells, particularly those involved in inflammation and immune regulation, and emphasised the innate immune component. Notably, various pro-inflammatory cytokines essential to host defence during CNS-TB infection were upregulated by astrocytes following the mycobacterial challenge. A robust type I IFN signature indicated that astrocytes activate IFN-related processes during mycobacterial infection. Although the overall gene signatures between BCG and *M. tuberculosis*-infected astrocytes were similar, there were definite distinctions in the gene expression profiles generated between the two strains suggesting that bacterial virulence influences gene expression. The transcriptomic data was validated by *in vitro* and *in vivo* experiments demonstrating specific astrocyte-induced cytokines and chemokines in response to mycobacterial infection. The elevation of proinflammatory gene expression by astrocytes, and their production and release, support their contribution to immune modulation through immune cell recruitment and activation at the site of infection. Furthermore, the murine models used herein highlight the importance of a functional peripheral immune system for effective astrocyte modulation of host immune response.

Moreover, the range of cytokines and chemokines produced by astrocytes during mycobacterial infection suggests that it is an influential cellular source capable of regulating BBB permeability by allowing immune cells to attach to and cross the BBB. In this way, they may modulate host immune responses and help regulate the severity of the disease. Although astrocytes may contribute to the development of CNS-TB pathology through proinflammatory factor secretion, the ability of astrocytes to regulate uncontrolled inflammation include the production of anti-inflammatory mediators and possibly signalling via negative feedback loops. This potential was demonstrated by their enhanced expression and production of well-described specific anti-inflammatory factors following mycobacterial infection. The data further showed increased expression of neurotrophic factors by astrocytes which can assist in the recovery and repair of the CNS during CNS-TB. By supporting the survival and function of neurons and modulating immune cell activity, astrocyte-derived neurotrophic factors can help to limit infection-induced damage and promote the resolution of inflammation.

The intricate interplay of the cytokines/chemokines creates a dynamic environment that needs careful modulation to achieve optimal outcomes. It is, therefore, imperative to maintain a delicate balance to facilitate pathogen clearance while preventing damage to host tissue caused by excessive inflammation. This study has presented the dichotomous behaviour of astrocytes during CNS-TB as they can contribute to the maintenance and protection of CNS function and host immune responses while potentially enhancing pathology during infection. Therapies selectively modulating astrocyte activity in response to CNS-TB infection may be possible by targeting specific molecular markers or receptors on these cells. Further investigation is, however, necessary to gain a comprehensive understanding of astrocytes during CNS *M. tuberculosis* infection and to explore the potential for developing more efficient and personalised treatments for this debilitating disease.

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APPENDIX

Table S-1 | Top 30 differentially expressed genes induced in astrocytes during BCG infection.

Gene Name	logFC	Adj. P Value	Gene Name	logFC	Adj. P Value
Cxcl1	6.29	2.37e-11	*Il1b*	4.15	6.49e-12
Csf3	5.74	4.65e-16	*Csf2*	4.14	2.18e-13
Cxcl2	5.56	6.19e-15	*2010005H15Rik*	4.08	7.38e-12
Il6	5.06	2.77e-13	*Mx1*	3.97	2.91e-10
Ccl7	4.98	4.62e-09	*Il19*	3.96	3.65e-09
Nos2	4.80	5.55e-12	*BC100530*	3.92	1.07e-12
Cxcl3	4.74	3.34e-13	*Rsad2*	3.89	1.68e-10
Tgtp2	4.64	1.1e-07	*H2-M2*	3.88	4.0e-11
Cxcl5	4.63	3.9e-09	*Ccl4*	3.80	3.47e-14
Gbp5	4.55	2.46e-09	*Ccl3*	3.77	1.18e-12
Gm5483	4.45	1.19e-13	*Ifi44*	3.76	3.39e-07
Tnf	4.43	6.19e-15	*Gbp6*	3.74	1.42e-09
Gbp4	4.34	1.21e-10	*Cxcl10*	3.77	5.31e-14
Il1a	4.21	1.29e-13	*Ccl2*	3.73	7.41e-08
Mmp13	4.21	1.76e-13	*Stfa3*	3.7	2.25e-11

Table S-2 | Top 30 differentially expressed genes induced in astrocytes *M. tuberculosis* BCG infection.

Gene Name	logFC	Adj. P Value	Gene Name	logFC	Adj. P Value
Cxcl1	5.11	7.64e-11	*Gbp6*	3.01	8.98e-10
Tgtp2	4.32	1.20e-09	*Gbp11*	2.95	1.27e-11
Cxcl5	4.06	1.31e-10	*Isg15*	2.91	8.65e-09
Ccl7	3.99	3.74e-09	*Gm9706*	2.90	1.83e-08
Nos2	3.75	8.26e-12	*Csf3*	2.85	6.75e-12
Gbp4	3.73	2.94e-11	*Clic5*	2.81	1.11e-11
Gbp5	3.67	1.79e-09	*Gm4951*	2.82	8.87e-09
Il6	3.48	1.13e-11	*Csf2*	2.79	1.16e-11
Cxcl2	3.43	9.18e-12	*Iigp1*	2.79	3.22e-10
Mx1	3.30	2.17e-10	*Apol9b*	2.77	2.60e-09
Rsad2	3.19	1.37e-10	*Cxcl10*	2.77	9.18e-12
Ifi44	3.14	1.69e-07	*Phf11d*	2.77	7.47e-09
Cxcl3	3.13	2.31e-11	*Tnfaip2*	2.77	9.18e-12
Ifi47	3.07	1.72e-08	*Zbp1*	2.74	2.53e-09
Ccl2	3.02	1.16e-07	*Gm4841*	2.74	2.14e-11

Table S-3 | KEGG enrichment analysis indicating top 20 perturbed pathways in astrocytes during *M. tuberculosis* infection (log2FC > 2, adj. p < 0.05).

Pathways induced in Astrocytes	KEGG ID	pSize	NDE	pNDE	tA	pPERT	pGFdr	Status
Cytokine-cytokine receptor interaction	4060	243	103	2,03e-11	71,26	5,00e-06	5,02E-13	Activated
Osteoclast differentiation	4380	121	67	1,97e-14	15,48	0,061	2,78E-12	Activated
Chemokine signalling pathway	4062	185	79	2,84e-09	56,79	5,00e-06	2,04E-11	Activated
Herpes simplex infection	5168	189	75	2,73e-07	37,37	5,00e-06	1,27E-09	Activated
Influenza A	5164	158	71	1,26e-09	17,06	0,003	2,71E-09	Activated
Leishmaniasis	5140	64	39	1,09e-10	5,86	0,223	1,35E-08	Activated
Toll-like receptor signalling pathway	4620	98	49	6,27e-09	24,52	0,005	1,48E-08	Activated
HTLV-I infection	5166	273	105	9,22e-09	17,87	0,028	9,76E-08	Activated
NF-kappa B signalling pathway	4064	91	45	3,95E-08	19,558	0,008	1,05E-07	Activated
NOD-like receptor signalling pathway	4621	56	34	1,96E-09	4,671	0,345	1,96E-07	Activated
Chagas disease (American trypanosomiasis)	5142	100	49	1,47E-08	13,785	0,077	2,90E-07	Activated
Tuberculosis	5152	173	71	1,20E-07	21,177	0,042	1,11E-06	Activated
Staphylococcus aureus infection	5150	49	29	6,75E-08	-9,193	0,095	1,28E-06	Inhibited
Pertussis	5133	72	38	4,84E-08	-2,370	0,653	5,29E-06	Inhibited
Rheumatoid arthritis	5323	80	38	1,58E-06	4,002	0,021	5,29E-06	Activated
MAPK signalling pathway	4010	265	95	1,92E-06	13,660	0,029	8,09E-06	Activated
Apoptosis	4210	84	35	1,25E-04	25,882	0,001	1,56E-05	Activated
Calcium signalling pathway	4020	184	74	1,74E-07	-2,175	0,729	1,56E-05	Inhibited
Measles	5162	131	53	7,50E-06	14,037	0,036	3,00E-05	Activated
B cell receptor signalling pathway	4662	76	37	1,02E-06	-6,445	0,299	3,19E-05	Inhibited