The characterisation of dendritic cell, microglial, macrophage and T cell responses during mycobacterial infection of the central nervous system.

by

Khanyisile Kgoadi

Submitted in fulfilment of the requirements for the degree:

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I, Khanyisile Kgoadi declare that the thesis which I hereby submit for Doctor of Philosophy degree in the Division of immunology, Institute of Infectious Disease and Molecular Medicine, Department of Pathology, at the University of Cape Town is my own work and has never been previously submitted by me for a degree at this or any other tertiary institute. I empower the university to reproduce any portion of the thesis or the whole thesis for research purpose in whatsoever manner.

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DEDICATION

I dedicate this thesis to Ephraim Ntshiyane Kgoadi (my dad), Donalia Kgoadi (my mum) and Chris Mthuthuzeli Ngaleka (my uncle) who have encouraged and supported my Education throughout my life. Thank you for all the sacrifices you have made for my Education and always expressing Pride and Happiness in all the achievements during my journey of becoming a global scientist. You are truly God's Blessings, Amazing guardians who raised me well, protected me and support my dreams. I love you and Thank you for everything! To my kid brother Sechaba Arthur Kgoadi aka "Chabbie", you served as a source of inspiration/motivation to my life and studies especially during my tough days, just the thought of your smile and laugh makes me happy and pushes me to soldier on. I hope I have set a good example for you my little talented, handsome, loving and caring little guy *Hugs* Always remain disciplined and Please follow your heart, passions and good things in life that bring you Joy and Happiness #ForeverLoveOfMyLife.

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PREFACE

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1) Khanyisile Kgoadi, Nai-jen-Hsu, Roanne Keeton and Muazzam Jacobs. Dendritic cells play a role in CNS protective immunity post BCG intracerebral infection. SAMRC Early Career Scientist Convention (ECSC) SAMRC conference centre, Parow, Cape Town (SA). 19-20 October 2016.

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7) Khanyisile Kgoadi, Nai-jen-Hsu, Roanne Keeton and Muazzam Jacob. The role of dendritic cells in CNS-TB following BCG intracerebral infection. 10th African Federation of Immunological Societies (FAIS) Congress. Hammamet, Tunisia. 3-7 December 2017. **Poster Presentation.**

8) Khanyisile Kgoadi, Roanne Keeton, Nai-jen Hsu, Avril Walters and Muazzam Jacobs. Influx of IL-1beta secreting dendritic cells and T cell activation in mice CNS-TB. ISNI 14th International Congress of Neuroimmunology. Brisbane, Australia. 27-31 August 2018. **Oral Presentation.**

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11) Khanyisile Kgoadi, Roanne Keeton, Avril Walters, Nai-jen Hsu and Muazzam Jacobs. Recruited brain conventional dendritic cells and T cells promote protection against CNS-TB. 17th International Congress of Immunology, Beijing, China. 19-23 October 2019. **Oral Presentation.**

12) Khanyisile Kgoadi, Roanne Keeton, Avril Walters, Nai-jen Hsu and Muazzam Jacobs. Dendritic cells, infiltrating macrophages and T cells contribute to protective immunity against CNS-TB. Keystone Symposia, Tuberculosis: Immunity and Immune Evasion (A2), Santa Fe, New Mexico, USA. 16-20 January 2020. **Poster presentation.**

LIST OF IMPORTANT ABBREVIATIONS

AFB	Acid-fast bacteria
APCs	Antigen presenting cells
BBB	Blood brain barrier
BCG	Bacille Calmette-Guèrin
CCR7	Chemokine Receptor-7
CD	Cluster of Differentiation
cDCs	Conventional dendritic cells
CFUs	Colony-forming units
CLNs	Cervical lymph nodes
CNS	Central nervous system
CNS-TB	Central nervous system tuberculosis
CSF	Cerebrospinal fluid
CTL	Cervical tuberculosis lymphadinitis
DCs	Dendritic cells
DC-TC	Dendritic cell-T cell
EPTB	Extra-pulmonary TB
ESAT-6	Early secretory antigenic target 6
HDT	Host-directed therapy
HIV	Human Immunodeficiency virus
IFNγ	Interferon gamma
IL	Interleukin
MHC	Major histocompatibility complex
M. bovis	*Mycobacterium bovis*
M. Tuberculosis	*Mycobacterium tuberculosis*
pDCs	plasmacytoid dendritic cells
PRRs	Pathogen recognition receptors
PTB	Pulmonary Tuberculosis
TB	Tuberculosis
TBM	Tuberculosis meningitis
TF	Transcription factor
TGFβ	Transforming growth factor- betta
TNFα	Tumor necrosis factor-alpha

Th1	T-helper 1
Treg	Regulatory T cells

LIST OF FIGURES

CHAPTER 1: ABSTRACT

Background: Tuberculosis (TB) remains a global health challenge and a quarter of the global population is infected with latent TB. It is a single infection that causes most deaths and was the number one cause of death in South Africa in 2017. Bacille Calmette-Guerin (BCG) remains the only licensed vaccine for protection against TB. Although TB primarily occurs as a pulmonary infection after inhalation of *Mycobacterium tuberculosis* (*M. tuberculosis*) bacilli, it can disseminate to other organs causing extra-pulmonary TB (EPTB). Approximately 5-15% of EPTB cases are attributed to central nervous system tuberculosis (CNS-TB) which commonly manifests as TB meningitis. CNS-TB is a severe form of TB associated with high morbidity and about 50% mortality due to inconclusive diagnosis and treatment challenges. Children and immunocompromised adults like those coinfected with HIV/AIDS are higher risk groups for the development of CNS-TB. Pathogenesis of CNS-TB occurs as a secondary infection during haematogenous dissemination of pulmonary TB to the brain parenchyma and meninges where inflammation occurs after rupture of rich foci into the subarachnoid space. Mechanisms by which *M. tuberculosis* infects the CNS and specific cell types targeted are not fully characterized. Little is understood of the cells that regulate CNS-TB, their respective functions, their cellular interactions, and contributions to the overall protection of the CNS. Most studies have focussed on microglia and macrophages as the preferential targeted antigen-presenting cells (APCs) by *M. tuberculosis* and neglected dendritic cells (DCs) to an extent because no consensus had been reached regarding the presence of DCs in a healthy CNS. Both myeloid (APCs) and T cells contribute to protection against CNS-TB. This study characterized the dendritic cell, microglial, macrophage, and T cell responses during mycobacterial infection of the CNS. We also investigated the modulation of T cells by DCs during CNS-TB.

Methodology and Results: Wild-type female C57BL/6J mice were intracerebrally (i.c.) infected with *M. tuberculosis* H37Rv or *Mycobacterium bovis* BCG while control animals were saline inoculated and naive mice. Mice were euthanized at weeks 2, 4, 6, and organs harvested for experimental analysis. Histology results detected acid-fast bacilli using Ziehl-Neelsen (ZN) stain in the brains of *M. tuberculosis* and BCG i.c. infected mice, respectively. This was accompanied by a high degree of inflammatory responses in the brain ventricles and meninges of infected mice as compared to saline control mice shown by Hematoxylin and Eosin (H & E) staining. Although controlled brain bacterial burdens were demonstrated from homogenates of *M. tuberculosis* or BCG infected mice, dissemination to the spleen and lungs occurred. The

histopathological results showed the successful reproduction of the murine CNS-TB infection model. For immunophenotyping, flow cytometry analysis of single-cell suspensions generated from brains and cervical lymph nodes were characterized for phenotypic and functional profiles. We detected the recruitment of macrophages and DCs to the brain from the periphery and an expansion of brain APCs (microglia, brain infiltrating macrophages, and DCs) during mycobacterial infection of the CNS. Brain APCs from infected animals displayed highly activated and mature phenotypes as shown by increased numbers of these cells expressing MHCII, co-stimulatory CD86 molecule, pro-inflammatory cytokines (IFNγ, TNFα, IL-1β, IL-6, IL-12) and an anti-inflammatory cytokine (IL-10) in comparison to saline control mice. We also demonstrated preferential recruitment of mature conventional DCs (CD11c+, MHCII+) that express chemokine receptor-7 (CCR7) to the brain and cervical lymph nodes (CLNs), a phenomenon that may have contributed to the recruitment and expansion of predominantly effector CD4+ T cells than CD8+ T cells (CD44+CD62L-) to the brain and CLNs during mycobacterial infection of the CNS. Increased numbers of recruited CD4+ T cells and CD8+ T cells expressed T-bet [T-helper (Th1) transcription factor) in the brain and CLNs post-infection. At week 4 post intracerebral infection, increased numbers of these T cells expressed both T-bet and FoxP3 (regulatory transcription factor) during CNS-TB and identified a higher frequency of polyfunctional IFNγ+TGF-β+CD4+ T cells than IFNγ+TGF-β+IL-10+CD4+ T cells. *M. tuberculosis*-infected DCs from CLNs of CNS-TB mice were cocultured with naïve CD3+ T cells to generate a DC-T cell coculture, cells were sorted using fluorescence-activated cell sorting (FACS). DC-T cell coculture demonstrated increased percentage expression of IFNγ, IL-4, IL-10 and TGF-β responses by CD4+ T cells and CD8+ T cells during CNS-TB. Our *in vitro* coculture findings validated *in vivo* findings of recruited brain CD4+ T cell cytokine responses that showed a combination of Th1 and regulatory T cell immune responses.

Conclusion: We successfully reproduced the CNS-TB murine model, which proved valuable in studying immune responses. The functional mature phenotypes of detected brain APCs (microglia, brain infiltrating macrophages, DCs) suggest their capabilities of inducing antigen-specific T cell responses that contributed to initiating and mediating immunity during mycobacterial infection of the CNS**.** Our study findings suggest protection against mycobacterial infection of the CNS was achieved by characterized cells based on reduced brain bacterial burdens and 100% animal survival rate. Detrimental disease outcome was prevented by the balance achieved between pro-inflammatory and anti-inflammatory responses. The

novel mechanism employed by conventional DCs during CNS-TB is modulating CD4+ and CD8+ T cell cytokine responses to Th1 and Treg polarization that achieved *M. tuberculosis* control in the brain. We demonstrated that DCs can be targeted for strategic therapeutic intervention against CNS-TB. Therefore; we support ongoing research that focuses on DCs for the development of tuberculosis vaccines and host-directed therapy. This study provided new knowledge on immune mechanisms and pathogenesis experienced during TBM, thus adding to the current gap of advancing basic and translational TBM research that will inform clinical interventions. These new insights have the potential to help reduce the high death and disability associated with CNS-TB.

CHAPTER 2: LITERATURE REVIEW

2.1) Introduction to Tuberculosis

Tuberculosis (TB) remains a global health challenge and a quarter of the global population is infected with latent tuberculosis (WHO, 2019). Tuberculosis is the leading cause of death from a single infectious agent and forms part of the top 10 killers in the world (Dye and Williams, 2010). One of the United Nations Sustainable Development Goal established in 2015 was to End TB by 2030 (UN TB summit, 2018). Although TB is an old bug that was discovered in 1882 by Robert Koch, it is still claiming millions of lives today. In 2018 the mortality rate was 1.5 million and the incidence was 10 million (WHO, 2019). Ethnicity plays a role in the level of increased susceptibility to tuberculosis and Black people are more susceptible than white people (Stead et al., 1990; Twaites et al., 2000). Among the high TB burden countries (Fig. 2.1), our country South Africa is one of the eight countries (Pakistan, Philippines, India, South Africa, Nigeria, Indonesia, Bangladesh, and China) that accounts for 66% of new tuberculosis cases per annum (WHO, 2019) and TB was the leading cause of death in South Africa in 2017 (TBfacts.org, 2018; Avert.org, 2020). South Africa was previously ranked fifth in the TB incidence cases and reported that 13 of the 15 countries of highest incidence cases of TB were in Africa, this was attributed to high HIV-TB coinfection rates which are common in Africa (Corbett et al., 2003; Cherian and Thomas, 2011). South Africa has a high prevalence of HIV-TB coinfection and TB is the leading cause of death in HIV-infected individuals because their immune system is compromised (SA National AIDS council, 2017; Avert.org, 15 April 2020). The risk of TB infection is increased in HIV/AIDS patients and sub-Saharan Africa has a prevalence of approximately 71% of HIV cases (James et al., 2018), therefore making HIV-TB co-infection an increased challenge in combating TB. Diagnostic challenges, co-infection with HIV, and the development of drug-resistant TB exacerbate high morbidity and mortality associated with TB.

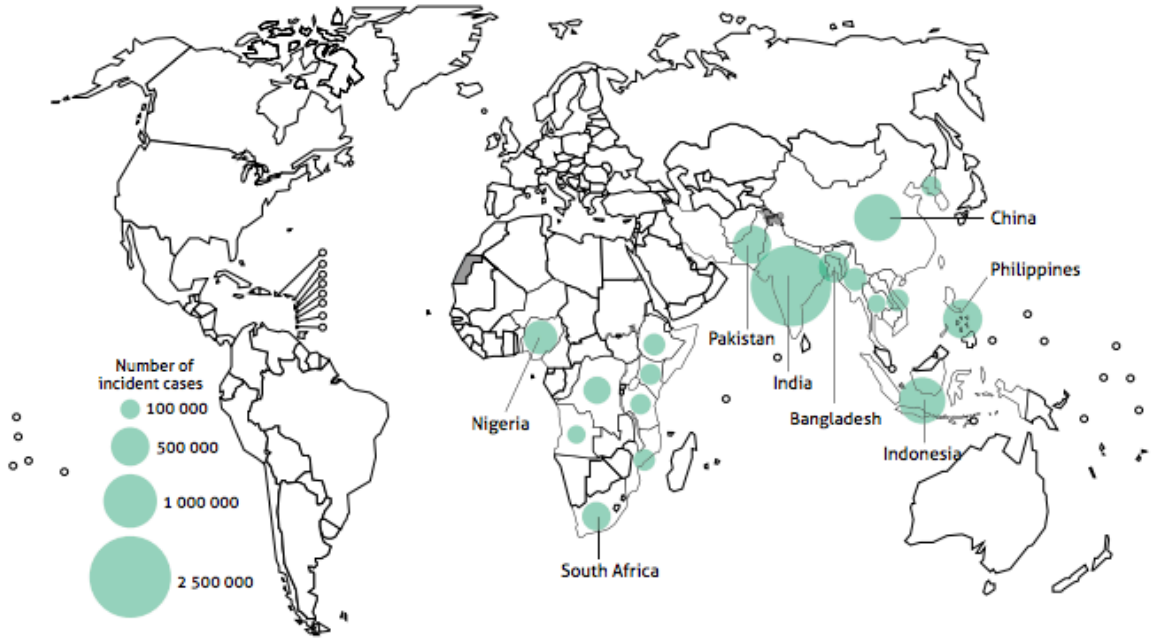


Figure 2.1: Tuberculosis incidence in 2018 showing the eight high burden countries per 100 000 population. The eight high burden countries account for two-thirds of the global tuberculosis burden (WHO Global TB Report, 2019).

2.2) Morphology of TB and Pathogenesis of pulmonary TB

TB is caused by *Mycobacterium tuberculosis* (*M. tuberculosis*) and primarily occurs as a pulmonary infection (Schluger and Rom, 1998; Corbett et al., 2003). This intracellular pathogen is a gram-positive bacterium that is rod-shaped and contains a cell wall that has peptidoglycans, lipids, and arabinomannans (Thwaites et al., 2000). *M. tuberculosis* is non-motile, aerobic and acid-fast bacilli that does not form spores and grow at a slow rate (Christie et al., 2008). Following inhalation of a few bacilli, innate immune cells (neutrophils, alveolar macrophages and dendritic cells) get infected with *M. tuberculosis* in the lungs (Davis et al., 2019). Activated alveolar macrophages in the lungs manage to control the growth of *M. tuberculosis* through innate immune responses when they phagocytose and kill the bacteria and latent pulmonary TB (LTB) is established in 5-10% of infected people (Mihret et al., 2012; Sáenz et al., 2013; Bini and Hernandez-Pando 2013; Gopal et al., 2014; Tsenova and Singhal. 2020). This macrophage activation is enhanced by proinflammatory cytokines (TNFα and IFNγ) that are secreted by T cells. Macrophages are promoted to recruit innate and adaptive immune cells to the site of infection when activated by TNFα and IFNγ to form a granuloma, a structure that can contain bacterial dissemination or promote disease progression (Saunders et al., 2000; Davis and Ramakrishnan, 2009; Flynn et al., 2011; Ehlers and Schaible; 2013;

Ndhlovu and Marakalala, 2016; Tsenova and Singhal, 2020). Approximately 90% of humans have LTB. However, failure of alveolar macrophages to control *M. tuberculosis* growth after phagocytosis leads to active pulmonary disease (Schluger and Rom., 1998; Christie et al., 2008; Be et al., 2009) and dissemination of bacteria to other organs causing extra-pulmonary TB (EPTB). The spread of TB among people in communities is fuelled by factors such as the existence of infectious co-morbidities, poverty that leads to malnutrition, homelessness, and poor public health infrastructure (Thwaites et al., 2000), and overcrowded places.

TB is treatable with a combination of antibiotic therapy for long periods of six to nine months and can be cured, however, drug resistance to these frontline drugs is increasing (Marino and Kirschner, 2016) leading to poor disease management and outcome. *Mycobacterium bovis* BCG is still the only FDA-licensed vaccine against tuberculosis that is administered globally, however, there is currently no efficacious vaccine available against TB and several vaccines under clinical trials (Marino and Kirschner, 2016). BCG vaccination has higher efficacy against childhood TB, while protection in adults is about 50% (Kaufmann 2000; Thwaites et al., 2000; Wang et al., 2002; Dye and Williams, 2010) and BCG also has high efficacy in EPTB like TB meningitis in children (Colditz et al., 1994; Twaites et al., 2000). EPTB occurs when TB disseminates from the lungs to the bloodstream, lymph nodes, and other organs (Rock et al., 1996; Drevets et al., 2004; Be et al., 2009) and it accounts for approximately 20% of all TB cases and it includes CNS-TB.

2.3) Central Nervous System Tuberculosis

CNS-TB is the most severe form of tuberculosis that forms about 1-10% of all TB cases and accounts for approximately 5-15% of EPTB cases (Garg et al., 1999; Farinha et al., 2000; Daikos et al., 2003; Sharma et al., 2005; Phypers et al., 2006; Rock et al., 2005 and 2008; Be et al., 2008 and 2009; Cherian and Thomas, 2011; Bartzatt et al., 2011; Jain et al., 2013; Qin et al., 2015; Chandra et al., 2017; Aher et al., 2018). It is associated with high morbidity and 30-57% mortality (Rock et al., 1996; Girgis et al., 1998; Yaramis et al., 1998; Christie et al., 2008; Cárdenas and Soto-Hernández, 2011; Be et al., 2012; Rohlwink et al., 2017) due to challenges involved in making a conclusive diagnosis (poor diagnosis and non-specific disease presentation) and treatment challenges (Rock et al., 2008; Marx and Chan, 2011; Nicholas et al., 2011; Qi et al., 2014) such as late treatment initiation and drugs that do not penetrate the blood-brain barrier (Be et al., 2012). A delay in diagnosis for more than one day increases

mortality (Karstaedt et al., 1999). Children and immune-compromised adults such as those infected with HIV are higher risk groups for CNS-TB development because of the immature immune system and low CD4+ T cells (Comstock et al., 1974; Farer et al., 1979; Dube et al., 1992; Brenguer et al., 1992; Bergemann and Karstaedt, 1996; Garg, 1999; Farinha et al., 2000; Rana et al., 2000; Thwaites et al., 2000; Katrak et al., 2000; Wells et al., 2007; Brown and Gray, 2008; Be et al., 2008; Garg, 2010; Cherian and Thomas, 2011; Be et al., 2012; Zucchi et al., 2012; Sáenz et al., 2013), especially in the developing world (Rock et al., 2008; Gandhi et al., 2006). Adults from Soweto (a township in South Africa) with high prevalence of HIV were reported more than two decades ago by Bergemann and Karstaedt (1996) to commonly die from tuberculosis meningitis (TBM). This is due to the fact that the prevalence of tuberculosis in HIV positive patients is 36% in Africaa and approximately 60% are attributed to South Africa (WHO, 2018). Additionally, factors such as malnutrition, age, alcoholism, malignancy, corticosteroids usage (adult immune-suppressive drugs), diabetes, and community disease prevalence also increase TBM development (Yaramis et al., 1998; Thwaites et al., 2000; Bidstrup et al., 2002; Phypers et al., 2006). Approximately 100 000 TBM cases occur per year (Gomez et al., 2014; Wilkinson et al., 2017). Mortality rates are at 40% for HIV negative patients and about 70 percent in patients with tuberculosis drug resistance (Gomez et al., 2014; WHO, 2018). This resulted in the intense-TBM project setting up clinical trials at selected African regions (South Africa, Ivory Coast, Uganda and Madagascar aimed at treating TBM patients who are HIV negative as well as HIV negative in an effort to reduce TMB mortality by 30%, some of which are currently underway (intense-tbm.org, accessed 25 March 2021).

The type of CNS-TB is determined by the location of the rich foci that normally seed in the brain, meninges, or spinal cord and the extent to which *M. tuberculosis* can be controlled (Rock et al., 2008) because CNS-TB manifests in different forms. There are two types of CNS-TB; intracranial and spinal (i.e spinal meningitis, non-osseous spinal tuberculoma, Pott's spine, and paraplegia), however for this study, our focus is on intracranial CNS-TB which can manifest as TB meningitis (TBM), TBM with miliary tuberculosis, CNS tuberculoma, tuberculous encephalopathy, tuberculous vasculopathy and tuberculosis abscess (Dastur and Lalitha, 1973; Garg, 1999; Farinha et al., 2000; Brown and Gray, 2008; Cherian and Thomas, 2011; Aher et al., 2018). Development of tuberculomas occurs in about 10% of TBM patients which can occur at several sites that include; brain, spinal cord, and subarachnoid space (Arseni, 1958; Bernaerts et al., 2003). However, CNS-TB mainly manifests as TBM, and TBM is associated with approximately greater than 25-30% mortality in humans (Hosoglu et al., 2002; Donald et

al., 2005; Christie et al., 2008; Garcia-Monco and Rodriguez-Sainz, 2018). TBM has also been established in animal models of CNS-TB (Tsenova et al., 1998 and 2005; Mazzolla et al., 2002; van Well et al., 2007; Be et al., 2008; Lee et al., 2009; Francisco et al., 2015; Tucker et al., 2016; Husain et al., 2017).

2.3.1) Diagnosis and Treatment of CNS-TB

Clinical presentation and neuroimaging are not sufficient for diagnosis of TBM and diagnosis require a combination of other tests including cerebrospinal fluid (CSF) as the standard diagnostic tool through culture or acid-fast bacilli staining (Ahuja et al., 1994; Cherian and Thomas, 2011; Aher et al., 2018). Symptoms of TBM in humans include; headache, vomiting, fever, changed consciousness levels, neck stiffness while seizures and nausea are also common in children (Sutlas et al., 2003; Rock et al., 2008; Christie et al., 2008; Cherian and Thomas, 2011; Aher et al., 2018). Additionally, computed tomography imaging features include; infarcts, exudates in basal cisterns, hydrocephalus, and tuberculoma formation and assist in diagnosing TBM (Ahuja et al., 1994; Cherian and Thomas, 2011). Whereas, Immunological/microbiological CNS-TB diagnosis includes the presence of increased protein (cytokines and chemokines), pleocytosis (abnormally increased numbers of lymphocytes), and low glucose concentrations in the CSF of patients (Saez-Llorens et al., 1990; Garg, 1999; Christie et al., 2008; Cherian and Thomas, 2011). Long CSF culture diagnosis is still the reference standard for TBM diagnosis, with the detection of acid-fast bacilli using Ziehl-Neelsen (ZN) stain in CSF yielding definitive diagnosis (Christie et al., 2008; Nelson and Zunt, 2011; Cherian and Thomas, 2011). ZN, auramine-rhodamine, and Kinyoun are standard staining techniques for tubercle bacilli in CSF using bacterial culture or smear examination (Cherian and Thomas, 2011). ZN stains the cell wall of *M. tuberculosis* by forming a complex with the cell wall to prevent discoloration that can result from alcohol or acid (Jenkins, 1994) and it remains one of the diagnostic tests that are effective and rapid (Thwaites et al., 2000). Inconclusive diagnosis remains a challenge for CNS-TB, one of the problems is that only less than two-thirds of cases can be diagnosed by CSF cultures which take long periods to test positive (Garcia-Monco and Rodriguez-Sainz, 2018).

Molecular and biochemical assays for diagnosing CNS-TB include; (1) polymerase chain reaction used for amplification of *M. tuberculosis* DNA detected in CSF and can be performed on all different manifestations of CNS-TB (Thwaites et al., 2004 and 2009; Christie et al., 2008; Takahashi et al., 2008 and 2012) and TBM patients tested more than 90% positive for CSF

PCR (Aher et al., 2018), (2) tuberculin skin test that contains positivity test ranging from 10-50% (Kilpatrik et al., 1996; Mahadevan et al., 2005; Christie et al., 2008), (3) interferon-gamma release assays (IGRAs) which are mostly employed by countries that have high TB burdens with low economic income because this assay cannot distinctly differentiate between latent and active tuberculosis (Cherian and Thomas, 2011). IGRAs measures increased IFNγ released by T cells *in vitro* after stimulation with *M. tuberculosis* specific antigens such as early secreted antigenic target 6 (ESAT-6) and culture filtrate protein 10 (Cherian and Thomas, 2011). Although IGRAs have high specificity for *M. tuberculosis* that is not affected by BCG vaccination unlike tuberculin skin test, it cannot provide a conclusive diagnosis for TBM because about 50% of patients who tested culture-positive for TBM showed no detection of *M. tuberculosis* specifc IFNγ production by lymphocytes in peripheral blood (Simmons et al., 2006; Farhat et al., 2006; Menzies et al., 2007; Pia et al., 2008). MTB/RIF and ultra MTB/RIF is a promising diagnostic tool for TBM.

Following diagnosis, CNS-TB treatment involves lengthy therapy with a combination of drugs (Be et al., 2009; Garcia-Monco and Rodriguez-Sainz, 2018; Cherian and Thomas, 2011) and the recommended first-line drug regimens for CNS-TB are rifampicin, isoniazid, pyrazinamide, and ethambutol (Thwaites et al., 2009). Adjunctive corticosteroids used for treating TBM patients improve disease outcomes through the mechanism of suppressing both inflammatory cytokines and inflammatory chemokines (Thwaites et al., 2004; Rock et al., 2005). However, corticosteroids also induce non-specific immune responses resulting in side effects that lead to their limited use (Ordonez et al., 2014). Another challenge with CNS-TB treatment is an increase in some strains of tuberculosis to multi-drugs like various second-line TB drugs which have further exacerbated the complications of CNS-TB (Thwaites et al., 2005; Be et al., 2009 and 2012). Although treatment of CNS-TB prevents deaths and disability in less than 50% of the patients (Girgis et al., 1998; Hosoglu et al., 2002; Thwaites and Hein, 2005; Be et al. 2009; Aher et al., 2018), most survivors (more than 25%) of TBM suffer from severe neurological disorders and disability (Hosoglu et al., 2002; Sutlas et al., 2003; Rock et al., 2008; Christie et al., 2008; Cárdenas et al., 2011; Be et al., 2012; van Laarhoven et al., 2019). This calls for urgent attention and the need for scientific improvements/advances in the diagnosis and treatment of CNS-TB, which can stem from a better understanding of the pathogenesis of CNS-TB to help improve patient disease outcomes. This includes the development of host-directed therapy (HDT) for CNS-TB (Tucker et al., 2016) because HDT

is a relatively new and promising treatment approach for TB (Tobin, 2015; Kolloli and Subbian, 2016). HDT is an adjunctive therapy that helps improve *M. tuberculosis* clearance and limit pathology damage by therapeutically modulating immune responses that are exerted by the host against *M. tuberculosis* (Hawn et al., 2013; Dorhoi and Kaufmann, 2014; Wilkinson, 2014; Zumla et al., 2016; Sharma et al., 2017). Potential advantages of HDTs include; shorter treatment designing to lessen the course of treatment, reduction of TB immunopathology by targeting pathways, increasing efficacy of drug-resistant regimens, and promotion of memory that reduces rates of relapses following anti-TB treatment (Palucci and Delogu, 2018; Tsenova and Singhal, 2020; Young et al., 2020). HDT is a promising relatively unexplored anti-TB therapy that is important to help achieve the End TB goal by WHO (WHO, 2018; Naicker et al., 2020).

2.3.2) CNS-TB Pathogenesis and Immune responses leading to meningitis development

CNS-TB occurs as a secondary infection following hematogenous dissemination of pulmonary infection to the brain meninges and parenchyma causing inflammatory meningitis after rupturing of rich focus into the CSF containing subarachnoid space (Rich and McCordork, 1933; Donald et al., 2005; Be et al., 2009) as shown in Figure 2.2. Rich and McCordock (1933) demonstrated TB meningitis using autopsy of TBM patients (children) and rabbits and that it occurs after several months following the establishment of active pulmonary disease. *M. tuberculosis* is a common intracellular pathogen infecting the CNS (Drevets et al., 2004) and is thought to be able to hide in the CNS for months and even years (Be et al., 2009). The CNS is a secondary site of *M. tuberculosis* infection resulting from migration from a different site in the host and increased risk of CNS-TB development following pulmonary infection can be attributed to the dissemination of higher numbers of bacteremia in the systemic circulation (Donald et al., 2005; Be et al., 2009**).** Approximately 25-30% of CNS-TB patients do not have active pulmonary TB (Bini and Hernandez-Pando 2013).

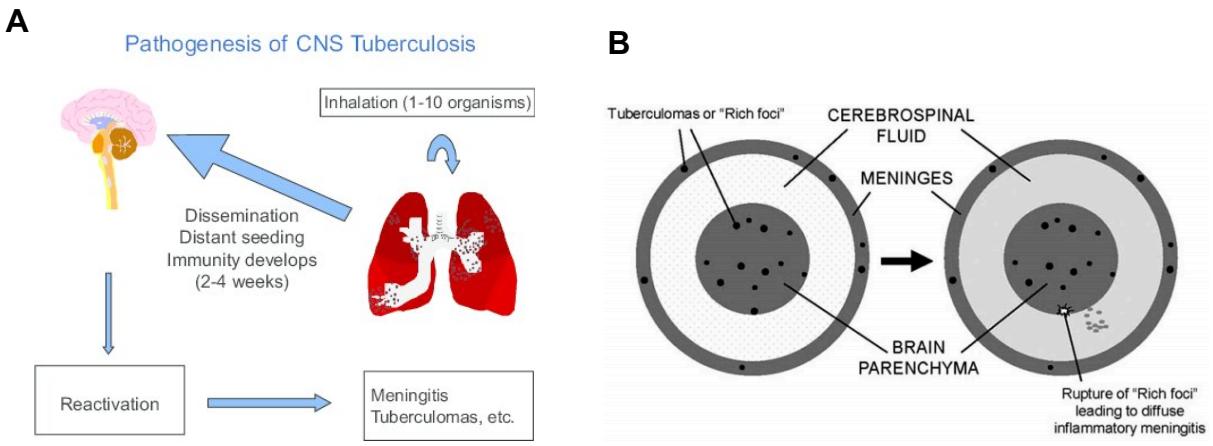


Figure 2.2: Pathogenesis of CNS-TB and resultant TB meningitis. (A) Inhalation of *M. tuberculosis* bacilli and establishment of pulmonary TB that led to hematogenous dissemination of bacilli to the brain and seeding. (A-B) Seeding of tuberculomas or "Rich foci" in the brain parenchyma and meninges which upon reactivation rupture in the subarachnoid space resulted in TB meningitis according to Rich and McCordock (1933). Figures are taken from Garcia-Monco (2014) and Be et al (2009).

A healthy CNS is thought to be immune-privileged because of the blood-brain barrier (BBB) formed around the brain parenchyma and meninges, the blood-CSF barrier formed at the choroid plexus, and lack of infiltrating leucocytes and MHCII expression (Fischer and Reichmann, 2001; Hatterer et al., 2006; Be et al., 2009; Wilson et al., 2010; Ransohoff and Engelhardt, 2012). Immune privilege refers to the limited immune responses to foreign antigens which is selective and modified (Ransohoff et al., 2003; Galea et al., 2007) and is crucial for limiting potential pathology damage resulting from inflammation caused to a non-regenerative organ such as the brain (Be et al., 2009). The CSF immune responses are much stronger than those of the brain parenchyma (Ransohoff et al., 2003). The BBB is composed of "tightly apposed brain microvascular endothelial cells that are held together by tight junctional complexes" and protects the CNS from systemic circulation (Rubin and Staddon, 1999; Hatterer et al., 2006; Be et al., 2008 and 2009). This prevents permeability to large molecules and pathogens that are in circulation limiting paracellular transport and transcellular movement but does not stop several bacteria or viruses from crossing the BBB that cause meningitis/encephalitis (Be et al., 2009). CNS-TB occurs when the BBB is breached by *M. tuberculosis* (Rich and McCordock, 1933; McGregor and Green, 1937; Drevets et al., 2004) as shown *in vitro* that *M. tuberculosis* evades the CNS and traverses the BBB (Bermudez et al., 2002; Jain et al., 2006). *M. tuberculosis* can cross the BBB either through infected monocytes,

neutrophils, leucocytes or in its free form as an organism (Wu et al., 2000; Bermudez et al., 2002; Be et al., 2009). The *M. tuberculosis* laboratory strain, H37Rv has been shown to evade and traverse the BBB of human brain microvascular endothelial cells more efficiently than non-pathogenic *M. smegmatis* owning to *M. tuberculosis* genes (Jain et al., 2006). These *in vitro* models are useful but their limitations of mimicking the complex inter-relationship among different cell types and compartments that generally encompass living organisms (Be et al., 2009) require *in vivo* models.

2.3.3) Animal models of CNS-TB

To mimic what happens during natural human pulmonary infection and CNS-TB infection, animal models have been widely used and constantly being developed to depict most characteristics of normal CNS-TB pathogenesis, but of course, limitations still exist in these models. These animal models of CNS-TB are required to increase our understanding of how *M. tuberculosis* evades the CNS to help us develop improved therapeutic interventions that induce protective immunity (Husain et al., 2017). Various types of mycobacterium species have been used to develop animal models of CNS-TB (Peterson et al., 1995; Mazzolla et al., 2002; Tsenova et al., 2007; Be et al., 2008; Lee et al., 2009). Regardless of these animal models not being able to reproduce some clinical human TBM characteristics for clinical signs of disease such as hydrocephalus which occurs when the inflammatory infiltrate obstructs CSF, intracranial vasculopathy, vasculitis causing infarction, cranial nerve palsies, and stroke (Ahuja et al., 1994; Leonard and Des Prez, 1999; Thwaites et al., 2000; van Well et al., 2007; Be et al., 2009; Christensen et al., 2011; Cherian and Thomas, 2011), they have been extremely useful in studying host-pathogen interactions.

Injection of *M. tuberculosis* through the tails of mice to achieve the normal hematogenous route of infection has proven ineffective in the development of CNS-TB in mice brains (Be et al., 2008). TBM has been induced in rabbits following intracisternal injection with *M. bovis* Ravenel (Kaplan et al. 2007, Tsenova et al., 1998 and 2005). Rabbit models that were intracisternally infected with either W4 or HN878 strains of *M. tuberculosis* resulted in increased bacterial burdens, increased TNFα in the CSF that was sustained, and severity of clinical symptoms (Tsenova et al., 2005). CNS-TB infection has been established in guinea pigs (*M. tuberculosis* H37Rv) and rabbits (*M. bovis)* by an intravenous infection that led to the development of granulomatous lesions (tuberculomas) in the brain parenchyma, TBM, and no

acute exudative meningitis (Rich and McCordock, 1933; Tsenova et al., 1998 and 2005). These findings confirmed that *M. tuberculosis* enters the CSF through the brain parenchyma and meninges of the BBB and not through the blood-CSF barrier (systemic circulation). Different routes of infection seem to result in different presentation of CNS-TB.

Murine models have been shown to share genetic and severity similarities with humans (Apt, 2011). A zebrafish model has also been used to study TBM post-infection with *Mycobacterium marinum* (Van Leeuwen et al., 2014). Be and colleagues (2008) showed that post intravenous *M. tuberculosis* infection, bacteria could be detected at all time points from whole brain tissue even though immune responses (measured by histopathology and cytokine profiles) induced in the brain did not differ significantly as compared to those in the lungs at similar time points. However, this model identified specific CNS phenotypes of *M. tuberculosis* mutants that were not found in the lungs. Van Well and colleagues (2007) developed a murine CNS-TB model to study pathogenesis in female C57BL/6 mice (6-week old) by infecting intracerebrally with a high dose (1×10^5) *M. tuberculosis* H37Rv in saline that led to TB meningitis and high infiltration of the cells into the CNS. This model showed that the inoculation site lied closely to subarachnoid space thus mimicking the Rich foci rupture to the CSF and proved valuable for studying innate immune responses of the host. Repeating of similar experiment with a higher dose of 5×10^5 reproduced similar results. Intracerebral or intracisternal infection models of TBM failed to mimic the natural route of CNS-TB in humans caused by *M. tuberculosis* and limited understanding of the immune mechanism employed by bacteria when they initially evade the CNS (Be et al., 2009). It has also been shown that different genotypes of *M. tuberculosis* and microbial factors cause CNS disease (Kim, 2003; Hernandez et al., 2010; van Leeuwen et al., 2019). Some CNS-TB models include; an aerosol challenge in guinea pigs using different strains of *M. tuberculosis* (CDC1551, H37Rv JHU, and TAMU) (Nicholas et al., 2011). Additionally, avirulent *M. bovis* BCG has been used to induce CNS-TB in murine models and shown to be efficient with the development of granulomas and meningitis (Mazzola et al., 2002; Lee et al., 2009; Zucchi et al., 2012). C57BL/6 were used in this study to develop CNS-TB infection because this mouse strain is resistant to *M. tuberculosis* and polarizes towards Th1 immune responses (Keller et al., 2004).

Different strains of mycobacteria have been utilized to establish animal models of CNS-TB. The differences in the genotype of *M. tuberculosis* strains may lead to virulence driven differences, differences in immune responses induced by the host, and disease outcome because

of different molecular and cellular profiles (McShane, 2003; Lopez et al., 2003; Tsenova et al., 2005; Gagneux and Small., 2007). In contrast, Maree and colleagues (2007) showed no correlation between *M. tuberculosis* genotype and TBM manifestations or disease outcome. A murine CNS-TB model developed by intravenous infection through the tail of BALB/c mice with a clinical isolate strain of *M. tuberculosis* (C3) isolated from CSF of CNS-TB patients showed a significant increase in brain bacterial burdens from day 30 to day 50 and an opposite observation in the lung resulting in increased mortality rates (Husain et al., 2017). This phenomenon is in contrast to observations found in mice infected with *M. tuberculosis* H37Rv (van Well et al., 2007; Be et al., 2008).

Communication between the CNS and immune cells makes a significant difference in homeostasis (tissue maintenance) and pathology (Mundt et al., 2019) because immune responses are not homogenous and can consist of antimicrobial, tissue repair responses, and regulatory responses (Kim et al., 2016). Trafficking of cells into the CNS is restricted even though it is hypothesized that *M. tuberculosis* can cross the BBB in its free form or through infected monocytes or neutrophils to evade the CNS (Ransohoff et al., 2003; Jain et al., 2006). However, host and microbial mechanisms associated with the process of *M. tuberculosis* evading the CNS remain mostly unknown (Jain et al., 2006). When immune responses are induced in the CNS by TB and the first line of defense is innate immunity facilitated by microglia (resident macrophages), peripheral macrophages, and dendritic cells that infiltrated the CNS (Barcia et al., 2013; Barichello et al., 2013). Although CNS-TB is the most lethal form of the disease, little is understood of the cells that regulate infection in the brain, their respective functions, and contributions of the various types of cells to the overall protection of the CNS and the inter-cellular interaction and cooperation amongst different cell types. Informative studies that address mechanisms of the host-pathogen relationship form the basis of intervention strategies that could be developed to the benefit of the host.

2.4) Cellular-mediated immunity during mycobacterial infection of the CNS: APCs and T cells

CNS infections are a common occurrence with a high mortality rate (Barichello et al., 2013) and cause meningitis (inflamed meninges), meningoencephalitis (inflamed brain), and myelitis (inflamed spinal cord) regardless of whether the source is viral, bacterial, fungal or parasitic origin (Somand and Meurer, 2009; D'Agostino et al., 2012). CNS infections are not common

and normally result from disseminated disease (Garcia-Monco and Rodriguez-Sainz, 2018) which is the case during CNS-TB. Wider ranges of mycobacteria infect the CNS (Busl and Bleck, 2013), but only a few cause diseases (van de Beek et al., 2007) and the most severe CNS infections (van de Beek et al., 2007; Somand and Muerer, 2009). Common bacterial CNS infections occurring in healthy and immune-compromised individuals include; *Streptococcus pneumonia, Neisseria meningitis, Listeria monocytogenes* (van de Beek et al., 2007; Somand and Muerer, 2009) and *Mycobacterium tuberculosis* noted as being the most common intracellular bacteria causing CNS infection (Drevets et al., 2004). *M. tuberculosis* contains virulence factors in the form of genes that are necessary for promoting CNS disease (Be et al., 2012). The immune status is a key determinant of the progress of CNS bacterial infections (Busl and Bleck, 2013) with the host's inflammatory response being the main cause of high mortality (Scheld et al., 2002). Increased frequencies of immune cells surveilling/infiltrating the CNS have been observed in both non-pathogenic inflammatory responses caused by traumatic brain injury (McKeating and Andrews, 1998) and pathogenic tuberculosis infection (Lee et al., 2009; Hernandez et al., 2016).

Macrophages, microglia and dendritic cells (DCs) in the brain increase during CNS Inflammation (Fischer and Reichmann, 2001), a group of antigen-presenting cells. The first line of defense against *M. tuberculosis* by the host is innate immune responses and if these responses are strong, protective and the strain is less virulent, they can eliminate *M. tuberculosis* and if not eliminated and remain in the host, latent TB infection is established that can lead to reactivation TB (Mihret, 2012; Sáenz et al., 2013). Alveolar macrophages and dendritic cells (DCs) are the first cells to encounter *M. tuberculosis* and they use pathogen recognition receptors (PRRs) [i.e. Toll-like receptors, complement, and C-lectin] to recognize the pathogen-associated molecular patterns (PAMPs) that are components of mycobacteria (Saijo and Glass, 2011; Kratky et al., 2011; Kleinnijenhuis et al., 2011; Sáenz et al., 2013). The CNS's first line of defense is innate immunity facilitated by 3 antigen-presenting cells (APCs); microglia (resident macrophages), peripheral macrophages, and DCs (Barcia et al., 2013; Barichello et al., 2013) that recognize bacterial PAMPs through binding with PRRs (Barcia et l., 2013).

2.4.1) Microglia and infiltrating macrophages in the CNS

Microglia are resident macrophages and innate immune cells in the CNS parenchyma (Sevenich, 2018). Microglia originate from embryonic yolk-sac progenitors during the process of embryogenesis (Ginhoux et al., 2010; Schulz et al., 2012) and are self-renewing cells (Gomez Perdighero et al., 2013). The human brain accounts for 0.5-16.6% of microglia while rodents consist of 5-20% microglia (Lawson et al., 1990 and 1992; Mittelbronn et al., 2001). These innate immune cells change functional states (morphology and physiology) during stimuli such as insult and their phenotype is normally disease dependent (Perry et al., 1985; Dalmau et al., 1998; Bachillar et al., 2018; Saijo and Glass, 2011). Microglia from the first line of defense against brain injury and infection, and also play a role in repair due to their major property of functional plasticity (Colton and Wilcock, 2010; Ransohoff and Perry, 2009). Human microglia express CD4, CD11c, and HLA-DR making them capable of acting as APCs (Ulvested et al., 1994) while mice microglia express CD11b, CD11c, and MHCII (Greter et al., 2015). Their morphology changes to one that looks like amoeboid when they are activated (Kozlowski and Weimer, 2012), and brain injury causes microglial activation (Schilling et al., 2003). Activated microglia present with increased expression of MHCII and this helps them perform their antigen presentation function of presenting antigens to T cells through interaction with TCR leading to T cell activation (Rock et al., 2004). Microglia are also suggested to act as APCs that play a role in the modulation of lymphocyte activation (Almolda et al., 2015). However, in comparison to dendritic cells (DCs), microglia are relatively poor APCs and rather induce effector cells to undergo apoptosis (Ford et al., 1996). Regardless, microglia act as central regulators of neuroinflammation, especially during bacterial infections (Peterson et al., 1995; Puliti et al., 1999; Rock et al., 2005; Prinz et al., 2011) and bacteria and proinflammatory factors cause the upregulation of chemokines by microglial cells (Zwijnenburg et al., 2006).

Microglia share properties with other macrophages from different tissues such as cytokine and chemokine profiles upon activation by different stimuli but also have distinct gene profiles from macrophages (Rock et al., 2004; London et a., 2013). Microglia and macrophages in the CNS cannot be distinguished by histology because they share similar markers; however, they can be classified and differentiated based on their level of CD45 expression using flow cytometry (Sedgwick et al., 1991; Hsieh et al., 2013). Figure 2.3 shows markers that are similar and shared between microglia and infiltrating macrophages.

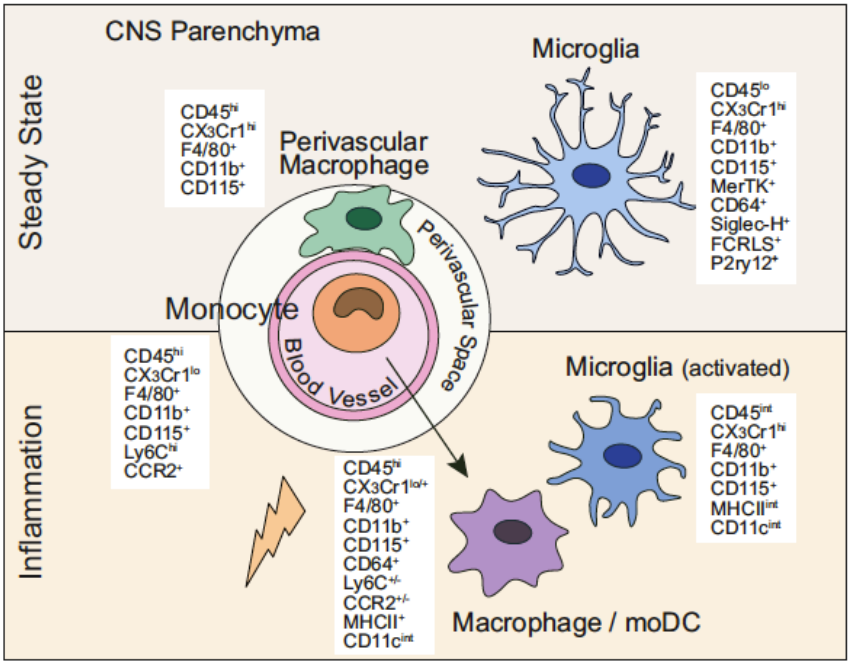


Figure 2.3: Lineage markers for myeloid cells in the CNS. Microglia, macrophages and DCs have diverse expression of markers and also share the expression of some surface markers during steady state and inflammatory conditions in the CNS. Figure taken from Greter et al., 2015.

Macrophages do not exist in a healthy brain parenchyma and are derived from either blood monocytes or microglia with each performing distinct functions (Gautier et al., 2012; Chiu et al., 2013; Butovsky et al., 2014; Yamasaki et al., 2014; Franco and Fernández-Suárez, 2015). Macrophages are phagocytic cells that act as initial innate immune defenders against intracellular mycobacteria like *M. tuberculosis* and can contain or kill *M. tuberculosis*; however, they can also be targeted by mycobacteria to be utilized as hosts through evasion mechanism such as prevention of fusion with lysosomes (Nguyen and Pieters, 2005; Weiss and Schaible, 2015). Blood-derived macrophages are early innate immune cell responders to acute traumatic brain injury (TBI) and are the major population to infiltrate the brain (Schilling et al., 2003; Kim et al., 2016). Monocyte-derived macrophages perform distinct functions from microglia and are mainly phagocytic and inflammatory (Shechter et al., 2009; London et al., 2011). Bone marrow-derived macrophages hematopoietic stem cells and differentiate throughout their life span (Ginhoux et al., 2010; Schutz et al., 2012). They remove pathogens and dead cells during homeostatic conditions in the periphery (Franco and Fernández-Suárez, 2015). During neurological injury, peripheral macrophages infiltrate the brain and contribute to disease progression (Ajami et al., 2007), however, alternatively activated macrophages have been shown to contribute to neuronal repair in the disease models of spinal cord injury (Schwartz, 2010; Shechter et al., 2009). Such findings support why Hsieh and colleagues

(2016) stated that a consensus has not been reached regarding the role of macrophages in the brain, whether they are protective or harmful. Human and mouse macrophages have been shown *in vitro* and *in vivo* to polarize into functionally distinct phenotypes and unique chemokine/cytokine profiles (Mantovani et al., 2004; Mosser and Edwards, 2008; Van Dyken and Locksley, 2013; Murray et al., 2014). Macrophages normally phagocytose bacteria and form a mature phagolysosome by the fusion of a phagosome (acidification) and lysosome causing the destruction of bacteria but this killing mechanism can be prevented when acidification of the phagosome is inhibited (Desjardins et al., 1994; Flynn and Chan, 2001; Behar et al., 2010; Mihret et al., 2012; Sáenz et al., 2013; Xu et al., 2015). Other macrophage killing mechanisms include; the generation of reactive oxygen or nitrogen intermediates such as nitric oxide (Mihret et al., 2012). The type of receptors utilized by mycobacteria to enter macrophages can be an early determinant of mycobacteria outcome (Kleinnijenhuis et al., 2011).

Macrophages and microglia can be classically activated (increased IFNγ and LPS induction) and correlate with Th1 immune responses to promote inflammation or alternatively activated (increased Arginase expressed, IL-4 and IL-13) correlate with Th2 immune responses to limit inflammation (suppression) and for repair tissue (Webb and Brooks, 1980; Kennard and Zolla-Pazner, 1980; Schebesch et al., 1997; Kodelja et al., 1998; Gordon, 2003; Gordon and Taylor, 2005; Martinez et al., 2009; Auffray et al., 2009; Colton et al., 2009; Chawla, 2010; Gordon and Martinez, 2010; Sica and Mantovani, 2012; Franco and Fernández-Suárez, 2015; Kim et al., 2016), the two being dominant subsets of activation. The proinflammatory M1 phenotype produces IL-1β, IL-6, TNFα and IL-12while M2 responds to IL-4 (*in vitro*), helminth infection or allergens (Nair et al., 2006; Nguyen et al., 2011). However, *in vivo* it has been demonstrated that macrophages are not restricted to polarizing into one phenotype, rather they possess plasticity of shifting between phenotypes (Mosser and Edwards, 2008; Chawla, 2010; Xue et al., 2014; Murray et al., 2014). This shifting polarization phenotype present during traumatic brain injury and ischemic stroke (Hu et al., 2012; Hsieh et al., 2013; Morganti et al., 2016; Kim et al., 2016). Cytokines for M1 activation of both microglia and macrophages include; IL-1β, IL-6, IL-12, INFγ, TNFα, and also Arginase surface marker for downregulation of nitric oxide synthesis. Whereas, M2 cytokines include IL-10 (M2a and M2b), TGF-β, IL-4Ralpha (M2b and M2c), and IL-4RA (M2a) (Hide et al., 2000; Mueller 2002; Kawanokuchi et al., 2006; Mosser and Edwards., 2008; Wang and Suzuki, 2007; David and Kroner, 2011; Prokop et al.,

2011; Hu et al., 2012; Fenn et al., 2012; La Flemme et al., 2012; Varnum and Ikezu, 2012; Chhor et al., 2013; Crain et al., 2013; Hanisch, 2013; Jaguin et al., 2013, Welser-Alves and Milner, 2013).

2.4.2) Microglia and macrophages during tuberculosis infection.

Microglia are said to be primary host cells of *M. tuberculosis* and mycobacterial infection of the CNS can be regulated by microglia through secretion of chemokines and cytokines (Curto et al., 2004; Rock et al., 2004 and 2005; Yang et al., 2007; Hernandez Pando et al., 2010). Human and mice microglial cells are easily and majorly infected by *M. tuberculosis*, making them potential principal target cells of *M. tuberculosis* and they secrete pro-inflammatory molecules/factors such as inducible nitric oxide synthetase (iNOS) to kill mycobacteria (Peterson et al., 1995; Curto et al., 2004; Rock et al., 2005; Zucchi et al., 2012). During CNS-TB, microglia is one of the inflammatory cells preferentially infected and activated by *M. tuberculosis* and may produce mediators; IL-1α, IL-1β, IL-6, and TNFα (Gao et al., 2003; Liu and Hong et al., 2003; van Well et al., 2007). Human microglia preferentially infected with *M. tuberculosis* H37Rv *in vitro* produce increased levels of pro-inflammatory cytokines (IL-1β, IL-6 and TNFα) and chemokines (CCL2, CCL5, CXCL10) post-infection (Dastur et al., 1995; Rock et al., 2005).

Macrophages are crucial sources of antimycobacterial peptides making them primary players in inducing protective innate immune responses against *M. tuberculosis* (Sáenz et al., 2013). Macrophages infected with *M. tuberculosis* undergo detrimental necrosis or beneficial apoptosis to kill mycobacteria during pulmonary infection (Hope et al., 2004; Sáenz et al., 2013). However, *M. tuberculosis* can subvert this killing mechanism (Rios-Berrera et al., 2006) and replicate inside macrophages which are unique to macrophages and replicates inside macrophages that are newly recruited (Dannenberg, 1991; Rock et al., 2005; Sáenz et al., 2013). Early secretory antigenic target (ESAT-6) causes interference of toll-like receptor signaling in macrophages through the prevention of myeloid differentiation factor 88 (MyD88) when mycobacteria are maintained in the cell host (Pathak et al., 2007). Alveolar macrophages protect against pulmonary TB when activated by producing nitric oxide, oxygen radicals, and phagosome-lysosome complex (Bini and Hernandez-Pando, 2013). Macrophages and DCs phagocytose *M. tuberculosis* (Guirado et al., 2013) and phagocytosis leads to the production of cytokines/chemokines that recruit leucocytes to the site of infection. Additionally, activated

macrophages that produce upregulated IL-1β, TNFα and IL-6 promote the formation of granulomas during BCG infection (Krishnan et al., 2013). While macrophages that produce IL-10 and TGF-β suppress T cell immune responses during *M. tuberculosis* infection (Weiss and Schaible, 2015). Macrophages are one of the key cell types that present *M. tuberculosis* antigens on MHCI and MHCII to T cells to induce immune responses (Satake et al., 2017). Microglia and infiltrating brain macrophages have been implicated as cells preferentially targeted by invading *M. tuberculosis* (Peterson et al., 1995; Rock et al., 2005; Yang et al., 2007) although Wolf and colleagues (2007) showed that myeloid dendritic cells are infected at a higher frequency by *M. tuberculosis* compared to alveolar macrophages during pulmonary infection.

2.4.3.1) Dendritic cells in systemic circulation

Dendritic cells (DCs) were originally identified in 1973 by Steinman and Cohn in mouse spleens as cells with dendrites found in the peripheral lymphoid organs. Hart (1997) wrote a beautifully detailed review that described dendritic cells as unique lymphocytes that control immune responses. In this review, Hart argues that DCs cannot be solely defined based on their distinct morphology and instead suggested some criteria such as; a specific cell surface antigen phenotype, specialized phagocytic activity, and their ability to stimulate primary T cell responses. Following the definition of DCs by Steinman and Cohn, the epidermal cells that were initially described by Paul Langerhans in 1868 showed similar properties to DCs, as well as cells from other tissues leading to the conclusion that there are different DC lineages (Hart, 1997; Merad et al., 2013). DCs also share some similar markers with monocytes, macrophages, and microglia (Ludewig et al., 2016). However, due to DCs sharing similar features to monocytes and macrophages, it still makes it difficult to have a distinctive DC lineage. Reaching a consensus on DC lineage and baseline levels is also a challenge. Both Hart and Fabre (1981) as well as Mckenzie and colleagues (1984) demonstrated that DC frequencies differ even in inbred mice and rats. Humans present with varying amounts of circulating DCs (Hart, 1997) and aging also plays a role in decreasing DC frequencies (Darden et al. 1990; Aidong et al. 1994). DCs are considered as professional APCs that enhance T cell recognition of antigens in lymph nodes (Steinman, 1991; Liu, 2001; Flynn, 2004; Marino et al., 2004) that use pattern recognition receptors (PRRs) to recognize invading pathogens and produce inflammatory cytokines for host defense when activated by microbial products (Sato and Fujita, 2007). DCs can host intracellular bacteria and modulate host immune responses

(Giacomini et al., 2001). DCs take up antigens by phagocytosis or pinocytosis, process antigens then deliver them to major histocompatibility complex (MHC) molecules and present them to T cell receptors (TCR) on naive T cells (Falo et al., 1992). Inaba and Steinman (1986) also showed that DCs express high levels of MHCII and effectively active primary T cell responses. The functionality of DCs is dependent on their maturation stage and expression of costimulatory molecules that are absent in resting DCs. Of all the three costimulatory molecules expressed by DCs, namely; (1) CD40 which binds to CD40L on T cells, (2) CD80 (B7.1), and (3) CD86 (B7.2) that both bind to CD28 on T cells required for initiation of T cell immune responses, and CD86 is the most abundantly expressed (Fanslow et al. 1994; Kawamura and Furue, 1995). Some cytokines secreted by DCs include IL-1α, IL-1β, TNFα, IL-6, IL-12, IL-10, IFNγ (Hart, 1997) and chemokines such as MIP1α, MIP1β and RANTES (Zhou and Tedder, 1995).

DCs confer diverse functions due to the heterogeneous subsets that are derived from different lineages and possess differing maturity and functional plasticity (Sato and Fujita, 2007). Different DC phenotypes exist, namely; classical DCs (cDCs) and plasmacytoid DCs (pDCs) (Colonna et al., 2004; Wu and Liu., 2007). Plasmacytoid DCs are mostly type-1 IFN producing cells that are present in small amounts in blood and lymphoid organs, have low levels of CD11c, MHCII, costimulatory molecules, and are poor antigen presenters (Wu and Liu., 2007: Merad et al., 2013). While classical/conventional DCs (cDCs) are specialized APCs that induce T cell immune responses and can be of myeloid or lymphoid origin (Merad et al., 2013), with DCs displaying similar functions but different phenotypes based on different location such as peripheral organs vs lymphoid organs (Gurka et al., 2015). Classical DCs express the surface markers CD11c, CD45, MHCII, and conditionally the tyrosine kinase receptor fms-like tyrosine kinase 3 (Merad et al., 2013). Mouse conventional DCs express CD11c and high MHCII expression (Gurka et al., 2015), and C57BL/6 mice express two types of splenic cDCs; (1) 20% of CD8+ DCs that cross-present antigens on MHCI to CD8+ T cells (Kurts et al., 1996; Pooley et al., 2001; Schulz et al., 2002; Schnorrer et al., 2006) which induce cytotoxic CD8+ T cells when the stimulus is a pathogen invading the host cell and (2) 60% of CD4+ DCs and the remainder are double negative DCs (Vremec et al., 2000). Distinguishing resident, migratory, and inflammatory DCs is challenging because the levels of surface markers used to identify DCs differ depending on the steady-state vs inflammatory condition. The location also plays a role in defining DC phenotypes, CD8+ DCs are of lymphoid origin while CD103+ DCs

are of non-lymphoid origin (Merad et al., 2013), however, CD8a DCs and CD103+ DCs are still not sufficient to distinguish peripheral vs lymphoid organ DCs (Gurka et al., 2015). DCs can be classified as mature or immature based on phenotypic markers and can be matured by TNFα, IL-1β, and IL-6 (e Sousa, 2006) and human DCs can be mature by cytokine cocktails with combinations of TNFα, IL-1β, IL-6, and prostaglandin E2 or TNFα, IL-1b, IFN-α, IFNγ and (Satake et al., 2016). Immature DCs express low levels of MHCII, CCR7, CD80/86, etc while mature DCs express high levels of these molecules and cytokines; IL-1, IL-6, IL-12, and TNFα (Henderson and Watkins, 1997; Banchereau et al., 2000; Banchereau and Steinman, 1998; Mihret et al., 2012). DCs are potent inducers of antitumor and antimycobacterial immune responses and a crucial source for IL-12 production (Sousa et al., 1997). They initiate cell-mediated immunity, however, they do not possess strong bacteria-killing capacity and have limited capacity for migration to draining lymph nodes (Buettner et al., 2005). DC functions include; inducing innate immune responses, regulating T cell responses (Sato and Fujita, 2007), migrating to lymph nodes for presentation antigens to T cells for activation or maintaining immune tolerance to self-tissue (Banchereau and Steinman, 1998), and differentiating T cell immune responses to a particular polarization (Th1, Th2, Th17 or Treg) (Liu, 2001). DCs are capable of priming both T-helper (Th) cells and cytotoxic T cells (Banchereau and Steinman, 1998; Kalinski et al., 1999; Nakamura et al., 2003), thus making them target for vaccines and immune modulation therapies (Satake et al., 2017). These DC functions make them an interesting and important cell type to study in infections/diseases for the benefit of the host in hopes of inducing protective immunity.

2.4.3.2) DCs in the CNS

A consensus has not been reached regarding the existence of DCs in a healthy CNS. There is a high restriction for the presence of DCs in a homeostatic human brain and low expression of MHCII leading to limited antigen presentation in the brain parenchyma (Galea et al., 2007). Functional DCs are present in the CNS during inflammation (Fischer and Reichmann, 2001). The CNS has long been thought to be immune-privileged because of the protective barriers (BBB and blood-CSF barrier) from systematic circulation (Hickey, 2001; Pachter et al., 2003; Be et al., 2009; Clarkson et al., 2012), which led to the belief that the CNS is devoid of immune cells like DCs. Perry (1998) showed that DCs are absent from brain parenchyma while others have proposed that DCs migrate from the periphery to the CNS during inflammation through adhesion molecules, chemokines/cytokines, and growth factors (Clarkson et al., 2012;

D'Agostino et al., 2012). Other studies demonstrated the differentiation of microglia into DCs in the presence of granulocyte-macrophage colony-stimulating factor (Fischer and Reichman et al., 2001), and some studies have shown that DCs surveil a healthy CNS in compartments such as choroid plexus, meninges (Matyszak and Perry, 1996; McMenamin, 1999; D'Agostino et al., 2012), ventricles, parenchyma and cerebrospinal fluid (Karman et al., 2004; Anandasabapathy et al., 2011). The discovery of the CNS lymphatic system by Louveau and colleagues (2015) showed that CNS immune surveillance occurs, and it reduced the long-standing belief of "CNS immune-privileged" to the selective permeability of molecules that enter the CNS. It has now become widely accepted that immune cells such as DCs and T cells exist in a healthy CNS. Karman and colleagues (2007) reported on the accumulation of DCs in the CNS post microinjection of protein antigen and drainage of the antigen by DCs into the CLNs where they activated antigen-specific T cells which were ultimately recruited to the brain. There is an accumulation of DCs in the CSF and perivascular spaces during neuro-infections (Serafini et al., 2000; Pashenkov et al., 2001; Pashenkov et al., 2002; Suter et al., 2003). Figure 2.4 shows DCs in the steady state and during neuroinflammation in the CNS. High levels of cytokines and myeloid DCs have been detected in meningitis patients (Pashenkov et al., 2002); however, the function of DCs in bacterial CNS inflammation has not been thoroughly explored. DCs initiate cell-mediated immunity, however, they do not possess strong bacteria-killing capacity and have limited capacity for migration to draining lymph nodes (Buettner et al., 2005). CNS inflammatory responses mostly result in detrimental consequences for the host and this makes the existence of an intricate relationship between the CNS and immune system of great benefit to the host for achieving improved health. The immunological role of DCs has been explored in BCG and *M. tuberculosis* CNS infections in mice (Matyszak and Perry, 1996; Mazzolla et al., 2002; Lee et al., 2009; Francisco et al., 2015) but not fully characterized. Also, an in vitro BBB for CNS-TB established that dendritic cell migration is hindered by *M. tuberculosis* but increases the initiation of DCs to interact with other cells for aggregate formation (Gilpin et al., 2019). More studies on the role of DCs during CNS-TB will provide new knowledge into understanding induction of protective T cell immune responses. This new knowledge on the role of dendritic cells and their interaction with T cells within the CNS will significantly broaden the overall understanding of host pathogen-relationship with respect to tuberculosis health challenges.

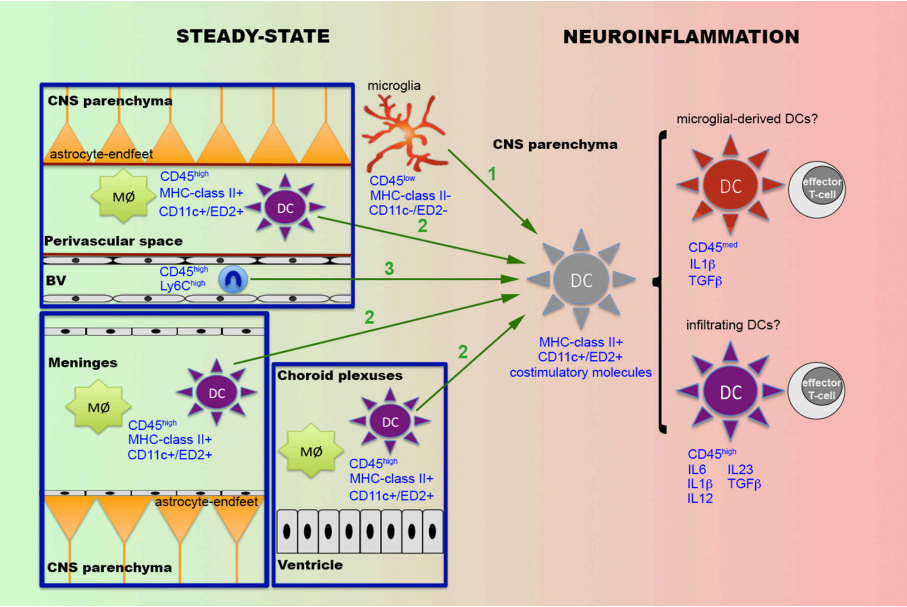


Figure 2.4: Dendritic cells in the CNS during steady state and Neuroinflammation. Presence of DCs (CD45highMHCII+CD11c+/ED2+) in the choroid plexus, perivascular space and meninges of the CNS together with microglia (Mϕ) during homeostasis. DCs also present in the CNS parenchyma which may have infiltrated from the periphery during neuroinflammation as a consequence of either infection, traumatic brain injury or other inflammatory conditions. Figure taken from Almolda et al., 2015.

2.4.4) Dendritic cells in tuberculosis and as targets for TB vaccines/therapy

DCs can act as host cells for mycobacteria and are mediators of immune responses (Jiao et al., 2002; Hickman et al., 2002). Communication between cells is crucial in achieving protection against TB (Domingo-Gonzalez et al., 2016). Dendritic cell maturation can be impaired by *M. tuberculosis* infection leading to reduction in the production of IL-12 and ultimately inability for DCs to activate T cells (Triccas et al., 2002). However, myeloid DCs are not completely inhibited by *M. tuberculosis* because they can produce cytokines inducing Th1 immune responses (Giacomii et al., 2001; Hickman et al., 2002). Small amounts of bacterial numbers can cause delayed T cell immune responses, whereas, large amounts can inhibit DC function or cause T cell exhaustion (Shaler et al., 2002; Day et al., 2011) and human DCs have also been shown to die following infection with *M. tuberculosis* (Ryan et al., 2011). During *M. tuberculosis* infection, DCs form a bridge between innate and adaptive immune responses, and unlike macrophages, they recognize PAMPs through dectin-1, TLR9, and DC-SIGN (Ernst, 1998; Kleinnijenhuis et al., 2011; Sáenz et al., 2013). DCs activate T cells when they present antigens to naïve T cells (Sáenz et al., 2013). During pulmonary infection, DCs initiate adaptive immune responses in the lymph nodes when they present live *M. tuberculosis* antigens which are transported from the lungs through chemokine receptor 7 (CCR7) or chemokine receptor-

5 (CCR5) (Bini and Hernandez-Pando, 2013; Sáenz et al., 2013), and increased levels of CCR7 produced by *M. tuberculosis* infected DCs are important for migration of T cells and NK cells (Sasindran and Torrelles, 2011). Human DCs are activated by both *M. tuberculosis* and BCG (Henderson et al., 1997; Thurnher et al., 1997). Similar to macrophages, DCs are the other key cell type that present *M. tuberculosis* antigens on MHCI and MHCII to T cells to induce immune responses (Satake et al., 2017). Proinflammatory cytokines (IL-1 and IL-6) secreted by both macrophages and DCs recruit cells to the site of TB infection (Giacomini et al., 2001). To induce effective immunity against TB, DCs must be activated early and migrate to draining lymph nodes to activate T cells (Choi et al., 2017). DCs polarised for Th1 immune responses secrete high levels of IL-12p70 in PBMCs of TB patients (Satake et al., 2017). This IL-12p70 activates CD4+ T cells and also activates CD8+ T cells to undergo expansion into effector and memory T cells (Chang et al., 2004; Lee et al., 2007). Mice that had their DCs depleted resulted in increased bacterial loads in the lungs and spleen because CD4+ T cell responses were impaired (Tian et al., 2005) while DC deficient mice presented with BCG-osis (Hambleton et al., 2011).

The delay in the accumulation of activated *M. tuberculosis-specific* CD4+ T cell immune responses in the lungs of mice that normally occurs (Reiley et al., 2008; Wolf et al., 2008) is suggested to be caused by the *M. tuberculosis* inhibiting the early presentation of antigens by APCs (Harding and Boom, 2010; Urdahl, 2014; Srivastava et al., 2016). During pulmonary TB infection, DCs that are infected by *M. tuberculosis* is needed to traffic antigens from the lung to the lymph nodes to prime T cell responses (Srivastava et al, 2014). DCs transfer antigens to bystander DCs in the LNs instead of directly and effectively presenting antigens to *M. tuberculosis* specific CD4+ T cells because of the non-efficiency of infected DCs (Srivastava et al, 2014). Understanding host-pathogen interaction leads to innovative TB treatment strategies like immunotherapy. Griffiths and colleagues (2016) showed that DC transfer confers superior vaccine-induced *M. tuberculosis* control suggesting that DCs should be targeted to accelerate T cell activation. Khan and colleagues (2016) showed that the dosage of anti-tuberculosis drugs utilized to achieve effective *M. tuberculosis* killing was reduced by increasing the efficacy of dendritic cells. Research findings of DC vaccines that were loaded with either TB immunogenic proteins or peptides did not show consistent results in TB murine models (Moll, 2004; Brinkman et al., 2003). While Malowany and colleagues (2005) previously showed that a modified dendritic cell vaccine was a much more potent activator of both CD4+ T cell and CD8+ T cells than peptide/protein vaccines, suggesting that a genetically

modified DC-based TB vaccine offers superior protection against TB. A study by Choi and colleagues (2018) showed that *M. tuberculosis* protein Rv3841 is known to play an important role in *M. tuberculosis* growth "functionally" activates dendritic cells (increased costimulatory molecule, increased proinflammatory cytokines) and contributes to inducing Th1 immunity. Recombinant live mycobacterial vaccines offer improved protection (Griffiths et al., 2016) and DC transfer confers superior vaccine-induced *M. tuberculosis* control, accelerates vaccine CD4+ T cell activation, mediates the control of CD4+ T cell dependency. Activating CD103+ DCs improve *M. tuberculosis* control by vaccines. Dendritic cell-T lymphocyte interaction is a relatively new explored research area in the context of CNS-TB and not well characterized.

2.4.5) Adaptive Immunity: T cells and TB

T cells are activated when the T cell receptors bind to the peptides presented on MHCII molecules and the stimulation of co-stimulatory molecules (CD80/86) presented by APCs (Wlodrczyk et al., 2014). Pro-inflammatory cytokine, IL-12 that is mainly produced by macrophages and DCs and plays a crucial role in connecting the innate and adaptive immune responses (Vignali and Kuchroo, 2012; Gately et al., 1991; O'Shea and Paul, 2002). IL-12 deficiency in mice leads to *M. tuberculosis* susceptibility (Khader et al., 2006; Holscher et al., 2001) because IL-12 important for protection against *M. tuberculosis* infection (Cooper et al., 2002). During pulmonary infection, myeloid DCs present *M. tuberculosis* antigens to CD4+ T cells in the lymph nodes which proliferate and get activated then migrate to the lungs to control bacterial replication (Sáenz et al., 2013). The amount of bacteremia in the lymph nodes is suggested to determine *M.tuberculosis* disease outcome (Reiley et al., 2008). Regulatory T cells (Tregs) limit the function of effector T cells during mycobacterial infection of the lungs (Sáenz et al., 2013). FoxP3+ T cells are crucial for regulating inflammation in the CNS through immune suppression (Liu et al., 2006) and intracerebral T cell immune responses are inhibited by TGF-β (Streilein et al., 1992). During *M. tuberculosis* infection, Tregs either prevent tissue damage by regulating inflammation caused by mycobacteria or they can cause inhibition of Th1 cells to regulate protective immune responses that are being induced (Hickman et al., 2002; Sáenz et al., 2013). Tregs are increased in patients with active pulmonary TB and they cause suppression of IFNγ and prevention of effector cell recruitment to sites of infection (Ribeiro-Rodriques et al., 2006; Guyot-Revol et al.,2006; Chen et al., 2007; Ozeki et al., 2010). Treg depletion in mice (*in vivo*) and humans (*in vitro*) has been shown to increase *M.tuberculosis* specific immune responses (Ribeiro-Rodriguez et al., 2006; Hougardy et al., 2007; Ozeki et

al., 2010; Chiacchio et al., 2009). T cells enter the brain predominantly via the choroid plexus but can gain direct access through the transversal of the blood-brain barrier (Emma et al., 2010). Th1 immune responses are required for protection against TB (Lande et al., 2003; Desvignes et al., 2012; Rai et al., 2016) and *M. tuberculosis* specific CD4+ T cell responses occur at week 2 (Thwaites et al., 2000). T cells secrete IFNγ and TNFα that signal activation macrophages to perform their function of killing mycobacteria and recruit cells to the site of infection (Thwaites et al., 2000; Flynn and Chan, 2001; Gopal et al., 2014). Both conventional and unconventional T cells are required for acquired TB immunity (Boom et al., 2003). CD4+ T cells remain the primary T cells required for protection against *M. tuberculosis* infection (Kaufmann et al., 2005). Activation of CD4+ T cells by the presentation of antigens by macrophages on MHCII leads to the secretion of IFNγ , TNFα, and IL-12 which activate macrophages to kill *M.tuberculosis* at the site of infection (Flynn and Chan, 2001; Mihret, 2012; O'Gara et al., 2013). Figure 2.5 shows the APCs presenting antigens to CD4+ T cell then activating them to differentiate into different subsets of T-helper cells which secrete cytokines. Memory CD4+ T cells (40-50%) and CD8+ T cells (20-30%) are present in the CSF of healthy humans (Kivisäkk et al., 2003; de Graaf et al., 2011). T cell antigens boost protective immunity but do not induce complete sterilizing immunity (Olsen et al, 2001).

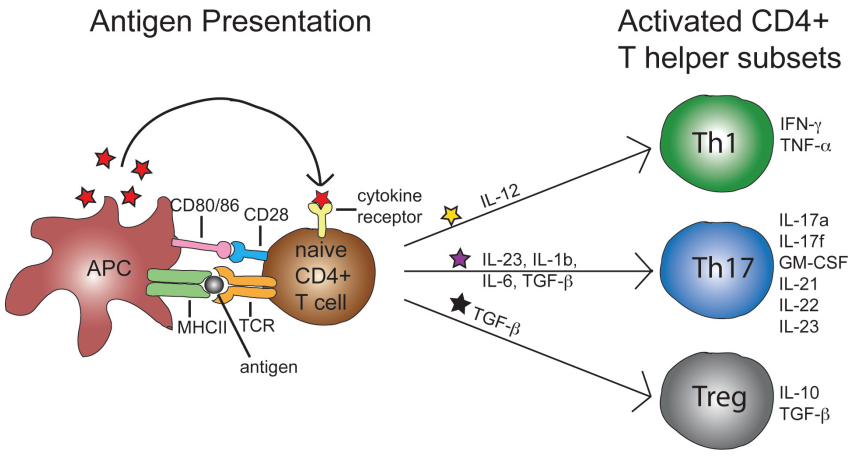


Figure 2.5: Diagram showing APCs activating and differentiating CD4+ T cells that produce pro-inflammatory and anti-inflammatory cytokines. APCs presenting antigens through MHCII to the T cell receptor (TCR) on naïve CD4+ T cells and activating them to proliferate when also signalled by APC CD80/86 binding to CD28 on T cells. Cytokine signalling from APCs contributing to polarization of effector CD4+ T cells into T-helper cell subsets of Th1/Th2 and Th17 that secrete pro-inflammatory and anti-inflammatory cytokines. Figure taken from Rodgers and Miller (2012).

2.5) Chemokines and Cytokines during mycobacterial infection of the CNS.

Chemokine and cytokine productions mediate host responses to TB in the brain that lead either to protective host defense or neuronal damage (Curto et al., 2004). Cytokines are small soluble proteins that influence the function of cells and are important for controlling disease outcome (Domingo-Gonzalez et al., 2016), these signaling molecules that modulate the innate and adaptive immune responses in an autocrine or paracrine manner (Doll et al., 2014) which bind to specific receptors (four major receptor groups are present) according to several structural homologies in the extracellular domain and signaling mechanisms they employ (Renauld, 2003; Liongue and Ward, 2007). While, chemokines are a large family of polypeptides that are characterized by four conserved cysteine residues that can be divided into different families like CC, XC and CXC (Fernandez and Lolis, 2002; Mèlik-Parsadaniantz and Rostène, 2008). Non-cell specific chemokine production (MIP-2, MCP-1, and KC) measured from brain homogenates of CNS-TB mice were significantly different from those of uninfected mice for more than 24 weeks but cytokine concentrations (IFNγ, TNFα, IL-6) did not differ 3 weeks post-infection (van Well et al., 2007). These chemokines are implicated in leukocyte recruitment to the CNS (MIP-2 and KC) and mononuclear phagocyte recruitment that causes activation of macrophages.

Figure 2.6 shows the pathogenesis of TBM and the cytokines produced in the brain during TBM. Non-immune and immune cells in the brain produce TNFα (Hsu et al., 2017) and microglia are major producers of TNFα during CNS-TB (Curto et al., 2004; Spanos et al., 2015). TNFα is an important contributor to CNS mycobacterial pathogenicity and protection (Mastroiani et al., 1997; Tsenova et al., 1998 and 1999; Thwaites et al., 2003; Curto et al., 2004; Kruglov et al., 2011). TNFα and IFNγ production are crucial for controlling *M. tuberculosis* infection and recruiting effector cells to the site of infection (Co et al., 2004; Allie et al., 2013; Segueni et al., 2016) and TBM patients present with high levels of IFNγ and TNFα in the CSF (Mastroianni et al., 1997). The levels of TNFα and IFNγ in CSF of TBM patients could not be correlated to clinical disease severity, whereas in non-TBM meningitis cases there is a correlation between TNFα levels and clinical disease outcome (Mustafa et al., 1989; Saez-Llorens et al., 1990; Mastroianni et al., 1997; Thwaites et al., 2003). These levels get reduced by anti-TB drugs but remain in high amounts after six months including high IL-1β, IL-6, IL-18, and IL-10 (Mastroianni et al., 1997; Babu et al., 2008; Misra et al., 2010). However, a contrasting study also found that TNFα and IFNγ are not detectable 1-2 months in patients

treated with antibiotics in combination with corticosteroids (Mansour et al., 2005). In rabbit models of TBM, TNFα levels have been shown to increase two hours after infection and significantly decrease after eight days (Tsenova et al., 1998). While levels in human patients have been shown to moderately increase over several weeks (Mastroianni et al., 1997) which is in contrast to Thwaites and colleagues (2003) who did not find any further increase in TNFα levels of TBM patients after 7 days. IL-6 stimulates IFNγ production in mice but is not crucial for protection against *M. tuberculosis* (Saunders et al., 2000). However, the absence of IL-6 in a large mycobacterial dose of intravenous *M. tuberculosis* infection results in susceptibility during mycobacterial infection while low dose infection does not lead to the death of mice even with high bacterial burdens (Ladel et al., 1997; Saunders et al., 2000). IL-10 production is capable of suppressing immune responses against *M. tuberculosis* infection in the lungs (Scott-Browne et al., 2007) but an increase in IL-10 in TB patients after anti-TB treatment is associated with the "recurrence" of pulmonary TB (Lago et al., 2012). During pulmonary TB, DCs secrete IL-12 inducing Th1 immune responses in mice, whereas, macrophages do not secrete IL-12 to promote Th1 polarization (Hickman et al., 2002). During pulmonary tuberculosis, mice deficient in IL-12p40 subunit are susceptible to *M. tuberculosis* because of reduced antigen-specific production of IFNγ by T cells (Cooper et al., 1997; Cooper et al., 2002), and IL-12p40 deficient humans are also predisposed to *M. tuberculosis* (Domingo-Gonzalenz et al., 2016). However, it is not known how the presence or absence of IL-12 influences disease outcomes during CNS-TB. Murine models of mice, IL-1β knockout (KO) mice showed that *M. tuberculosis* infection causes increased bacterial burdens and mortality indicating susceptibility because IL-1β is important for promoting resistance to *M. tuberculosis* (Juffermans et al., 2000; Sugawara et al., 2001; Fremond et al., 2007; Mayer-Barber et al., 2010).The chemokine and cytokine responses induced by *M. tuberculosis* target cells that protect against CNS-TB has not been completely elucidated.

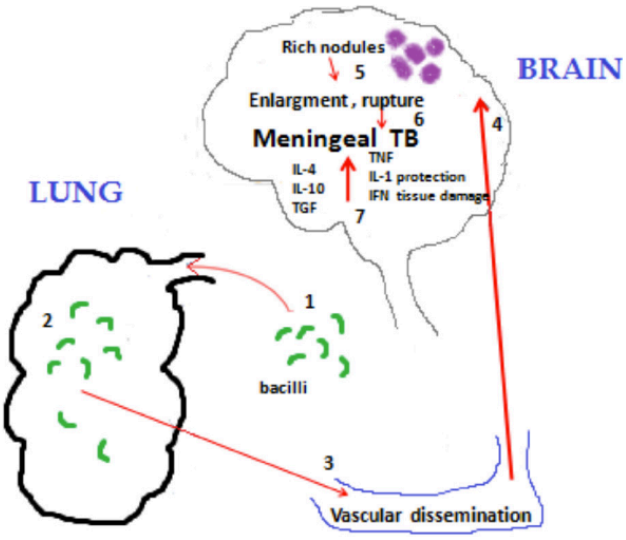


Figure 2.6: Pathogenesis of TB meningitis and production of cytokines. *M. tuberculosis* bacilli inhaled into the lungs and spreads to the brain through hemotegenous dissemination to seed and cause meningeal TB upon rupture of Rich nodules resulting in increase of pro-inflammatory (IFNγ, IL-1, and TNF) and anti-inflammatory cytokines (IL-10, TGF-β, and IL-4). Figure taken from Bini and Hernandez-Pando (2013).

2.6) Hypotheses

1) Brain APCs (microglia, macrophages and DCs), and T cells are differentially infected by *M. tuberculosis* or BCG post intracerebral infection and their resulting phenotype and functional profiles play a protective role in initiating and mediating cellular immune responses against mycobacterial infection of the CNS.

2) During CNS-TB, *M. tuberculosis* infection modifies dendritic cell phenotype and function which in turn alters T cell phenotype and function to favour immunity during dendritic cell - T cell interaction.

2.7) Research Objectives

1) To investigate the immunological roles of antigen-presenting cells (microglia, macrophages, and DCs) and T cells using *in vivo* murine models of CNS-TB infection.

2) To investigate the functional and phenotypic immune effects of dendritic cell, microglial, macrophage and T cell responses as outcomes during mycobacterial infection of the CNS.

3) To investigate modulation of T cells by DCs during CNS-TB.

2.8) Aims

1) To establish CNS-TB infection in mice through intracerebral infection with *M. tuberculosis* H37Rv and BCG, respectively.

2) To determine the kinetic recruitment of dendritic cells (DCs), macrophages, and T cells to the brain and cervical lymph nodes (CLNs) post *M. tuberculosis* and BCG intracerebral infection, respectively.

3) To determine the presence of DCs in a healthy CNS and characterize the cellular phenotype of DCs recruited to the brain and CLNs post *M. tuberculosis* and BCG intracerebral infection, respectively.

4) To characterize the phenotype and functional profiles (maturation and cytokines) of microglia and infiltrating DCs and macrophage post *M. tuberculosis* and BCG intracerebral infection, respectively.

5) To determine the T cell subsets, activation states and transcription factor profiles in the brain and CLNs post *M. tuberculosis* and BCG intracerebral infection, respectively.

6) To determine the polyfunctional cytokine profiles of recruited of brain CD4+ T cells during CNS-TB.

7) To investigate the modulation of T cells by DCs during CNS-TB using a dendritic cell-T cell coculture.

CHAPTER 3: MATERIALS AND METHODS

3.1) Animal Ethics Approval

Wild type C57BL/6J mice use in this study were purchased from The Jackson Laboratory (Bar Harbor, Maine, US). The breeding and maintenance of mice was conducted under specific pathogen-free (SPF) conditions at the University of Cape Town's Research Animal Facility (Cape Town, South Africa). The Animal Research Ethics Committee (AEC) approved all animal procedures (AEC reference number: 015/014) at the University of Cape Town in accordance to the South African National Standard. Performance of all animal procedures was authorized by the South African Veterinary Council (SAVC accreditation: AR15/14173). Adult female mice were aged 8-12 weeks were used for all experiments and were maintained under biosafety levels 2 and 3 conditions (BSL2 & BSL3).

3.2) Mycobacterial Preparation

The strain of *M. tuberculosis* H37Rv and *Mycobacterium bovis* Bovine BCG were acquired from the Trudeau mycobacterial culture collection (Trudeau Institute, Saranac Lake, New York, USA) and they were individually grown to log phase in Middlebrook 7H9 broth (Difco™ Becton, Dickinson and Company, Sparks, MD 21152, USA) that contained 10% of oleic acid-dextrose-catalase (OADC) supplement (Difco, Detroit, MI), 0.5% glycerol (Merck, Darmstadt, Germany) and 0.05% of Tween-80 (Sigma, MO, USA) at 37 °C for 19-21 days. Following incubation, 1 ml aliquots of inoculum were respectively aliquoted into 2 ml cryovial tubes (Nalge Nunc International, Naperville, IL, USA) and stored in the freezer at −80 °C. The concentration of the stocks was determined by thawing the 1 ml frozen aliquot and passaging it 30 times through a 29.5-gauge syringe that was fitted with a 0.5 ml syringe (Omnican®, B. Braun, Melsungen, AG, Germany) for disruption of clumps. For each strain, 100 ul of the inoculum was plated on 90 mm compartmentalized plates (Sterilin, Bibby Sterilin Ltd., Stone, Staffs, UK) in 10-fold serial dilutions of saline (0.9%)-Tween 80 (0.05%) on 7H10 Middlebrook agar (Difco™ Becton, Dickinson and Company, Sparks, MD 21152, USA) that was supplemented with 10% OADC and 0.5% glycerol. We sealed plates containing serial dilutions of the inoculum in a plastic bag and cultured at 37°C for 19-21 days. After incubation, colony-forming units (CFU) were counted manually under the biosafety level 2 cabinet, and mycobacterial concentrations were calculated.

3.3) Mycobacterial preparation on the day of infection

Frozen 1 ml aliquots of live-attenuated *M. tuberculosis* H37Rv or BCG stocks (with pre-determined concentrations) were first thawed at room temperature followed by centrifugation at 10 000 rpm for 5 min at 4 °C. The supernatants were discarded, and pellet resuspended in 1 ml of sterile 0.9% saline [Sodium chloride (Saarchem, Merck Chemicals Pty, Ltd, South Africa) in distilled H_20]. Then the inoculum was passed 30 times through a 29,5-gauge needle fitted on a 0.5 ml syringe (Omnican®, B. Braun, Melsungen, AG, Germany) to disrupt the clumps (for BCG only) or by adding glass beads and vortexing (for BCG or *M. tuberculosis* H37Rv). The inoculum was finally diluted in saline to a volume of 0.5-1 ml to the desired concentration that produced a concentration of 1 x 10^5 CFU per 3 ul volume. The inoculum was plated in serial dilutions and cultured as described in section 3.2 for confirmation of concentration.

3.4) Intracerebral infection

Prior to inoculation, mice were anesthetized intraperitoneally with a ketamine [Bayer (Pty) Ltd, Germany] and Xylazine (Intervet, Zurich-Switzerland) (100mg/ml:10mg/kg) cocktail and were administered pre-emptive analgesic Buprenorphine [Temgesic, Ricket Benkiser Healthcare SA, (PTY) LTD] (0.1mg/kg) subcutaneously as a painkiller. Then mice were prepared for surgery by shaving the hair off their head area that will undergo surgery with a clipper (Moser 1556 Trimmer, Germany) then making a 1cm incision on the head skin using a sterile surgical scalpel blade (AZDENT, model 15#, China). A burr hole was then constructed on the skull using a driller (Dremel 300i, Wisconsin, USA) about 0.2 mm anterior to the bregma and 2mm to the left of the midline of the skull exposing the dura mater. Intracerebral infection of mice was performed using a stereotaxic approach of directly injecting a dose of 1 x 10^5 CFUs of live- attenuated *M. tuberculosis* H37Rv or BCG (contained in 3 ul saline suspension) to a depth of 2.0 mm into the cerebral cortex using a single insertion needle. A Hamilton syringe (Gastight no. 1701, Hamilton, Bonaduz, Switzerland) was used to inject the inoculum solution at a rate of 0.1ul per minute and delivered in 3ul saline suspension while keeping the needle in place for 10min for the allowance of absorption of the solution. The burr hole was sealed with sterile bone wax (Ethicon LLC, China) and the skin sutured with a sterile 5.0 non-observable suture (Ethicon LLC, China). Animals were then kept warm with a lamp until recovery before being returned to their cages.

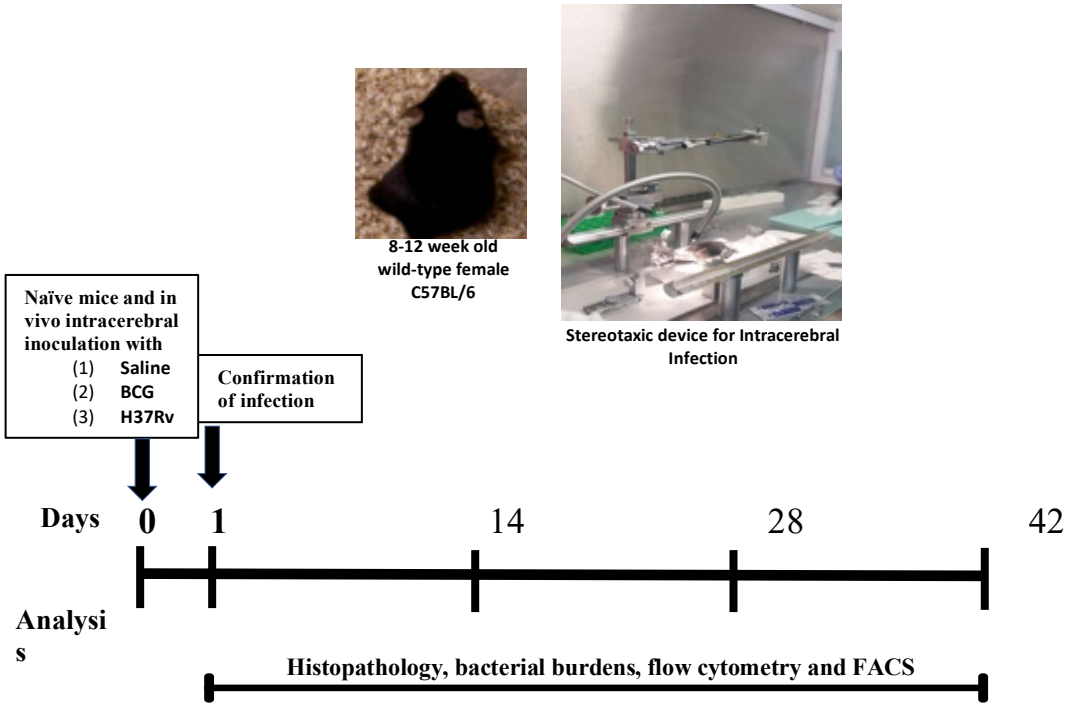


Figure 3.1: Flow diagram of experimental methodology showing intracerebral inoculation of female wild-type C57BL/6 mice with *M. tuberculosis*, BCG, or saline followed by the experimental analysis of harvested organs at defined time-points.

3.5) Animal welfare

The clinical severity and welfare of the animals were monitored post intracerebral inoculation to take care of their well-being. For relief from pain that was caused by the surgery procedure, buprenorphine was administered 2 x 8-12 hours apart post-operative to all mice that underwent surgery and once daily for the first 3 days post-operative. Mice were then monitored and weighed twice a day 3 days post-surgery. The mice welfare monitoring for behavior during infection utilized the following scoring scale: No visible discomfort or stress = 0; Mild discomfort/stress = 1; Moderate discomfort/stress =2; Distress = D. Finally, mice (mycobacterial infected, saline inoculated, and naïve) were monitored twice daily and weighed once a week for the duration of infection/experiment until predetermined sacrifice time intervals.

3.6) Bacterial Burdens

Mice were intracerebrally infected with *M. tuberculosis* and BCG, respectively and saline inoculated. Then mice were euthanized at defined experimental time points (Day 1, then 2, 4 & 6 weeks) using 5% halothane (2-bromo-2-chloro-1,1,1-trifluoro-ethane) (Safeline Pharmaceuticals Pty Ltd, South Africa) in the air and death confirmed by cervical dislocation. Harvested brains, spleens, and lungs were homogenized respectively in sterile 0.9% saline (0.9g NaCl in dH_2O) using a glass tissue Grinder-homogeniser (Kimix Chemicals and Laboratory Suppliers, South Africa). Ten-fold dilutions of the homogenates were prepared in a solution of 0.9% NaCl/0.04% Tween 80 (v/v) and 100ul were plated in duplicates on Middlebrook 7H10 agar (Difco^{TM} Becton, Dickinson and Company, Sparks, MD 21152, USA) plates supplemented with 10% of OADC and 0.5% of glycerol then incubated at 37 °C for 19-21 days. Colonies grown on agar plates for each organ were counted manually and the concentration of bacterial burdens determined. See Appendix A for reagent preparations.

3.7) Sample Preparation for Histological analysis

Mice were intracerebrally infected with *M. tuberculosis* and BCG, respectively. Naive mice and saline inoculated mice were used as controls. Then mice were euthanized at defined experimental time points (2, 4 & 6 weeks) using 5% halothane (2-bromo-2-chloro-1,1,1-trifluoro-ethane) (Safeline Pharmaceuticals Pty Ltd, South Africa) in the air. Brains, spleens, lungs, and cervical lymph nodes were harvested from naïve mice, saline inoculated mice, and mycobacterial infected mice and fixed in 10% formalin [formaldehyde solution (Sigma Aldrich, Saint Louis, MO, USA) in PBS at pH 7.4. See Appendix A for reagent preparation. The organs were then embedded in paraffin wax overnight (Histosec Pastilles, Merck, Germany) after dehydration in an automated tissue processor (Shandon Elliot, Shandon Southern Instruments, Ltd, Surrey, UK). Tissue dehydration steps were as follows; (1) 1 x 70% alcohol for 2hrs, then (2) 2 x 96% alcohol for 2hrs, followed by (3) 4 x 100% alcohol and finally (4) 2hrs in Xylol. Fixed tissues were microscopically sectioned into 4 μm using microtone (Leica, model RM-2125, Wetzlar, Germany) and dewaxed at 56°C overnight for different histology staining.

3.7.1) Haematoxilin and Eosin Staining

Haematoxylin and Eosin (H&E) staining detects of immune cell infiltration responses and was carried out according to Culling (1974) protocol. Dewaxed tissue sections were washed as

follows; (1) 3 x 100% ethanol for 1min, (2) 2 x with 96% ethanol for 1min, (3) for 1 min with 70% ethanol and finally rinsed with tap water. Then the tissue sections were incubated for 9 min with haematoxylin then rinsed with tap water. This was followed by the washing of tissues with Scotts water for 3 min then 2 min with tap water. Counter staining was then carried out with 1% eosin solution for 2 min. After staining, the tissues were washed with tap water and dehydrated with 70% and 90% alcohol and finally with Xylol. Tissues were then mounted with Canada Balsam (Sigma-Aldrich, Kempton Park, South Africa) and slides covered with Entellan ((Merck, Darmstadt, Germany). See Appendix B for reagent preparation. Slides were analyzed under a microscope (Nikon Eclipse 90i) using the Nikon NIS element.

3.7.2) Ziehl-Neelsen Staining

Ziehl-Neelsen (ZN) staining detects presence of acid-fast bacteria. The ZN staining was modified for acid fast bacilli from Culling (1974) and Charletons protocols. Slides were placed on a rack across basin. Filtered carbol fuchsin solution was used to cover slide sections. The slides were heated gently until the steamed arose, then the slides were left to stand for 5 min and this procedure was repeated twice before being rinsed with tap water. To decolorize, slides were washed with 1% acid alcohol solution (1% hydrochloric acid in 70% alcohol) until excess pink color was removed. Then the slides were decolorized in 20% sulphuric acid for 20 min and washed with running tap water for 10 min. Rinsed sections were then covered with drops of Loeffler's methylene blue for 30-60 seconds 1 min and rinsed with water before being dehydrated with different concentrations of alcohol (70%, 90%, 96% and 100%). Finally, slides were dipped for 1 min in Xylol before being mounted in Entellan (Merck, Darmstadt, Germany). See Appendix B for reagent preparation. Slides were analyzed under a microscope (Nikon Eclipse 90i) using the Nikon NIS element.

3.8) Flow Cytometry experiments

Mice were intracerebrally infected with *M. tuberculosis* and BCG, respectively. Naive mice and saline inoculated mice were used as controls. Then mice were euthanized at defined experimental time points (2, 4 & 6 weeks) using 5% halothane (2-bromo-2-chloro-1,1,1-trifluoro-ethane) (Safeline Pharmaceuticals Pty Ltd, South Africa) in the air and death was confirmed by cervical dislocation. Harvested brains and cervical lymph nodes were collected in 1x PBS buffer (pH. 7.4). Single-cell suspensions of the tissues were generated using sterile 70 μm strainer (Corning cell strainer, Merck, Darmstadt, Germany) and cells were stained for flow cytometry. Cells fixed in FACS fixation buffer were added to a hemocytometer and counted under the Nikon Eclipse e100 microscope. Surface and/or intracellular staining was

conducted on the following cell types; APCs (dendritic cells, microglia, macrophages) and T cells (subsets, transcription factors and cytokines). Antibody cocktails were used to stain different panels. Table 3.1 shows the antibodies, dilutions, their fluorochrome, clones, and manufacturing company.

3.8.1) APCs and intracellular cytokine staining

Approximately 2 x 10^6 brain and cervical lymph node cells were separately added to V-shaped 96 well plates. Cells were surface stained with a 25 ul antibody cocktail diluted in FACS blocking solution [FACS buffer/block mix (1/100 αFcyRII)] for the following microglia/macrophage and dendritic cell markers differently combined; CD45, CD11b, CD11c, MHCII, CD86 and PDCA-1 and then incubated on at 4^0 C in the dark for 30 min. FACS buffer was then added to cells and cells centrifuged at 1500 rpm for 5 min at 4^0 C using a Beckman allegra^{TM} 25R refrigerated centrifuge. Following centrifugation, the supernatants were discarded. Then cell pellet re-suspended in permeabilization buffer (0.1% saponin in FACS buffer), and incubated at 4^0 C in the dark for 20 min. After permeabilization, cells were centrifuged at 1500 rpm for 5 min at 4^0 C and the supernatant discarded. The pellet was stained for intracellular cytokines with different combinations of cytokines that included; TNFα, IFNγ, IL-1β, IL-6, IL-12 & IL-10 in a 25ul antibody cocktail diluted in permeabilization buffer, then incubated in the dark for 40min at 4^0 C. Finally, the cells were washed in permeabilization buffer and fixed in 2% paraformaldehyde fixation buffer, then and stored at 4^0 C in the dark until flow acquisition. Both surface and intracellular staining included fluorescence minus one (FMO) controls. See Appendix C for reagent preparations. Samples were filtered through a 40 µm strainer (Corning cell strainer, Merk, Darmstadt, Germany) and transferred to FACS tubes (Falcon, ThermoFisher Scientific,MA, USA) prior to acquisition on a BD LSRFotessa^{TM} Flow cytometer cell analyzer (Becton, Dickinson and Company, USA).

3.8.2) T cells and Transcription factor staining

The staining protocol utilized was according to the manufacturer's instructions for the BD Pharmingen transcription factor buffer set (Catalog no. BDB562574, BD Biosciences, USA), however it was modified/adjusted to accommodate using 96 well plates instead of FACS tubes. Approximately 2 x 10^6 brain and cervical lymph node cells were separately added to V-shaped 96 well plates. The cells were surface stained with 25 ul antibody cocktail diluted in in FACS blocking solution [FACS buffer/block mix (1/100 αFcyRII)] for the following T cell surface

markers; CD3, CD4, CD8, CD44 and CD62L and then incubated on at 4^0 C in the dark for 30 min. Thereafter, 200 ul of FACS buffer was added and cells centrifuged at 1500 rpm for 5 min at 4^0 C. The supernatants were discarded and 100 ul of 1x Fix/Perm buffer was added. Cells were then incubated at 4^0 C in the dark for 40 min. The sample was then centrifuged, and pellet re-suspended in 200 ul of Perm/Wash buffer. The samples were then centrifuged and supernatant discarded. Following centrifugation, intracellular staining was carried out using a 25 ul of antibody cocktail containing transcription factors (T-bet, GATA3, RORγT and FoxP3) dissolved in Perm/Wash buffer was added to the cells and incubated for at 4^0 C in the dark for 40 min. Then the samples were centrifuged again, and pellet re-suspended in 200 ul Perm/Wash buffer. The wash step was repeated, and cell pellets were re-suspended in 200 ul FACS buffer. Cells were stored at 4^0 C in the dark until flow acquisition. Both surface and intracellular staining included FMO controls. See Appendix B for reagent preparations. Samples were filtered through a 40 μm strainer (Corning cell strainer, Merck, Darmstadt, Germany) prior to acquisition on a BD LSRFotessa^{TM} Flow cytometer cell analyzer (Becton, Dickinson and Company, USA).

3.8.3) T cell Re-stimulation and intracellular cytokine staining

Only brain cells from *M. tuberculosis* intracerebrally infected mice were used for the T cell re-stimulation assay. Tissue culture round bottom sterilin 96 well plates were coated with 50 ul of αCD3/CD28 at 4^0 C overnight. The next day, the αCD3/CD28 cocktail was aspirated before adding the cells. Approximately 2 x 10^6 cells in 100 ul of 1x PBS buffer were added to the wells. The following stimulants of 2x working solutions (ESAT-6 and H37Rv) diluted in complete re-stimulation medium were added to cells that did not contain stimulant αCD3/CD28 that and media only served as a control. Cells were stimulated for 6 hrs at 37^0 C in a CO2 incubator, then the reaction was stopped with brefeldin after 2 hrs of incubation. The cells were mixed well with multichannel then centrifuged at 1500 rpm for 5 min at 4^0 C and supernatant discarded. Cell pellets were re-suspended in 300 ul FACS buffer and transferred to V-shaped 96 well plates, where 1 x 10^6 cells were plated for FACS controls vs 2 x 10^6 cells for positive experimental samples. T cell surface staining for; CD3, CD4, CD8, CD44, CD62L and intracellular staining for the following cytokines; TNFα, IFNγ, TGF-β, IL-17, IL-10 and IL-4 were carried out using the above modified BD Pharmingen transcription factor buffer set protocol (3.8.2). See Appendix D for reagent preparations. Samples were filtered through a 40

μm strainer (Corning cell strainer, Merck, Darmstadt, Germany) prior to acquisition on a BD LSRFotessa^{TM} Flow cytometer flow analyser (Becton, Dickinson and Company, USA).

3.9) Dendritic cell-T cell coculture and flow cytometry staining

Wild-type female C57BL/6J mice (8-12w old) were intracerebrally infected with *M. tuberculosis* H37Rv and naïve mice were used for controls. Mice were euthanized at week 4 after infection with 5% halothane as described in section 3.8. Only CLNs were harvested from CNS-TB mice and spleens from naïve mice were collected in 1x PBS buffer (pH. 7.4|) and single-cell suspensions generated using a 70 μm strainer (Corning cell strainer, Merck, Darmstadt, Germany). Approximately 2 x 10^6 cells were added to FACS tubes. The cells were then surface stained with a 25 ul antibody cocktail diluted in in blocking solution [FACS buffer/block mix (1/100 αFcyRII)] for dendritic cell markers (CD45, CD11b and CD11c) and T cell markers (CD3, CD4, CD8 and CD44). Then the cells were incubated on at 4^0 C in the dark for 30 min. After incubation, FACS buffer was then added and cells were centrifuged at 1500 rpm for 5 min at 4^0 C. *M. tuberculosis* infected DCs (CD45+CD11b-CD11c+) from CLNs and CD3+ T cells from naïve spleens were sorted using a FACSAria^{TM} Fusion cell sorter (BD Becton, Dickinson and Company, USA) in the BSL-3 facility. Sorted cells were collected in complete restimulation medium. Sorted cells fixed in FACS Fixation buffer were added to the hemocytometer and counted under the microscope. Cells were then centrifuged at 1500 rpm for 5 min at 4^0 C. Supernatants were discarded and cells were pooled from 5-6 mice. Cells were seeded in 96 well tissue culture plates at a DC:TC ratio of 1:5 and incubated at 37^0 C in a CO_2 incubator overnight. The next day, samples were centrifuged at 1500 rpm for 5 min at 4^0 C and resuspended in Perm/Fix buffer. Intracellular staining of T cell cytokines; IFNγ, TGF-β, IL-4 and IL-10 was carried out using the modified BD Pharmingen transcription factor buffer set protocol (section 3.8.2). Samples were stored at 4^0 C in the dark until flow cytometry acquisition. Samples were filtered through a 40 μm strainer (Corning cell strainer, Merck, Darmstadt, Germany) prior to acquisition on a BD LSRFotessa^{TM} Flow cytometer cell analyser (Becton, Dickinson and Company, USA).

Table 3.1: Flow cytometry antibodies used in this study.

Antibody & Dilution	**Clone**	**Channel**	**Source**	**Reactivity**
Dendritic cell, microglia, and macrophage surface staining				
CD45 **(1/100)**	30-F11	BV510	BD Biosciences	Rat Anti-Mouse
CD11b **(1/100)**	M1/70	PERCP.Cy5.5	BD Biosciences	Rat Anti-Mouse
CD11c **(1/100)**	HL3	APC-Cy7	BD Biosciences	Hamster Anti-Mouse
PDCA-1 (CD317/BST2) **(1/50)**	eBio927	FITC	eBioscience	Anti-mouse
PDCA-1 (CD317/BST2) **(1/50)**	eBio927	FITC	Invitrogen (ThermoFisher Scientific)	Anti-mouse
MHCII (1A/I-E) **(1/100)**	M5/114.15.2	BV711	BD Biosciences	Rat Anti-mouse
CD86 **(1/50)**	GL-1	V450	BD Biosciences	Rat Anti-mouse
CCR7 (CD197) **(1/100)**	4B12	BV605	BD Biosciences	Rat Anti-Mouse
Dendritic cell, microglia, and macrophage intracellular cytokine staining				
IL-1β **(1/50)**	NJTEN3	PE-Cy7	eBioscience	Anti-Mouse
Il-12p40/p70 **(1/50)**	C15.6	PE	BD Biosciences	Rat Anti-Mouse
TNFα **(1/50)**	MP6-XT22	AL647	BD Biosciences	Rat Anti-Mouse
IL-10 **(1/50)**	JES5-16E3	BV605	BD Biosciences	Rat Anti-Mouse
IFNγ **(1/50)**	XMG1.2	PE	BD Biosciences	Rat Anti-Mouse
IL-6 **(1/50)**	MP5-20F3	APC	BD Biosciences	Rat Anti-Mouse
T cell surface staining				
CD3e **(1/100)**	145-2C11	BV421	BD Biosciences	Hamster Anti-Mouse
CD4 **(1/100)**	RM4-5	AL700	BD Biosciences	Rat Anti-Mouse
CD8a **(1/100)**	53-6.7	APC-Cy7	BD Biosciences	Rat Anti-Mouse

CD44 **(1/100)**	IM7 (RVO)	BV786	BD Biosciences	Rat Anti-Mouse
CD44 **(1/100)**	IM7 (RVO)	FITC	BD Biosciences	Rat Anti-Mouse
CD62L **(1/100)**	MEL-14	BV786	BD Biosciences	Rat Anti-Mouse
CD62L **(1/100)**	MEL-14	BV605	BD Biosciences	Rat Anti-Mouse
T cell transcription factor intracellular staining				
T-bet **(1/50)**	O4-46	PE	BD Biosciences	Mouse Anti-T-bet
GATA3 **(1/50)**	L50-823	PE-Cy7	BD Biosciences	Mouse Anti-GATA3
RORγT **(1/50)**	Q31-378	PERCP.Cy5.5	BD Biosciences	Mouse Anti-RORγT
FoxP3 **(1/50)**	MF23 (RVO)	AL647	BD Biosciences	Mouse Anti-FoxP3
T cell intracellular cytokine staining				
IL-2 **(1/50)**	JES6-5H4	FITC	BD Biosciences	Rat Anti-Mouse
IFNγ **(1/50)**	XMG1.2	PE-Cy7	BD Biosciences	Rat Anti-Mouse
IL-4 **(1/50)**	11B11	BV605	BD Biosciences	Rat Anti-Mouse
IL-10 **(1/50)**	JES5-16E3	BV711	BD Biosciences	Rat Anti-Mouse
TGFβ (LAP) **(1/50)**	TW7/16B4	PE	BD Biosciences	Mouse Anti-Mouse
Il-17A **(1/50)**	TC11-18H10	PERCP.Cy5.5	BD Biosciences	Rat Anti-Mouse

3.9) Data and Statistical analysis

Flow cytometry data was acquired and analysed on a BD FACSDiva™ Software (Becton, Dickinson and Company, USA) and further analysed using FlowJo version 9.9.6 (Becton, Dickinson and Company, New Jersey, USA). FlowJo files were further analysed using Pestle and SPICE v5.1 software (**http://exon.niaid.nih.gov/spice/**). Statistical analysis was performed using the GraphPad Prism 6.0 (GraphPad Software Inc, La Jolla, USA). The student's test and Mann-Whitney U test was used for all the statistical analyses among/between different experimental groups and different time points for bacterial burdens determination and flow cytometry analysis. Data are represented as the mean ± standard error of the mean. For all tests, $p \leq 0.05$ was considered statistically significant.

CHAPTER 4: RESULTS

The results chapter is divided into three sections; (1) bacterial burdens, (2) histopathology and (3) cellular immune responses post experimental *M. tuberculosis* H37Rv and *M. bovis* Bacille Calmette-Guerrin (BCG) intracerebral infection. In order for us to conduct studies to achieve our objectives and aims, we firstly had to reproduce the already established murine model of central nervous system tuberculosis (CNS-TB) that portrays characteristics defined in literature. This could be achieved by intracerebral infection of wild-type C57BL/6J mice with mycobacteria and analysis of both bacterial burdens and histopathology. Bacterial burdens and development of brain pathology results showing CNS inflammation as infection progresses serve as indicators of disease outcome in the brain. Our objective was to investigate whether mice are protected against mycobacterial infection of the CNS following establishment of *M. tuberculosis* H37Rv or BCG brain infection and to identify the roles of immune cells involved during infection. We had to first validate the existing CNS-TB murine model according to previous studies (Matyszak and Perry, 1995; Mazzolla et al., 2002; van Well et al., 2007; Lee et al., 2008; Rock et al., 2008; Be et al., 2009) to increase credibility of our research findings when we investigate cellular immune responses post infection. We wanted to determine the overall contribution of immune cells (microglia, macrophages, dendritic cells and T cells) in regulating mycobacterial infection of the CNS.

4.1) CNS-TB infection model reproduced and immune responses induced.

Murine models of CNS-TB have been established by directly depositing *M. tuberculosis* or BCG into the brain cortex during intracerebral infection (Matyszak and Perry, 1996; van Well et al., 2007; Mazzolla et al., 2002; Lee et al., 2009; Zucchi et al., 2012; Francisco et al., 2015; Hsu et al., 2017). Depending on the strain of mice or mycobacteria utilized to reproduce the CNS-TB model, mycobacterial replication in the CNS post intracerebral infection can either be detrimental to the host or it can be contained/controlled. For instance, BALB/c mice that developed CNS-TB infection post intravenous infection with *M. tuberculosis* C3 strain (clinical strain) showed increased mortality compared to controls (Husain et al., 2017) while wild-type C57BL/6J mice intracerebrally infected with laboratory strain, H37Rv *M. tuberculosis* showed resistance against CNS-TB as evidenced by decline in bacterial burdens (van Well et al., 2007). Some gene deficient mouse strains also show sensitivity to infection as shown by others and work emanating from our research group showed that, lack of TNFα

results in severe CNS-TB disease outcome (Francisco et al., 2015; Hsu et al., 2017). Therefore, our objective was to reproduce and validate the CNS-TB murine model through intracerebral infection with virulent H37Rv *M. tuberculosis* or avirulent *M.bovis* BCG through determination the bacterial growth kinetics and histopathology.

4.1.1) *M. tuberculosis* H37Rv replication controlled in the brain post intracerebral infection and disseminates.

For the purpose of this project, we first had to confirm previously established CNS-TB murine model by intracerebral (i.c.) infection with H37Rv *M. tuberculosis* or BCG at the infection dose of 1 x 10^5 colony forming units (CFU). CNS-TB infection of wild-type C57BL/6J mice was initially confirmed by the presence of H37Rv *M. tuberculosis* or BCG CFUs from whole mouse brain homogenates at day 1 post intracerebral infection (Fig. 4.1A and 4.2A).

Brain bacterial burdens significantly increased from day 1 to day 14 (25-folds, $p<0.01$), then peaked at day 14 during CNS-TB (Fig. 4.1A). This was followed by a significant decrease at day 28 from day 14 (7.8 folds; $p<0.01$) post infection. And finally, no kinetic change in bacterial burdens occurred between day 28 and day 42 post infection, suggesting bacterial persistence because of this sustained growth. However, CFUs at day 42 were significantly lower than those that peaked at day 14 (11-folds, $p<0.01$) during CNS-TB. Although not exact time points were measured by van Well and colleagues (2007) in the CNS-TB murine model achieved by intracerebral *M. tuberculosis* infection of C57BL/6J mice, they also observed a decreased kinetic trend of brain CFUs from day 21 to day 49 post infection. Their findings showed bacterial burden decrease that continued until 12 weeks post infection and was followed by increases that lasted until 24 weeks, however our study was limited to 6 weeks and it made it impossible for us to predict the same bacterial reproduction at later time points. While the CNS-TB model produced through intravenous infection by Be and colleagues (2008) in BALB/c mice showed similar brain bacterial growth kinetics as our findings. Dissemination of mycobacteria from the site of infection to other organs or tissue is a common occurrence and has been documented murine models of CNS-TB infection (Mazzolla et al., 2002; van Well et al., 2007; Lee et al., 2009; Francisco et al., 2015; Deveci et al., 2016), it occurs through the blood or lymphatics.

Dissemination to the spleen and lungs occurred from day 1 post *M. tuberculosis* i.c. infection (Fig. 4.1B and C). Spleen CFUs significantly increased from day 1 to day 14 (101-folds, $p<0.01$) and peaked at day 14, followed by a significant decrease from day 14 to day 28 (3-folds, $p<0.01$) and finally plateaued between day 28 and 42 post infection (Fig. 4.1B). There was also a significant decrease in spleen CFUs from day 14 to day 42 (4.6-fold, $p<0.01$) during CNS-TB. We are unable to compare spleen kinetics to work by Well and colleagues (2007) because they only measured spleen CFUs from week 7 onwards. During CNS-TB, spleen bacterial burdens followed a closely similar kinetic trend as the brain but the brain as an infection site contained the highest number of CFUs compared to the spleen.

We also measured lung *M. tuberculosis* bacterial loads following dissemination and detected a large significant increase from day 1 to day 14 (257-folds, $p<0.01$) then another significant but smaller increase occurred from day 14 to day 42 (3.6-folds, $p<0.01$) post infection (Fig. 4.1C). This exponential increase in lung CFUs indicates that pulmonary infection was not controlled during CNS-TB, phenomenon that has been observed by previous studies (van Well et al., 2007; Be et al 2008). Our brain and lung bacterial kinetics were also similar to those observed in rabbit models of paediatric TB meningitis model (Tucker et al., 2016) and rabbits have been recommended as better models for TBM because of their close resemblance of clinical and histological evidence to human disease (Rock et al., 2008). Our findings share some characteristics similar to previous murine studies of bacterial detection in the brain and controlled bacterial growth kinetics post *M. tuberculosis* i.c. infection (van Well et al., 2007; Be et al., 2009) suggesting we reproduced the CNS-TB model. As a control for contamination, saline inoculated brains, spleens and lungs were plated and no bacteria grew from all organ homogenates of saline infected mice (data not shown). One parameter indicative of protective immunity is decreased bacterial burdens, which we observed in the brain and spleen.

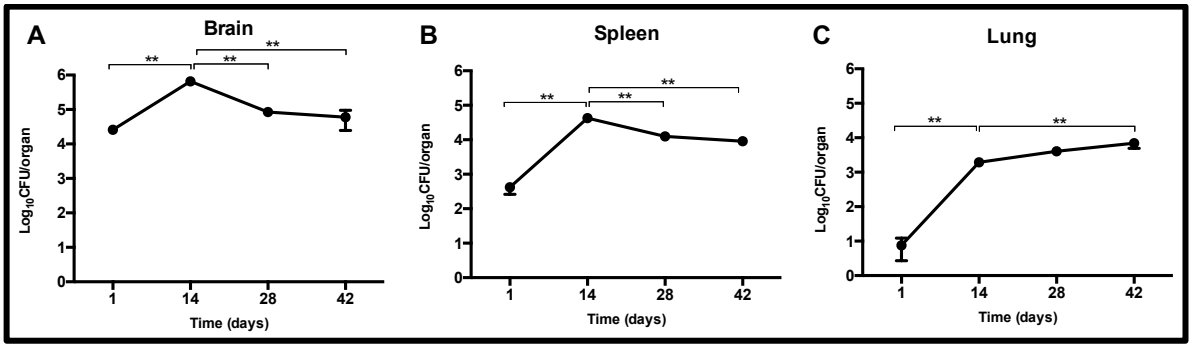


Figure 4.1: *M. tuberculosis* H37Rv controlled in the brain during CNS-TB and disseminates. Wild-type female C57BL/6J mice (4-6 mice/group) were intracerebrally infected with 1 x 10^5 CFU of M. tuberculosis H37Rv and bacterial burdens from (A) brains, (B) Spleens, (C) lungs were determined at days 1, 14, 28 and 42 post infection. Graphs represent two experiments. Data is representative of the Mean +/- SEM. *p<0.05, **p<0.01, Mann-Whitney test.

4.1.2) *M. bovis* BCG replication controlled in the brain post intracerebral infection and disseminates.

To determine the impact of virulence, mice were also infected with *M.bovis* BCG and CNS infection was confirmed by the presence of bacteria day 1 post i.c. infection (Fig. 4.2A), similar to previous studies with heat killed BCG (Matyszak and Perry, 1995) even though we used a live-attenuated BCG strain. Brain bacterial burdens significantly increased from day 1 to day 14 (23-folds, $p<0.05$), then peaked at day 14 post BCG infection (Fig. 4.2B). This was followed by a significant decrease from day 14 to day 28 (9.7-folds, $p<0.01$) post infection and finally no kinetic change occurred between day 28 to day 42, a trend that was similar to M. tuberculosis CNS infection (results section 4.1.1). Mazzolla and colleagues (2002) observed similar kinetics in different mouse strains (BALB/c and DBA/2 mice) but their last time point was day 35. Lee and colleagues (2009) also observed decreasing brain CFUs during CNS-TB infection, even though experimental time points differed as they were from day 21 to day 35 post infection.

From day 1 post infection, we detected disseminated BCG in the spleen and lungs (Fig. 4.2B and C), similarly observed by previous studies (Mazzolla et al., 2002; Lee et al., 2009). In the spleen, there was a significant increase in CFUs from day 1 to day 14 (9.5-folds, $p<0.01$) post infection (Fig. 4.1B), which was followed by a non-significant increase from day 14 to day 28 and finally a significant decrease from day 28 to day 42 (4.5-folds, $p<0.01$) post infection. Lee and colleagues (2009) also observed a decrease in BCG CFUs from day 21 to day 35 while Mazzolla and colleagues (2002) also observed a decrease in spleen CFUs from day 28 to day

35 that had peaked at day 21. Our findings and other studies show that spleen CFUs increase and peak between day 14 and 21 then decrease as infection progresses. The lungs also showed a significantly large kinetic increase in bacterial burdens from day 1 to day 14 (6863-folds, $p<0.05$) and a significant decrease from day 28 to day 42 (27-folds, $p<0.05$) post infection. This is in contrast to our finding of *M. tuberculosis* i.c. infection that showed exponential growth of mycobacteria as the infection progressed. We still detected BCG CFUs at day 42 and BCG that was intracranially infected has been shown not clear in the CNS during acute infection at 42 days (Matyszak and Perry, 1995). Our BCG bacterial growth findings are in agreement with previous studies suggesting that we managed to reproduce the murine CNS-TB infection model.

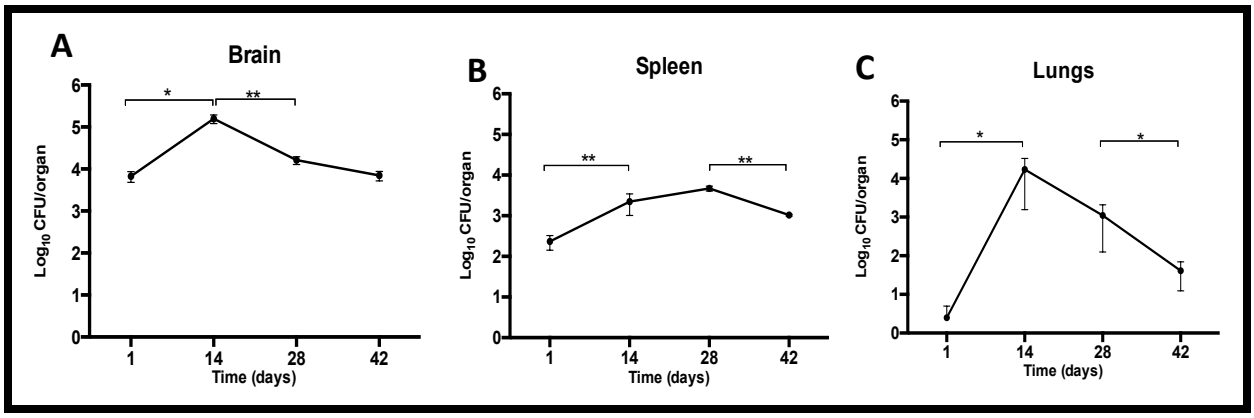


Figure 4.2: *M. bovis* BCG controlled in the brain post intracerebral infection and disseminates. Wild-type female C57BL/6J mice (4-6 mice/group) were intracerebrally infected with 1 x 10^5 CFU of *M.bovis* BCG and bacterial burdens from (A) brains, (B) Spleens, (C) lungs were determined at days 1, 14, 28 and 42 post infection. Graphs represent two independent experiments. Data is representative of the Mean +/- SEM. *p<0.05, **p<0.01,

M. tuberculosis and BCG intracerebral infection has been reported to cause CNS inflammation and can be associated with brain injury (Matyszak and Perry, 1995; van Well et al., 2007; Lee et al., 2009; Be et al., 2009). In results section 4.1, CNS mycobacterial infection was confirmed in mice brains by measured CFUs. The aim of this experiment was to investigate the inflammatory responses caused by mycobacterial infection of the CNS. We examined histopathology post *M. tuberculosis* H37Rv, BCG or saline intracerebral inoculation. This was determined by tissue histology using hematoxylin-eosin (H&E) which determines inflammation through visualization of tissue lesions and morphology (Fischer et al., 2008; Li et al., 2018) and Ziehl-Neelsen staining, a stain that detects acid fast bacilli (Koch and Cote, 1965; Greenwood and Fox, 1973). Saline inoculated mice were used as controls for inflammatory responses caused by non-pathogenic brain injury which mice experienced as a result of the surgery procedure.

4.1.3) Acid fast bacteria detected in brain post *M. tuberculosis* and BCG i.c. infections

Acid-fast bacteria were detected in the brain ventricles of both H37Rv *M. tuberculosis* or BCG infected mice as evidenced by Ziehl-Neelsen stain (Fig. 4.3.1-A and B) similar to previous studies (Mazzolla et al., 2002; Lee et al., 2009; Francisco et al., 2015). Through visualization of ZN staining under the microscope, *M. tuberculosis* i.c. infected mice presented with higher number of acid-fast bacteria compared to BCG infected mice, suggesting virulence was a factor in the amount of bacteria present.

4.1.4) Inflammatory responses detected in the brain ventricles and meninges post *M. tuberculosis* and BCG i.c. infections

H&E staining showed higher inflammatory responses in the brain tissue of *M. tuberculosis* H37Rv infected mice and BCG infected mice compared to saline control mice 6 weeks post infection (Fig. 4.3.2-A1, A2 and A3). We showed week 6 representative H & E images because acute inflammatory responses were shown to subside 3-4 weeks post BCG intracerebral infection and accompanied by the repair of the BBB (Matyszak and Perry, 1995). Brain meninges of H37Rv infected mice were inflamed showing evidence indicative of TB meningitis (Fig. 4.3.2-A3). Brain ventricles and meninges of the saline control mice showed lower infiltration of leucocytes compared to mycobacterial infection (Fig. 4.3.2-A2 and A3). Surgery procedure caused non-pathogenic inflammation as observed in saline inoculated mice

but it was mild compared to the higher degree of inflammation that was increased by pathogenic mycobacterial infection.

During CNS-TB, an exponential increase in lung CFUs was observed (Fig. 4.3.2B), which was not surprising as this is when lungs were harvested, also supported by visual inspection of lesions indicating diseased state. And a study by Be and colleagues (2008) also showed a number of lesions post 14 days of intravenous infection and as infection progressed during CNS-TB. Histology analysis revealed granulomas in the diseased *M. tuberculosis* lungs 6 weeks post intracerebral infection located in the air spaces of the lungs (Fig. 4.3.2B3) and were absent in saline inoculated and BCG infected lung tissues (Fig. 4.3.2-B1 and B2). Granulomas are hallmarks of TB disease that normally contain *M. tuberculosis,* but they also facilitate disease progression (Saunders et al., 1999; Russell, 2007; Miranda et al., 2012; Subbian et al., 2015; Ndlovu and Marakalala., 2016; Tsenova and Singhal, 2020), in our study they appear to have failed to contain mycobacteria in the lungs.

Taken together, our data for *M. tuberculosis* or BCG CNS infections suggests that we successfully reproduced murine CNS-TB infection using different strains of mycobacteria as evidenced by detection of acid-fast bacteria in infected brains, controlled brain bacterial loads accompanied by bacterial dissemination and finally, inflamed meninges indicative of TB meningitis. Our models share characteristics with previous murine models of CNS-TB infection (Mazzolla et al., 2002; van Well et al., 2007; Lee et al., 2009; Francisco et al., 2015), therefore validating our models. Finally, we also showed that there is tissue-specific mechanism of limiting bacterial growth in mice that is also influenced by virulence property of mycobacteria. Our study mice post *M. tuberculosis* and BCG intracerebral infection respectively showed 100% survival rate of the study mice throughout the course infection.

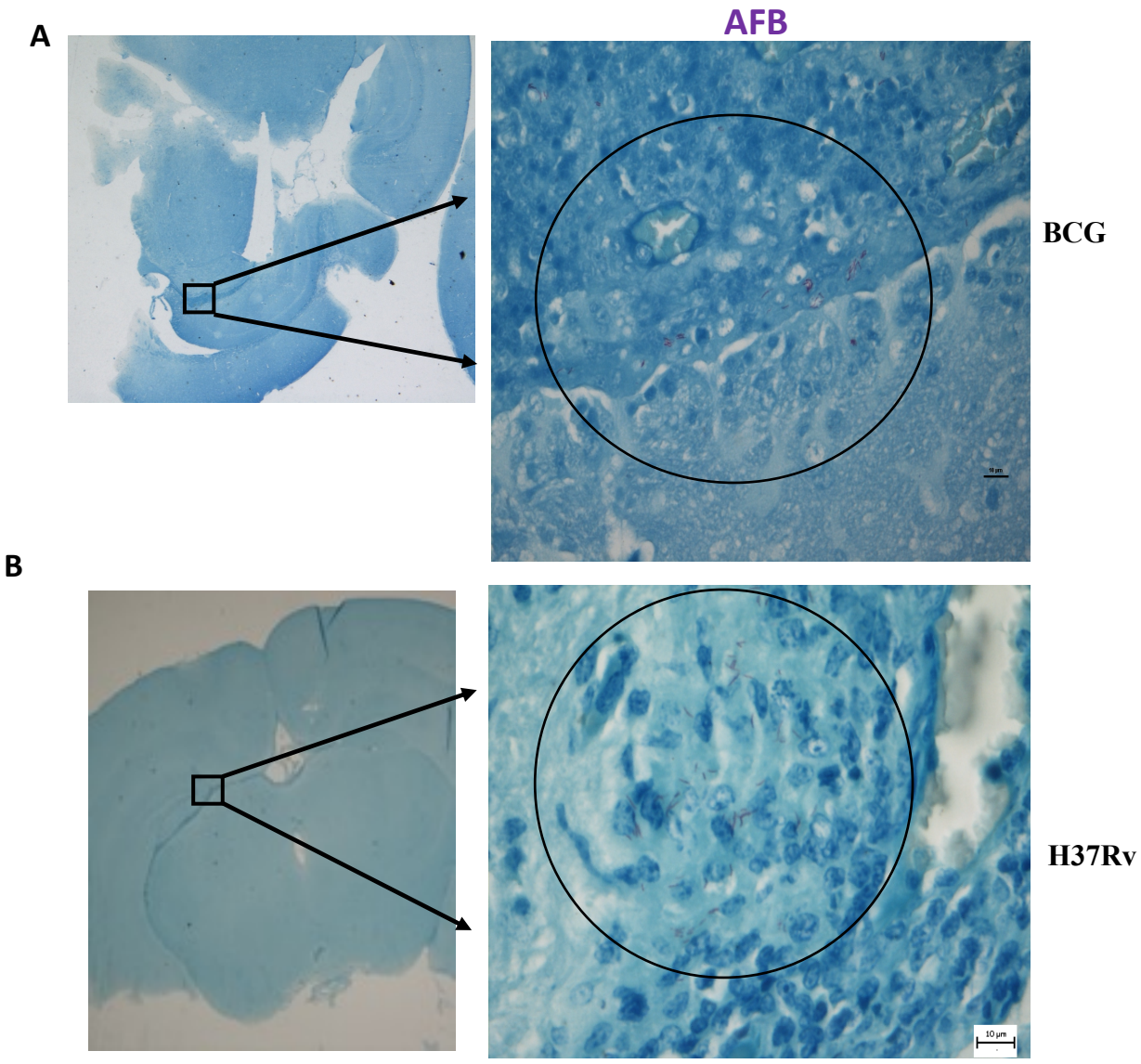


Figure 4.3.1: Detection of acid-fast mycobacteria in the brains of BCG or *M. tuberculosis* H37Rv intracerebrally infected mice. Wild-type female C57BL/6J mice (n=4-5 mice/group) were intracerebrally infected with 1 x 10^5 CFUs of BCG or *M. tuberculosis* H37Rv.Mice were euthanized at weeks 2, 4 & 6 and histological analysis conducted. Acid-fast bacteria in the brain ventricles of **(A)** BCG infected mice and **(B)** *M. tuberculosis* infected mice 6 weeks post infection. Representative microscopy images of 4 independent experiments at 40X magnification.

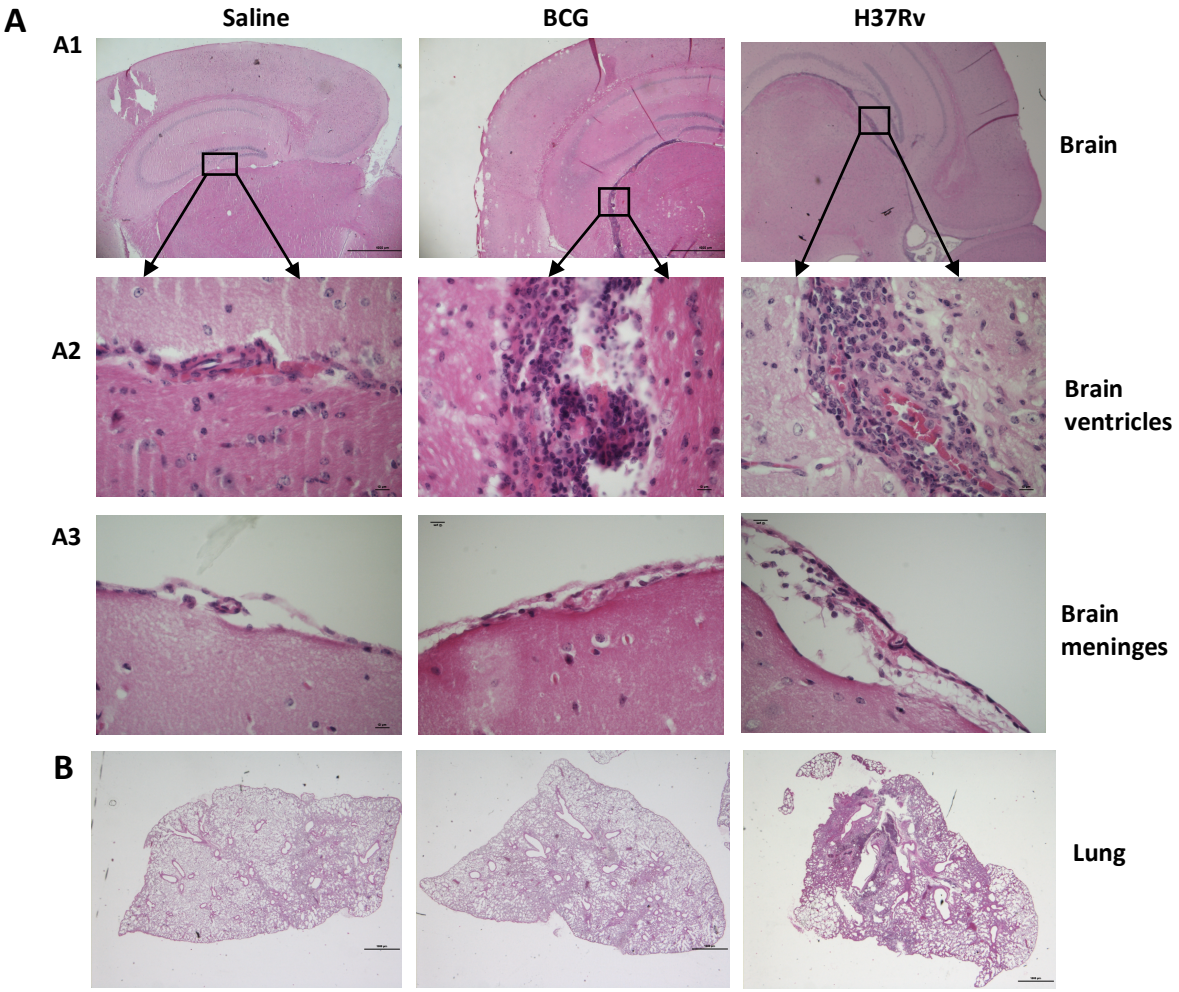


Figure 4.3.2: *M. tuberculosis* H37Rv and BCG intracerebral infection induced inflammatory responses in the brain and granulomas in the lungs. Wild-type female C57BL/6J mice (n=4-5 mice/group) were intracerebrally inoculated with saline, 1 x 10^5 CFUs of BCG or H37Rv *M. tuberculosis.* Mice were euthanized at weeks 2, 4 & 6 and histological analysis conducted. **(A)** Inflammatory responses in the (A1) brain ventricles and (A2) inflamed brain meninges, 40X magnification. **(B3)** Granulomas in lung air spaces during CNS-TB but not (B1) saline or (B2) BCG infected mice. (1X magnification) **(A-B)** microscope images taken 6 weeks post infection. Representative graphs of 3 independent experiments for BCG and 2 independent experiments for H37Rv.

Brain neuroinflammation, which are inflammatory responses in the brain that can either be beneficial or detrimental to the host (DiSabato et al., 2016) resulted from intracerebral infection with *M. tuberculosis* and BCG and caused immune responses. Following these inflammatory responses, we wanted to determine the cellular-mediated immune responses.

4.2) Induction and promotion of protective immune responses by brain APCs and CLN DCs against mycobacterial infection of the CNS.

Following our validation of the CNS-TB murine model, we demonstrated that both H37Rv *M. tuberculosis* and *M. bovis* BCG intracerebral infection causes CNS inflammation as evidenced by inflammatory responses in the brain ventricles and meninges (Section 4.2) and this resulted in induction of immune responses. Even though *M. tuberculosis* is a common pathogen infecting the CNS (Drevets et al., 2004), immune mechanisms and specific cell types involved in protective immunity against mycobacteria are not fully characterized. Both *M. tuberculosis* and BCG replication was controlled in the brain post intracerebral infection (Section 4.1) suggesting that immune responses induced in wild-type female C57BL/6J mice were protective against CNS-TB. In this regard, we examined the repertoire of cells present during the course of mycobacterial infection of the CNS that contributed to the overall protection of the CNS.

The first line of defense in the CNS is innate immunity which is facilitated by antigen presenting cells (APCs), namely; (1) microglia (resident-macrophages), (2) peripheral macrophages and (3) dendritic cells (DCs) (Natarajan et al., 2011; Barcia et al., 2013; Barichello et al., 2013). We firstly analysed the immunological roles of these three APCs in the CNS from wild-type C57BL/6J mice following intracerebral infection with either *M. tuberculosis* or BCG and characterized their phenotype and functional profiles using flow cytometry. Microglia and infiltrating macrophages have been widely studied in murine and rabbit models of mycobacterial (BCG or *M. tuberculosis)* CNS infection (Matyszak and Perry,1996; Lee et al., 2008; Lee et al., 2009; Francisco et al., 2015; Tucker et al., 2016; Hsu et al., 2017), whereas few studies have investigated DCs using CNS-TB murine models (Matyszak and Perry., 1995; Lee et al., 2008) including work from our research group (Francisco et al., 2015; Hsu et al., 2017). We therefore analysed the frequency of APCs in the CNS of wild-type C57BL/6J mice following intracerebral infection with either H37Rv *M. tuberculosis* or BCG and characterized their phenotype and functional phenotype using flow cytometry.

During CNS inflammation, there is a presence of APC subpopulations performing specific or distinct functions and it is now widely acceptable that immune APCs (macrophages and dendritic cells) infiltrate the CNS subsequent to mycobacterial infection (Matyszak and Perry, 1995; Fischer and Reichman, 2001; Lee et al., 2008; Wlodarczyk et al., 2014; Francisco et al.,

2015; Tucker et al., 2016; Hsu et al., 2017). This leads to inflammation that results in the expansion of microglia, macrophages and CD11c+ brain cells (Fischer and Reichmann et al., 2001). Our objective was to characterize the phenotype and functional profiles of microglia, brain infiltrating macrophages and dendritic cells subsequent to *M. tuberculosis* or BCG CNS infection.

4.2.1) Macrophages recruited to the brain and undergo expansion together with microglia post *M. tuberculosis* or BCG i.c. infection.

As brain-resident macrophages, microglia play a crucial and central role in regulation of neuroinflammation caused by bacterial infections (Peterson et al., 1995; Rock et al., 2005; Prinz et al., 2011; Spanos et al., 2015) and are first line responders during CNS insult and infection (Aloisi, 2001; Sevenich, 2018). Microglia share the role of regulating CNS inflammation with infiltrating macrophages microglia due to shared properties with peripheral macrophages that infiltrate the CNS (Rock et al., 2004; Sevenich, 2018). Human microglia have been shown to more efficiently phagocytose *M. tuberculosis* than avirulent strains of mycobacteria such as *M. bovis* BCG, however; *M. tuberculosis* has been shown to replicate within macrophages after being ingested (Peterson et al., 1996; Curto et al., 2004; Rock et al., 2008). The first aim was to determine the kinetics of microglia and brain infiltrating macrophages at different time intervals for a period of 6 weeks post H37Rv *M. tuberculosis* or BCG intracerebral infection using flow cytometric analysis.

Representative flow cytometry plots showing gating strategy of microglia (CD11b+CD45int) and brain infiltrating macrophages (CD11b+CD45high) of all 4 experimental groups (naïve, saline inoculated, BCG and *M. tuberculosis* H37Rv infected mice) (Fig. 4.4-A1 and A2). Data obtained showed that *M. tuberculosis* H37Rv or BCG i.c. infection resulted in the infiltration of peripheral macrophages to the brain and expansion of both these infiltrating macrophages and microglia (Fig. 4.4). We found no significant difference between the number of microglia in naïve (uninfected) mice compared to week 2 saline mock control (saline inoculated) mice (Fig. 4.4B). The number of microglia from saline control mice significantly increased ($p<0.05$) from week 2 to week 4 then significantly decreased ($p<0.01$) from week 4 to week 6 (Fig. 4.4B) likely due to inflammatory responses caused by the surgery procedure in the brain (site of infection). When we assessed microglia as a result of BCG i.c. infection, we found that the number of microglia from BCG infected mice were only significantly higher than microglia

from saline control mice at week 4 ($p<0.05$) and week 6 ($p<0.01$) but not at week 2 post intracerebral infection (Fig. 4B). Kinetics of microglia from BCG infected mice showed significant increases from week 2 to week 4 ($p<0.01$) and from week 2 to week 6 ($p<0.01$). During CNS-TB, the number of microglia from *M. tuberculosis* infected mice were significantly higher ($p<0,01$) than those of saline control mice at all three time points (weeks 2, 4 & 6) (Fig. 4.4B), however there was no kinetic change in the levels of microglia from *M. tuberculosis* infected mice throughout the course of infection. The number of microglia post intracerebral infection with *M. tuberculosis* remained high. To determine whether virulence was a factor with microglial cell responses during mycobacterial infection of the CNS, we compared the amount of microglia from mice i.c. infected with virulent H37Rv *M. tuberculosis* infected mice with microglia from mice that were i.c. infected with avirulent *M. bovis* BCG. Even though mice were infected with the same dose of H37Rv *M. tuberculosis* or BCG, we found that the number of microglial cells from *M. tuberculosis* infected mice were significantly higher ($p<0.01$) than those from BCG infected mice throughout the course of infection (Fig. 4.4B). A phenomenon similarly observed in human microglial cells that were shown to efficiently phagocytose *M. tuberculosis* than avirulent mycobacteria (Curto et al., 2004), suggesting microglia infection by mycobacteria is virulence dependent. Taken together, these findings suggest that microglia expansion occurs post intracerebral infection with BCG or *M. tuberculosis.*

Macrophages from systemic circulation were the major cell population that infiltrate the CNS and like microglia, they are also innate responders to brain injury and infection (Kim et al., 2016). We observed a recruitment of peripheral macrophages to the brain (infiltrating macrophages) during mycobacterial infection of the CNS (Fig. 4.4A, C). Like microglia, there were no significant difference between the number of infiltrating macrophages in naïve mice compared to week 2 saline control mice (Fig. 4.4C). The small number of infiltrating macrophages from saline control mice significantly decreased ($p<0.01$) from week 2 to 4 and also from week 2 to 6 post infection (Fig. 4.4C). We then assessed infiltrating macrophages as a result of BCG i.c. infection and found that the number of infiltrating macrophages from BCG infected mice was significantly higher than saline control mice during the entire course of infection ($p<0.01$ at weeks 2, 4 and 6) (Fig. 4.4C). Kinetics showed a significant increase ($p<0.01$) in the number of infiltrating macrophages from BCG i.c. infected mice from week 2 to week 4, however no change in the number of infiltrating macrophages was observed from week 4 to week 6 post infection (Fig. 4.4C).

During CNS-TB, the number of infiltrating macrophages from *M. tuberculosis* infected mice were significantly higher than those from saline control mice at week 2 ($p<0.01$), week 4 ($p<0.05$) and week 6 ($p<0.01$) post i.c. infection (Fig. 4.4C). We did not find any change in kinetics of *M. tuberculosis* infected mice during CNS-TB. Like microglia, we checked if virulence was a factor for infiltrating macrophage responses during mycobacterial infection of the CNS by comparing the number of infiltrating macrophages from mice i.c. infected with *M. tuberculosis* with mice i.c. infected with BCG. We found significantly increased numbers of infiltrating macrophages of *M. tuberculosis* i.c. infected mice compared to BCG mice at week 2 ($p<0.01$), week 4 ($p<0.05$) and week 6 ($p<0.01$) (Fig. 4.4B).

Macrophages are recruited to the brain post *M. tuberculosis* or BCG intracerebral infection and accumulate in the brain following expansion. Brain infiltrating macrophages appeared to be specific to mycobacterial infection of the CNS because there were minimal to negligible levels of macrophages found in saline control mice as compared to significantly increased numbers in both H37Rv *M. tuberculosis* or BCG infected mice. Microglial cells were the larger cell population in the brain compared to infiltrating macrophages.

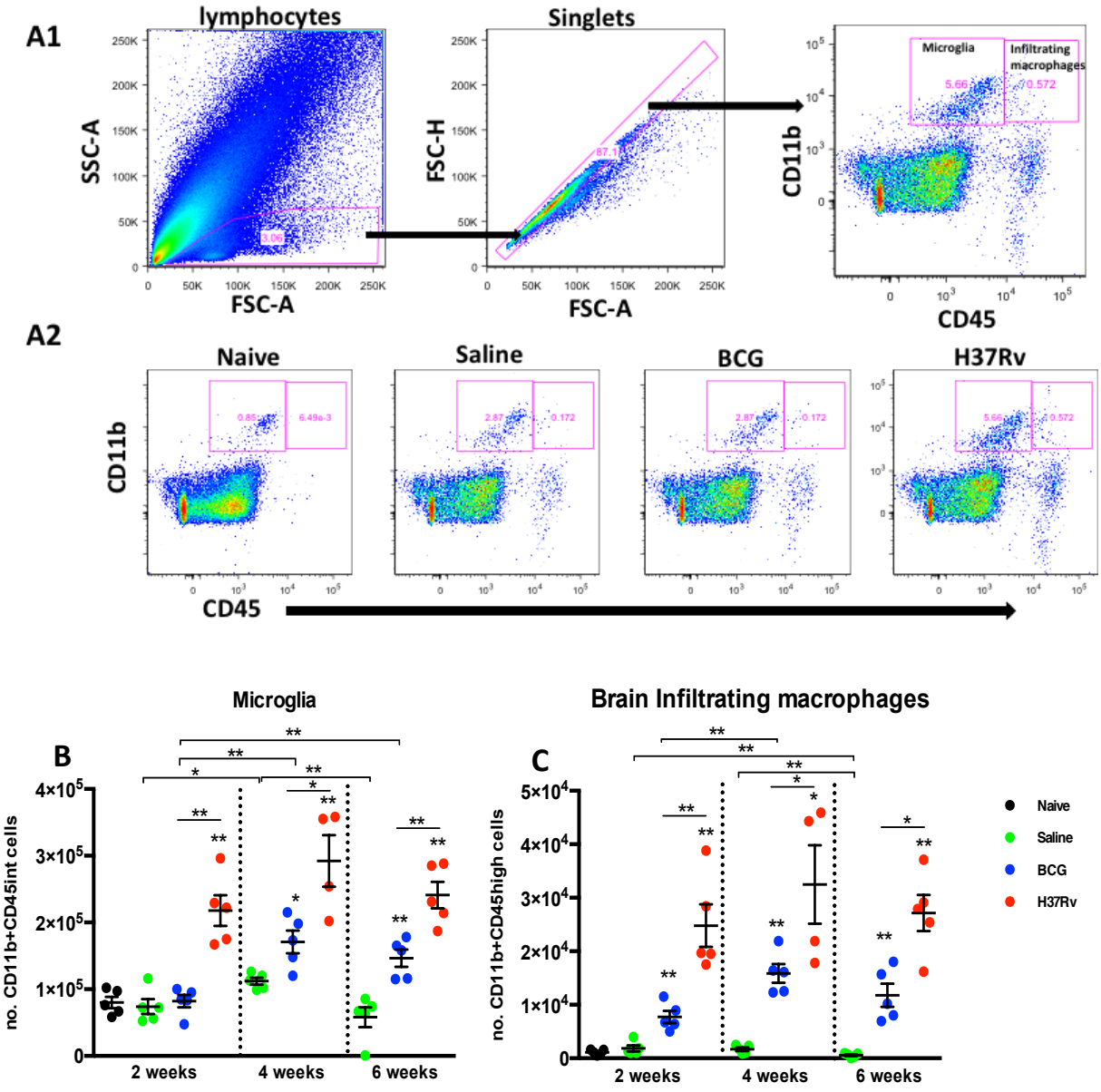


Figure 4.4: Recruitment of macrophages to the brain post BCG and *M. tuberculosis* H37Rv intracerebral infection and expansion of both infiltrating macrophages and microglia. Wild-type female C57BL/6J mice (n=4-5 mice/group) were intracerebrally inoculated with saline, 1 x 10^5 CFUs of BCG or H37Rv *M. tuberculosis* and not inoculated (naïve mice). Mice were euthanized at weeks 2, 4 & 6 and immune cells analysed by flow cytometry. **(A1)** Flow cytometry plots showing gating of microglia (CD11b+CD45int) and infiltrating macrophages (CD11b+CD45high) from (A2) naïve mice, saline mice, BCG and *M. tuberculosis* infected mice. Expansion of (B) microglia and **(C)** infiltrating macrophages in *M. tuberculosis* and BCG infected mice and controls. Graphs representative of 3 independent experiments for BCG and 2 independent experiments for H37Rv. Data represents the Mean +/- SEM. *p<0.05, **p<0.01 vs saline controls. Mann-Whitney test.

4.2.2) Microglia and infiltrating macrophage display activated and mature phenotypes post *M. tuberculosis* or BCG i.c. infection.

We demonstrate that microglia and infiltrating macrophages are increased in the brain post *M. tuberculosis* or BCG intracerebral infection in C57BL/6J mice (Fig. 4.4). We then asked whether these cells expressed maturation markers required for mediating immunity. Maturation markers commonly expressed by microglia and brain infiltrating macrophages are major histocompatibility complex (MHCII) and co-stimulation molecule CD86 (Matyszak and Perry, 1995; Lee et al., 2008; Hsieh et al., 2013). Both these surface markers are needed for induction of adaptive T cell immune responses. MHCII is for antigen presentation to T cells (Rock et al., 2004) and CD86 is a costimulatory molecule that binds T cell receptors to promote T cell expansion and differentiation (Lavacentia, 1997; Gimmi et al., 1991), which in combination induce T cell immune responses.

Figure 4.5.1A shows representative flow cytometry plots gated on MHCII+ and CD86+ microglia during mycobacterial infection of the CNS. We observed no differences in the number of microglia expressing MHCII (MHCII+microglia) between naïve mice and week 2 saline inoculated mice (Fig. 4.5.1B). Flow cytometry gating strategy showing gating of MHCII and CD86 on microglia provided in Appendix E. Similar to previous studies where a small subset of microglia were shown to express low levels of MHCII in naïve C56BL/6 mice while more than 97% do not express MHCII (Mrdjen et al., 2018; Mundt et al., 2019). Kinetics of the very low numbers of MHCII+microglia from saline control mice demonstrated significant increase from week 2 to week 4 ($p<0.05$), that was followed by a significant decrease at week 6 ($p<0.05$) post inoculation (Fig. 4.5.1B), a trend similar to Zucchi and colleagues (2012). The numbers of MHCII+microglia from naïve mice and saline control mice were extremely low compared to mice intracerebrally infected with either *M. tuberculosis* or BCG. We found increased numbers of MHCII+microglia from BCG infected mice that were significantly higher at week 2 ($p<0.01$), week 4 ($p<0.01$) and week 6 ($p<0.01$) post i.c. infection compared to saline control mice (Fig. 4.5.1B). These numbers of MHCII+ microglia from BCG infected mice were significantly increased from week 2 to week 4 ($p<0.01$) and again from week 2 to week 6 ($p<0.01$) post infection. During CNS-TB, we found increased numbers of MHCII+microglia from *M. tuberculosis,* H37Rv infected mice to be significantly higher than saline control mice at week 2 ($p<0.01$), week 4 ($p<0.05$) and week 6 ($p<0.05$) (Fig. 4.5.1B). We observed no changes in the kinetics of MHCII+microglia during CNSTB. The number of MHCII+microglia from *M. tuberculosis* infected mice were significantly higher than BCG

infected mice at week 2 ($p<0.01$), week 4 ($p<0.05$) and week 6 ($p<0.05$) post intracerebral infection (Fig. 4.5.1B).

When we looked at the expression of co-stimulatory molecule CD86 by microglia, we found a significant higher ($p<0.01$) number of microglia expressing CD86 (CD86+microglia) between naïve mice and week 2 saline inoculated mice (Fig. 4.5.1C). However, we found no differences in numbers of CD86+microglia between mice infected with either *M. tuberculosis* or BCG at week 2 and week 4 post infection and saline inoculated mice (Fig. 4.5.1C). We also did not observe changes in kinetics of CD86+microglia from saline inoculated mice, *M. tuberculosis* infected mice of BCG infected mice at all experimental time points. We found significantly higher ($p<0.01$) numbers of CD86+microglia from H37Rv *M. tuberculosis* or BCG infected mice compared to saline control mice (Fig. 4.5.1C). Finally, virulence was not a factor between the two strains of mycobacteria in terms of CD86+microglia.

Taken together, our data shows that microglia are highly activated (MHCII+) and only express very little CD86 later during mycobacterial infection of the CNS.

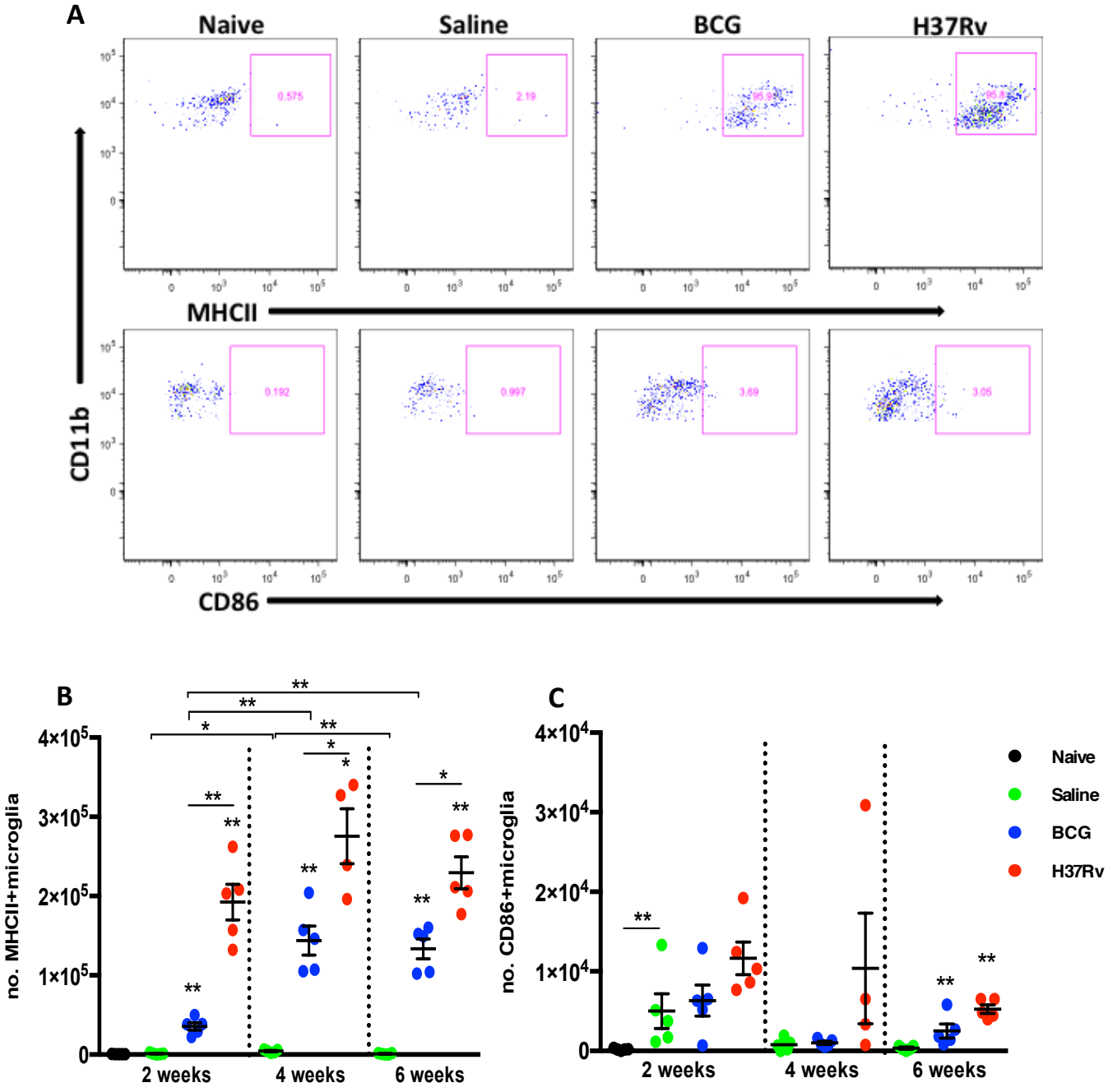


Figure 4.5.1: Activation and maturation of microglia post BCG and *M. tuberculosis* H37Rv intracerebral infection.

Wild-type female C57BL/6J mice (n=4-5 mice/group)were intracerebrally inoculated with saline, 1 x 10^5 CFUs of either BCG or H37Rv *M. tuberculosis* and not inoculated (naïve mice). Mice were euthanized at weeks 2, 4 & 6 and immune cells analysed by flow cytometry. Flow cytometry plots showing **(A)** MHCII+microglia and CD86+microglia of the four experimental groups. Number of (B) activated (MHCII+) microglial cells and (C) CD86+microglial cells post BCG or *M. tuberculosis* CNS infection. Graphs are representative of 3 independent experiments for BCG and 2 independent experiments for H37Rv. Data represents the Mean +/- SEM. *p<0.05, **p<0.01 vs saline controls. Mann-Whitney test.

Figure 4.5.2A shows representative flow cytometry plots gated on MHCII+ and CD86+ brain infiltrating macrophages during mycobacterial infection of the CNS (Fig. 4.5.2A). Flow cytometry gating strategy showing gating of MHCII and CD86 on infiltrating macrophages provided in Appendix E. We found no difference in the number of brain infiltrating macrophages expressing MHCII (MHCII+macrophages) between naïve mice and week 2 saline inoculated mice (Fig. 4.5.2B). MHCII+macrophages from saline control mice were negligible and showed no kinetic change at all time points post inoculation (Fig. 4.5.2B). The number of MHCII+macrophages from BCG i.c. infected mice were significantly increased ($p<0.01$) at week 2, week 4 and week 6 post infection compared to saline control mice (Fig. 4.5.2B), a trend similar to microglia. The number of MHCII+ macrophages from BCG infected mice significantly increased from week 2 to week 4 ($p<0.01$) and was followed by a significant decrease from week 4 to week 6 ($p<0.05$) post infection. Whereas, during CNS-TB we have the number of MHCII+macrophages from *M. tuberculosis* infected mice were significantly increased at week 2 ($p<0.01$), week 4 ($p<0.05$) and week 6 ($p<0.01$) post infection compared to saline control mice (Fig. 4.5.2B). However, there was no differences in kinetics of MHCII+macrophages during CNS-TB. The number of MHCII+ macrophages from *M. tuberculosis* infected mice were significantly higher than BCG infected mice at week 2 ($p<0.01$), week 4 ($p<0.05$) and week 6 ($p<0.05$) post intracerebral infection (Fig. 4.5.2B), indicating that virulence was a factor in inducing immune responses.

We found no difference in the number of brain infiltrating macrophages expressing CD86 (CD86+macrophages) between naïve mice compared to week 2 saline inoculated mice (Fig. 4.5.2C). The number of CD86+macrophages were significantly higher in mice that were intracerebrally infected with mycobacteria compared to saline control mice (Fig. 4.5.2C). The number of CD86+macrophages was significantly increased in BCG i.c. infected mice at week 2 ($p<0.05$), week 4 ($p<0.01$) and week 6 ($p<0.01$) post infection compared to saline control mice (Fig. 5.2C). Both saline inoculated mice and BCG CNS infected mice showed no kinetic changes during the entire experimental time points (Fig. 4.5.2C). Whereas, during CNS-TB, we found the number of CD86+macrophages from *M. tuberculosis* infected mice to be significantly increased at week 2 ($p<0.01$), week 4 ($p<0.05$) and week 6 ($p<0.01$) compared to saline control mice (Fig. 4.5.2C). We observed a significant increase ($p<0.05$) in CD86+macrophages from week 2 to week 6 during CNS-TB (Fig. 4.5.2C). Virulence was a factor for CD86 responses because we detected higher numbers of CD86+macrophages from

M. tuberculosis i.c. infected mice compared to BCG infected mice at week 2 ($p<0.01$) and week 4 ($p<0.05$) post infection (Fig. 4.5.2C).

Both microglia and brain infiltrating macrophages expressed high levels of MHCII during mycobacterial infection of the CNS implying that they were activated and capable of presenting BCG and *M. tuberculosis* antigens to T cells to induce adaptive immune responses, however mycobacterial infection resulted in low numbers of both cell types expressing CD86 (<15%), suggesting induction of non-robust T cell responses.

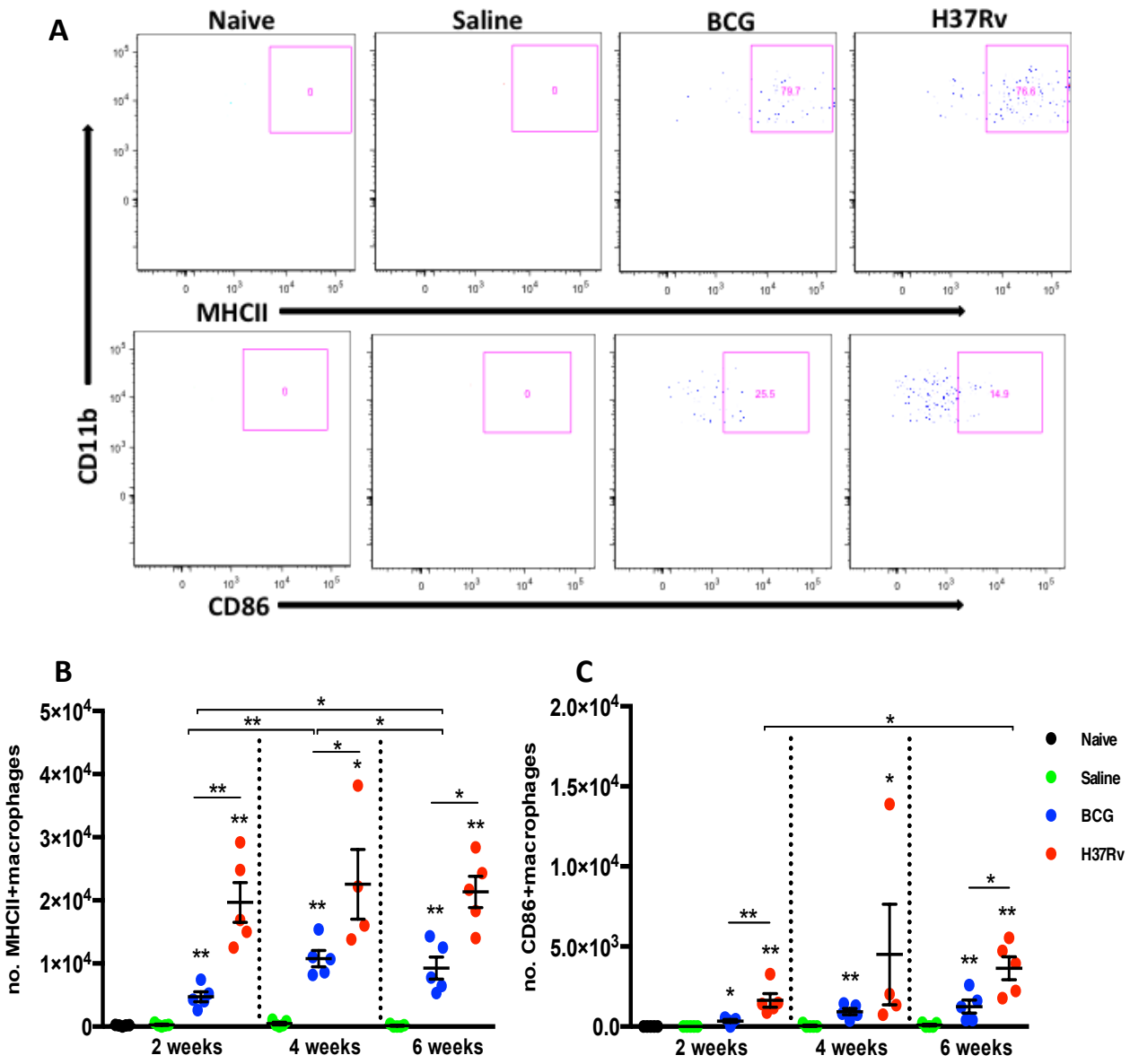


Figure 4.5.2: Activation and maturation of brain infiltrating macrophages post BCG and *M. tuberculosis* H37Rv intracerebral infection. Wild-type female C57BL/6J mice (n=4-5 mice/group)were intracerebrally inoculated with saline, 1 x 10^5 CFUs of either BCG or H37Rv *M. tuberculosis* and not inoculated (naïve mice). Mice were euthanized at weeks 2, 4 & 6 and immune cells analyzed by flow cytometry. Flow cytometry plots of **(A)** MHCII+macrophages and CD86+macrophages of the four experimental groups. The number of (B) MHCII+macrophages and **(C)** CD86+macrophages post BCG or *M. tuberculosis* CNS infection. Graphs are representative of 3 independent experiments for BCG and 2 independent experiments for H37Rv. Data represents the Mean +/- SEM. *p<0.05, **p<0.01 vs saline controls. Mann-Whitney test.

4.2.3) Increased numbers of microglia express IL-12 and IFNγ post *M. tuberculosis* of BCG i.c. infection.

We showed that microglia and brain infiltrating macrophages are activated and display a mature phenotype during *M. tuberculosis* H37Rv or BCG intracerebral infection. During tuberculosis infection, cytokines are able to control infection and prevent pathology damage caused by response to inflammation (Flynn and Chan, 2001; Flynn and Chan, 2003; Cooper, 2009). We performed intracellular cytokine staining of microglia and brain infiltrating macrophages using flow cytometry to further characterize their functional profiles. The secretion of cytokines and chemokines is how microglia regulate immune responses against mycobacteria (Curto et al., 2004; Rock et al., 2004 and 2005) and are secreted by microglia after *M. tuberculosis* infection (Peterson et al., 1995; Rock et al., 2005). Representative flow cytometry plots showing gating strategy of a number of microglia from naïve mice, saline inoculated mice, *M. tuberculosis* and BCG intracerebrally infected mice expressing pro-inflammatory cytokines, IL-12 and IFNγ (Fig. 4.6A). Flow cytometry gating strategy of cytokines gated on microglia provided in Appendix F.

Activated microglia secrete proinflammatory cytokines, that include IL-12 (Becher et al., 1996; Park & Shin, 1996; Aloisi et al., 1997; Stalder et al., 1997) and production of IL-12 by microglia during CNS-TB has not been determined. Our study measured the number of microglia expressing IL-12 (IL-12+microglia) and results obtained showed that at 2 weeks post i.c. procedure, saline treated control mice had increased number of microglia expressing IL-12, equivalent to BCG-infected mice when compared to naïve controls (Fig. 4.6B). However, only the number of IL-12+microglia from week 2 saline inoculated mice were significantly higher ($p<0.01$) than naïve mice (Fig. 4.6B). Week 6 saline inoculated mice presented with significantly lower ($p<0.01$) numbers of IL-12+microglia compared to week 2 and week 4 saline inoculated mice. The number of IL-12+microglia was significantly increased at week 4 ($p<0.01$) and week 6 ($p<0.05$) in BCG intracerebrally infected mice as compared to saline treated mice (Fig. 4.6B). Kinetics revealed a significant increase in the number of IL-12+microglia from BCG infected mice from week 2 to week 4 ($p<0.01$), that was followed by a significantly drastic decrease at week 6 ($p<0.01$) post infection, with week 6 numbers being lower than week 2 (Fig. 4.6B). Whereas, *M. tuberculosis* mice presented with significantly increased numbers of IL-12+microglia at week 2 ($p<0.01$), week 4 ($p<0.05$) and week 6 ($p<0.01$) during CNS-TB compared to saline control mice (Fig. 4.6B). And finally, the number

of IL-12+ microglia from *M. tuberculosis* infected mice showed significant decreases from week 4 to week 6 ($p<0.05$) and from week 2 to week 6 ($p<0.01$) post infection. Looking at virulence as a factor, the number of IL-12+microglia from BCG infected mice were significantly lower relative to week 2 *M. tuberculosis* infected mice which had double the number of IL-12+microglia (Fig. 4.6B). It has been shown that some microglia favour Th1 T cell immune responses via IL-12 production in the CNS (Suzumura et al., 1998), which appears to be what is happening during H37Rv *M. tuberculosis* and BCG CNS infection.

Microglia produce IFNγ during CNS-TB (Rock et al., 2005). When we measured the number of microglia expressing IFNγ; At week 2 post i.c. procedure, there was no difference in the number of microglia expressing IFNγ (IFNγ+microglia) among the three treatment groups, namely; naïve mice, saline mice and BCG infected mice (Fig. 4.6C). The kinetics of IFNγ+microglia from saline control mice showed two significant increases, the first from week 2 to week 4 ($p<0.01$) and another from week 2 to week 6 ($p<0.01$) post inoculation (Fig. 4.6C). The number of IFNγ+microglia from BCG infected mice was significantly higher ($p<0.05$) than saline treated mice only at week 6 post i.c. procedure (Fig. 4.6C). There were no differences in the number of IFNγ+microglia between BCG and saline control mice at weeks 2 and 4 post infection Whereas, the number of IFNγ+microglia from H37Rv *M. tuberculosis* infected mice were significantly increased ($p<0.01$) compared to saline treated mice at week 2 post i.c. procedure (Fig. 4.6C) unlike BCG infected mice. During CNS-TB, the number of IFNγ+microglia were significantly higher at week 2 ($p<0.01$), week 4 ($p<0.05$) and week 6 ($p<0.01$) post infection compared to saline inoculated mice (Fig. 4.6C). No kinetic changes were observed in the number of IFNγ+microglia post H37Rv *M. tuberculosis* or BCG intracerebral infection and neither were there any differences in the number of IFNγ+microglia between H37Rv *M. tuberculosis* and BCG infected mice during the entire course of infection (Fig. 4.6C).

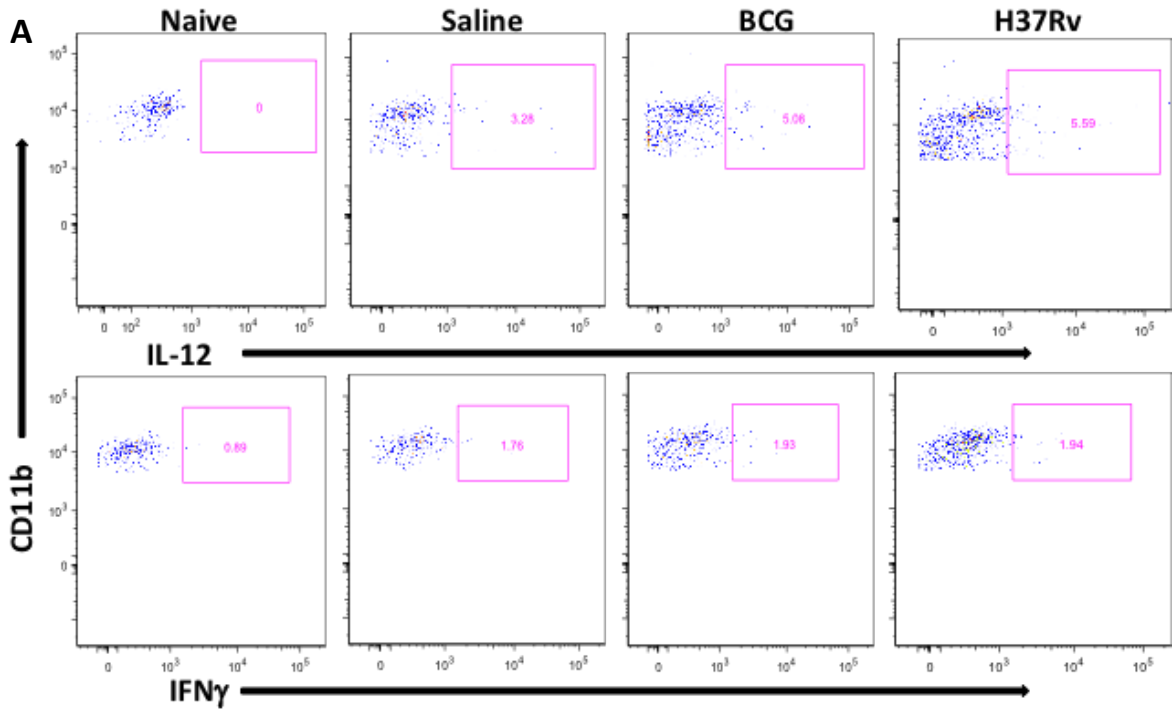
Microglia have been shown to be major producers of TNFα post BCG intracerebral (i.c.) infection (Lee et al., 2009) but not post *M. tuberculosis* intracerebral infection. We found that at week 2 post i.c. procedure, saline treated mice did not show different levels in the number of microglia expressing TNFα (TNFα+microglia) when compared to naïve mice (Fig. 4.6D).We also did not detect any differences in the number of TNFα+microglia post BCG or H37Rv *M. tuberculosis* infection during the entire course of infection when compared to saline

treated mice (Fig. 4.6D). However, we observed kinetic changes in the number of TNFα+microglia post i.c procedure for all treatment groups (saline treated, BCG and H37Rv *M. tuberculosis* infected groups) (Fig. 4.6D). The number of TNFα+microglia from saline control mice significantly decreased ($p<0.05$) at two time points, firstly from week 2 to week 6 and secondly from week 4 to week 6 post inoculation. While both *M. tuberculosis* and BCG infected mice showed a significant decrease in the number of TNFα+microglia from week 4 to week 6 ($p<0.05$). Virulence was not a factor for TNFα+microglia during mycobacterial infection of the CNS. It appears as though the number of microglia expressing TNFα during mycobacterial infection of the CNS may have not had any significant contribution to disease outcome.

Proinflammatory cytokine, IL-6 has been implicated in protection against pulmonary TB and microglia secrete IL-6 in response to *M. tuberculosis* infection (Yang et al., 2007). The number of microglia expressing IL-6 (IL-6+microglia) were significantly high ($p<0.01$) in week 2 saline treated mice compared to naïve mice (Fig. 4.6E). However, there were no differences observed in the number of IL-6+microglia between mycobacterial infected mice and saline control mice at weeks 2 and 4 (Fig. 4.6E). It was only at week 6 post *M. tuberculosis* and BCG infection, respectively that we observed significantly increased ($p<0.05$) numbers of IL-6+microglia compared to saline treated mice (Fig. 4.6E). And when we looked at kinetics of IL-6+microglia during the course of the i.c procedure, the number of IL-6+microglia showed no kinetic changes for saline treated mice and during CNS-TB but there was a significant increase ($p<0.05$) in BCG infected mice from week 2 to week 6 post infection (Fig. 4.6E). The expression of IL-6 by a number of microglial cells suggests that microglia may contribute to innate immunity later during mycobacterial infection of the CNS.

Production of IL-1 and IL-10 by microglia have been shown to be inhibited by *M. tuberculosis* after infection (Curto et al., 2004). IL-1β is a proinflammatory cytokine that plays an important role in protection against pulmonary TB (Mayer-Barber et al., 2011) however microglia have not been identified as producers of IL-1β during CNS-TB. We did not find any differences in the number of the microglia expressing IL-1β (IL-1β+microglia) among all the different experimental groups (naïve, saline, BCG and *M. tuberculosis*) at all the different time points of the experiment (Fig. 4.6F). Neither did we find any differences in the number of IL-1β+microglia as a result of virulence (Fig. 4.6F). Additionally, there were no kinetic changes

in the levels of IL-1β+microglia for saline, BCG or *M. tuberculosis* infected mice. It appeared as though the number of microglia expressing IL-1β did not play a role during mycobacterial infection of the CNS. There were no differences in the number of microglia expressing IL-10 (IL-10+microglia) among all the experimental groups (naïve, saline, BCG and H37Rv) post i.c. procedure at all experimental time points (Fig. 4.6G). No kinetic changes were observed in saline inoculated mice. BCG infected mice showed a significant decrease ($p<0.05$) in the number of IL-10+microglia from week 2 to week 4, while *M. tuberculosis* infected mice showed a significant increase ($p<0.01$) from week 2 to week 6 during CNS-TB (Fig. 4.6F-G). This suggests that the IL-10 produced by microglia does not have an effect on CNS infection caused by BCG or H37Rv *M. tuberculosis* infection.



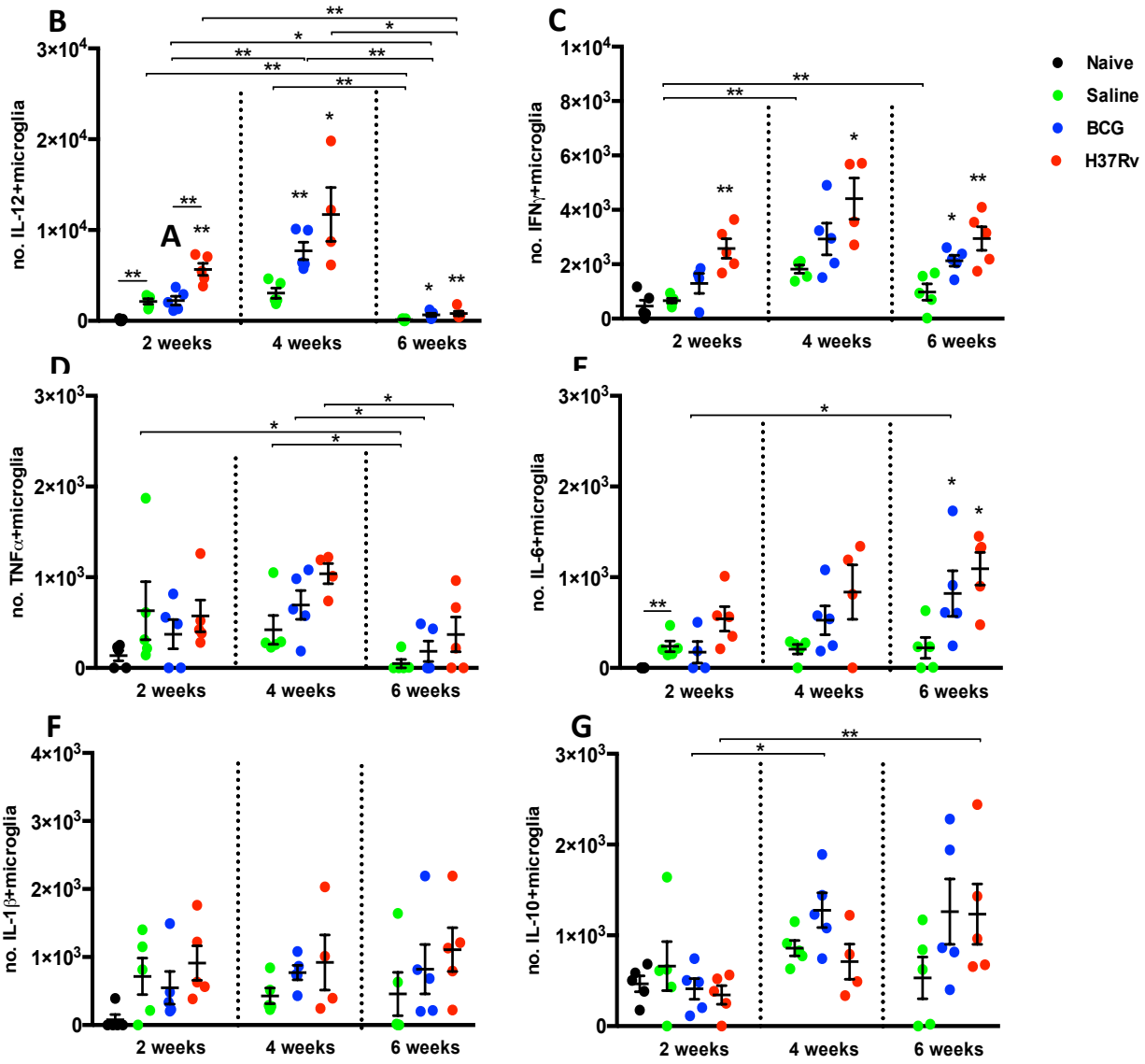


Figure 4.6: Increased numbers of microglia expressed IL-12 and IFNγ post *M. tuberculosis* H37Rv or BCG intracerebral infection that favour antimycobacterial immune responses. Wild-type female C57BL/6J mice (n=4-5 mice/group) were intracerebrally inoculated with saline, 1 x 10^5 CFUs of BCG or H37Rv M. tuberculosis and not inoculated (naïve mice). Mice were euthanized at weeks 2, 4 & 6 and immune cells analysed by flow cytometry. **(A)** Flow cytometry plots showing IL-12+microglia and IFNγ+microglia of naive, saline, BCG and *M. tuberculosis* inoculated mice. Increased numbers of microglia from BCG and H37Rv infected mice, respectively expressed pro-inflammatory cytokines **(B)** IL-12, **(C)** IFNγ and **(D)** (IL-6) during mycobacterial infection of the CNS. No differences in the number of microglia expressing anti-inflammatory cytokines **(D)** TNFα, **(F)** IL-1β and anti-inflammatory **(G)** IL-10. Graphs are representative of 3 independent experiments for BCG and 2 independent experiments for H37Rv. Data represents the mean +/- SEM. *p<0.05, **p<0.01 vs saline controls. Mann-Whitney test.

4.2.4) Increased numbers of brain infiltrating macrophages express pro-inflammatory and anti-inflammatory cytokines post *M. tuberculosis* or BCG i.c. infection.

As we have shown that macrophages from the periphery infiltrate the brain during *M. tuberculosis* or BCG intracerebral infection, we then asked whether these cells also express functional cytokines and followed the same approach as we did for microglia by staining for intracellular cytokines and flow cytometric analysis. Representative flow cytometry plots showing gating strategy of brain infiltrating macrophages from naïve mice, saline inoculated mice, *M. tuberculosis* and BCG intracerebrally infected mice expressing pro-inflammatory cytokines (IFNγ, TNFα, IL-1β, IL-6 and IL-12) and anti-inflammatory cytokine (IL-10) (Fig. 4.7A). Flow cytometry gating strategy of cytokines gated on brain infiltrating macrophages provided in Appendix F.

IL-12 has been shown to be induced after phagocytosis of *M. tuberculosis* by macrophages and DCs (Ladel et al., 1997; Henderson et al., 1997). IL-12 plays a crucial role for protection against pulmonary TB (Cooper et al., 1995; Cooper et al., 1997). However, its role in CNS-TB remains unknown. Lack of IL-12 has been associated with lack of control of bacterial growth in liver, spleen and lung tuberculosis (Cooper et al., 2007). We found no differences in the number of brain infiltrating macrophages expressing IL-12 (IL-12+macrophages) between week 2 saline inoculated mice and naïve mice (Fig. 4.7B). Although the number of IL-12+macrophages from saline inoculated mice appeared to be lower at week 4 and week 6 compared to week 2, there were no significant kinetic changes. The number of week 2 saline inoculated mice were equivalent to week 2 BCG i.c. infected mice with no significant difference between the two groups and no differences were observed between the two groups also at week 4 and week 6 (Fig. 4.7B). Additionally, the number of IL-12+macrophages from BCG infected mice remained unchanged throughout the course of infection. Even though the number of IL-12+macrophages from week 2 *M. tuberculosis* infected mice appeared to be double compared to BCG infected mice, they were not significantly different from week 2 saline inoculated mice (Fig. 4.7B). However, the number of IL-12+macrophages from *M. tuberculosis* infected mice were significantly higher at week 4 ($p<0.05$) and week 6 ($p<0.01$) during CNS-TB compared to saline inoculated mice (Fig. 4.7B) and there no kinetic changes observed during CNS-TB. Virulence was not a factor with regards to the number of IL-12+macrophages between *M. tuberculosis* and BCG infected mice during the entire course of intracerebral infection.

IFNγ and TNFα production are crucial for controlling *M. tuberculosis* infection and recruiting effector cells to the site of infection (Co et al., 2004; Allie et al., 2013; Segueni et al., 2016). Lee and colleagues showed that macrophages in the brain produce TNFα post BCG intracerebral infection, however during CNS-TB it has not been shown. We measured the the number of macrophages expressing these two pro-inflammatory Th1 cytokines post BCG and M. tuberculosis intracerebral infection, respectively. The number of brain infiltrating macrophages expressing IFNγ (IFNγ+macrophages) were barely detectable in saline inoculated mice and naïve mice at all experimental time points with no differences observed between week 2 saline inoculated mice (Fig. 4.7B). Neither were there any kinetic changes in saline control mice post inoculation at all experimental time points. The number of IFNγ+macrophages from BCG infected mice were only significantly higher ($p<0.01$) than saline control mice at week 4 post but not at weeks 2 and 6 post infection (Fig. 4.7C). Although BCG infected mice presented with an increasing trend in the number of IFNγ+macrophages from week 2 to week 4 ($p<0.05$) that was followed by a non-significant decrease at week 6 post infection. Whereas, *M. tuberculosis* infected mice presented with a number of IFNγ+macrophages that were significantly higher at week 2 ($p<0.01$), week 4 ($p<0.01$) and week 6 ($p<0.01$) during CNS-TB compared to saline control mice (Fig. 4.7C). There were no kinetic changes in the number of IFNγ+macrophages during CNS-TB. *M. tuberculosis* infected mice contained significantly higher numbers of IFNγ+macrophages than BCG infected mice at week 2 ($p<0.05$), week 4 ($p<0.05$) and week 6 ($p<0.01$) post intracerebral infection, indicating that virulence played a role in the magnitude of immune responses. Brain infiltrating macrophages presented with undetectable numbers of TNFα (TNFα+macrophages) at weeks 4 and week 6 post inoculation, even though the low numbers of TNFα+macrophages week 2 saline inoculated were significantly higher ($p<0.05$) than naïve mice (Fig. 4.7D). In contrast, we detected high numbers of TNFα+macrophages during mycobacterial infection of the CNS. We found the number of TNFα+macrophages from BCG infected mice were only significantly higher ($p<0.05$) at week 4 but not at weeks 2 and 6 post infection as compared to saline inoculated mice (Fig. 4.7D). Although week 4 BCG infected mice presented with higher numbers of TNFα+macrophages compared to lower levels at weeks 2 and 4 post infection, there were no kinetic changes during the entire course of infection. Whereas, *M. tuberculosis* infected mice expressed high numbers of TNFα+macrophages at week 4 ($p<0.05$) and week 6 ($p<0.05$) during CNS-TB compared to saline inoculated mice but not at week 2 during CNS-

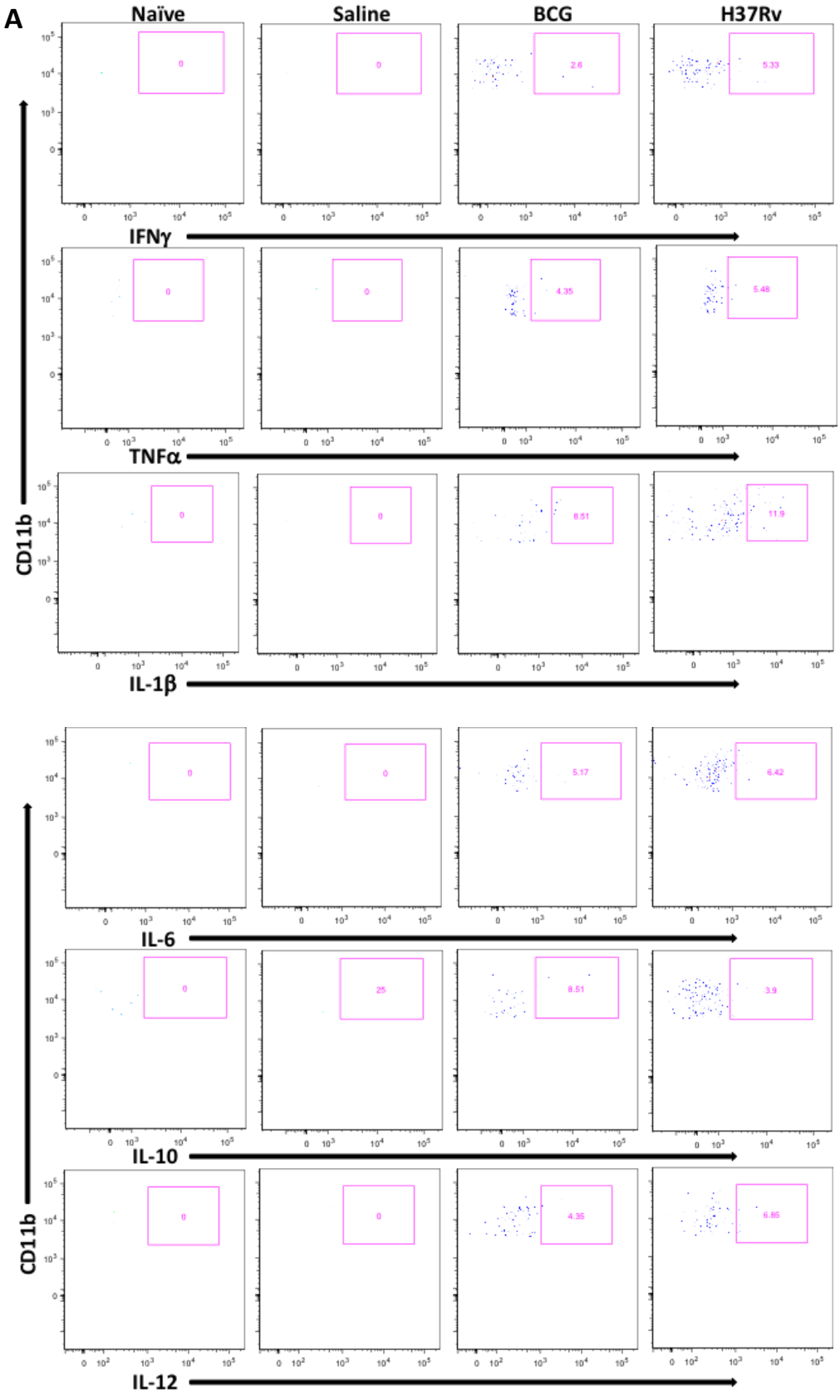
TB (Fig. 4.7D). We observed a significant decrease in the number of TNFα+macrophages from week 4 to week 6 during CNS-TB. No kinetic changes occurred during CNS-TB. It was only at week 2 that the responses of IL-12+macrtophages were virulence driven as evidenced by *M. tuberculosis* mice presenting with significant higher ($p<0.01$) numbers of TNFα+macrophages than BCG infected mice (Fig. 4.7D).

When we looked at IL-6, a pro-inflammatory cytokine that macrophages also produce for contribution to innate immunity (Ladel et al., 1997; Saunders et al., 2000; Giacomini et al., 2001), which has not yet been documented to be produced by brain infiltrating macrophages. We detected extremely low numbers of brain infiltrating macrophages expressing IL-6 (IL-6+macrophages) from week 2 saline control mice that were high compared to the undetected numbers at week 4 and week 6 post saline inoculation (Fig. 4.7E). And naïve mice also presented with undetectable numbers of TNFα+macrophages that did not differ from saline inoculated mice. During mycobacterial infection of the CNS, BCG infected mice presented with significantly high numbers of IL-6+macrophages at week 2 ($p<0.05$) and week 4 ($p<0.05$) but not at week 6 when compared to saline inoculated mice (Fig. 4.7E). Furthermore, no kinetic changes were observed post BCG intracerebral infection during the entire course of infection. Whereas, *M. tuberculosis* infected mice presented with increased numbers of IL-6+macrophages at week 2 ($p<0.01$), week 4 ($p<0.05$) and week 6 ($p<0.01$) during CNS-TB as compared to saline control mice (Fig. 4.7E), and no kinetic changes were observed during CNS-TB. Mycobacterial virulence affected the response of brain infiltrating macrophages that expressed IL-6 because *M. tuberculosis* infected mice showed significantly increased numbers of IL-6+macrophages at week 2 ($p<0.05$) and week 6 ($p<0.01$) compared to BCG infected mice.

IL-1β is another proinflammatory cytokine that protects against TB (Mayer-Barber et al., 2011), however the production of IL-1β by macrophages during CNS-TB has not been studies. We found low numbers of brain infiltrating macrophages expressing IL-1β (IL-1β+macrophages) from week 2 saline inoculated mice, however they were significantly increased ($p<0.05$) compared to naïve mice (Fig. 4.7F).While week 4 and week 6 saline inoculated mice contained undetectable numbers of IL-1β+macrophages and we found no kinetic changes in saline control mice. In contrast, mycobacterial infection of the CNS resulted in increased numbers of IL-1β+macrophages (Fig. 4.7F). BCG infected mice presented with

significantly higher numbers of IL-1β+macrophages at week 4 ($p<0.01$) and week 6 ($p<0.01$) but not week 2 post infection as compared to saline inoculated controls (Fog. 4.7F). We also found no differences in the kinetics of IL-1β+macrophages from BCG infected mice. Whereas, *M. tuberculosis* infected mice presented a number of IL-1β+macrophages that were significantly increased at week 2 ($p<0.01$), week 4 ($p<0.05$) and week 6 ($p<0.05$) during CNS-TB compared to saline control mice (Fig. 4.7F). No kinetic changes in the number of IL-1β+macrophages were observed during CNS-TB. The number of IL-1β+macrophages from *M. tuberculosis* infected mice were significantly increased at week 2 ($p<0.05$) and week 6 ($p<0.05$) but not week 4 post infection (Fig. 4.7F), indicating virulence was a factor during immune responses.

We found no differences in the number of brain infiltrating macrophages expressing anti-inflammatory cytokine IL-10 (IL-10+macrophages) among all the four experimental groups (naïve, saline inoculated, *M. tuberculosis* and BCG infected) at week 2 and at week 4 (Fig. 4.7G). Neither did we observe any kinetic changes in any of the three experimental groups (saline inoculated, *M. tuberculosis* and BCG infected). BCG infected mice showed no differences in the number of IL-10+macrophages at week 6 post infection compared to saline controls. However, the number of IL-10+macrophages from *M. tuberculosis* infected were significantly increased ($p<0.01$) at week 6 post infection compared to saline control mice (Fig. 4.7G). Virulence was not a factor in immune responses of IL-10+macrophages between *M. tuberculosis* infected mice and BCG infected mice.


A
Naïve
Saline
BCG
H37Rv
IFNγ
TNFα
IL-1β
IL-6
IL-10
IL-12
CD11b
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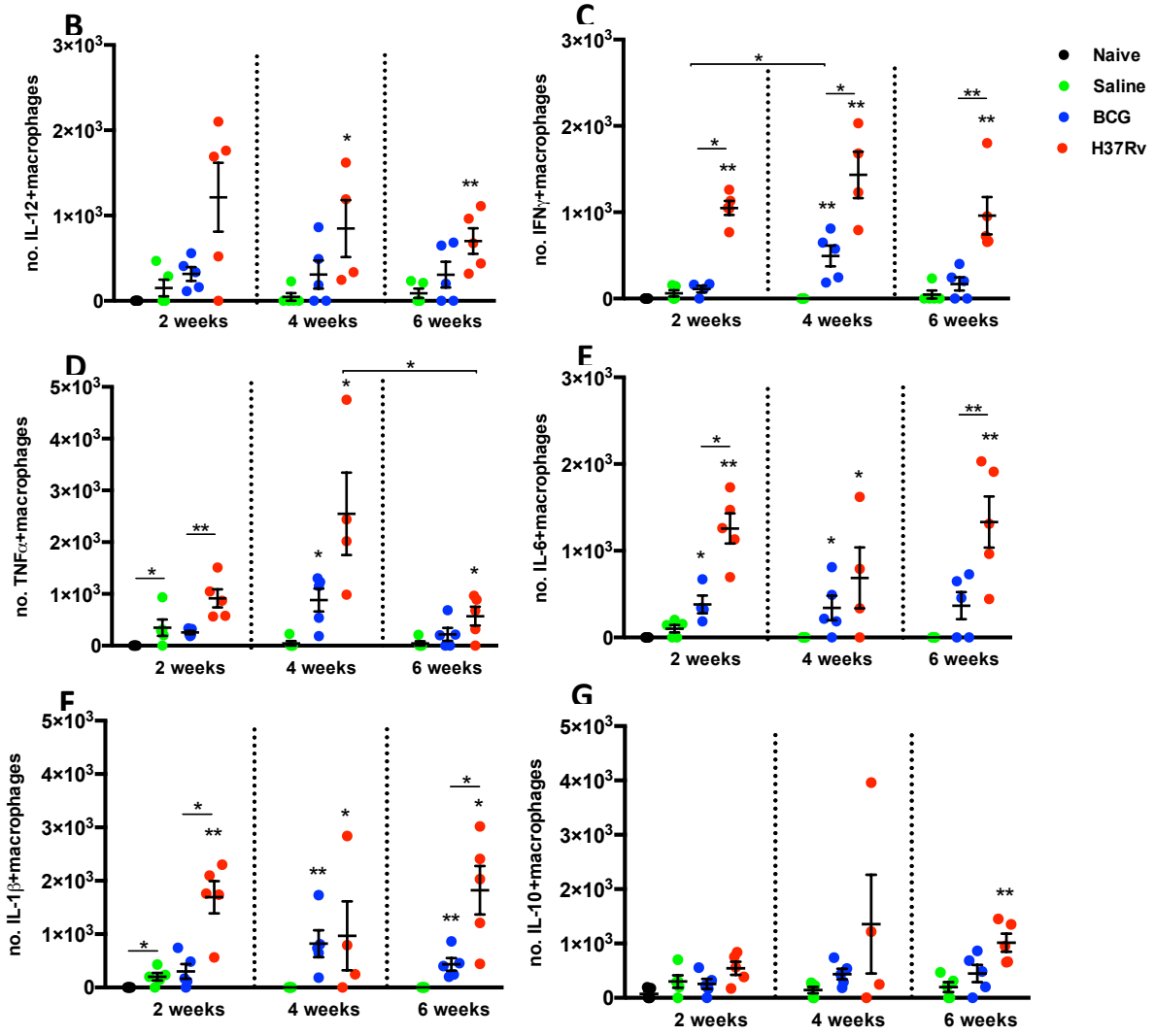


Figure 4.7: Increased numbers of brain infiltrating macrophages expressed pro-inflammatory cytokines (IFNγ, TNFα, IL-1β, IL-6 and IL-12) associated with antimycobacterial immune responses post BCG and *M. tuberculosis* H37Rv intracerebral infection. Wild-type female C57BL/6J mice (n=4-5 mice/group) were intracerebrally inoculated with saline, 1 x 10^5 CFUs of BCG or H37Rv and not inoculated (naïve mice). Mice were euthanized at weeks 2, 4 & 6 and immune cells analysed by flow cytometry. **(A)** Flow cytometry plots showing macrophages from naive, saline, BCG and *M. tuberculosis* inoculated mice that expressed IFNγ, TNFα, IL-1β, IL-6, IL-10 and IL-12. Increased numbers of macrophages from BCG and H37Rv infected mice, respectively expressed pro-inflammatory cytokines **(B)** IL-12, **(C)** IFNγ, **(D)** TNFα, **(E)** IL-6, **(F)** IL-1β and anti-inflammatory **(G)** IL-10 during mycobacterial infection of the CNS. . Graphs are representative of 3 independent experiments for BCG and 2 independent experiments for H37Rv. Data represents the mean +/- SEM. *p<0.05, **p<0.01 vs saline controls. Mann-Whitney test.

4.2.6) Kinetic recruitment of mature and functional brain dendritic cells promotes protective immune responses against mycobacterial CNS infection.

Controversies regarding the nature of APCs in the brain such as DCs of the CNS still exist because their roles are not well understood (Karman et al., 2004). In this study, two APCs (microglia and brain infiltrating macrophages) were phenotypically and functionally characterised (section 4.3.1). In this section the focus is on DCs which seem to have not been widely researched during mycobacterial infection of the CNS and yet DCs have been shown to be infected by *M. tuberculosis* at a higher frequency in pulmonary TB and as potential target cells for inducing antigen specific CD4+ T cell immunity against TB (Wolf et al., 2007; Choi et al., 2017). Controversies surrounding the presence of DCs in a healthy CNS led to limited research on the role of DCs during CNS-TB. Perry and Matyszak (1996) qualitatively characterized the roles of DCs during BCG CNS infection using microscopic analysis while Lee et al (2009) qualitatively and quantitatively characterized maturation of DCs including work from our research group (Francisco et al., 2015). When it comes to CNS-TB, the functional roles and cellular mechanism that DCs play in the outcome of *M. tuberculosis* and BCG CNS infection has not yet been fully studied. Therefore, the objective was to further characterize the immunological roles of DCs during CNS-TB, by phenotypically and functionally characterizing recruited brain DCs in wild-type C57BL/6J mice using flow cytometry.

4.2.6.1) Dendritic cells recruited to the brain and accumulate post *M. tuberculosis* H37Rv or BCG i.c. infection.

The first aim was to determine the kinetic recruitment of DCs to the brain post *H37Rv M. tuberculosis* and BCG intracerebral (i.c.) infection. Representative flow plots showing classification of total brain DCs (CD11b-CD45+CD11c+) during mycobacterial infection of the CNS (Fig. 4.8A). DCs were recruited to the brain post H37Rv *M. tuberculosis* and BCG intracerebral (i.c.) infection (Fig. 4.8A and B). There was no difference in the number of DCs between naive mice and week 2 saline control mice and no kinetic changes in saline inoculated mice was observed among different time points (Fig. 8B). However, the number of DCs that were recruited to the brain post BCG i.c. infection were significantly higher than saline control mice at weeks 4 ($p<0.01$) and 6 ($p<0.01$) but not at week 2 regardless of week 2 BCG DC numbers being almost double that of week 2 saline control mice (Fig. 4.8B). No kinetic changes were detected in the number of recruited DCs throughout the course of BCG CNS infection.

During CNS-TB, the number of DCs recruited to the brain were significantly higher in *M. tuberculosis* infected mice compared the saline control mice at weeks 2 ($p<0.01$), week 4 ($p<0.01$) and week 6 ($p<0.01$) (Fig. 4.8B). Total number of brain DCs from *M. tuberculosis* infected mice were significantly higher than those from BCG infected mice at week 2 ($p<0.05$) and week 6 ($p<0.05$) post infection suggesting virulence is a factor (Fig. 4.8B). Similar to BCG infection kinetics, we observed no kinetic change in DC recruitment during CNS-TB. We found that mycobacterial infection of the CNS with BCG or H37Rv results in recruitment and accumulation of DCs in the CNS as shown in previously studies (Matyszak and Perry, 1996; Lee et al., 2008; Francisco et al., 2015; Clarkson et al., 2017) and that virulence is a factor between the two strains as evidenced by the magnitude of the immune responses.

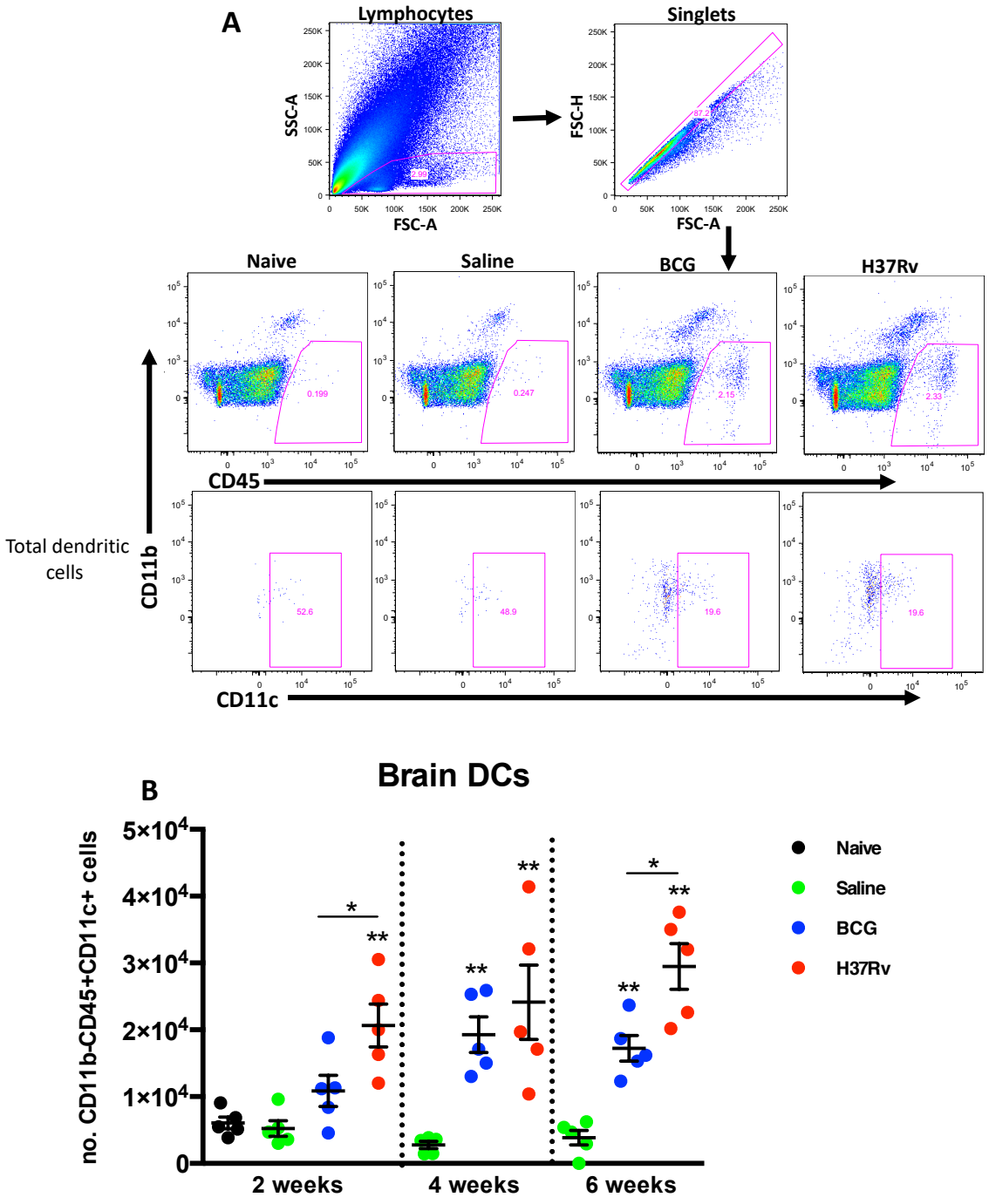


Figure 4.8: Dendritic cells recruited to the brain and accumulate post *M. tuberculosis* H37Rv or BCG intracerebral infection. Wild-type female C57BL/6J mice (n=4-5 mice/group) were intracerebrally inoculated with saline, 1 x 10^5 CFUs of BCG or H37Rv *M. tuberculosis* and not inoculated (naïve mice). Mice were euthanized at weeks 2, 4 & 6 and immune cells analyzed by flow cytometry. (A) Flow cytometry plots showing gating of total brain dendritic cells (DCs; $CD45^+$ $CD11b^-$ $CD11c^+$), during CNS-TB. **(B)** DCs recruited to the brains post *M. tuberculosis* and BCG CNS infection. Graphs representative of 3 independent experiments for BCG and 2 independent experiments for H37Rv. Data represents the Mean +/- SEM. *p<0.05, **p<0.01 vs saline controls. Mann-Whitney test.

4.2.6.2) Conventional DCs preferentially recruited to the brain post *M. tuberculosis* or BCG i.c. infection.

DCs remain potent APCs in the CNS compared to other APCs (Anandasabapathy et al., 2011; Mundt et al., 2019). The type of DC subsets dominating during pathogenic immune responses determines the functional specialization of DCs that will dictate disease outcome (Macri et al., 2018). The type of DC subsets present during infection dictates the direction of the immune responses. The second aim was to characterize the cellular phenotype of DCs that are recruited to the brain during mycobacterial infection of the CNS to determine which DC subset is preferentially recruited post H37Rv *M. tuberculosis* of BCG intracerebral infection. We identified conventional DCs (cDCs) and plasmacytoid DCs (pDCs) in C57BL/6J mice using flow cytometric analysis.

Figure 4.9A shows the representative flow plots of cDCs (CD11b-CD11c+MHCII+) and pDCs (CD11b-CD11c+PDCA-1+) recruited to the brain post H37Rv *M. tuberculosis* or BCG intracerebral infection. We detected low numbers of DCs in naïve mice which were pDCs and no cDCs were present (Fig. 4.9B). This finding is in partial agreement with work by Mundt and colleagues (2019) who characterized steady state DC subsets in the CNS that consisted two classes of cDCs (CD11blow-cDCs1 and CD11b+cDC2s) and three classes of pDCs (B220+, Siglec-H and Ly6C+) and this could be a result of using different classification markers. Both conventional DCs and plasmacytoid DCs got recruited to the brain post BCG and H37Rv M. tuberculosis i.c. infection (Fig. 4.9B). Saline inoculated mice contained slightly higher numbers of pDCs than naïve mice at week 2 but not at weeks 4 and 6 as levels decreased (Fig. 4.9B) and these saline pDCs were lower than those detected during mycobacterial infection of the CNS. These saline control mice also contained a number of cDCs that were lower than those of mycobacterial CNS infection. A high number of conventional DCs were recruited to the brain and accumulated during mycobacterial infection of the CNS compared to pDCs (Fig. 4.9A and B). There was an increasing trend of CDs in BCG i.c. infected mice from week 2 to week 4 followed by a decrease at week 6 post infection. Whereas, during CNS-TB, the number of cDCs recruited the brain showed increasing trend throughout the course of infection. These findings showed that cDCs were preferentially recruited to the brain post *M. tuberculosis* or BCG intracerebral infection as evidence by the dominant infiltration of cDCs to the brain.

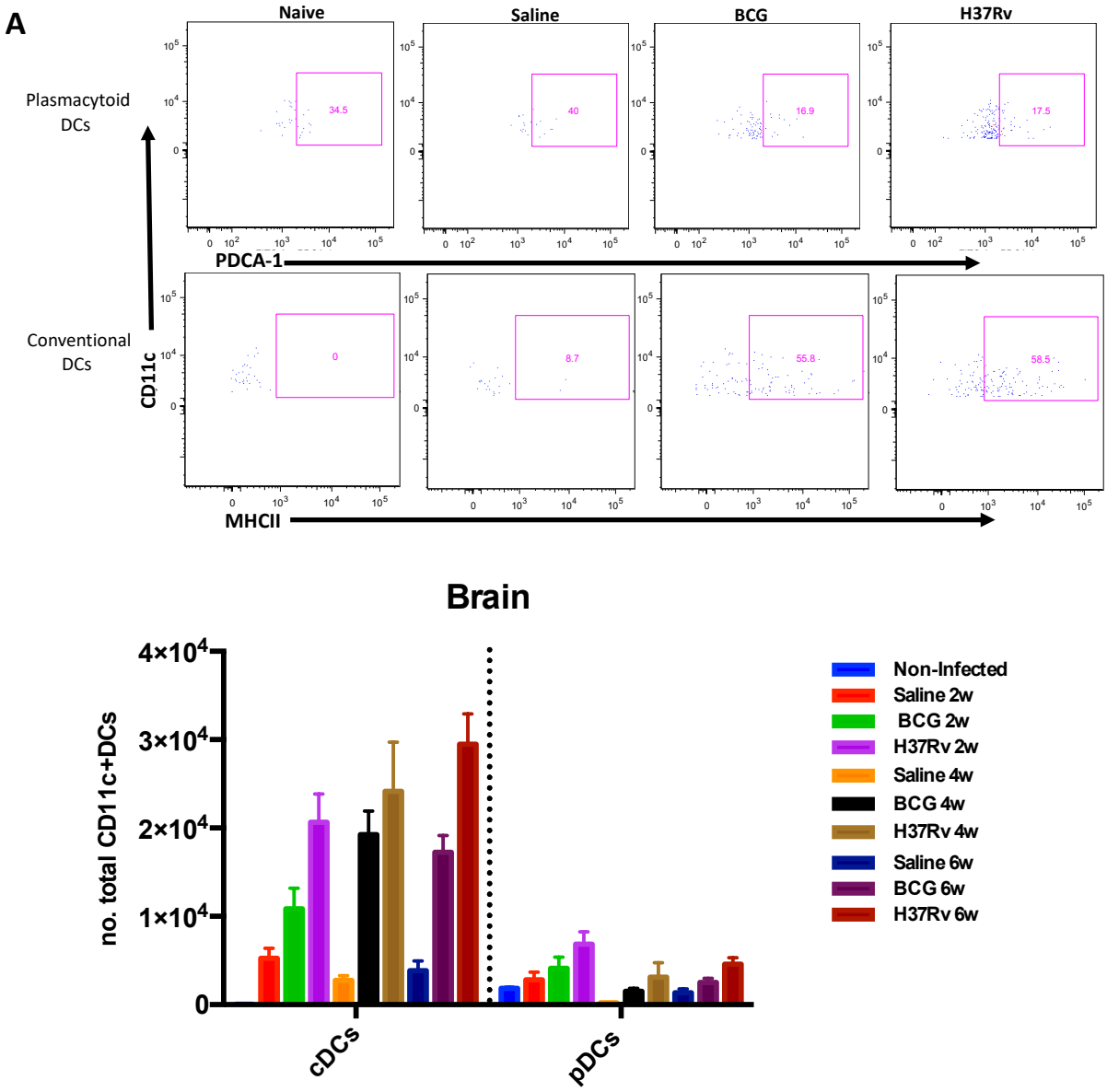


Figure 4.9. Conventional DCs preferentially recruited to the brain post *M. tuberculosis* H37Rv or BCG intracerebral infection. Wild-type female C57BL/6J mice (n=4-5 per/group) were intracerebrally inoculated with saline and 1 x 10^5 CFUs of H37Rv *M. tuberculosis* or BCG. Mice were euthanized at weeks 2, 4, and 6 post infection. **(A)** Flow cytometry plots showing gating of conventional DCs (cDCs; CD11c+MHCII+) and plasmacytoid DCs (pDCs; CD11c+PDCA-1+). **(B)** cDCs preferentially recruited to the brain during CNS-TB. Graphs are representative of 3 independent experiments for BCG and 2 independent experiments for H37Rv. Data represents the mean +/- SEM.

4.2.6.3) Functionally mature DCs recruited to the brain post *M. tuberculosis* or BCG i.c. infection.

It was established that conventional DCs were mainly recruited to the brain post *M. tuberculosis* or BCG intracerebral infection, then the next step was to determine the functionality of recruited DCs. DC activation and maturation are required to achieve *M. tuberculosis* clearance through adaptive immunity (Choi et al., 2018). DC maturation determines the functionality of DCs and can be measured by the upregulation of MHCII expression, costimulatory molecules (CD80/86) and chemokine receptor-7 (CCR7), a migratory DC molecule that traffics DCs to draining lymph nodes (Sanchez-Sanchez et al., 2006; Gunn, 2003). Steady state DCs express MHCI and MHCII molecules which are increased (or highly upregulated) when DCs get activated (Macri et al., 2018). We determined the activation and maturation status of brain DCs during mycobacterial infection of the CNS by measuring the expression of MHCII, CD86 and CCR7 using flow cytometry.

Figure 4.10A shows the representative plot of total brain DCs expressing MHCII, CD86 and CCR7 post *M. tuberculosis* and BCG infection, respectively. Conventional DCs classified as expressing MHCII represented activated DCs. See Appendix G for gating strategy of MHCII+, CD86+ and CCR7+ recruited brain DCs. Recruited brain DCs have been shown to express increased levels of MHCII during mycobacterial infection of the CNS (Lee et al., 2009; Francisco et al., 2015). Naïve mice presented with the least number of total brain DCs expressing MHCII (MHCII+DCs) that were negligible and equivalent to saline control mice for all saline inoculated time points (2, 4 and 6 weeks) as compared to mycobacterial infection of the CNS (Fig. 4.10B). This was expected for naïve and saline control mice because they are non-pathogenic. During BCG CNS infection, the number of recruited brain MHCII+DCs were significantly higher compared to saline control mice at week 2 ($p<0.01$), week 4 ($p<0.01$) and week 6 ($p<0.01$) (Fig. 4.10B). These BCG i.c. infected mice showed significant ($p<0.01$) kinetic increase from week 2 to week 4 and from week 2 to week 6 ($p<0.01$). Whereas, *M. tuberculosis* infected mice also expressed significantly high numbers of recruited brain MHCII+DCs ($p<0.01$) compared to saline control mice at week 2 ($p<0.01$), week 4 ($p<0.01$) and week 6 ($p<0.01$) during CNS-TB and these MHCII+DCs showed an increasing kinetic trend that was not significant as the infection progressed (Fig. 4.10B). When we compared the number of brain MHCII+DCs during mycobacterial infection of the CNS, we found that *M. tuberculosis* infected mice expressed significantly higher numbers than BCG mice at weeks 2 ($p<0.01$) and week 6 ($p<0.05$) post infection (Fig. 4.10B). This showed that mycobacterial

infection of the CNS activated DCs as shown by previous studies (Lee et al., 2009; Francisco et al., 2015).

DCs also express costimulatory molecules that include CD86 (Azuma et al., 1993), which produce costimulatory signals that are required for T cell proliferation and T cell cytokine production (Gimmi et al., 1991; Freeman et al., 1991; Linsley and Ledbetter, 1993). The expression of CD86 by DCs has been shown to be increased in the brains of mice infected with mycobacteria (Lee et al., 2009; Francisco et al., 2015). We detected low numbers of DCs expressing CD86 (CD86+DCs) from the brains of naïve mice and saline inoculated mice as compared to mycobacterial infection of the CNS (Fig. 4.10C). We found that naïve mice expressed significantly lower numbers of brain CD86+DCs compared to week 2 saline control mice (Fig. 4.10C), probably because intracerebral saline inoculation caused inflammation that increased CD86 expression. We observed a significant decrease in the number of recruited brain CD86+DCs in saline control mice from week 2 to week 6 showing reduction in inflammatory responses following wound healing. BCG intracerebral infection caused significant increases in the number of recruited brain CD86+DCs compared to saline control mice at weeks 4 ($p<0.01$) and week 6 ($p<0.01$) but the increase was not significant at week 2 post infection (Fig. 4.10C). When looking at kinetics of BCG i.c. infection, we found that the number of recruited brain CD86+DCs significantly decreased only from week 4 to week 6 post infection to levels that were lower than week 2 saline control mice. During CNS-TB, *M. tuberculosis* infected mice expressed significantly higher numbers of recruited brain CD86+DCs at weeks 2 ($p<0.01$), week 4 ($p<0.01$) and week 6 ($p<0.01$) post intracerebral infection (Fig.10C). However, no changes in the kinetics of recruited brain CD86+DCs occurred during CNS-TB (Fig. 4.10C). Finally, there was no difference in the number of recruited brain CD86+DCs between *M. tuberculosis* and BCG intracerebral infection suggesting that virulence was not a factor for CNS-TB infection outcome and that CNS inflammation was sustained.

We further investigated the migratory capacity of these recruited brain DCs to see if they were capable of trafficking *M. tuberculosis* antigens to cervical lymphoid organs by measuring the expression of DC migratory chemokine CCR7. CCR7 deficient DCs accumulate in the CNS while CCR7+DCs migrate to cervical lymph nodes (CLNs) during CNS autoimmune disease (Clarkson et al., 2017; Louveau et al., 2018). Increased numbers of recruited brain DCs expressed CCR7 (CCR7+DCs) during mycobacterial infection of the CNS compared to low

numbers expressed by saline inoculated mice (Fig. 4.10D). Both *M. tuberculosis* i.c. infected mice presented with high numbers of CCR7+DCs compared to saline controls at week 2 ($p<0.01$), week 4 ($p<0.05$) and week 6 ($p<0.01$) during CNS-TB. Kinetics revealed a significant decrease in the number of recruited brain CCR7+DCs from week 2 to week 4 ($p<0.05$) that was followed by an increase at week 6 ($p<0.05$) during CNS-TB (Fig. 4.10D). BCG i.c. infected mice presented with high numbers of recruited brain CCR7+DCs compared to saline controls at week 2 ($p<0.01$), week 4 ($p<0.01$) and week 6 ($p<0.01$) post infection (Fig. 4.10D). Whereas, BCG infected mice presented with increasing numbers of CCR7+DCs as infection progressed (Fig. 4.10D). Virulence was only a factor at week 2 post intracerebral infection, where *M. tuberculosis* infected mice presented with significantly higher ($p<0.01$) numbers of recruited brain CCR7+DCs compared to BCG infected mice (Fig. 4.10D). Our data is in agreement with previous studies that show that mature DCs express high levels of MHCII, and costimulatory molecules CD86 (Banchereau et al., 2000) and increased expression of CCR7 in the brain DCs shows that they possess migratory capabilities. This data suggests that recruited brain DCs were mature and functional during mycobacterial infection of the CNS.

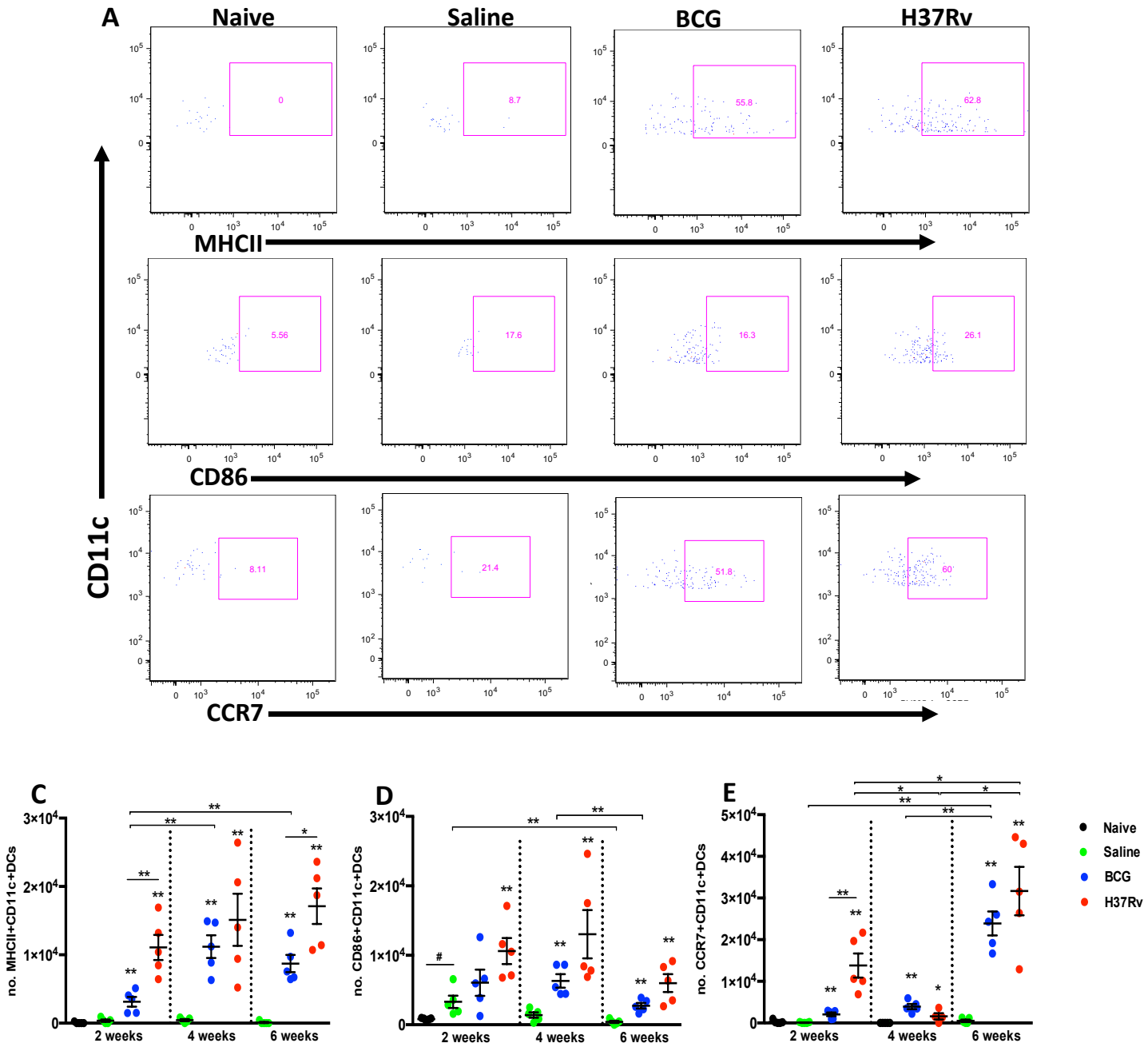


Figure 4.10: Functionally mature DCs recruited to the brain post *M. tuberculosis* and BCG intracerebral infection. Wild-type female C57BL/6J mice (n=4-5 mice/group) were intracerebrally inoculated with saline, 1 x 10^5 CFUs of BCG or H37Rv *M. tuberculosis* and not inoculated (naïve mice). Mice were euthanized at weeks 2, 4 & 6 and immune cells analyzed by flow cytometry. **(A)** Flow cytometry classification of MHCII+, CD86+ and CCR7+ brain DCs. **(B)**MHCII+ and **(C)** CD86+ brain DCs increased during CNS-TB. Percentage of brain DCs expressing CCR7 during CNS-TB. Graphs are representative of 3 independent experiments for BCG and 2 independent experiments for H37Rv. Data represents the mean +/- SEM. *p<0.05, **p<0.01 vs saline controls. Mann-Whitney test.

4.2.6.4) Recruited brain DCs from *M. tuberculosis* or BCG i.c. infected mice expressed proinflammatory and anti-inflammatory cytokines.

Cytokine signalling by dendritic cells is another method in which DCs direct immune responses. To further characterize the functional profile of DCs recruited to the brain during mycobacterial infection of the CNS, cytokines expressed by *M. tuberculosis* or BCG i.c. infected mice were measured by flow cytometric intracellular staining. Currently, no research has investigated the cytokines expressed by DCs during CNS-TB. Representative flow cytometry plots showing gating of brain dendritic cell cytokines IL-1β, IFNγ, TNFα, IL-6, IL10 and IL-12 during mycobacterial infection of the CNS (Fig. 11A). Appendix G provides the flow cytometry gating strategy of the cytokines expressed by recruited brain DCs. A significant number of DCs expressed proinflammatory cytokines IL-1β, TNFα, IFNγ, IL-6, IL-12 and anti-inflammatory IL-10 post *M. tuberculosis* or BCG intracerebral infection (Fig. 4.11B-G).

DCs are known to produce proinflammatory cytokines like IL-1 and IL-6 that play a crucial role in helping with the recruitment of cells to the site of infection during pulmonary TB infection (Giacomini et al., 2001). Production of both these cytokines by DCs during CNS-TB has not been investigated. The number of recruited brain DCs expressing IL-1β (IL-1β+DCs) was not different between naïve mice and week 2 saline control mice and there were no kinetic changes in the number of IL-1β+DCs in saline inoculated mice at all time points of the experiment (Fig. 4.11B). Whereas, mycobacterial infection of the CNS caused significant increases of IL-1β+DCs compared to saline control mice (Fig. 4.11B). BCG i.c. infected mice showed significantly higher numbers of recruited brain IL-1β+DCs compared to saline inoculated mice at week 2 ($p<0.05$) and week 6 ($p<0.0001$) but not at week 4 post infection (Fig. 4.11B). These IL-1β+DCs numbers significantly decreased in BCG infected mice from week 4 to week 6 ($p<0.05$) post infection. While during CNS-TB, the number of recruited brain IL-1β+DCs was significantly higher in *M. tuberculosis* infected mice than saline control mice at week 2 ($p<0.05$), week 4 ($p<0.05$) and week 6 ($p<0.0001$) post infection (Fig. 4.11B). A significant increase ($p<0.01$) in the number of IL-1β+DCs from *M. tuberculosis* infected mice occurred from week 2 to week 6 post infection. To determine if virulence was a factor in the expression of IL-1β by recruited brain DC, we compared numbers of IL-1β+DCs between *M. tuberculosis* and BCG infection and found that *M. tuberculosis* mice presented with

significantly higher numbers at weeks 2 ($p<0.05$) and week 6 ($p<0.001$) post infection (Fig. 4.11B).

IL-12 secreted by DCs is critical for a robust T cell response and serves as the predominant source of interferon IFNγ (Macatonia et al., 1995). IL-12 secreted by DCs normally plays an important role in signalling T cells to polarize towards T-helper 1 (Th1) immune responses (Gee et al., 2009), which is protective against tuberculosis. Limited knowledge exists about the production of IL-1 by DCs during CNS-TB. The number of recruited brain DCs expressing IL-12 (IL-12+DCs) were significantly higher ($p<0.05$) in week 2 saline control mice than naïve mice with no observed kinetic change in saline inoculated mice at all time points of the experiment (Fig. 4.11C). Surprisingly, intracerebral infection with BCG did not causes any significant change between BCG infected mice and saline controls during the entire course of infection (Fig. 4.11C), even though significant kinetic changes occurred in BCG infected mice with an increase was observed from week 2 to week 4 ($p<0.01$) that was followed by a significant decrease ($p<0.0001$) from week 4 to week 6 post infection (Fig. 4.11C).Whereas, recruited brain DCs from *M. tuberculosis* infected mice expressed increased numbers of IL-12+DCs during CNS-TB; which were significantly higher than those from saline control mice at week 2 ($p<0.05$), week 4 ($p<0.0001$) and week 6 ($p<0.01$) post infection (Fig. 4.11C). However, there was no kinetic change during CNS-TB. And virulence was a factor because the number of IL-12+DCs recruited to the brain in *M. tuberculosis* infected mice was significantly higher than those from BCG infected mice at week 2 ($p<0.001$), week 4 ($p<0.05$) and week 6 ($p<0.001$) post CNS intracerebral infection (Fig. 4.11C).

IFNγ, a proinflammatory cytokine that is critical for protection against pulmonary TB was expressed by a high number of recruited brain DCs during CNS-TB (Fig. 4.11D). DCs have not been shown to produce IFNγ during CNS-TB. The number of recruited brain DCs expressing IFNγ (IFNγ+DCs) did not differ between naïve mice and saline inoculated mice, and there was no kinetic change in the numbers IFNγ+DCs from saline inoculated mice at all time points of the experiments (Fig. 4.11D). While BCG infected expressed high numbers of IFNγ+DCs that significantly differed ($p<0.05$) from saline control mice only at week 2 post infection and no difference occurred in the kinetics of IFNγ+DCs OF BCG infected mice throughout the course of infection (Fig. 4.11D). Whereas, during CNS-TB, *M. tuberculosis* infected mice expressed significantly higher numbers of IFNγ+DCs than saline inoculated mice

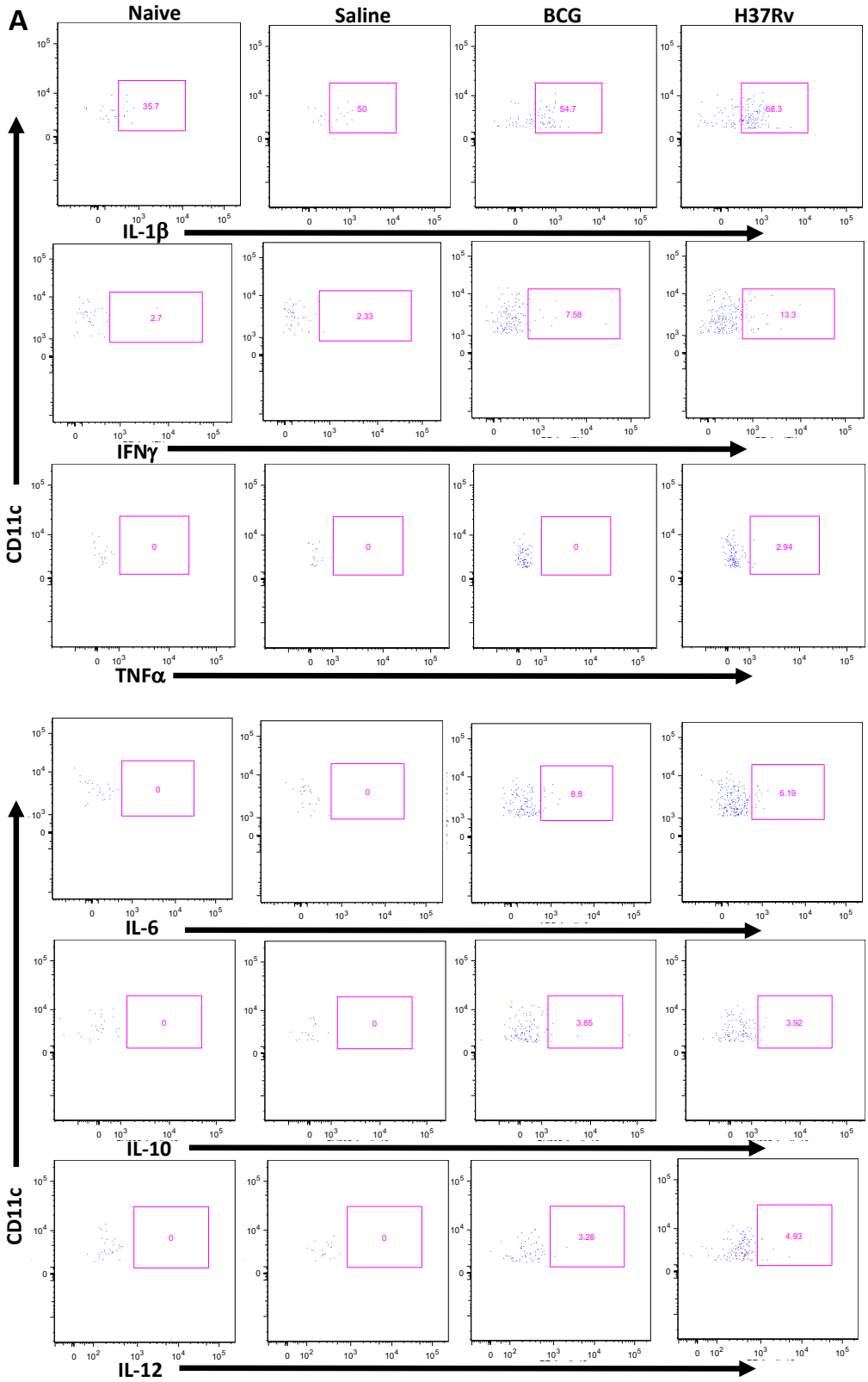
week 2 ($p<0.0001$), week 4 ($p<0.0001$) and week 6 ($p<0.001$) post infection, however; this was not accompanied by any kinetic change for the duration of infection (Fig. 4.11D). Finally virulence was a factor between two strains of mycobacteria because the number of IFNγ+DCs from *M. tuberculosis* infected were significantly higher than BCG infected mice at week 2 ($p<0.01$), week 4 ($p<0.0001$) and week 6 ($p<0.05$) post intracerebral infection (Fig. 4.11D). (Fig.11D).

Another proinflammatory cytokine with dual functionality of protection against pulmonary and CNS-TB or pathogenicity is TNFα (Thwaites et al, 2003; Co et al., 2004; Allie et al., 2013Curto et al., 2004; Francisco et al., 2015; Hsu et al., 2017). Lee and colleagues identified DCs as one of the source of TNFα production during BCG CNS infection but not during *M. tuberculosis* CNS infection.. week 2, all experimental groups; naïve mice, saline inoculated mice, BCG or *M. tuberculosis* i.c. infected mice did not show any difference in the number of recruited brain DCs expressing TNFα (TNFα+DCs) among each other (Fig. 4.11E). We observed no differences in the number of TNFα+DCs between saline inoculated mice and BCG infected mice at all time points of the experiment (Fig. 4.11E), nor were they any kinetic changes. However, there were significantly higher numbers of TNFα+DCs from M. tuberculosis infected mice compared to saline control mice at week 4 ($P<0.05$) and week 6 ($p<0.01$) during CNS-TB (Fig. 4.11E). These *M. tuberculosis* infected mice showed significant increase in the number of TNFα+DCs from week 2 to week 4 ($p<0.01$) and also from week 4 to week 6 ($p<0.05$) post infection. Additionally, virulence was a factor only at week 6 post infection because *M. tuberculosis* infected mice presented with higher numbers of TNFα+DCs compared to BCG infected mice. This might suggest that the TNFα expressed by recruited brain DCs played a protective role against CNS-TB because brain bacterial burdens were decreased, and mice survived until experimental end points.

Proinflammatory cytokine, IL-6 that has been implicated in protection against *M. tuberculosis* pulmonary infection but not BCG infection (Ladel et al., 1997) and has been identified to remain high in tb patients (Mastroianni et al., 1997; Misra et al., 2010). We investigated whether DCs are contributors to IL-6 responses during CNS-TB. We found no difference in the number of recruited brain DCs that expressed IL-6 (IL-6+DCs) between naïve mice and week 2 saline inoculated mice (Fig. 4.11F), and saline inoculated mice only showed a significant decrease in the number of IL-6+DCs from week 2 to week 6 ($p<0.05$) post

inoculation. When we looked at mycobacterial infection, BCG infected mice expressed significantly higher ($p<0.001$) numbers of IL-6+DCs than saline inoculated mice only at week 6 post infection (Fig. 4.11F), while earlier time points showed no differences between the two groups. Whereas during CNS-TB, all *M. tuberculosis* infected mice presented with significantly higher numbers of IL-6+DCs at week 2 ($p<0.01$), week 4 ($p<0.05$), and week 6 ($p<0.001$) post infection compared to saline inoculated mice (Fig. 4.11F). Finally, we observed a significant increase ($p<0.05$) in the number of IL-6+DCs from week 2 to week 4 post infection. However, *M. tuberculosis* infected mice and BCG infected mice did not show any differences in the number of IL-6+DCs post infection.

Pro-inflammatory cytokine IL-10 has been implicated in suppressing immune responses during pulmonary TB (Goverman et al., 1995) and IL-10 levels are increased in TBM patients (Donald et al., 1995; Misra et al., 2010; Visser et al., 2015). We investigated if DCs contribute to elevated levels of IL-10 during CNS-TB infection in mice. At week 2, all experimental groups; naïve mice, saline inoculated mice, BCG or *M. tuberculosis* i.c. infected mice did not show any difference in the number of recruited brain DCs expressing IL-10 (IL-10+DCs) among each other (Fig. 4.11G). Interestingly, later during infection the number of IL-10+DCs expressed by BCG infected mice were significantly higher at week 4 ($p<0.01$) and week 6 ($p<0.05$) post infection compared to saline inoculated mice (Fig. 4.11G). We found a significant increase in the number of IL-10+DCs from BCG infected mice from week 2 to week 4 (triple-fold, $p<0.001$) that was followed by a significant decrease ($p<0.01$) at week 6 post infection. Similar to BCG infected mice, *M. tuberculosis* infected mice also presented with significantly high numbers of IL-10+DCs at similar time points; at week 4 ($p<0.05$) and week 6 ($p<0.0001$) post infection compared to saline inoculated mice (Fig. 4.11G). During CNS-TB, the number of IL-10+DCs significantly increased from week 2 to week 4 ($p<0.01$) and from week 2 to week 6 ($p<0.001$) post infection. Virulence was a factor at only week 6 post infection because the number of IL-10+DCs was significantly higher ($p<0.001$) in *M. tuberculosis* infected mice compared to BCG infected mice. These findings suggest that early innate TB immunity induced by DCs was not inhibited by IL-10 and that later in the course of infection DCs expressed IL-10 that may have assisted in regulating antimycobacterial immune responses.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Naive
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BCG
H37Rv
IL-1β
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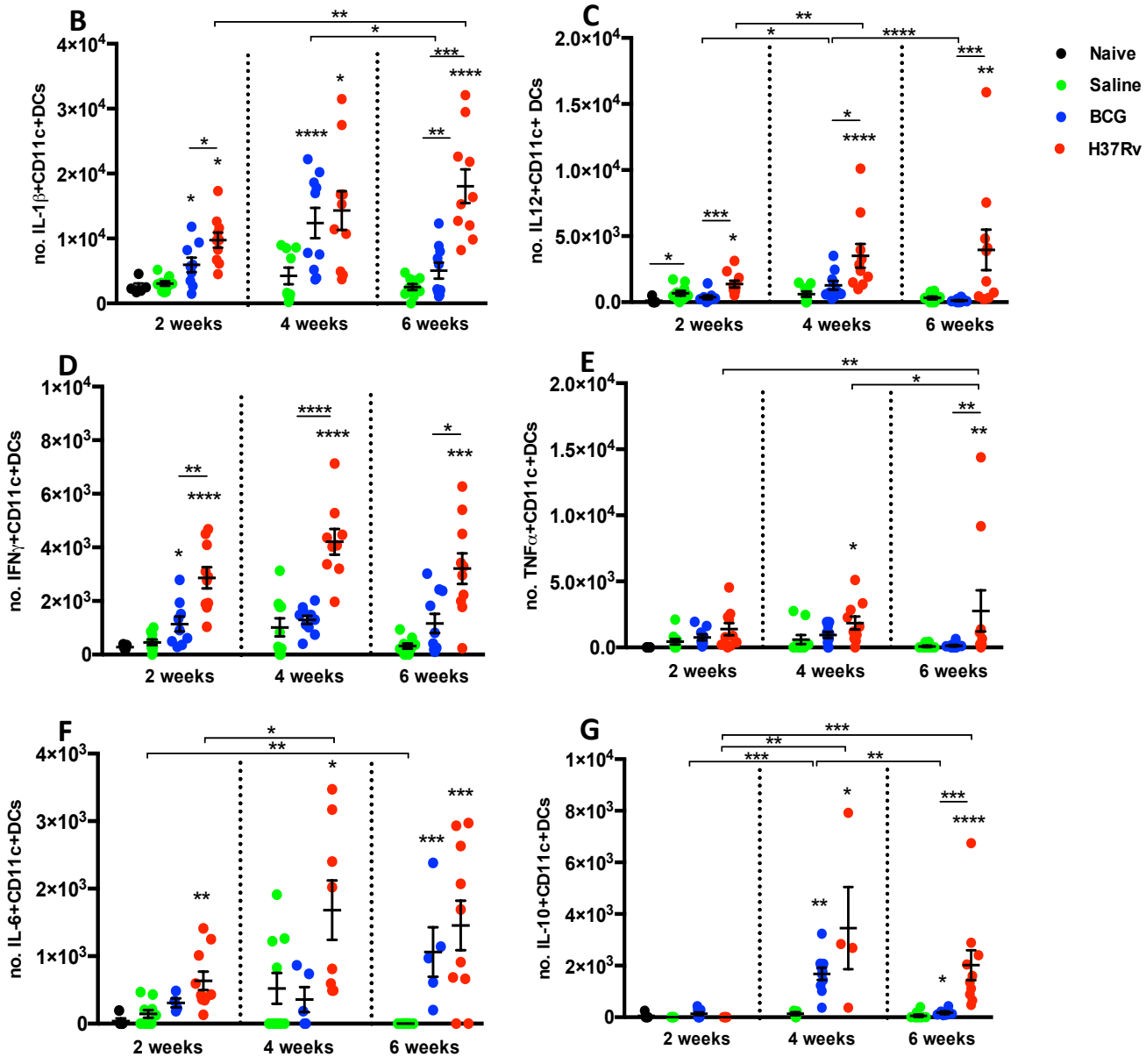


Figure 4.11: Recruited brain DCs from BCG or *M. tuberculosis* H37Rv intracerebrally infected mice expressed proinflammatory and anti-inflammatory cytokines involved in antimycobacterial immunity. Wild-type female C57BL/6J mice (n=4-10 mice/group) were intracerebrally inoculated with saline, 1 x 10^5 CFUs of BCG or H37Rv *M. tuberculosis* and not inoculated (naïve mice). Mice were euthanized at weeks 2, 4 & 6 and immune cells analyzed by flow cytometry. **(A)** Flow cytometry plots of IFNγ, IL-1β, IL-6, IL-10, TNFα, and IL-12. Production of proinflammatory cytokines **(B)** IL-1β, **(C)** IL-12, **(D)** IFNγ, **(E)** TNFα, **(F)** IL-6 and **(G)** anti-inflammatory IL-10 post *M. tuberculosis* and BCG CNS infection. Graphs are representative of pooled data from 2 independent experiments. Data represents the mean +/- SEM. *p<0.05, **p<0.01,***p<0.001, ****p<0.0001 vs saline controls. Mann-Whitney test.

4.2.6.5) Conventional and mature DCs recruited to the CLNs post *M. tuberculosis* or BCG i.c. infection.

During pulmonary infection, DCs initiate adaptive immune responses in the lymph nodes when they present live *M. tuberculosis* antigens which are transported from the lungs through CCR7 or CCR5 (Sáenz et al., 2013). During pulmonary TB, *M. tuberculosis* infected dendritic cells migrate to the draining lymph nodes to activate T cells post *M. tuberculosis* infection (Reiley et al., 2008; Wolf et al., 2008). Whereas, DCs that capture CNS antigens migrate to cervical lymph nodes (CLNs) to present antigens to T cells (Kida et al., 1993; Weller, 1998; Hatterer et al., 2006; Mohammad et al., 2014) because antigen presentation of neuroinflammatory diseases mainly occurs in CLNs (Phillips et al., 1997; de Vos et al., 2002). DCs in the CNS that express CCR7 like our data have been shown to drain antigens into CLNs (Clarkson et al., 2017; Louveau et al., 2018). This suggests that recruited brain CCR7+DCs that were detected in this study showed capabilities of trafficking *M. tuberculosis* and BCG antigens to CLNs. Therefore, the first aim was to determine the recruitment and migration of DCs to cervical lymph nodes in wild-type C57BL/6J mice post H37Rv *M. tuberculosis* and BCG intracerebral infection using flow cytometry.

We found that CLNs from *M. tuberculosis* infected mice were relatively swollen and larger than saline control mice at week 4 post intracerebral infection (Fig. 4.12.1) and infected mice presented with enlarged neck mass. CLNs are commonly affected by tuberculosis (Jha et al., 2001). DC recruitment has been shown to cause lymph node swelling when immune responses are underway (Acton et al., 2014) so measured DCs recruited to the CLNs.

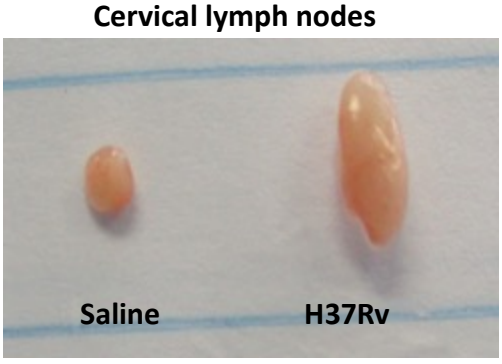


Figure 4.12.1. Cervical lymph nodes (CLNs) enlarged and swollen during CNS-TB. Wild-type female C57BL/6J mice (n=4-5 per/group) were intracerebrally inoculated with saline and 1 x 10^5 CFUs of H37Rv *M. tuberculosis*. Mice were euthanized at weeks 4 post infection. Cervical lymph nodes (CLNs) of *M. tuberculosis* infected mice became large and swollen post infection during CNS-TB.

Figure 4.12.2A-B shows the gating strategy of the representative flow plots of total cervical lymph node DCs (CD45+CD11b-CD11c+) post H37Rv *M. tuberculosis* or BCG intracerebral infection. We detected no difference in the number of total DCs recruited to the CLNs post saline inoculation compared to naïve mice (Fig. 4.12.2B). Additionally, there was no change in the kinetics of saline control mice at all experimental time points. Surprisingly, BCG i.c. infected mice presented with total number of DCs in the CLNs that were not significantly different from saline control mice at weeks 4 and 6 post infection even though they appeared to be slightly higher in numbers (Fig. 4.12.2C). BCG CNS infection resulted in significantly higher numbers of total DCs in the CLNs only at week 2 ($p<0.01$) and these numbers were significantly decreased ($p<0.05$) from week 2 to week 4 post infection. However; during CNS-TB, the number of total DCs recruited to the CLNs were significantly high compared to saline controls during the entire course of infection (Fig. 4.12.2B). Total DCs in CLNs of *M. tuberculosis* infected mice significantly higher than saline control mice at week 2 ($p<0.05$), week 4 ($p<0.01$) and week 6 ($p<0.0001$) post infection and showed no kinetic changes during CNS-TB. These findings show recruitment and accumulation of DCs in the CLNs during CNS-TB, however the number of DCs in the CLNs were too high to only be attributed from those that drained from the brain and this suggesting DCs from other tissues like the spinal cord also drained into CLNs.

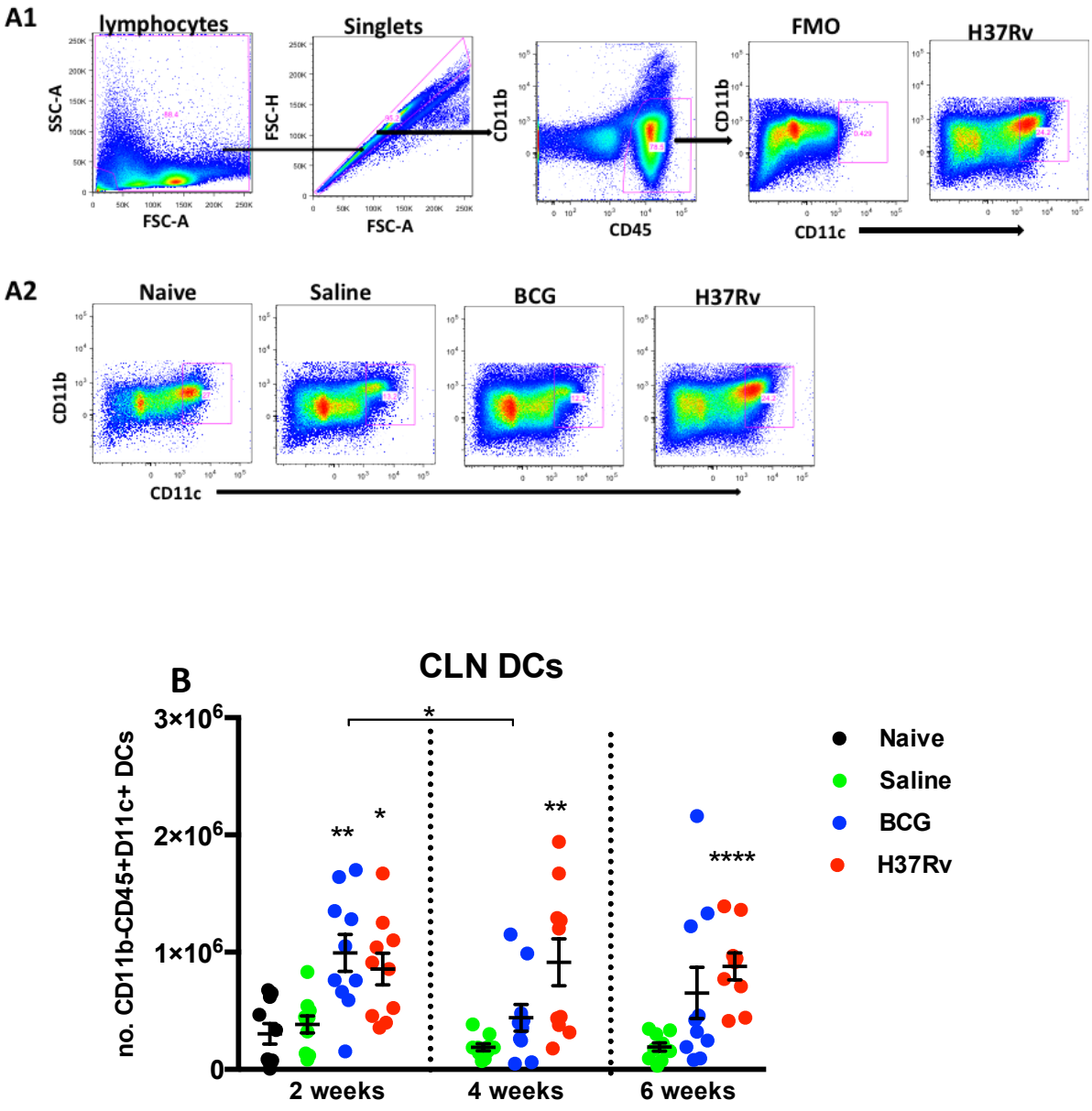


Figure 4.12.2. DCs recruited to the CLNs and accumulate post H37Rv *M. tuberculosis* and BCG intracerebral infection. Wild-type female C57BL/6J mice (n=4-10 per/group) were intracerebrally inoculated with saline and 1 x 10^5 CFUs of H37Rv *M. tuberculosis* or BCG. Mice were euthanized at weeks 2, 4 and 6 post infection and immune cells analyzed by flow cytometry. Flow cytometry plots showing gating of **(A)** total CLNs dendritic cells (DCs; $CD45^+$ $CD11b^-$ $CD11c^+$) during mycobacterial CNS infection. **(B)** High DCs during *M. tuberculosis* and BCG infection. Graphs are representative of 3 independent experiments for BCG and 2 independent experiments for H37Rv. Data represents the mean +/- SEM. *p<0.05, **p<0.01 vs saline controls. Mann-Whitney test.

4.2.6.6) Conventional DCs preferentially recruited to the CLNs post *M. tuberculosis* or BCG i.c. infection.

Brain DCs displayed migratory capabilities to the cervical lymph nodes during mycobacterial infection of the CNS. Our aim was to determine whether any DC subset in the CLNs was preferentially recruited post BCG and *M. tuberculosis* i.c. infection. DC cellular phenotypes of cDCs and pDCs were identified using flow cytometry.

Figure 4.13A shows the representative flow plots of cDCs (MHCII+CD11c+) and pDCs (PDCA-1+CD11c+) in the CLNs during mycobacterial infection of the CNS. Unlike recruited brain DCs that only presented with pDCs from naïve mice, DCs from naïve mice in the CLNs mostly composed of high numbers of cDCs and low numbers of pDCs (Fig. 4.13B). This phenomenon was similar the case for saline inoculated mice that also consisted of extremely high numbers of cDCs compared to low numbers of pDCs. Lastly, we detected mainly high numbers of cDCs than pDCs during mycobacterial infection of the CNS and these numbers were high for both H37Rv *M. tuberculosis* or BCG infected mice compared to low numbers in saline control mice (Fig. 4.13B). The kinetics of both cDCs and pDCs in the CLNs showed a decrease from week 2 to week 4 that was followed by an increase at week 6 for both tuberculosis strains. These findings showed that conventional DCs were preferentially recruited to the brain during mycobacterial infection of the CNS and also during non-pathogenic inflammation suggesting that recruitment of DCs to the CLNs is not antigen driven.

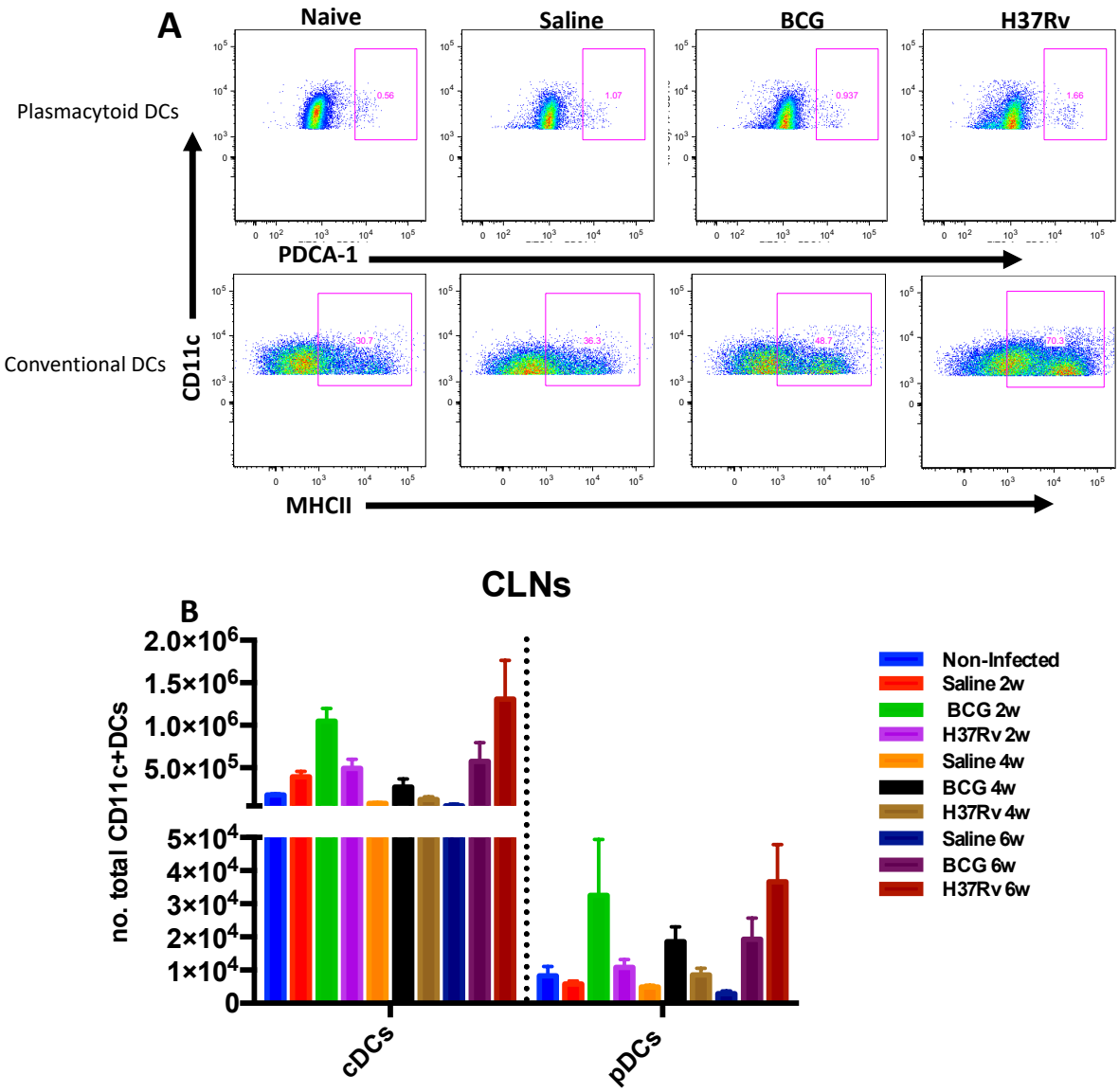


Figure 4.13: Conventional DCs preferentially recruited to the CLNs post *M. tuberculosis* H37Rv or BCG intracerebral infection. Wild-type female C57BL/6J mice (n=4-10 mice/group) were intracerebrally inoculated with saline, 1 x 10^5 CFUs of BCG or H37Rv *M. tuberculosis* and not inoculated (naïve mice). Mice were euthanized at weeks 2, 4 & 6 and immune cells analyzed by flow cytometry. **(A)** Flow cytometry plots showing of conventional DCs (cDCs; CD11c+MHCII+) and plasmacytoid DCs (pDCs; CD11c+PDCA-1+) of mice post *M. tuberculosis* or BCG CNS infection. **(B)** cDCs preferentially recruited to the brain during CNS-TB. Graphs are representative of 3 independent experiments for BCG and 2 independent experiments for H37Rv. Data represents the mean +/- SEM.

4.2.6.7) Activated and mature DCs drain into CLNs post *M. tuberculosis* or BCG i.c. infection.

DCs in the CLNs have not been investigated during mycobacterial infection of the CNS have not yet been characterized. We then assessed the activation status of total DCs in the CLNs by the expression of MHCII (MHCII+DCs) during *M. tuberculosis* or BCG CNS infection because it has never been investigated. The number of MHCII+DCs from week 2 saline control mice were significantly higher ($p<0.01$) than naïve mice (Fig. 4.14A) and kinetics of saline inoculated mice showed decreases at week 4 and week 6 that were lower than week 2. During BCG CNS infection, the number of these week 2 saline control MHCII+DCs was significantly lower ($p<0.05$) than BCG infected mice but at weeks 4 and 6, there were no significant differences between the two groups (Fig. 4.14B). We observed a significant decrease in the number of cervical lymph node MHCII+DCs from week 2 to week 4 ($p< 0.05$) in BCG infected mice, week levels were the lowest compared to week 2 or week 6 post BCG infection. Whereas, *M. tuberculosis* infected mice presented with high numbers of MHCII+DCs in the CLNs at week 2 ($p<0.01$), week 4 ($p<0.05$) and week 6 ($p<0.0001$) compared to saline controls during CNS-TB (Fig. 14.4B). However, there were no kinetic changes in the number of these MHCII+DCs during CNS-TB but *M. tuberculosis* infected mice contained higher numbers of MHCII+DCs than BCG infected mice at week 4 ($p<0.01$) and week 6 ($p<0.05$) post infection. These findings suggest that activated DCs in the CLNs included those that drained from the brain during CNS-TB and that antigen specific DCs are activated. MHCII+DC levels were significantly high in *M. tuberculosis* infected mice compared to BCG infected mice at week 4 ($p<0.01$) and week 6 ($p<0.05$) during CNS-TB. We detected higher brain bacterial burdens in *M. tuberculosis* infected mice compared to BCG and it has been suggested the higher numbers of bacteraemia in the lymph nodes play a role in determining disease outcome during pulmonary TB (Reiley et al., 2008). Also, infected DCs have been shown to transfer antigens to bystander uninfected DCs for presentation to T cells (Srivastava et al, 2014). It was not surprising that virulence was a factor between activated DCs in the CLNs between *M. tuberculosis* infection and BCG during mycobacterial infection of the CNS because of robust immune responses induced by a virulent strain.

Expression of CD86 by DCs in the CLNs has never been investigated during *M. tuberculosis* or BCG CNS infection. When we measured the number of DCs expressing CD86 (CD86+DCs), naïve mice expressed significantly higher ($p<0.01$) levels of CD86 than saline control mice 2 weeks post infection (Fig. 4.14C), this is not expected and could be a result of

technical or experimental error. The number of CD86+DCs from saline control mice did not show any kinetic change post inoculation at all time points (Fig. 4.14C). BCG intracerebral infection did not result in significant increase in the number of CD86+DCs in the CLNs compared to saline control mice at week 2 and week 4 but was significantly higher ($p<0.01$) at week 6 post infection (Fig. 4.14B). When we looked at the number of CD86+DCs during BCG i.c. infection, we found a significant increase ($p<0.01$) only from week 4 to week 6 post infection. During CNS-TB, *M. tuberculosis* infected mice expressed significantly higher numbers of CD86+DCs at week 4 ($p<0.05$) and week 6 ($p<0.0001$) post intracerebral infection (Fig. 4.14C). Additionally, the number of CD86+DCs in the CLNs during CNS-TB showed increasing kinetics from week 2 to week 4 ($p<0.05$) and from week 2 to week 6 ($p<0.001$) post infection. Finally, there was no difference in the number of recruited CD86+DCs in the CLNs between *M. tuberculosis* and BCG intracerebral infection suggesting that virulence was not a factor during mycobacterial infection of the CNS.

CCR7 upregulation is induced in trafficking of DCs to lymph nodes (Khader et al, 2006) and our data showed that recruited brain DCs expressed high numbers of CCR7 like previous studies (Clarkson et al., 2017; Louveau et al., 2018). To identify these migratory recruited brain DCs, we determined the expression of CCR7+DCs in the CLNs during mycobacterial infection. Naïve mice presented with twice as higher numbers of CCR7+DCs in the CLNs compared to the low numbers in week 2 saline inoculated mice (Fig. 4.14D). Surprisingly, we found that these week 2 saline inoculated mice presented with equivalent numbers of CCR7+DCs as those from *M. tuberculosis* or BCG i.c. infected mice (Fig. 4.14D). This may suggest that during week 2 inflammatory responses resulting from mycobacterial infection of the CNS had not led to significant trafficking of DCs to CLNs instead only injury driven. *M. tuberculosis* i.c. infected mice presented with significantly increased numbers of CCR7+DCs compared to saline control mice at week 4 ($p<0.01$) and week 6 ($p<0.01$) with a significant kinetic increase observed from week 2 to week 6 ($p<0.05$) during CNS-TB (Fig. 4.14D). Whereas, BCG i.c infected mice also presented with similar findings as during CNS-TB, wherein they presented with significantly increased numbers of CCR7+DCs compared to saline control mice at week 4 ($p<0.01$) and week 6 ($p<0.01$) with a significant kinetic increase observed from week 2 to week 6 ($p<0.05$) post infection. The observed mature DC phenotypes we observed in the brain and CLNs during mycobacterial infection of the CNS are characteristic of cells that induce protective adaptive T cell immune responses against mycobacteria, suggesting they contribute to immunity.

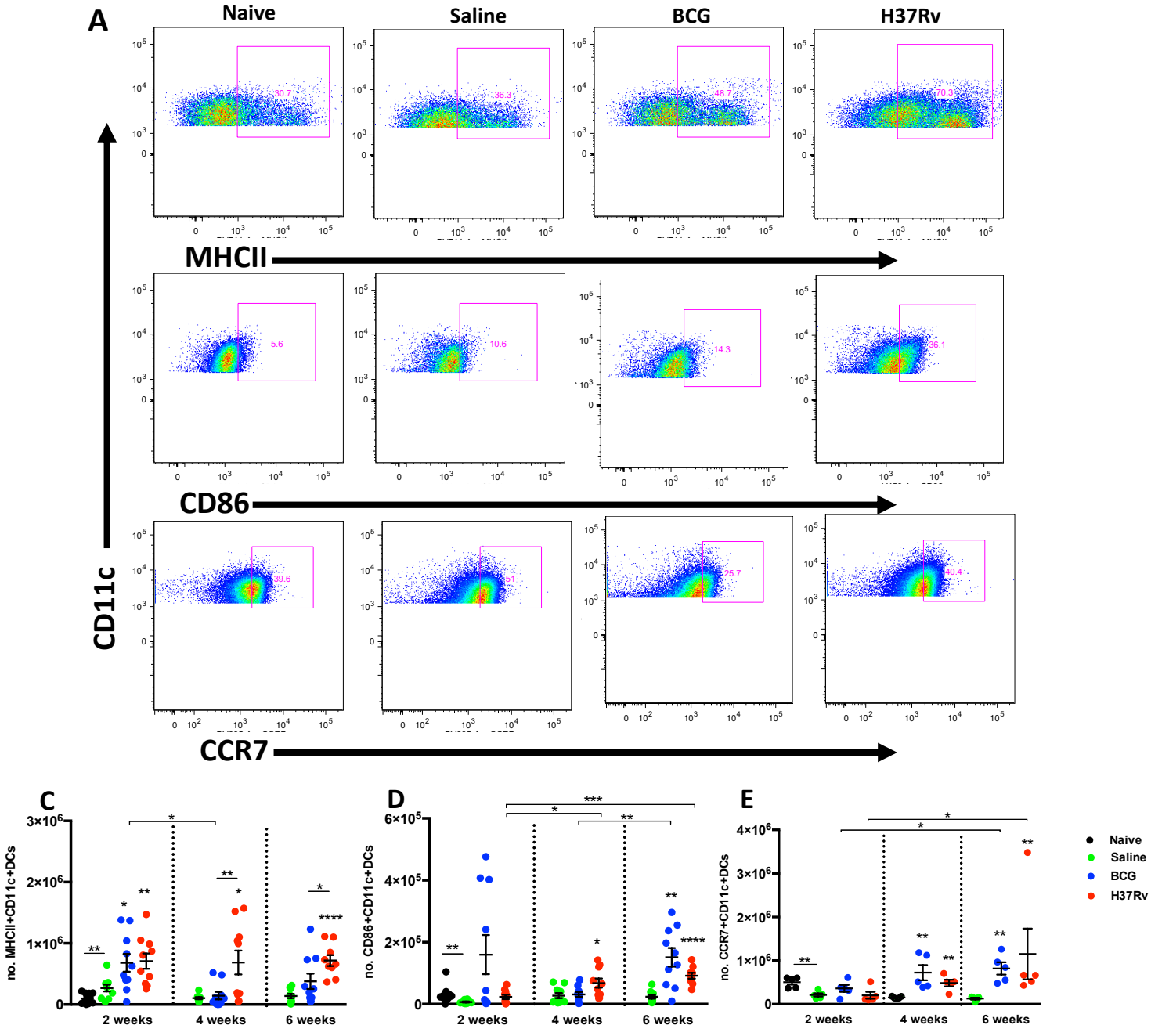


Figure 4.14: Activated and mature DCs drain into CLNs *post M. tuberculosis* H37Rv or BCG intracerebral infection.

Wild-type female C57BL/6J mice (n=4-5 mice/group) were intracerebrally inoculated with saline, 1 x 10^5 CFUs of BCG or H37Rv *M. tuberculosis* and not inoculated (naïve mice). Mice were euthanized at weeks 2, 4 & 6 and immune cells analyzed by flow cytometry.(A) Flow cytometry plots showing MHCII+, CD86+ and CCR7+ DCs in the CLNs post *M. tuberculosis* or BCG CNS infection. (B) MHCII+ DCs in BCG and *M. tuberculosis* infected mice. (C) CD86+ DCs during CNS-TB. (D) CCR7+ DCs in the CLNs. Graphs are representative of 3 independent experiments for BCG and 2 independent experiments for H37Rv. Data represents the mean +/- SEM. *p<0.05, **p<0.01 vs saline controls. Mann-Whitney test.

4.2.6.8) Increased numbers of CLN DCs expressed proinflammatory and anti-inflammatory cytokines post *M. tuberculosis* or BCG i.c. infection.

Proteins from CSF have been shown to drain into CLNs (Harling-Berg et al., 1989; Yamada et al., 1991) such as cytokines. The production of cytokines by CLNs during CNS-TB has not yet been elucidated. We measured the number of DCs expressing cytokines in the CLNs during mycobacterial infection of the CNS to determine the functionality of DCs as professional APCs. Figure 4.15A shows representative flow plots of cytokines IL-1β, IL-12, TNFα and IL-10 expressed by DCs in the CLNs during mycobacterial infection of the CNS. The cytokine profiles of CLN DCs during CNS-TB has not been extensively characterized.

The production of IL-1β is a protective proinflammatory cytokine against pulmonary TB (Mayer-Barber et al., 2011). The number of DCs in the CLNs expressing IL-1β (IL-1β+DCs) of saline inoculated mice were lower compared to naïve mice but not significant (Fig. 4.15B). Additionally, there were no changes in the number of IL-1β+DCs from saline inoculated mice among all experimental time points. Surprisingly, BCG infected mice did not present with high numbers of IL-1β+DCs compared to saline inoculated mice during mycobacterial infection as expected and no kinetic changes were observed during infection (Fig. 4.15B). It was only during CNS-TB that *M. tuberculosis* infected mice showed significantly higher numbers of IL-1β+DCs in the CLNs at week 2 ($p<0.05$), week 4 ($p<0.05$) and week 6 ($p<0.0001$) post infection compared to saline inoculated mice. Also, virulence was not a factor in the number of IL-1β+DCs in the CLNs between BCG infected and *M. tuberculosis* infected mice.

IL-12 produced in the CLNs by innate cells contributes to the induction of Th1 immune responses during pulmonary TB (Lozza et al., 2014; Wlodarczyk et al., 2015). When we compared 3 experimental groups, naïve mice, week 2 saline inoculated mice and week 2 BCG infected mice, we found no differences in the number of DCs expressing IL-12 (IL-12+) in the CLNs among the three groups (Fig. 4.15C). Whereas, BCG infected mice presented with significantly high numbers of IL-12+DCs only at week 6 ($p<0.001$) post infection compared to saline control mice. Additionally, we observed no kinetic changes in the number of IL-12+DCs of both saline inoculated mice and BCG infected mice among measured time points. It was interesting to find that that it was only during CNS-TB that the numbers of IL-12+DCs in the CLNs were significantly higher in *M. tuberculosis* infected mice at week 2 ($p<0.05$), week 4 ($p<0.0001$) except at week 6 post infection when compared to saline control mice (Fig.

4.15C). We also observed kinetic decreases in the number of IL-12+DCs in the CLNs during CNS-TB as infection progressed. There was a significant decrease from week 2 to week 4 ($p<0.001$) and from week 4 to week 6 ($p<0.001$) post *M. tuberculosis* infection. Virulence did not cause significant variation between *M. tuberculosis* responses and BCG responses.

TNFα production is associated with protective immunity against TB. We detected no differences in the number of DCs expressing TNFα (TNFα+DCs) in the CLNs among the 4 experimental groups; naïve mice, week 2 saline control mice, week 2 BCG infected mice and week 2 *M. tuberculosis* infected mice (Fig. 4.15D). However, we found significantly high numbers of TNFα+DCs in the CLNs at week 4 ($p<0.001$) and week 6 ($p<0.05$) in BCG infected mice compared to saline controls. These TNFα+DCs numbers significantly increased from week 2 to week 4 ($p<0.0001$) then decreased from week 4 to week 6 ($p<0.05$) post infection (Fig.15D). While during CNS-TB, the number of TNFα+DCs in the CLNs was significantly higher than saline control mice at week 4 ($p<0.0001$) post *M. tuberculosis* infection (Fig. 4.15D), this was accompanied by significant increase in the number of TNFα+DCs from week 2 to week 4 post infection.

IL-10 dampens immune responses as an anti-inflammatory cytokine. Surprisingly, naïve mice presented with higher numbers of IL-10+DCs compare to week 2 saline control mice (Fig. 4.15E), these numbers were even higher than those of mycobacterial infection at week 2 post infection. While saline inoculated mice showed no kinetic changes at all experimental time points even though the number of IL-10+DCs appeared high at week 6 post inoculation. BCG infected mice presented with significantly high numbers of IL-10+DCs ont at week 4 post infection when compared to saline inoculated mice (Fig. 4.15E), with no kinetic changes. During CNS-TB, we found significantly high numbers of IL-10+DCs in *M. tuberculosis* infected mice at week 2 ($p<0.05$) and week 4 ($p<0.001$) but not week 6 post infection (Fig. 4.15E). This was accompanied by a significant kinetic decrease in the number of IL-10+DCs from week 2 to week 4 ($p<0.01$) followed by an extremely large significant increase from week 4 to week 6 ($p<0.001$) during CNS-TB. Finally, virulence caused a significant higher immune response in *M. tuberculosis* infected mice than BCG infected mice at week 6 ($p<0.001$) post infection (Fig. 4.15E).

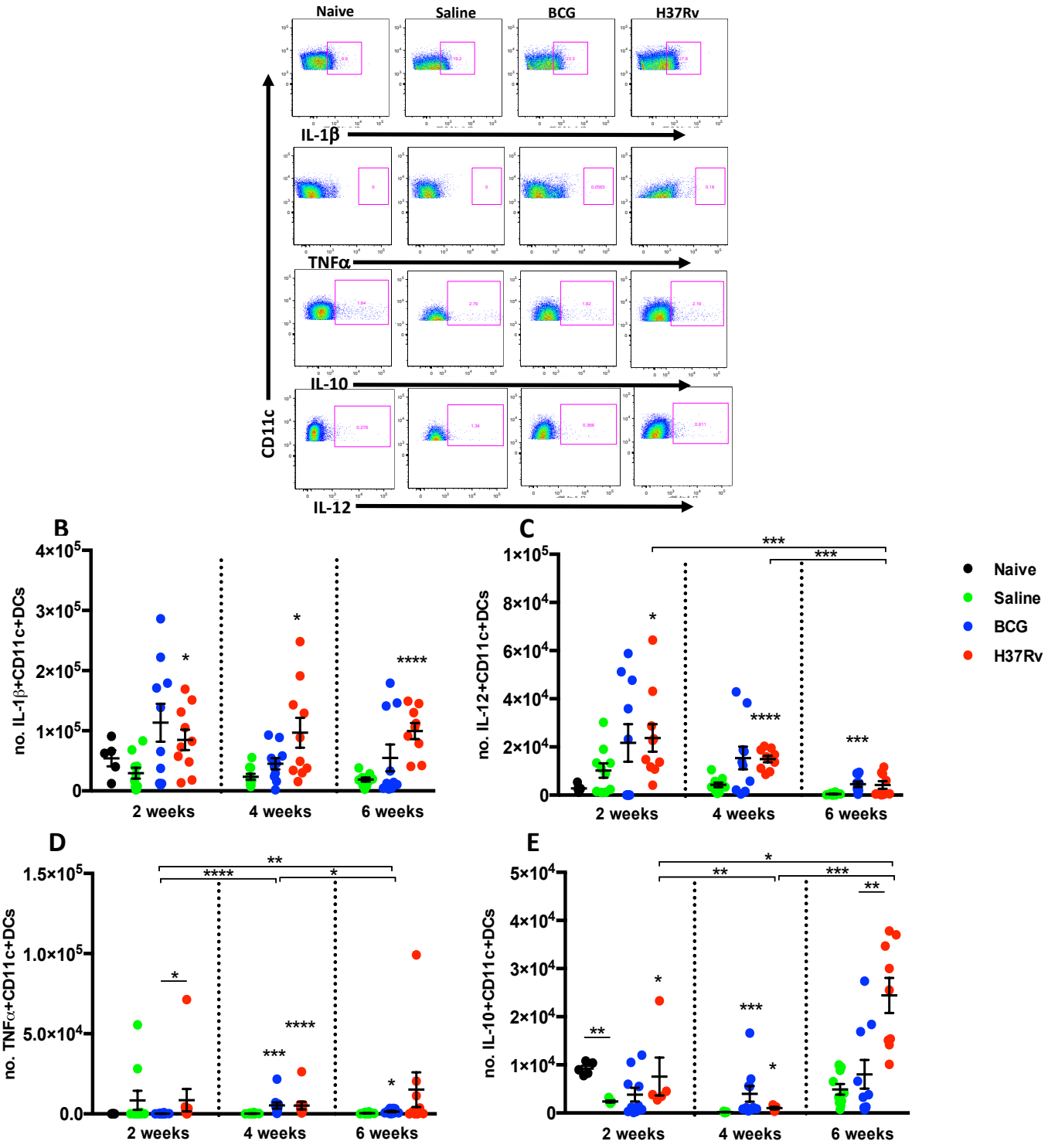


Figure 4.15: Increased numbers of CLN DCs expressed proinflammatory and anti-inflammatory cytokines associated with Th1 immunity during mycobacterial infection of the CNS. Wild-type female C57BL/6J mice (n=4-10 mice/group) were intracerebrally inoculated with saline, 1 x 10^5 CFUs of BCG or H37Rv *M. tuberculosis* and not inoculated (naïve mice). Mice were euthanized at weeks 2, 4 & 6 and immune cells analyzed by flow cytometry. **(A)** Flow cytometry gating of IL-1β, TNFα, IL-10 and IL-12 expressed by DCs in CLNs. **(B)** Proinflammatory cytokines **(B)** IL-1β, **(C)** IL-12, **(D)** TNFα and **(E)** anti-inflammatory IL-10 by DCs post *M. tuberculosis* or BCG CNS infection. Graphs are representative of pooled data from 2 independent experiments. Data represents the mean +/- SEM. *p<0.05, **p<0.01,***p<0.001, ****p<0.0001 vs saline controls.

APCs must present an abundant amount of bacterial antigen to T cells in the draining lymph nodes to reach a threshold capable of activating specific CD4+ T cell immune responses (Wolf et al., 2008). In this study, CNS inflammation that was caused by intracerebral infection with *M. tuberculosis* H37Rv and BCG caused immune responses that resulted in recruitment, expansion and activation of mature APCs, with DCs migrating to cervical lymph nodes.

4.3) Regulated Th1 immune responses induced during mycobacterial infection of the CNS.

T cells survey a healthy CNS (Ousman and Kubes, 2012) and CNS insult results in the recruitment of naïve bystander T cells to the brain (Krakowski and Owens, 2000), whether it is caused by infection or injury. Studies have shown that T cells infiltrate the CNS post BCG infection (Matyszak and Perry, 1996; Lee et al., 2009) and H37Rv *M. tuberculosis* infection (Hernandez et al., 2016; Hsu et al., 2017). However, these recruited brain T cells have not been fully characterized in context to CNS-TB and how they contribute to disease outcome. T cell responses are critical for *M. tuberculosis* control (Feldman et al., 1938; Srivastava et al., 2016). Therefore, our objective was to evaluate adaptive cellular mediated immune responses by investigating the phenotype and functional profile of T cells that are recruited to the brain and cervical lymph nodes post H37Rv *M. tuberculosis* or BCG intracerebral infection. This was achieved by flow cytometric analysis of T cells cells from the brains and cervical lymph nodes of H37Rv *M. tuberculosis* or BCG intracerebrally infected wild-type C57BL/6J mice.

4.3.1) Predominant influx and accumulation of CD4+ T cells than CD8+ T cells to the brain post *M. tuberculosis* or BCG i.c. infection.

Our first aim was to determine the recruitment kinetics of T cells to the brains of wild-type C57BL/6J mice post H37Rv *M. tuberculosis* or BCG intracerebral (i.c.) infection using flow cytometry. Figure 4.1A shows the representative flow cytometry plots for the gating strategy of brain CD4+ (CD3+CD4+) T cells and CD8+ (CD3+CD8+) T cells post H37Rv or BCG intracerebral infection. There was significant influx in the number of CD4+ and CD8+ T cells to the brains of both H37Rv *M. tuberculosis* and BCG infected mice post i.c. infection, with the dominant population being CD4+ T cells (Fig. 4.16B and C). There was minimal recruitment in the number of CD4+ T cells to the brains of week 2 saline inoculated mice that was significantly higher ($P<0.01$) than those in naïve mice (Fig. 4.16B). The numbers of CD4+ T cells from saline inoculated mice did not significantly increase from week 2 to week 4 but

significantly decreased ($p<0.01$) from week 4 to week 6, however; week 6 CD4+ T cells were significantly lower ($p<0.01$) in numbers compared to week 2 (Fig. 4.16B). While we observed significantly higher numbers of CD4+ T cells recruited to the brains of BCG i.c. infected mice at week 2 ($p<0.05$) and week 6 ($p<0.01$) as compared to saline inoculated mice but no differences at week 4 (Fig. 4.16B). This was accompanied by significant kinetic increases from week 2 to week 4 ($p<0.05$) and from week 2 to week 6 ($p<0.01$) post infection. And then during CNS-TB, CD4+ T cell recruitment to the brains of H37Rv *M. tuberculosis* infected mice was significantly higher than those in saline control mice at week 2 ($p<0.01$) and week 4 ($p<0.01$) and these levels significantly decreased ($p<0.05$) from week 2 to week 6 post infection (Fig. 4.16B). The number of CD4+ T cells that were recruited to the brains of H37Rv *M. tuberculosis* infected mice were significantly higher than those of BCG infected mice at week 2 ($p<0.01$), week 4 ($p<0.05$) and week 6 ($p<0.05$) post intracerebral infection (Fig. 4.16B). These findings suggest that virulence was a factor and that immune responses were affected differently by the strain of mycobacteria utilized for CNS infection.

We found that CD8+ T cells were also recruited to the brain post H37Rv *M. tuberculosis* and BCG intracerebral infection (Fig. 16C). The number of CD8+ T cells recruited to the brains of saline inoculated mice were significantly higher ($p<0.01$) than naïve mice (Fig. 4.16C), and these numbers significantly decreased from week 4 to week 6 ($p<0.01$) and also from week 2 to week 6 ($p<0.01$) post infection. Brains of BCG infected mice showed a higher recruitment in the number of CD8+ T cells than saline control mice at week 2 ($p<0.01$) and week 6 ($p<0.01$) post infection but not at week 4 (Fig. 4.16C). This was accompanied by significant kinetic increases from week 2 to week 4 ($p<0.01$) and from week 2 to week 6 ($p<0.01$) post infection. Whereas, the number of CD8+ T cells recruited to the brain during CNS-TB were significantly higher than saline inoculated mice throughout the entire course of infection (Fig. 4.16C). H37Rv *M. tuberculosis* infected mice showed significantly higher numbers of CD8+ T cells recruited to the brain than saline control mice at week 2 ($p<0.01$), week 4 ($p<0.05$) and week 6 ($p<0.01$) post infection, with kinetic increase only observed from week 2 to week 4 ($p<0.05$) post infection. Finally, the number of CD8+ T cells from H37Rv *M. tuberculosis* infected mice were significantly higher than those from BCG infected mice at week 2 ($p<0.01$), week 4 ($p<0.01$) and week 6 ($p<0.05$) post intracerebral infection (Fig. 4.16C).

CD4+ T cells and not CD8+ T cells were dominantly recruited to the brains of *M. tuberculosis* and BCG infected mice post intracerebral infection. Taken together, these findings suggest that

virulence was a factor for in the recruitment of both brain CD4+ T cells and CD8+ T cells because H37Rv *M. tuberculosis* infected mice presented with higher numbers of T cells compared to BCG infected mice throughout the entire course of CNS infection. This clearly indicates that immune responses were affected differently by the strain of mycobacteria utilized for CNS infection.

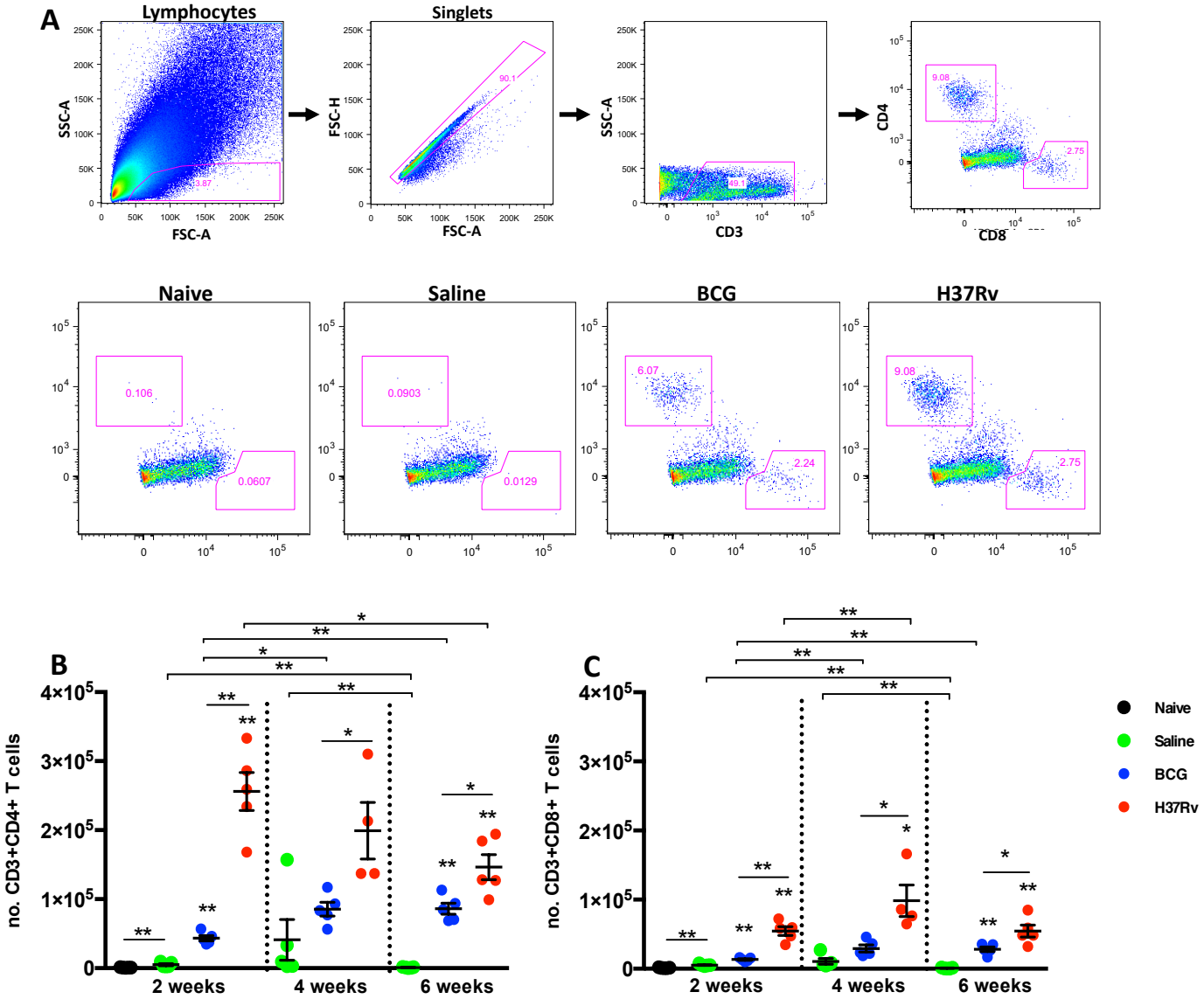


Figure 4.16: Infiltration of dominantly CD4+ T cells than CD8+ T cells to the brain post *M. tuberculosis* H37Rv and BCG intracerebral infection. (A, D) Wild-type female C57BL/6J mice (n=4-5 mice/group) were intracerebrally inoculated with saline, 1 x 10^5 CFUs of BCG or H37Rv *M. tuberculosis* and not inoculated (naïve mice). Mice were euthanized at weeks 2, 4 & 6 and immune cells analyzed by flow cytometry**. (A)** Flow cytometry gating of brain CD4+ (CD3+CD4+), CD8+ (CD3+CD4+) T cells of different groups post *M. tuberculosis* and BCG CNS infection. Graphs are representative of 3 independent experiments for BCG and 2 independent experiments for H37Rv. Data represents the mean +/- SEM. *p<0.05, **p<0.01 vs saline controls. Mann-Whitney test.

4.3.2) Predominant influx and accummulation of effector CD4+ T cells and effector CD8+ T cells to the brain of *M. tuberculosis* or BCG i.c. infected mice.

After characterisation of T cell subsets (CD4+ and CD8+ T cells) that were recruited to the brain, our second aim was to characterise the subpopulations of these brain T cells post H37Rv *M. tuberculosis* and BCG intracerebral i.c. infection to help us understand which T cell immune responses were elicited. CNS T cell subpopulations have not been characterised in context to CNS-TB. This was achieved by surface staining of naïve T cells (Tnaive), effector T cells (Teff) and central memory T cells (Tcm) by flow cytometry. All these three subpopulations got recruited to the brain during CNS inflammation. Representative flow cytometry plots of showing classification of the three T cell subpopulations; Tnaive (CD44-CD62L+), Teff (CD44+CD62L+) and Tcm (CD44+CD62L+) recruited to the brain during mycobacterial infection of the CNS (Fig. 4.17A).

For T cells to be able to carry out their effector functions, they must proliferate and become activated. With saline treated control mice, brain CD4+ T cells expressed low numbers for all the three subpopulations (Tnaive, Teff and Tcm) and this was only observed at week 4 post inoculation as compared to mycobacterial infection while negligible levels were observed at weeks 2 and 6 (Fig. 4.17B). Whereas, CD8+ T cells of control mice expressed negligible levels of all subpopulations at weeks 2, 4 and 6 (Fig. 4.17C). However, mycobacterial infection of the CNS with H37Rv *M. tuberculosis* or BCG resulted in the expansion of effector T cells for both CD4+ T cells and CD8+ T cells that were recruited to the brain post intracerebral infection (Fig. 417B and D). We determined the development of these effector T cells over time to have an indication of mycobacterial control in the brain post intracerebral infection. These findings showed kinetic increases of predominately effector T cells in the brains of H37Rv *M. tuberculosis* from 2 weeks to 4 weeks followed by a decrease at week 6 during CNS-TB (Fig. 4.17B and D), whereas BCG infected mice showed a kinetic increase throughout the course of infection (Fig. 4.17B and D). Evaluation of virulence showed that H37Rv *M. tuberculosis* infection resulted in more robust effector T cell immune responses than BCG CNS infection with the following fold changes observed for CD4+ T cells; 5-fold change at week 2, 2.5-fold change at week 4 and 1.7-fold change at week 6 post intracerebral infection (Fig. 4.17B). While CD8+ T cells showed a 5-fold change at week 2, 3.9-fold change at week 4 and 1.9-fold change at week 6 post infection (Fig. 4.17D).

We detected small to negligible numbers of Tnaive and Tcm cells that were expressed by brain CD4+ and CD8+ T cells of H37Rv *M. tuberculosis* or BCG infected mice compared to the high numbers of effector T cells (Fig. 4.17B and D). It was not surprising that mycobacterial infection resulted in expression of low numbers of naive T cells because brain infection attracts activated/effector T cells to the site of infection to fight off the infection. Also, negligible numbers of central memory T cells were detected in the brain as expected because not only is the brain not a secondary lymphoid organ but also only a few studies have reported on resident brain memory T cells (Steinbach et al., 2016; Szabo et al., 2019) during CNS infection. Taken together, the predominant subpopulation in the brain during mycobacterial infection of the CNS is effector T cells which are capable of promoting immunity against CNS-TB infection.

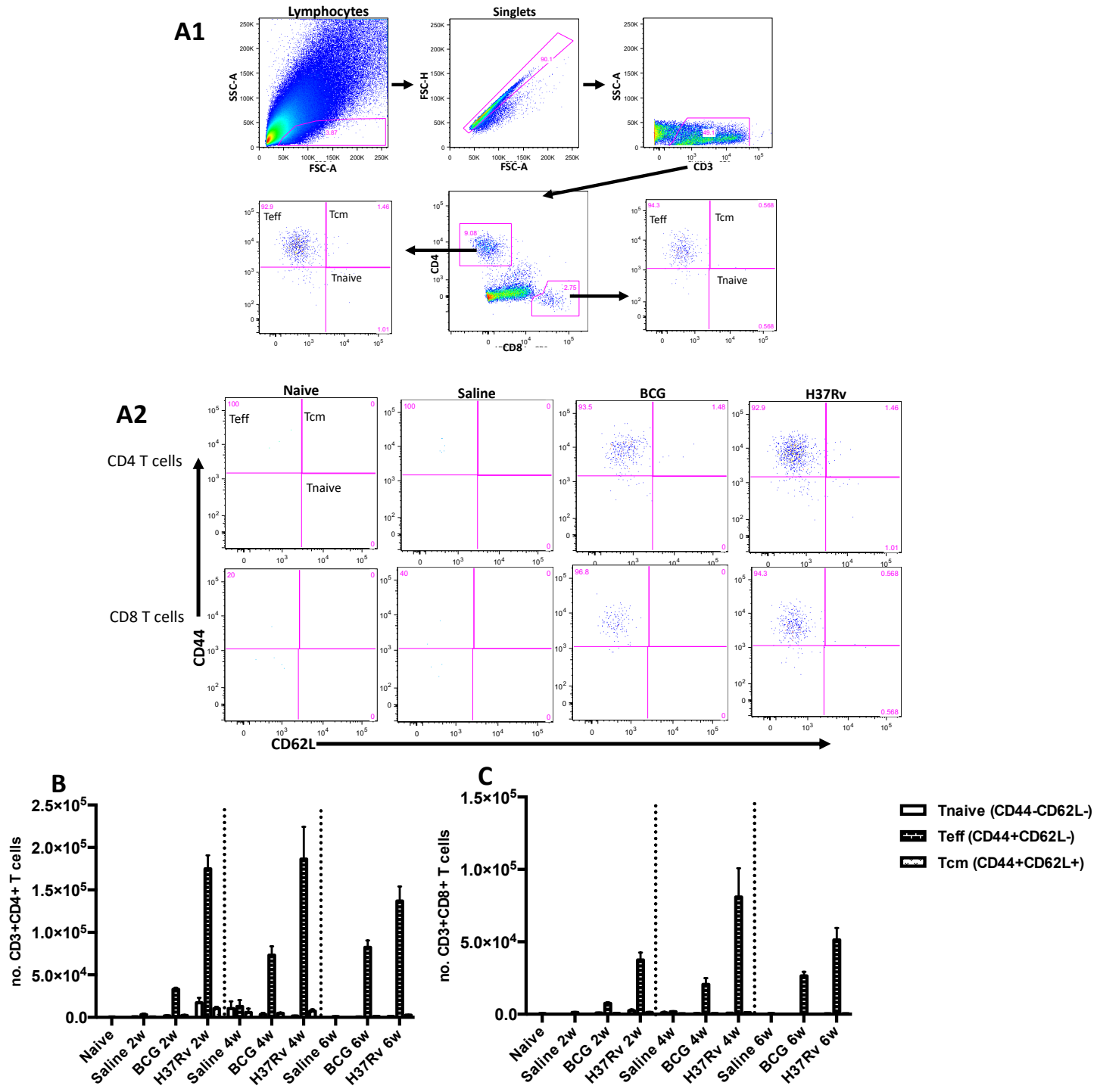


Figure 4.17: Predominant influx of effector T cells to the brain of post *M. tuberculosis* H37Rv or BCG intracerebral infection. Wild-type female C57BL/6J mice (n=4-5 mice/group) were intracerebrally inoculated with saline, 1 x 10^5 CFUs of BCG or H37Rv *M. tuberculosis* and not inoculated (naïve mice). Mice were euthanized at weeks 2, 4 & 6 and immune cells analyzed by flow cytometry**. (A)** Flow cytometry gating of three brain CD4+ and CD8+ T cell subpopulations [naïve T cells (Tnaive: CD44-CD62L+); effector T cells (Teff: CD44+CD62L-) and central memory T cells (Tcm: CD44+CD62L+)]. **(B)** Brain CD4+ T cell and **(C)** CD8+ T cell subpopulations infiltrating the brains of H37Rv *M. tuberculosis* and BCG infected mice. Graphs are representative of 3 independent experiments for BCG and 2 independent experiments for H37Rv.

4.3.3) Predominant influx and accumulation of CD4+ T cells than CD8+ T cells to the CLNs post *M. tuberculosis* or BCG i.c. infection.

During infection, naïve T cells get recruited to the secondary lymph nodes where the innate adaptive crosstalk occurs for induction of adaptive immune responses that determine infection outcome. CNS antigens drain into CLNs, where T cells proliferate and get activated by APCs and we detected CD4+ and CD8+ T cell recruitment to the brain during mycobacterial infection of the CNS, suggesting they these T cells possibly originated from CLNs. Our aim was to determine the recruitment of T cells to the CLNs post H37Rv *M. tuberculosis* and BCG intracerebral infection using flow cytometry.

Figure 4.18A shows representative flow cytometry plots gated on CD4+ T cells (CD3+CD4+) and CD8+ (CD3+CD8+) T cells in the CLNs of H37Rv *M. tuberculosis* mice and BCG intracerebrally infected mice. There were no significant differences in the number of CD4+ and CD8+ T cells that were recruited to the CLNs between week 2 saline control mice and naïve mice (Fig. 4.18B and D). Additionally, no kinetic changes were observed in the levels of CD4+ and CD8+ T cells of saline control mice. Whereas, the number of CD4+ T cells and CD8+ T cells that were recruited to the CLNs during mycobacterial infection of the CNS were significantly higher compared to saline control mice during the entire course of infection (Fig. 4.18B and C). With CD4+ T cells accumulating in the CLNs of BCG i.c. infected mice being significantly higher ($p<0.01$) than those of saline control mice at weeks 2, 4 and 6 post infection (Fig. 4.18B). The number of these CD4+ T cells significantly increased from week 2 to week 4 ($p<0.01$), followed by no kinetic change between week 4 and week 6 post infection. However, a significant increase in the number of CD4+ T cells was observed from week 2 to week 6 ($p<0.01$) in the CLNs and CD4+ T cell numbers at weeks 4 and 6 were significantly higher than those at week 2 post BCG CNS infection. During CNS-TB, higher numbers of CD4+ T cells were recruited to the CLNs and accumulated (Fig. 4.18B), with *M. tuberculosis* infected mice presenting with significantly higher ($p<0.01$) numbers of CD4+ T cells at week 2, 4 and 6 post infection compared to saline control mice (Fig.18B). The number of these CD4+ T cells initially significantly increased from week 2 to week 4 ($p<0.05$) and were followed by a significant decrease from week 4 to week 6 ($p<0.05$) post *M. tuberculosis* i.c. infection. And finally, no differences were detected in the numbers CD4+ T cells in the CLNs between *M. tuberculosis* and BCG infected mice (Fig. 4.18B), suggesting virulence was not a factor.

BCG CNS infection also resulted in the recruitment and accumulation of CD8+ T cells in the CLNs (Fig.18C). CLNs of BCG infected mice presented with significantly higher ($p<0.01$) numbers of CD8+ T cells compared to saline control mice at weeks 2, 4 and 6 post intracerebral infection (Fig. 4.18C). The number of these CD8+ T cells significantly increased from week 2 to week 4 ($p<0.01$), followed by no kinetic change between week 4 and week 6 post BCG intracerebral infection. However, a significant increase in the number of CD8+ T cells was observed from week 2 to week 6 ($p<0.01$) in the CLNs and CD8+ T cell numbers at weeks 4 and 6 were significantly higher than those at week 2 post BCG CNS infection. This trend was similar to what we observed with CD4+ T cells in the CLNs. And during CNS-TB, the number of CD8+ T cells in the CLNs of *M. tuberculosis* infected mice were significantly higher ($p<0.01$) compared to saline control mice at weeks 2, 4 and 6 post infection (Fig. 4.18C), which showed a significantly kinetic increase ($p<0.01$) only from week 2 to week 4 post *M. tuberculosis* infection. And finally, no differences were detected between the number of CD8+ T cells in the CLNs of *M. tuberculosis* and BCG infected mice (Fig. 4.18C), suggesting virulence is not a factor.

Taken together, these findings show that there is an infiltration of CD4+ and CD8+ T cells in the CLNs during mycobacterial infection of the CNS, with CD4+ T cells being the dominant population. And this recruitment is not virulence driven.

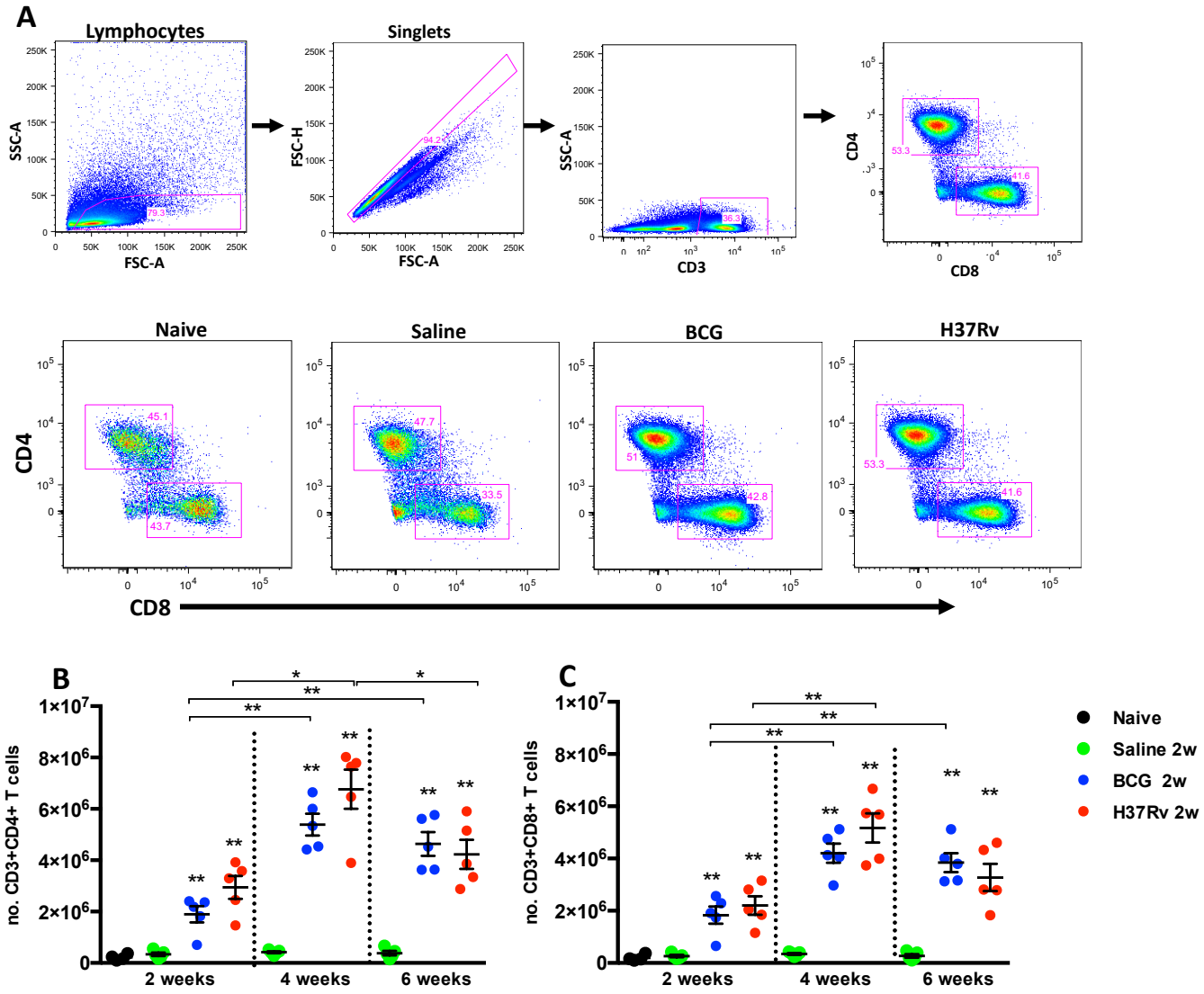


Figure 4.18: *M. tuberculosis* H37Rv and BCG intracerebral infection resulted in recruitment and expansion of CD4+ and CD8+ T cells in CLNs. Wild-type female C57BL/6J mice (n=4-5 mice/group) were intracerebrally inoculated with saline, 1 x 10^5 CFUs of BCG or H37Rv *M. tuberculosis* and not inoculated (naïve mice). Mice were euthanized at weeks 2, 4 & 6 and immune cells analysed by flow cytometry**.** Flow cytometry gating of CLNs **(A)** CD4+ (CD3+CD4+), CD8+ (CD3+CD4+) T cells post *M. tuberculosis* and BCG i.c infection. **(B)** High CD4+ and **(C)** CD8+ T cells accumulated in CLNs. Graphs are representative of 3 independent experiments for BCG and 2 independent experiments for H37Rv. Data represents the mean +/- SEM. *p<0.05, **p<0.01 vs saline controls. Mann-Whitney test.

4.3.4) Predominant influx and accumulation of effector CD4+ T cells than CD8+ T cells in the CLNs post *M. tuberculosis* or BCG i.c. infection.

T cells are primed by DCs in the draining lymph nodes leading to expansion and induction of effector T cell polarization and generation of memory T cells (Lozza et al., 2014). We showed that mature brain DCs post H37Rv *M. tuberculosis* or BCG intracerebral infection were capable of trafficking CNS mycobacterial antigens to the CLNs through migratory CCR7 expression. We also detected mainly mature DCs (CCR7+MHCII+CD86+) in the CLNs. These DCs could present antigens to CD3+ T cells through MHCII+ expression, and signal T cell co-stimulation by the binding of dendritic cell CD86 molecule to CD28 ligand on T cells leading to activation of effector T cells. These activated T cells migrate to the site of infection to perform their specific effector functions and we found mainly effector CD4+ and CD8+ T cells in the brain. Therefore, our aim was to assess the functionality of the CD4+ and CD8+ T cells that were recruited to the CLNs by determining the different T cell subsets that were present during mycobacterial infection of the CNS. This was achieved by surface staining of naïve T cells (Tnaive), effector T cells (Teff) and central memory T cells (Tcm) by flow cytometric analysis of wild-type C57BL/6J mice post *M. tuberculosis* or BCG intracerebral infection.

Representative flow cytometry plots of showing classification of the three T cell subpopulations; Tnaive (CD44-CD62L+), Teff (CD44+CD62L+) Tcm (CD44+CD62L+) in the CLNs during mycobacterial infection of the CNS (Fig. 4.19A). Both naïve mice and saline inoculated mice expressed low to negligible numbers of CD4+ T cell subpopulations in the CLNs at all time points compared to those observed during mycobacterial infection of the CNS (Fig. 4.19B). We detected the highest number of naïve CD4+ T cells as the dominant T cell subpopulation expressed in the CLNs of *M. tuberculosis* or BCG infected mice, a phenomenon that was apparent throughout the course of infection (Fig. 4.19B). The second highest number of CD4+ T cell subpopulation in the CLNs was effector T cells and the lowest was central memory T cells, which were evident at week 4 post *M. tuberculosis* or BCG intracerebral infection. This data showed that during mycobacterial infection of the CNS, CLNs present with activated T cells that are equipped with performing specific effector functions.

Similar to what we observed with CD4+ T cells, we found low to negligible numbers of CD8+ T cells expressed by naïve mice and saline inoculated mice compared to *M. tuberculosis* or BCG infected mice at all time points (Fig. 4.19B and C). However, unlike CD4+ T cells that expressed naïve T cells as the highest subpopulation during mycobacterial infection of the

CNS, effector T cells were the highest subpopulation expressed by CD8+ T cells in the CLNs throughout the course of infection (Fig.19B and 19C). The number of naïve CD8+ T cells was high in M. tuberculosis or BCG intracerebrally infected mice compared to saline inoculated mice and the second highest subpopulation in the CLNs (Fig. 4.19C). Interestingly, these naïve CD8+ T cells decreased in numbers at week 6 post *M. tuberculosis* or BCG intracerebral infection as compared to weeks 2 and 4. The number of central memory CD8+ T cells was a evidently pronounced at week 4 during mycobacterial infection of the CNS as compared to other time points (Fig. 4.19C).

Taken together, these findings suggest that mycobacterial infection of the CNS results in the activation and expansion of effector CD4+ and CD8+ T cells in the CLNs which potentially favour antimycobacterial adaptive immune responses.

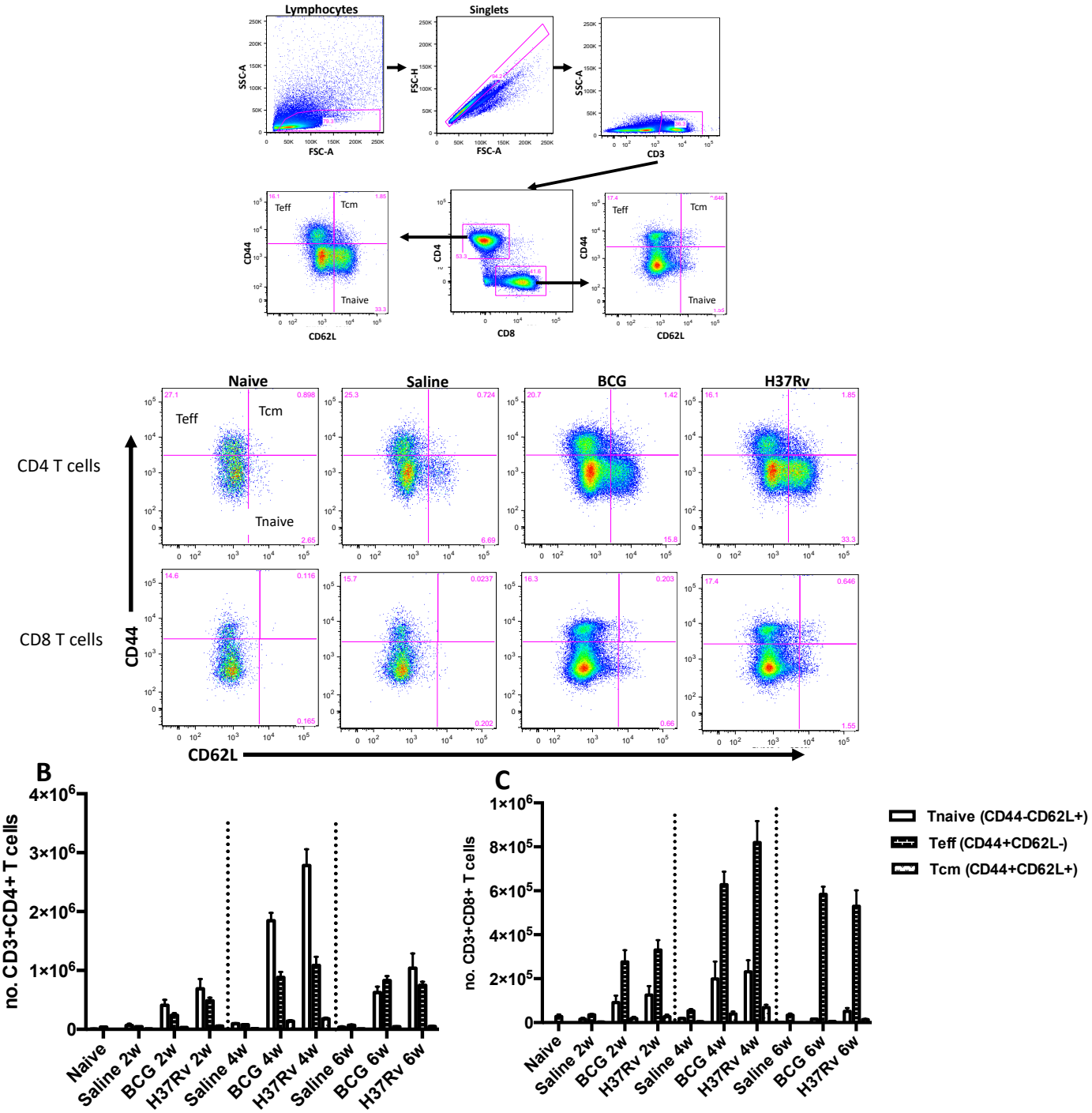


Figure 4.19: Expansion of effector CD4+ T cells and CD8+ T cells in the CLNs post *M. tuberculosis* H37Rv or BCG intracerebral infection. Wild-type female C57BL/6J mice (n=4-5 mice/group) were intracerebrally inoculated with saline, 1 x 10^5 CFUs of BCG or H37Rv *M. tuberculosis* and not inoculated (naïve mice). Mice were euthanized at weeks 2, 4 & 6 and immune cells analyzed by flow cytometry. **(A)** Flow cytometry gating of CD4+ T cell subsets naïve T cells (Tnaive: CD44+CD62L-), effector T cells (Teff; CD44+CD62L+) and central memory T cells (Tcm: CD44-CD62L) and subsets of different groups post *M. tuberculosis* or BCG CNS infection. **(B)** CD4+ T cells in CLNs post infection**.** Representative graph of 3 independent experiments for BCG and 2 independent experiments for H37Rv.

4.3.4) Increased numbers of recruited brain T-bet+CD4+ T cells and T-bet+CD8+ T post *M. tuberculosis* or BCG i.c. infection.

We already showed that majority of the cells that were recruited to the brain were effector CD4+ T cells and CD8+ T cells. During pulmonary *M. tuberculosis* infection, a Th1 immune response is desirable to illicit a dominant TB specific T cell immune response (Lozza et al., 2014; Wlordarczyk et al., 2015). Unlike systemic circulation, the CNS is immune privileged suggesting some levels of regulatory T cells are also required to prevent excessive neuroinflammation. Both the activation of T cells and T helper cell differentiation depend on changes in gene expression, which is regulated by transcription factors (TFs) during immune responses (Liberman et al., 2003; Brown et al., 2019). However, the T cell transcription factor profiles that dominate during mycobacterial infection of the CNS have not been fully characterized. This is why we investigated the transcription factor (TF) profile of C57BL/6J mice post H37Rv *M. tuberculosis* and BCG intracerebral infection using flow cytometry. We measured the expression of TF that represent an array of various T-helper (Th) immune responses; namely T-bet (Th1 TF), GATA3 (Th2 TF), RORγT (Th17 TF) (Littman and Rudensky, 2010; Kanno et al., 2012) and FoxP3 (Treg TF) (Hickman et al., 2002; Liu et al., 2006; Sáenz et al., 2013) during mycobacterial infection of the CNS.

Figure 4.20A shows the representative flow cytometry plots of the gating strategy for T cell TFs (Fig. 20A1) and gating of the number of T cells (CD4+ T cells and CD8+ T cells) expressing T-bet and FoxP3 (Fig. 4.20-A2 and A3) during mycobacterial infection of the CNS. The brains of naïve mice contained low numbers of CD4+ and CD8+ T cells and this led to undetectable levels of TFs (Fig. 4.20B and C). Increased numbers of recruited brain CD4+ T cells and CD8+ T cells from *M. tuberculosis* or BCG infected mice expressed high T-bet (T-bet+CD4+ T cells and T-bet+CD8+ T cells) throughout the course of infection compared to saline inoculated mice (Fig. 4.20B and C). Among the four TFs (T-bet, GATA3, RORγT and FoxP3), the highest number of recruited brain CD4+ T cells and CD8+ T cells expressed T-bet than any other TF during mycobacterial infection of the CNS (Fig. 4.20B and C). Unlike mycobacterial infection, we observed low to negligible numbers of T-bet+CD4+ T cells and T-bet+CD8+ T cells from saline inoculated mice as expected from controls because there was no infection by any pathogen (Fig. 4.20B and C). We found negligible to no numbers of recruited brain CD4+ T cells or CD8+ T cells expressing GATA3 (GATA3+CD4+ T cells and GATA3+CD8+ T cells), Th2 TF post *M. tuberculosis* or BCG intracerebral infection (Fig. 20B

and C). Additionally, there were low and negligible numbers of recruited brain CD4+ T cells and CD8+ T cells that expressed RORγT (RORγT+CD4+ T cells and RORγT+CD8+ T cells), a Th17 TF during CNS-TB but not post BCG intracerebral infection (Fig. 4.20B and C). While CD4+ T cells and CD8+ T cells from saline inoculated mice mainly presented with an undetectable number of cells expressing RORγT when compared to CNS-TB mice.

4.3.5) Increased numbers of recruited brain FoxP3+CD4+ T cells during CNS-TB.

During CNS-TB, medium numbers of recruited brain CD4+ T cells expressed FoxP3 (Foxp3+CD4+ T cells), a regulatory TF post *M. tuberculosis* i.c. infection which was between 1-3 folds lower than T-bet+CD4+ T cells throughout the course of infection (Fig. 4.20B). However, there were no number of CD4+ T cells nor CD8+ T cells from BCG i.c. infected mice that expressed FoxP3. Increased numbers of recruited brain CD8+ T cells only expressed high T-bet and no other TFs (GATA3, RORγT and FoxP3) in BCG i.c. infected mice, while *M. tuberculosis* infected mice also showed extremely low to undetectable numbers of CD8+ T cells expressing FoxP3 and RORγT (Fig. 4.20C). The expression of T-bet in the brain was virulence driven in terms of the magnitude of immune responses induced because higher numbers of *M. tuberculosis* infected mice expressed T-bet compared to BCG infected mice. T-bet is needed for resistance against tuberculosis and is a crucial immune cell transcription factor for the development of Th1 responses in both innate and adaptive immunity (Sullivan et al., 2005; Stolarczyk et al., 2014). Our findings showed dominant T-bet+ CD4+ T cells and T-bet+CD8+ T cells in the brain during mycobacterial infection of the CNS suggesting induction of immunity against *M. tuberculosis* or BCG i.c. infection. Our data showed increased numbers brain recruited Foxp3+CD4+ T cells and little RORγT+CD4+ T cells. In conclusion, the TF profile we detected of recruited brain DCs during CNS-TB suggests that polarization of T cells towards regulated Th1 immune responses was favoured, which are protective against *M. tuberculosis* and BCG infection. These findings attest that CD4+ T cells and CD8+ T cells contributed to the controlled bacterial loads in the brain.

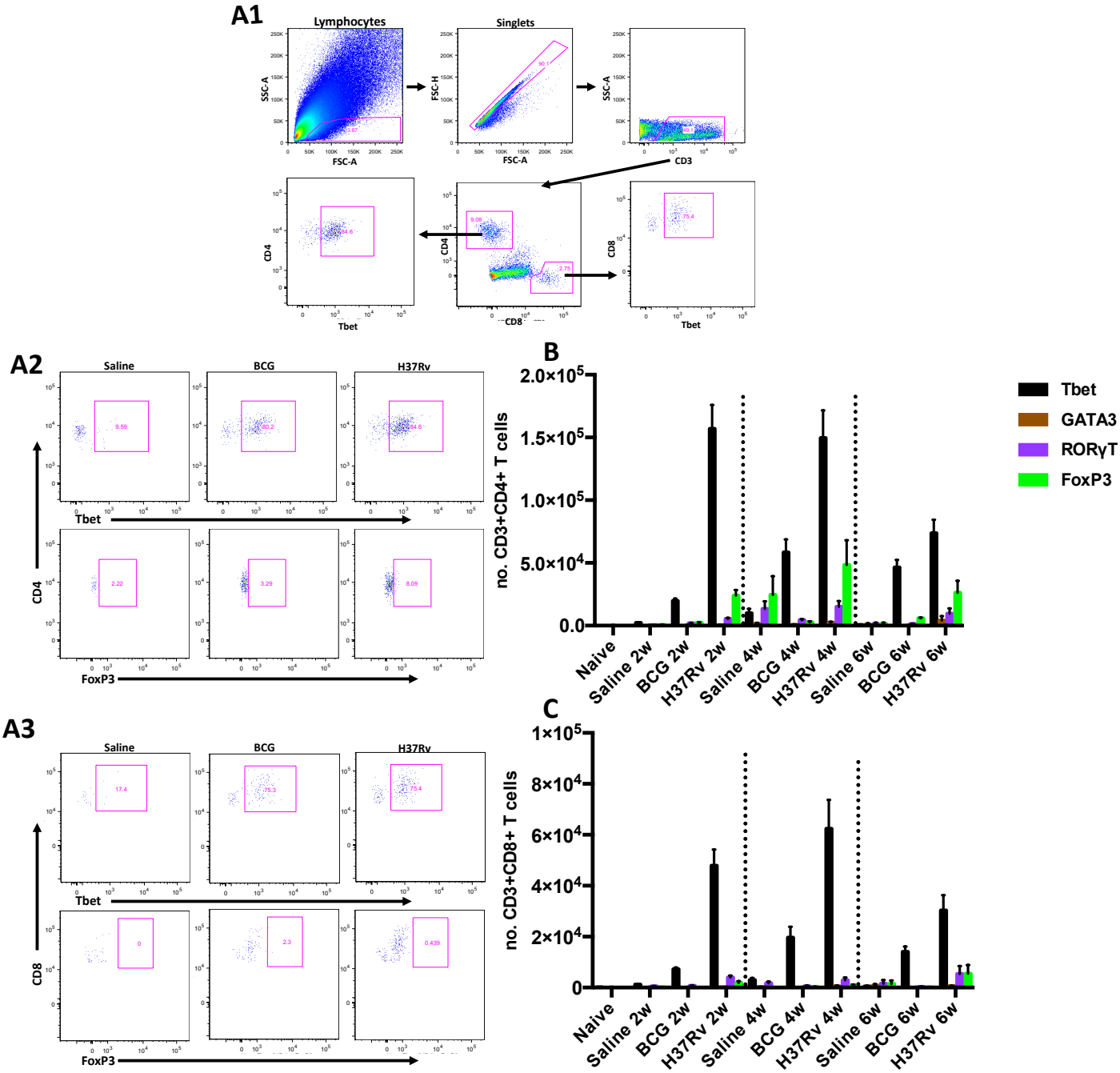


Figure 4.20: Increased numbers of recruited brain CD4+ T cells and CD8+ T cells expressing T-bet and FoxP3+CD4+ T cells post *M. tuberculosis* H37Rv or BCG intracerebral infection. Wild-type female C57BL/6J mice (n=4-5 mice/group) were intracerebrally inoculated with saline, 1 x 10^5 CFUs of BCG or H37Rv *M. tuberculosis* and not inoculated (naïve mice). Mice were euthanized at weeks 2, 4 & 6 and immune cells analyzed by flow cytometry**.** Flow cytometry gating strategy of **(A1)** CD4+ and CD8+ T cell TFs and flow plots of **(A2)** CD4+ T cell and **(A3)** CD8+ T cells expressing T-bet and FoxP3 post *M. tuberculosis* or BCG intracerebral infection. Number of **(B)** CD4+ T cells and **(C)** CD8+ T cells expressing TFs; T-bet, GATA3, RORγT and FoxP3+ CD4+ T cells during CNS-TB-infection. Graphs are representative of 3 independent experiments for BCG and 2 independent experiments for H37Rv. Data represents the mean +/- SEM.

4.3.6) Increased numbers of T-bet+CD4+ T cells, T-bet+CD8+ T cells and FoxP3+CD4+T cells recruited to CLNs post *M. tuberculosis* or BCG i.c. infection.

This study demonstrated that CLNs CD4+ T cells and CD8+ T cells were primed and activated post *M. tuberculosis* and BCG intracerebral infection. We further wanted to determine the polarization of these T cells because their specific effector functions depend on direction of polarization. The TF profile of T cells in the CLNs post *M. tuberculosis* or BCG intracerebral infection has not been explored. Our objective was to determine the T cell TF profile in the CLNs of wild type C57BL/6J by measuring T-bet, GATA3, RORγT and FoxP3 during CNS-TB infection using flow cytometry, like we did with brain T cells.

We showed the representative flow cytometry gating strategy of T cell TFs in the CLNs (Fig. 21A1) and plots of the amount of CD4+ T cells and CD8+ T cells in the CLNs of mice expressing T-bet and FoxP3 post *M. tuberculosis* or BCG intracerebral infection (Fig. 4.21-A2 and A3). Due to the low numbers of CD4+ T cells and CD8+ T cells present in naïve mice, it was difficult to detect measurable numbers of T cells expressing TFs (Fig. 4.21B and C), an observation similar to the brain. Extremely low to undetectable numbers of CD4+ and CD8+ T cells expressed the four TFs in saline inoculated mice because there was no infection in the control animals (Fig. 4.21B and C). However, the outcome was different for mycobacterial infection of the CNS, where increased numbers of both CD4+ T cells and CD8+ T cells expressed T-bet, Foxp3, RORγT and no GATA3 as compared to saline inoculated mice (Fig. 4.21B and C). Similar to the what was observed in the brain, the highest/larger number of T cells expressed T-bet (T-bet+CD4+ T cells and T-bet+CD8+ T cells) in the CLNs post *M. tuberculosis* and BCG intracerebral infection compared to other TFs (Fig. 4.21B, C), therefore favouring induction of Th1 immune responses. Additionally, virulence appeared not to be a factor between *M. tuberculosis* and BCG infection because of no difference in the number of -T-bet+ T cells during mycobacterial infection of the CNS for most time points of the experiment (Fig. 4.21B and C). An exception occurred during week 4 during CNS-TB, where an equivalent number of CD4+ T cells expressed equal numbers of T-bet and FoxP3 cells post *M. tuberculosis* intracerebrally infected mice (Fig. 4.21B) as evidenced by no significant differences ($p>0.05$), suggesting major regulation of T-bet+CD4+ T cells by FoxP3+CD4+ T cells during CNS-TB infection. Unlike mycobacterial infection, low to negligible numbers of CD4+ T cells from CLNs of saline inoculated mice expressed T-bet compared to mycobacterial infection (Fig. 4.21B). Whereas, a high number of CD8+ T cells from CLNs of *M. tuberculosis*

and BCG intracerebrally infected mice expressed T-bet (T-bet+CD8+T cells) as compared to saline inoculated mice. T-bet+CD8+ T cells were accompanied by minimal numbers of FoxP3+CD8+ T cells at week 4 during CNS-TB and very little to no numbers of RORγT+CD8+ T cells post *M. tuberculosis* or BCG intracerebral infection (Fig. 4.20C). The observed CD4+ T cell and CD8+ T cell transcription factor profiles in the CLNs during mycobacterial infection of the CNS favoured dominant Th1 immune responses, which appeared to be regulated only at week 4 post infection. And the absence of prominent transcriptional factor profiles in saline control mice supports that expression of transcription factors was specifically induced by mycobacterial and not injury. Taken together, it suggests induction of protective immunity against CNS-TB infection.

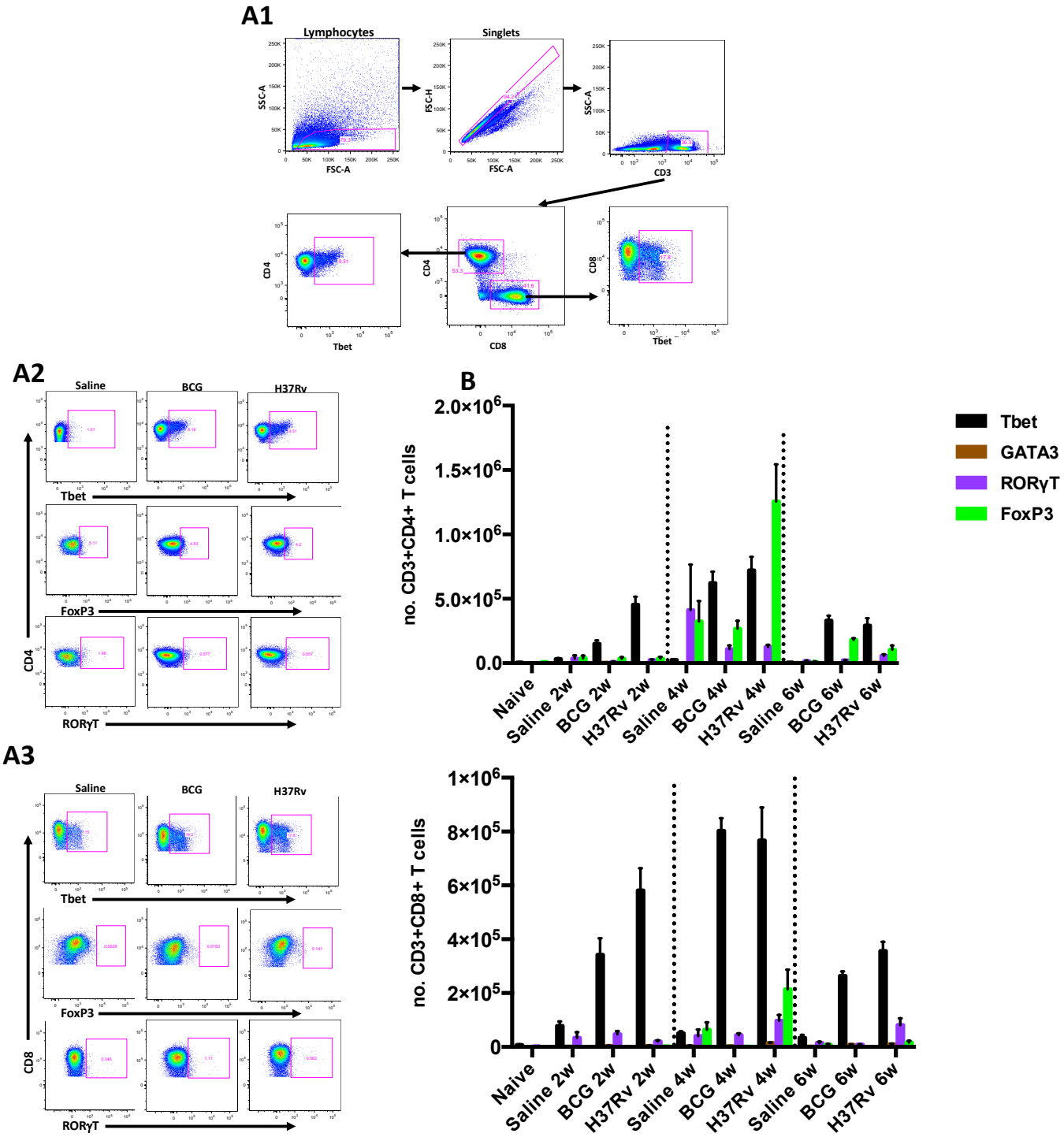


Figure 4.21: Increased numbers of recruited CLN CD4+ T cells and CD8+ T cells expressed T-bet and FoxP3 post *M. tuberculosis* H37Rv or BCG intracerebral infection favouring regulated Th1 immune responses. Wild-type female C57BL/6J mice (n=4-5 mice/group) were intracerebrally inoculated with saline, 1 x 10^5 CFUs of BCG or H37Rv *M. tuberculosis* and not inoculated (naïve mice). Mice were euthanized at weeks 2, 4 & 6 and immune cells analyzed by flow cytometry**.** Flow cytometry gating strategy of (A1) CD4+ and CD8+ T cell TFs and flow plots of (A2) CD4+ T cell and (A3) CD8+ T cells expressing T-bet, RORγT and FoxP3 post M. tuberculosis or BCG intracerebral infection. Number of **(B)** CD4+ T cells and **(C)** CD8+ T cells expressing TFs; T-bet, GATA3, RORγT and FoxP3+ CD4+ T cells during CNS-TB-infection. Graphs are representative of 3 independent experiments for BCG and 2 independent experiments for H37Rv. Data represents the mean +/- SEM.

4.4) *In vitro* re-stimulants caused no alterations in numbers of *in vivo* CD4+ T cell recruited to the brain during CNS-TB.

CD4+ T cells are at the centre of protective immune responses and they help enhance the killing of mycobacteria by macrophages through cytokine production (Zhu and Paul., 2008). During CNS-TB, we detected high expression of IL-12 and IL-1β by recruited brain DCs and cervical lymph node DCs, cytokines that are known to polarize T cells towards Th1 immune responses (Wlodarczyk et al., 2014). We also demonstrated that during CNS-TB, increased numbers of effector CD4+ T cells that were recruited to the brain and CLNs expressed T-bet and FoxP3 at week 4 post *M. tuberculosis* intracerebral infection. These two transcription factors are associated with activation and differentiation of T cells to T-helper-1 cells (Th1) and regulatory T cells (Tregs), respectively (Hickman et al., 2002; Liu et al., 2011; Sáenz et al., 2013; Lozza et al., 2014). T-bet transcribes proinflammatory Th1 cytokines while FoxP3 transcribes regulatory T cell cytokines(Szabo et al., 2000; Suffia et al., 2006; Belkalid., 2008; Yang et al., 2015). Additionally; Th1, Th2, Th17 and Tregs are lineages of CD4+ T cell subsets (Zhu and Paul, 2008). Our aim was to characterize the functional cytokine profiles of recruited brain CD4+ T cells, including their polyfunctional cytokine profiles during CNS-TB using flow cytometry. This was conducted by re-stimulating equal density of total brain cells harvested from week 4 CNS-TB infected mice and culturing them *in vitro* overnight with different antigens/stimulants (ESAT-6, H37Rv, αCD3/C28 and only media) followed by quantifying the number of CD4+ T cells that expressed cytokines.

Figure 4.22 shows the representative flow plots of the recruited brain CD4+ T cells during CNS-TB following *in vitro* restimulations. These brain CD4+ T cells from *M. tuberculosis* intracerebrally infected mice were significantly higher than saline inoculated mice after being treated with αCD3/C28 ($p<0.0001$), ESAT-6 ($p<0.001$) and media only ($p<0.0001$) but not those treated with H37Rv (Fig. 4.22.2A). This could be attributed to cells getting infected *in vitro* by *M. tuberculosis* H37Rv. Additionally, these H37Rv *M. tuberculosis* treated saline cells expressed significantly higher ($p<0.001$) numbers of CD4+ T cells than naïve mice unlike other treatments that showed equivalent number of CD4+ T cells between naïve mice and saline control mice (Fig. 4.22.2A).

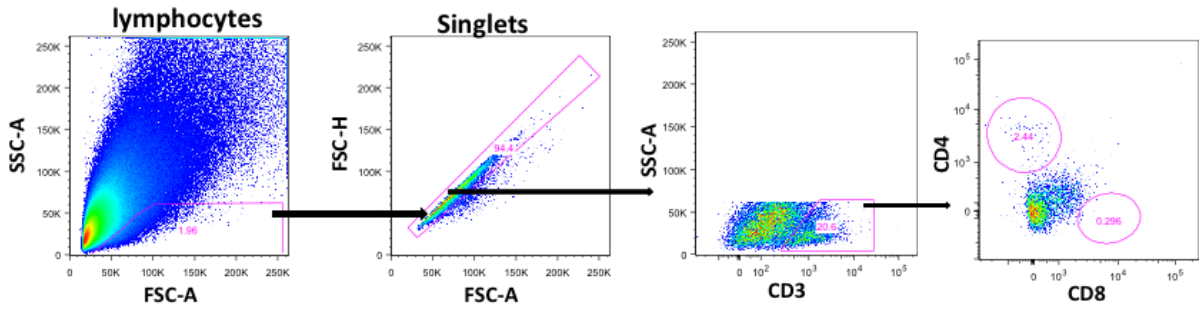


Figure 4.22: Representative flow plots gating strategy of re-stimulated recruited brain CD4+ T cells. Wild-type female C57BL/6J mice (8-12w old) were intracerebrally inoculated with saline or 1 x 10^5 CFUs H37Rv *M. tuberculosis*. Then euthanized at week 4 and immune cells analyzed by flow cytometry. **(A)** Cells were re-stimulated with αCD3/CD28, H37Rv, ESAT-6 and only media. Increased numbers of cells expressed CD4+ T cells.

4.4.1) Pro-and anti-inflammatory cytokine responses of CD4+ T cells recruited to the brain post *M. tuberculosis* i.c. infection not affected by *in vivo* re-stimulants.

We detected significantly higher numbers of CD4+ T cells from CNS-TB infected mice expressing proinflammatory and anti-inflammatory cytokines as compared to saline inoculated mice (Fig. 4.22.2B-G). The order of these cytokines in terms of ranking from high levels to low were as follows; TGFβ> IL-10> IFNγ > IL-4> TNFα > IL-17.

In order for *M. tuberculosis* to be contained and controlled, CD4+ T cells must produce IFNγ, or a combination IL-2, TNFα and IFNγ (Sugawara et al., 2000; Bozzano et al., 2014). Figure 4.22.1A shows the representative flow plots of *in vivo* recruited brain CD4+ T cells that expressed IFNγ and TNFα after *in vitro* restimulation conditions. IFNγ is a pro-inflammatory cytokine that is mainly produced by T cells and natural killer cells and is important for protection against *M. tuberculosis* (Orme et al., 1993; Flynn et al., 1993). We detected significantly higher numbers of recruited CD4+ T cells expressing IFNγ (IFNγ+CD4+ T cells) in *M. tuberculosis* infected mice compared to saline controls for all re-stimulations; only media ($p<0.001$), ESAT- 6 ($P<0.001$), H37Rv ($p<0.05$) and αCD3/C28 ($p<0.01$), representing the third highest cytokine expressed during CNS-TB (Fig. 4.22.1D). While a low number of recruited brain CD4+ T cells expressed increased numbers of TNFα (TNFα+CD4+ T cells), another Th1 proinflammatory cytokine during CNS-TB (ranking fifth of all cytokines), however, these numbers were significantly increased compared to saline controls for media

only ($p<0.05$) and ESAT-6 ($p<0.05$) re-stimulations but not H37Rv and αCD3/C28 (Fig. 4.22.2E). Production of IFNγ and TNFα controls mycobacterial infection in systemic circulation by inducing protective immunity (Co et al., 2004). Taken together, the increased numbers of IFNγ+CD4+ T cells and TNFα+CD4+ T cells suggest that the mice were protected against CNS-TB by the Th1 immune responses.

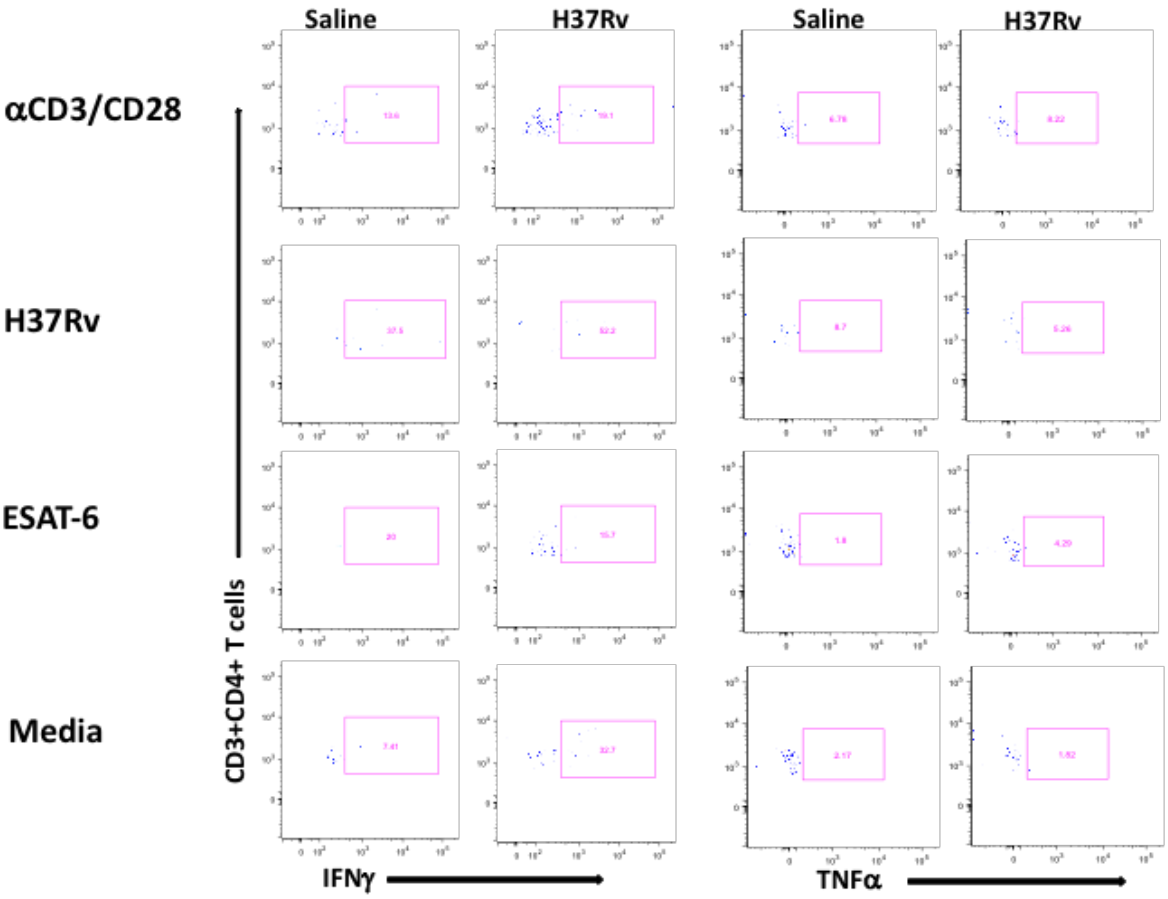


Figure 4.22.1A: Representative flow plots showing of pro-inflammatory cytokines IFNγ and TNFα expressed by recruited brain CD4+ T cells. Wild-type female C57BL/6J mice (8-12w old) were intracerebrally inoculated with saline or 1 x 10^5 CFUs H37Rv *M. tuberculosis*. Infected and naïve mice were euthanized at week 4 and immune cells analyzed by flow cytometry. **(A)** Cells were re-stimulated with αCD3/CD28, H37Rv, ESAT-6 and only media. Increased numbers of cells expressed IFNγ and TNFα during CNS-TB.

Regulatory T cells perform anti-inflammatory functions and are directed by TGFβ and IL-10 (Goverman, 2007). Figure 4.22.1B shows the representative flow plots of *in vivo* recruited brain CD4+ T cells that expressed TGFβ and IL-10 after *in vitro* restimulation conditions. We detected the largest number of recruited brain CD4+ T cells expressed TGFβ (TGFβ+CD4+ T cells), an anti-inflammatory that was highly increased compared to other cytokines during CNS-TB (Fig. 4.22.2B-G). The number of TGFβ+CD4+ T cells were significantly higher in *M. tuberculosis* i.c. infected mice compared to saline control mice for all re-stimulations; αCD3/C28 ($p<0.001$), H37Rv ($p<0.001$), ESAT-6 ($p<0.001$) and only media ($p<0.0001$) (Fig. 4.22.2B). However, no differences were observed in the number of TGF-β +CD4+ T cells among different stimulants possibly because the cells were already stimulated to the maximum. TGF-β production has been shown to inhibit the proliferation of intracerebral T cell immune responses in the CNS (Streilein et al., 1992). We also found increased numbers or recruited brain CD4+ T cells also expressed IL-10 (second to TGF-β), an anti-inflammatory during CNS-TB compared to saline controls (Fig. 4.22.2C). IL-10+CD4+ T cells were significantly higher in *M. tuberculosis* i.c. infected mice than saline controls for H37Rv ($p<0.001$), ESAT-6 ($p<0.0001$) and only media ($p<0.0001$) but not for αCD3/C28 re-stimulation (Fig. 4.22.2C). IL-10 can be produced by T cells and dampens the immunity that is induced against TB (Redford et al., 2011; Pitt et al., 2012). This suggests that the specific T cell effector immune responses that were induced in the brain during CNS-TB are highly regulated by recruited brain TGFβ+CD4+ T cells and IL-10+CD4+ T cells.

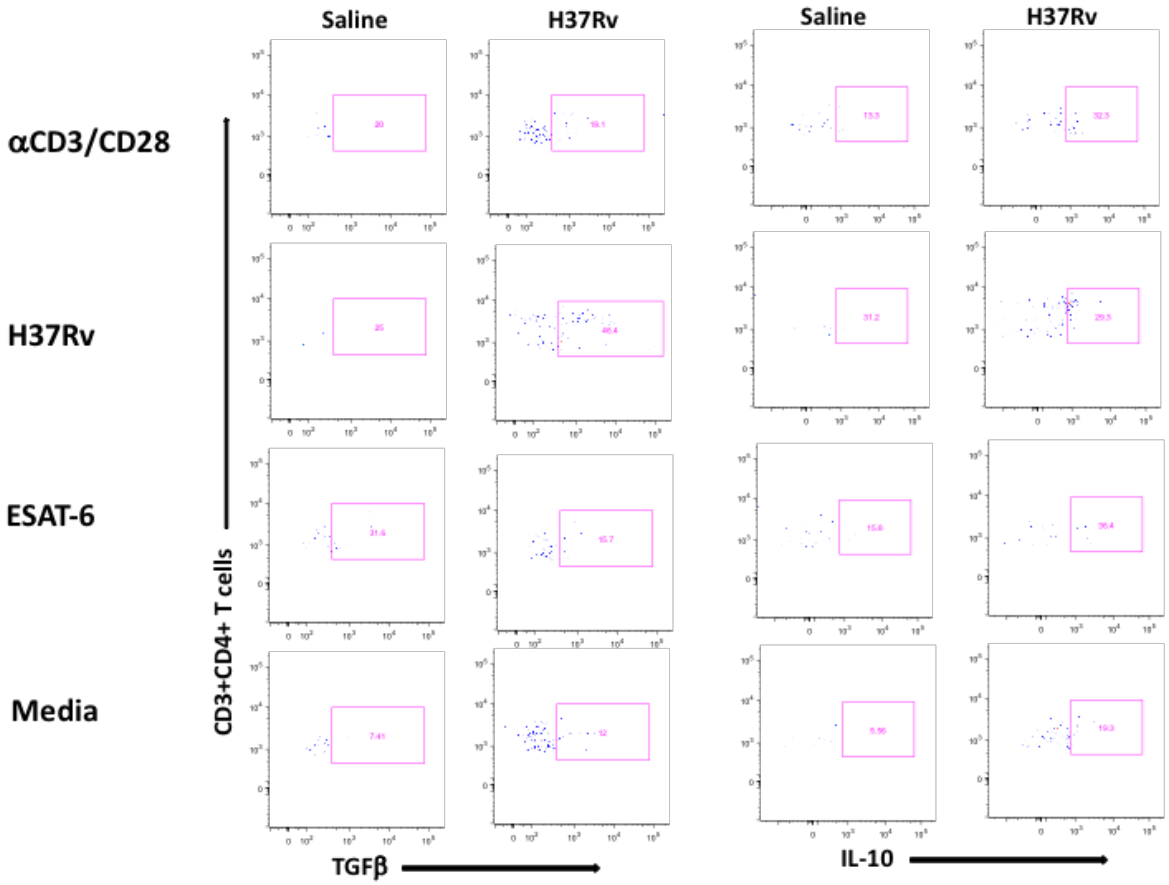


Figure 4.22.1B: Representative flow plots showing of anti-inflammatory cytokines TGFβ and IL-10 expressed by recruited brain CD4+ T cells. Wild-type female C57BL/6J mice (8-12w old) were intracerebrally inoculated with saline or 1 x 10^5 CFUs H37Rv *M. tuberculosis*. Inoculated, infected and naïve mice were euthanized at week 4 and immune cells analyzed by flow cytometry. **(A)** Cells were re-stimulated with αCD3/CD28, H37Rv, ESAT-6 and only media. Increased numbers of cells expressed TGFβ and IL-10 during CNS-TB.

We also detected a significant number of recruited brain CD4+ T cells expressing IL-4 (IL-4+CD4+ T cells), a Th2 cytokine during CNS-TB which was increased compared to saline control mice. The function of this proinflammatory cytokine that favours Th2 immune responses is protective in helminth infections (Sugawara et al., 2000) not bacterial infections. We detected higher numbers of recruited brain IL-4+CD4+ T cells during CNS-TB which were significantly higher than saline control mice following stimulations with only media ($p<0.05$), ESAT-6 ($p<0.01$) and αCD3/C28 ($p<0.01$) but not H37Rv re-stimulation (Fig. 4.22.2F). Levels of IL-4 expression ranked fourth among other cytokines. Additionally, a significant kinetic increase was observed in the expression of IL-4 by CD4+ T cells during CNS-TB between media only and αCD3/C28 re-stimulation (positive control). Previous studies have shown an increase in IL-4 production or associated cells during tuberculosis (Surcell et al., 1994; Hook et al., 1996). Our data also shows occurrence of that low levels of Th2 immune responses during CNS-TB even though we previously detected negligible levels of Th2 transcription factor GATA3 following *M. tuberculosis* intracerebral infection.

We also detected an increased number of recruited brain CD4+ T cells expressing IL-17 (IL-17+CD4+ T cells) during CNS-TB (Fig. 4.22.2G). This Th17 cytokine that can regulate the effector activity of other immune cells during tuberculosis also plays an important role in adaptive immune responses (Umemura et al., 2007; Song et al., 2016). The least number of recruited brain CD4+ T cells expressed IL-17 compared to other cytokines during CNS-TB (Fig. 4.22.2B-G). *M. tuberculosis* infected mice produced significantly higher levels of IL-17+CD4+ T cells compared saline control mice following stimulation with only for ESAT-6 ($p<0.05$) and αCD3/C28 ($p<0.01$) but not only media or H37Rv re-stimulation (Fig. 4.22.2G). And these levels were significantly increased ($p<0.05$) between media only and the positive control αCD3/C28 and also between H37Rv and αCD3/C28 re-stimulation (Fig. 4.22.2G). IL-17 has been implicated in potentially contributing to protective immunity against pulmonary model of BCG infection (Umemura et al., 2007). Whereas, in wild-type C57BL/6J mice and IL-17 deficient mice models of pulmonary TB, it was shown to not be required for protective immunity earlier or later during infection (Gopal et al., 2014). Our findings imply that IL-17+CD4+ T cells also played a role in the protective immune responses we observed in our CNS-TB model. Taken together, the cytokine signature of recruited brain CD4+ T cell favours highly regulated Th1 immune responses that are protective in the brain during CNS-TB.

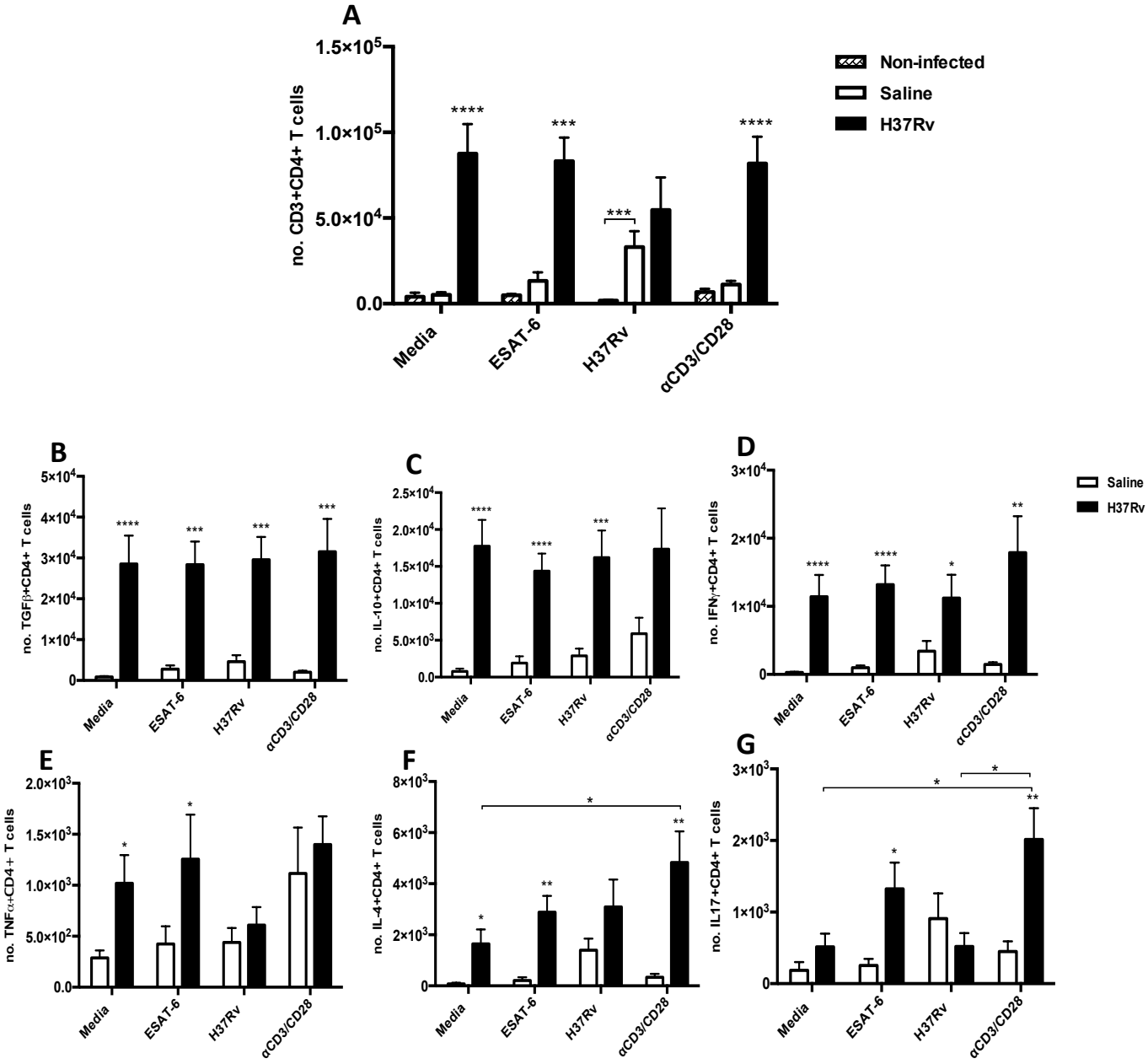


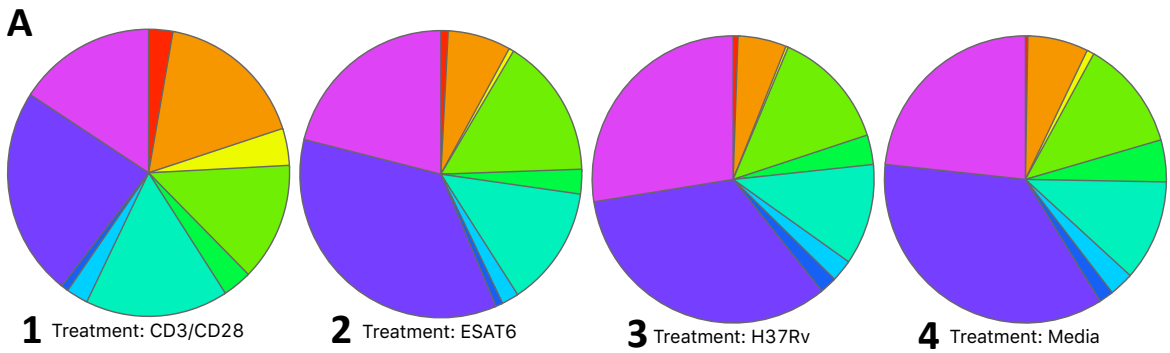
Figure 4.22.2: Increased numbers of recruited brain CD4+ T cells expressed Th1 proinflammatory and anti-inflammatory cytokines during CNS-TB not affected by *in vitro* re-stimulants. Wild-type female C57BL/6J mice (8-12w old) were intracerebrally inoculated with saline or 1 x 10^5 CFUs H37Rv *M. tuberculosis* and not inoculated (naïve). Infected and naïve mice were euthanized at weeks 4 post infection and immune cells analyzed by flow cytometry. Cells were re-stimulated with αCD3/CD28, H37Rv, ESAT-6 and only media. **(B-G)** Increased numbers of CD4+ T cells expressed TGFβ, IL-10, IFNγ, TNFα, IL-4 and IL-17 during CNS-TB compared to saline controls. Graphs represents pooled samples from two independent experiments. Data represents the mean +/- SEM.*p<0.05, **p<0.01, ***p<0.001, ****p <0.0001, Mann-Whitney test.

4.4.2) Polyfunctional TGFβ + IFNγ +CD4+ T cells recruited to the brain during CNS-TB reveal regulated Th1 immunity.

Production of multiple cytokines by the same immune cell has been associated with beneficial immune responses (Han et al., 2012) and cytokines produced dictate the polarization of immune cells. CD4+ T cells that produce a combination of proinflammatory cytokines IFNγ, TNFα, and IL-2 have been implicated in inducing protection against *M. tuberculosis* (Bozzano et al., 2014; Lewinsohn et al., 2017). A study by Lee and colleagues (2009) specifically looked at the co-expression of IL-17+ IFNγ+ CD4+ T cells post intracerebral infection with BCG and showed their preferential accumulation in the CNS. However, no studies have looked at polyfunctional cytokine profiles post *M. tuberculosis* intracerebral infection. It is within this context that we characterized the CD4+ T cell polyfunctional cytokine profiles during CNS-TB. Unlike *M. tuberculosis* intracerebral infection that resulted in the infiltration and accumulation of T cells in the brain, there were very numbers of CD4+ T cells in mice that were mock inoculated with saline (Fig. 4.22.1-2A), showing that T cells that infiltrated the brain during CNS-TB were specific to mycobacteria. This is the reason we only determined the polyfunctional profiles *M. tuberculosis* infected mice.

We further analysed the data of the recruited brain CD4+ T cells that were re-stimulated at week 4 post during CNS-TB using the SPICE software in order to generate cytokine polyfunctional profiles. Figure 4.22.3 shows the representative polyfunctional cytokine profiles of these brain CD4+ T cells during CNS-TB as depicted by a SPICE pie charts and bar graph. The pie charts is accompanied by pie chart results table that showed that the positive antigen control, αCD3/C28 produced a cytokine distributions that were significantly different from all other re-stimulations (only media, ESAT-6 and H37Rv) (Fig. 4. 22.3A). When looking at the percentages of cytokines produced by brain CD4+ T cells, the percentages of CD4+ T cells expressing TGF-β alone was the highest, followed by IFNγ and IL-10 as previously shown in our bar graph data (Fig. 4.22.2B), followed by expression of combinations of multiple cytokines (Fig. 4. 22.3B). The 3 major polyfunctional cytokine profiles were (1) TGFβ + IFNγ+, (2) TGFβ + IL-10+ and (3) TGFβ + IFNγ +IL-10 with pair one and pair two expressing relatively equivalent levels, while pair three contained less percentage expression (Fig. 4. 22.3B). IL-17+ polyfunctional combinations levels were negligible. IFNγ is a proinflammatory that functions for protection against TB while anti-inflammatory cytokines TGFβ and IL-10 function to regulate T cell immune responses. This testifies to the immune privilege of the CNS

as evidenced by the polyfunctional cytokine profiles that consisted of the combination of Th1 cytokine/s and regulatory cytokines during CNS-TB resulting in immunity in the brain.



Pie Category Test Results

Pie #	1	2	3	4
1		0.0050	0.0091	0.0075
2	0.0050		0.0862	0.5700
3	0.0091	0.0862		0.4398
4	0.0075	0.5700	0.4398	

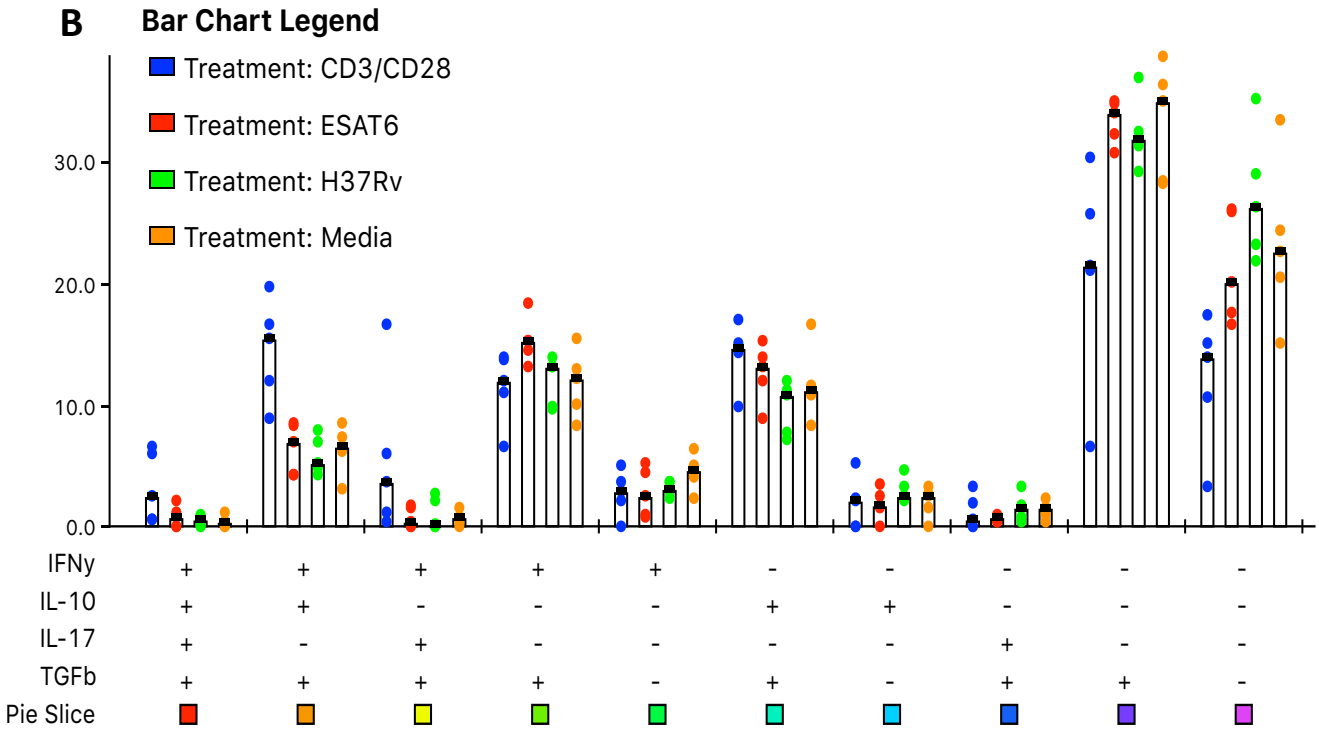


Figure 4.22.3: CD4+ T cells recruited to the brain during CNS-TB produced polyfunctional cytokines associated with regulated Th1 immunity. Wild-type female C57BL/6J mice (8-12w old) were intracerebrally infected with 1 x 10^5 CFU of H37Rv *M. tuberculosis* and euthanized at weeks 4 with uninfected mice. Immune CD4+ T cells were analyzed with flow cytometry. **(A)** Pie chart showed different polyfunctional cytokine distribution of brain CD4+ T cells during CNS-TB. **(B)** Polyfunctional profile showed the high percentage combinations of (1) TGFβ + IFNγ, (2) TGFβ + IL-10 then (3)TGFβ+ IFNγ+IL-10 during CNS-TB. Graph representative of two independent experiments. Data represents the mean +/- SEM. Student two tailed t-test. *p<0.05, **p<0.01.

4.5) H37Rv dendritic cell-T cell coculture demonstrates induction of regulated Th1 immune responses by *in vivo* CNS-TB infected DCs.

Both innate and adaptive immune responses are important for inducing protective immunity against mycobacterial infection (Sato and Fujita, 2007), and dendritic cells (DCs) are professional APCs that form a bridge between the innate and adaptive arm (Kapseberg, 2003). Dendritic cells and T lymphocytes are actively recruited to the brain during CNS-TB and may interact outside of a lymphoid environment. A complex relationship may therefore be at play between adaptive and innate immune responses within the CNS, and dendritic cells positioned as important players in directing such responses. DCs have the ability to differentiate T cell immune responses to a particular polarization (Th1, Th2, Th17 or Treg) (Liu, 2001) thus making them target cells of interest for therapeutic intervention. DCs are potent cells that have been shown to interact with T cells during pulmonary TB, whereby they prime and activate *M. tuberculosis* specific T helper 1 immune responses (Malowamy et al., 2005; Lozza et al., 2014; Choi et al., 2018), however the interaction of DCs and T cells in the context of CNS-TB is a relatively newly explored area. We hypothesise that *M. tuberculosis* infection modifies dendritic cell phenotype and function that in turn alters T cell regulation during CNS-TB. And our objective was to investigate the modulation of function and phenotype of T cells by dendritic cells during CNS-TB infection. We achieved this by investigating dendritic cell-T cell (DC-TC) interaction during CNS-TB by setting up a direct DC-TC coculture. We sorted DCs from cervical lymph nodes (CLNs) of wild-type C57BL/6J mice at week 4 post H37Rv H37Rv *M. tuberculosis* infection during CNS-TB and cocultured them overnight with CD3+ T cells from spleens of naïve wild-type C57BL/6J mice. And we determined the direction of T cell polarization using flow cytometric analysis.

Fig. 4.23.1A shows representative flow cytometry plots gated on CD3+CD4+ T cells and CD3+CD8+ T cells from naïve spleen T cells and those from the *M. tuberculosis* H37Rv DC-TC coculture during CNS-TB. CD3+ T cells from controls (naïve T cells only) or H37Rv DC-TC coculture consisted of high percentages of CD4+ T cells and lower percentages of CD8+ T cells (Fig. 4.23A and B). We observed no differences in the frequency of CD4+ and CD8+ T cells that expressed CD44 (an activation marker for T cells) between the coculture and naïve T cells (Fig. 23.1C). Appendix H provides flow cytometry gating strategy of CD4+ and CD8+ T cells expressing CD44. Although we did not find any differences in the frequency of activated T cells, we detected significant differences in the percentages of cells that expressed

cytokines between the two experimental groups following intracellular staining using flow cytometric analysis.

Figure 4.23.2A shows the representative flow cytometry plot of the four cytokines (IFNγ, TGFβ, IL-4 and IL-10) expressed by CD4+ T cells during the H37Rv *M. tuberculosis* DC-TC coculture assay and controls (T cells only). We found a higher frequency of CD4+ T cells expressing pro-inflammatory and anti-inflammatory cytokines in the DC-TC coculture. Cytokine expression of CD4+ T cells from the DC-TC coculture experiment was higher compared to controls (T cells only) for IFNγ (52,95% vs 27.93%, $p<0.05$), TGFβ (40.98% vs 14.80%, $p<0.05$), IL-10 (25.93% vs 1.24%, $p<0.001$) and IL-4 (6.24% vs 1.66%, $p<0.001$) shown in figure 4.23.2B. We also measured the cytokine expression of CD8+ T cells. Figure 4.23.3A shows the representative flow cytometry plot of the four cytokines (IFNγ, TGFβ, IL-4 and IL-10) expressed by CD8+ T cells during the H37Rv *M. tuberculosis* DC-TC coculture assay and controls (T cells only). Similarly, to CD4+ T cells cytokine trend, we found a higher frequency of CD4+ T cells expressing pro-inflammatory and anti-inflammatory cytokines in the DC-TC coculture. Cytokine expression of CD8+ T cells from the DC-TC coculture experiment was higher compared to controls for IFNγ (46,75% vs 22.43%, $p<0.05$), TGFβ (62.62% vs 34.93%, $p<0.01$), IL-10 (15.53% vs 0.85%, $p<0.001$) and IL-4 (17.98% vs 3.30%, $p<0.001$) shown in figure 4.23.3B.

The descending order for the percentages of the cytokines expressed by CD4+ T cells from the coculture from highest to lowest frequency was IFNγ>TGFβ>IL-10>1L-4 (Fig. 4.23.2B) while for CD8+ T cells it was TGFβ> IFNγ>IL-4>1L-10 (Fig. 4.23.3B). These findings show that DCs from CNS-TB infected CLNs induced a Th1 (IFNγ), Th2 (IL-4) and Treg (IL-10 and TGFβ) immune responses. The percentages of CD4+ and CD8+ T cells from the DC-TC coculture expressed a combination of both proinflammatory (IFNγ) and anti-inflammatory cytokines (IL-10 and TGFβ) suggesting that DCs from CNS-TB infected mice can prime and activate naïve T cells which differentiate to produce regulated Th1 immune responses.

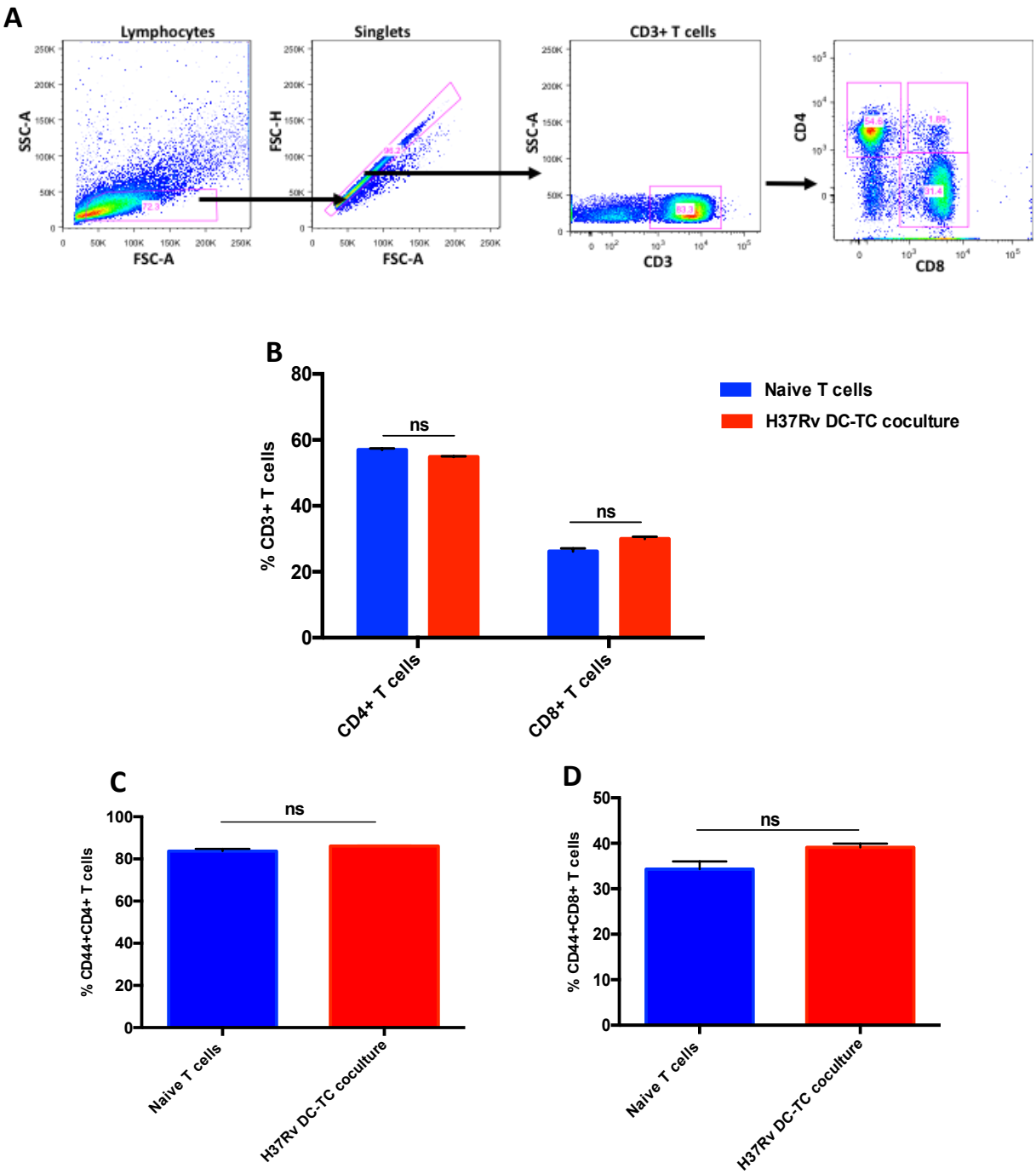
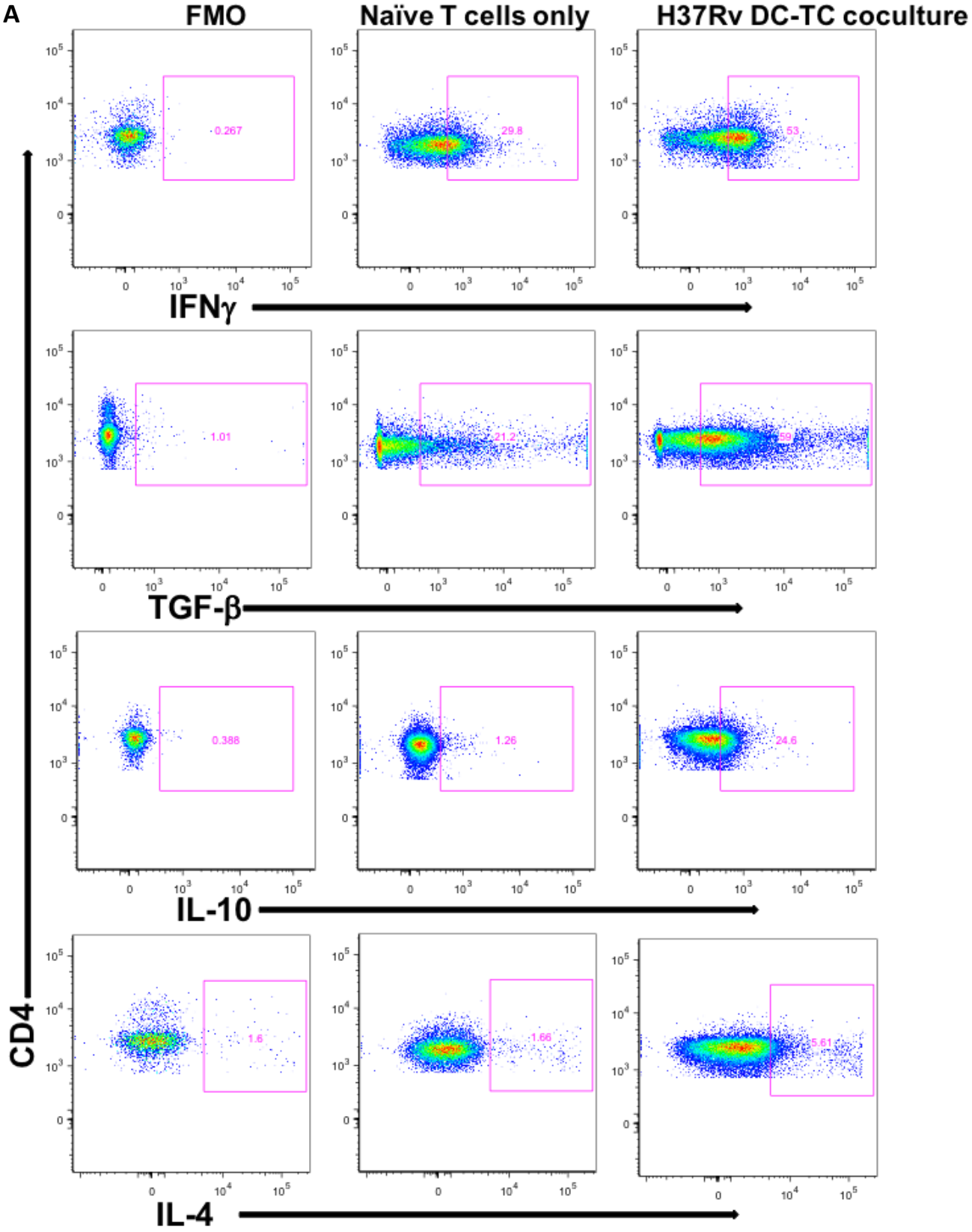


Figure 4.23.1 No differences in spleen CD4+ and CD8+ T frequency and activation induced by CNS-TB dendritic cells. Wild-type female C57BL/6J mice (8-12w old) were intracerebrally infected with 1 x 10^5 CFU of H37Rv *M. tuberculosis* and euthanized at weeks 4 with uninfected mice. FACS sorted DCs from CLNs of *M. tuberculosis* infected mice were cocultured with CD3+ T cells from spleens of uninfected mice. **(A)** Flow cytometry plots showing CD4+ T and CD8+ T cells **(B)** High percentages of CD4+ T cells than CD8+ T cells. **(C)** No change in CD44 expression by CD4+ T and CD8+ T cells. Data represents the Mean +/- SEM. *p<0.05, **p<0.01, ***p<0.001 using an unpaired student t-test.


A
FMO
Naïve T cells only
H37Rv DC-TC coculture
IFNγ
TGF-β
IL-10
IL-4
CD4
0.267
29.8
53
1.01
21.2
59
0.388
1.26
24.6
1.6
1.66
5.61

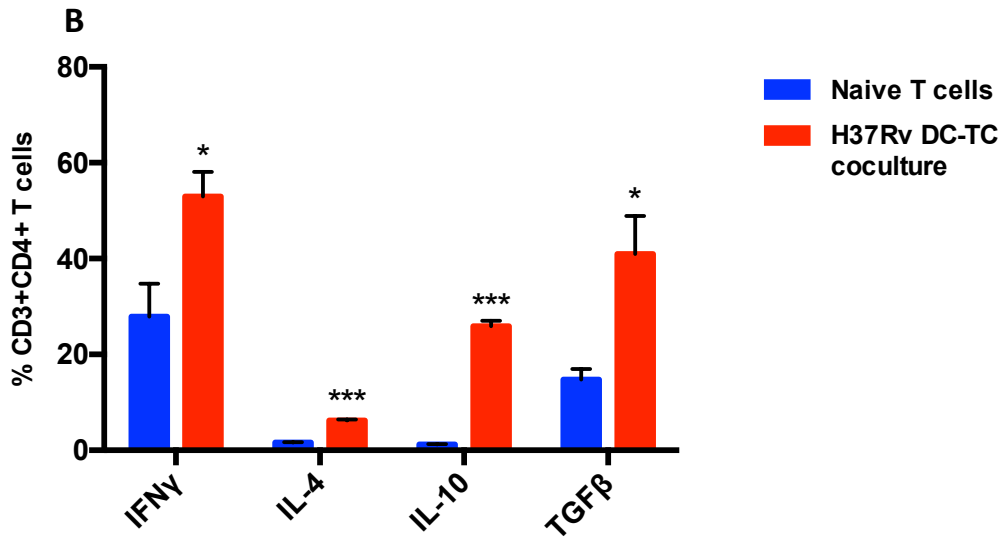
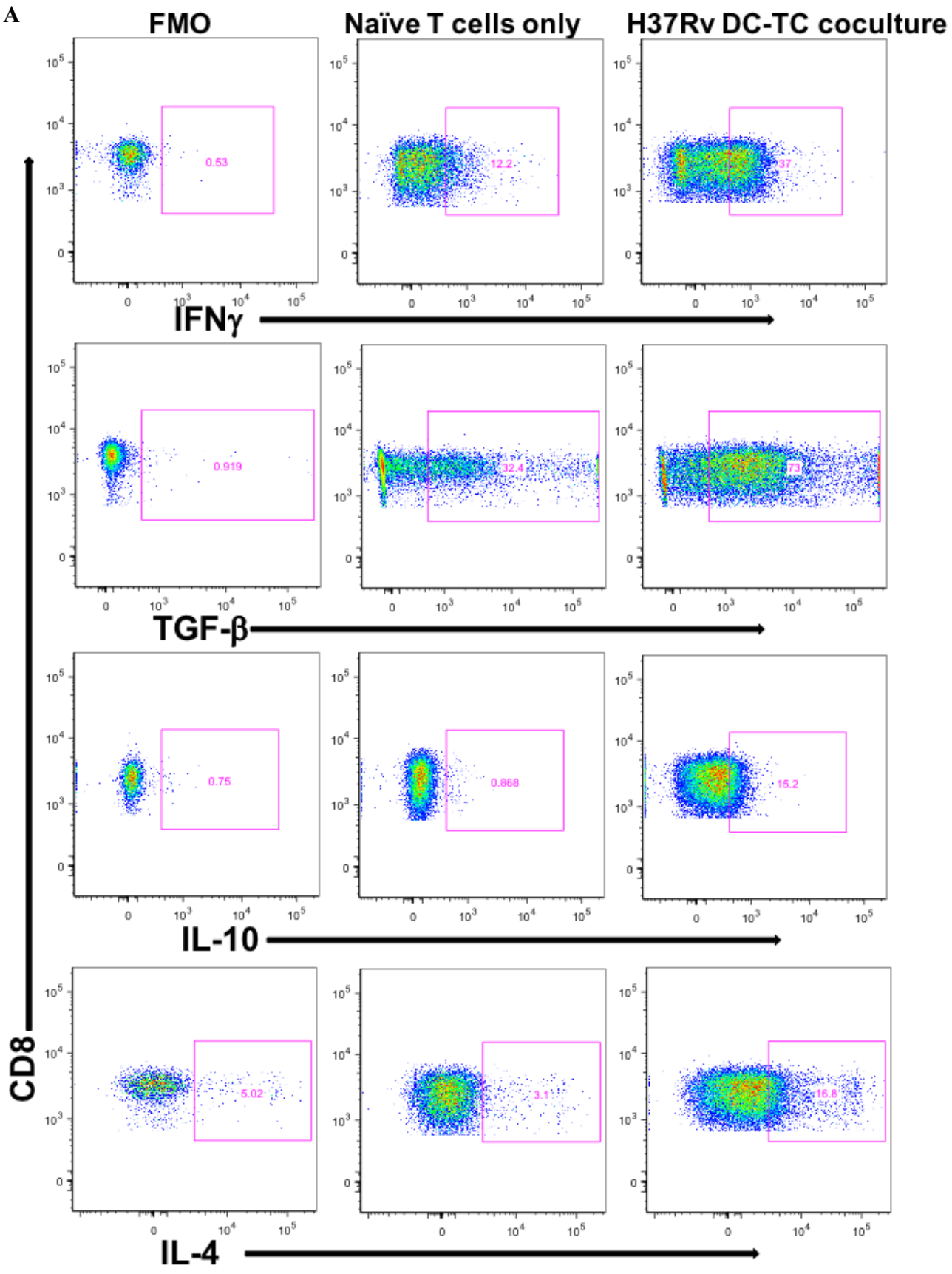


Fig 23.2. DCs from CNS-TB mice induced CD4+ T cells to secrete pro-inflammatory and anti-inflammatory cytokines polarized towards Th1 immunity. Wild-type female C57BL/6J mice (8-12w old) were intracerebrally infected with 1 x 10^5 CFU of H37Rv *M. tuberculosis* and euthanized at weeks 4 with uninfected mice. FACS sorted DCs from CLNs of *M. tuberculosis* infected mice were cocultured with CD3+ T cells from spleens of uninfected mice. **(A)** Flow cytometry plots showing proinflammatory (IFNγ) and anti-inflammatory (IL-4, IL-10 and TGFβ) gated on CD4+ T cells. (B) Increased percentage expression of IFNγ, IL-4, IL-10 and TGFβ by CD4+ T cells during CNS-TB. Data represents the Mean +/- SEM. *p<0.05, **p<0.01, ***p<0.001 using an unpaired student t-test.


A
FMO
Naïve T cells only
H37Rv DC-TC coculture
0.53
12.2
37
IFNγ
0.919
32.4
73
TGF-β
0.75
0.868
15.2
IL-10
5.02
3.1
16.8
IL-4
CD8

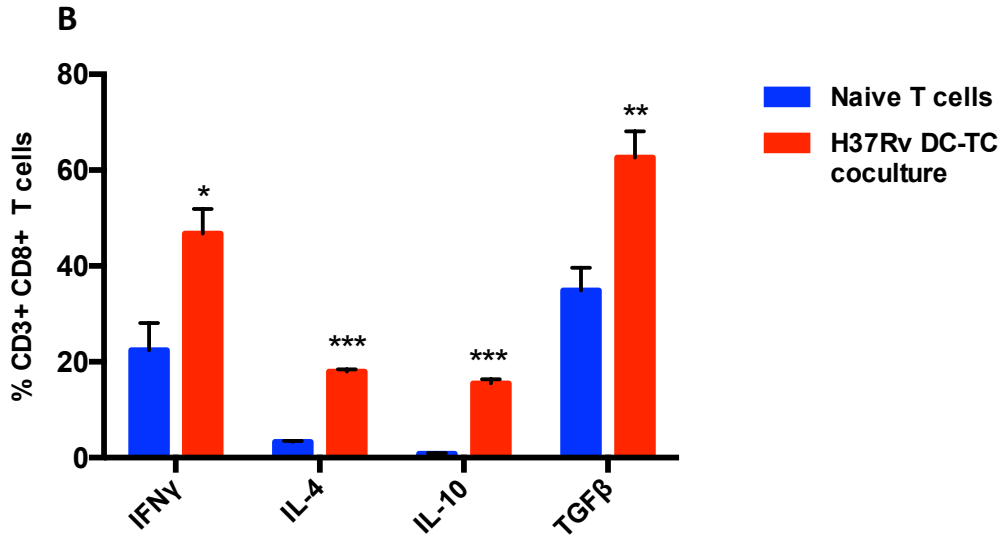


Fig 23.3. DCs from CNS-TB mice induced CD8+ T cells to secrete pro-inflammatory and anti-inflammatory cytokines polarized towards Th1 immunity. Wild-type female C57BL/6J mice (8-12w old) were intracerebrally infected with 1 x 10^5 CFU of H37Rv *M. tuberculosis* and euthanized at weeks 4 with uninfected mice. FACS sorted DCs from CLNs of *M. tuberculosis* infected mice were cocultured with CD3+ T cells from spleens of uninfected mice. **(A)** Flow cytometry plots showing proinflammatory (IFNγ) and anti-inflammatory (IL-4, IL-10 and TGFβ) gated on CD4+ T cells. **(B)** Increased percentage expression of IFNγ, IL-4, IL-10 and TGFβ by CD4+ T cells during CNS-TB. Data represents the Mean +/- SEM. *p<0.05, **p<0.01, ***p<0.001 using an unpaired student t-test.

CHAPTER 5: DISCUSSION

Small animal models enable us the platform to study immune responses during CNS-TB (Hernandez-Pando et al., 2011) for better understanding of host-pathogen interactions in the context of tuberculosis. The development of animal models is crucial for generating knowledge that will enable us to understand how *M. tuberculosis* evades the CNS to establish CNS infection, therefore leading to the development of improved therapeutic intervention strategies against CNS-TB (Husain et al., 2017). Rich and McCordock (1933) showed that tuberculous meningitis (TBM), a common manifestation of CNS-TB cannot be achieved by the natural route of secondary infection through hematogenous dissemination of pulmonary infection in rabbits and guinea pigs and that it required directly depositing bacilli in the CNS. In contrast, Donald and colleagues (2005) argued that hematogenous dissemination is critical for clinical TBM development in children, however; in animal models of CNS-TB infection, it has been established that either hematogenous dissemination or intracerebral/intracisternal infection can lead to TBM development (Tsenova et al., 1998 and 2005; Mazzolla et al., 2002; van Well et al., 2007; Be et al., 2008; Lee et al., 2009; Francisco et al., 2015; Tucker et al., 2016; Husain et al., 2017).

In this study, we reproduced the murine model of CNS-TB in wild type C57BL/6J after intracerebral infection with virulent *M. tuberculosis* H37Rv and avirulent *M. bovis* BCG, respectively like previous studies (Matyszak and Perry;1995; Lee et al., 2008; Mazzolla et al., 2002; van Well et al., 2007; Lee et al., 2008; Rock et al., 2008; Olin et al., 2008; Francisco et al., 2015; Hsu et al., 2017) and it approximated tuberculoma of the cortex which showed evidence of TBM. Model characteristics included; confirmation of CFUs from brains of *M. tuberculosis* or BCG i.c. infected mice as early as day 1 post intracerebral infection, acid-fast bacilli in the brain ventricles, and a high degree of inflammatory responses in brain ventricles and meninges, these observations were similar to previous studies in mice (Mazzolla et al., 2002; van Well et al., 2007; Lee et al., 2009; Francisco et al., 2015). CNS-TB does not always manifest as tuberculomas in humans and animals even though granuloma formation is crucial for the containment of mycobacteria. Mice did not show clinical symptoms of CNS-TB infection nor succumb to death, they appeared well and grew, findings similar to previous studies (van Well et al., 2007; Olin et al., 2008), regardless of detection of inflammatory responses in the brain by histology as observed by Rock and colleagues (2008). The surgical intervention caused mild physical damage to the CNS, as previously demonstrated by Matyszak and Perry (1995), and was observed at days 1 to 2 post intracerebral inoculation and

as evidenced by mild discomfort and weight loss. Rock and colleagues (2008) noted the disadvantage of the lack of mortality in animal models which does not represent human TBM outcomes. Tuberculosis vaccine development requires animal models that closely simulate human disease and these models can study pathological changes, immune responses, and pathogenesis (Smith et al., 2000; Acosta et al., 2011; Gong et al., 2020). Therefore, improved CNS-TB murine models that closely mimic human disease still need to be improved/developed.

Bacterial growth was controlled in the brains of both *M. tuberculosis* H37Rv or BCG infected mice as shown by significant decreases in CFUs that peaked at week 2 post-infection and were followed by decreasing bacterial loads, similar to previous CNS-TB studies in mice (C57BL/6J, BALB/c, and DBA2) and rabbits (Mazzola et al., 2002; van Well et al., 2007; Be et al., 2008; Lee et al., 2009; Lee et al., 2009; Tucker et al., 2016) although analyzed experimental time points and infection duration differed in some studies. Dissemination of mycobacteria from the site of infection other organs or tissue is a common occurrence and has been documented in murine models of CNS-TB infection (Mazzolla et al., 2002; van Well et al., 2007; Lee et al., 2009; Francisco et al., 2015; Deveci et al., 2016). Dissemination of *M. tuberculosis* H37Rv and BCG from the brain to the spleen and lungs systemically or through cervical lymphatics because intracerebral infection caused mild CNS physical damage that resulted in vasculature damage. Additionally, the discovery of the CNS lymphatic system by Louveau and colleagues (2015) suggests the latter holds too. Spleen CFUs showed a relatively similar trend of bacterial control as the brain for both *M. tuberculosis* and BCG infected mice. However, only lungs from BCG infected mice controlled bacterial replication as opposed to *M. tuberculosis-infected* mice which presented with bacterial burdens that exponentially increased as infection progressed. Studies in mice (van Well et al., 2007; Be et al., 2008) and in rabbits (Tucker et al., 2016) showed similar lung bacterial burden kinetics during CNS-TB/TBM. This was not surprising because most mice in our study presented with lung pathology that showed lesions similar to what others observed in mice and rabbits (van Well et al., 2007; Be et al., 2008; Tucker et al., 2016). Even though the lungs were the secondary site of infection in our model unlike the ideal natural route of infection in humans where lungs are the primary infection site. Lung histopathological analysis revealed the development of granulomas similar to Be and colleagues (2008), with granulomas as hallmarks of TB that normally contain *M. tuberculosis* but they can also facilitate disease progression (Saunders et al., 1999; Russell, 2007; Silva Miranda et al., 2012; Subbian et al., 2015; Ndlovu and Marakalala, 2016). Lung granulomas appear to have failed to perform expected protective

functions against the development of pulmonary TB during CNS-TB. IFNγ disruption leads to the formation of defective granulomas and poor secretion of reactive nitrogen species required for killing bacteria (Cooper et al., 1993; Flynn et al., 1993) which we speculate could have been the case but further investigation is required. Lung bacterial burdens and histology of these two strains, where *M. tuberculosis* caused pulmonary infection and inflammatory lesions (granulomas) suggest that microbial virulence can be a key determinant in the establishment of infection at secondary sites.

Different routes of infection and different strains of mycobacteria have been shown to cause CNS tuberculosis in mice (Wu et al., 2000; Lee et al., 2009; Francisco et al., 2015; Husain et al., 2017). CNS-TB has been established by the natural route of infection (aerosol infection) with different strains of *M. tuberculosis in* guinea pigs and suggested an efficient and more realistic experimental animal model (Nicholas et al., 2011). It is ideal to develop experimental animal models of CNS-TB that use the natural route of disease development as it occurs in human disease to enable extrapolation of major findings, however; factors such as those mentioned by Pando (2011) highlighted how the protective properties of the CNS by the BBB cause a challenge for establishment of CNS-TB. Also, about 1% of all TB patients develop CNS-TB (Cherian and Thomas, 2011) and this decreases the probability of most of our experimental mice developing CNS-TB following aerosol inhalation and dissemination through the blood, a process that can take months to years. Our study showed that different strains of mycobacteria established CNS infection with controlled brain bacterial burdens, however, the virulent *M. tuberculosis* H37Rv strain presented with higher bacterial burdens than avirulent *M. bovis* BCG as expected. Nontuberculous mycobacteria meningitis is not a common CNS infection (Flor et al., 1996), although there have been few instances where it has been misdiagnosed as being caused by *M. tuberculosis* and it resulted in the death of patients from the administration of wrong therapy (Gyure et al., 1995; Weiss et al., 1995). Therefore, it is extremely important to establish the correct diagnosis of the mycobacteria that caused CNS-TB infection to prevent detrimental outcomes and provide proper treatment.

Induction of host cellular immune responses that inhibit mycobacterial replication are key in controlling *M. tuberculosis* infection and inducing immunity (Mihret et al., 2012; Satake et al., 2017). It has been established that the brain and the immune system communicate (Matyszak and Perry, 1995; Perry and Teeling., 2013; Louveau et al., 2015). We detected leucocyte infiltration in the brains of all mice infected with *M. tuberculosis* or BCG and they presented with higher meningeal inflammation (TBM) compared to saline inoculated control

mice. This observation is common in murine models of CNS-TB which present with leucocyte infiltration in the brain parenchyma and meninges (van Well et al., 2007; Lee et al., 2009; Francisco et al., 2015). Zaharie and colleagues (2020) detected the presence of innate (circulating macrophages and microglia) and adaptive (CD4+ and CD8+ T cells) in the brains of TBM patients when they analysed granuloma formation. In humans, poor outcome of TBM patients has been attributed to immune pathology (Wilkinson et al., 2017), which promotes the importance of studying the role of immune cells in CNS-TB disease. To date, very few studies have investigated immune responses that occur during CNS-TB infection using animal models (Matyszak and Perry; 1995; Tsenova et al., 2005; Lee et al., 2008; Lee et al., 2009; Zucchi et al., 2012; Francisco et al., 2015; Hsu et al., 2017; Tucker et al., 2016; Hernandez et al., 2018) because the focus has mainly been on the pulmonary infection (primary route of infection) even though CNS-TB is a devastating form of tuberculosis (Rock et al., 2008). Therefore, we examined immune responses induced during mycobacterial infection of the CNS by characterizing dendritic cell, microglial, macrophage, and T cells using flow cytometry. We showed changes in the cellular composition of innate and adaptive immune cells overtime during mycobacterial infection of the CNS, as similarly observed by Lee and colleagues (2009) who demonstrated this phenomenon post BCG intracerebral infection (Lee et al., 2009). Innate immune responses play a crucial role in defense against TB and ultimately TB pathogenesis (Young et al., 2020) which are normally facilitated by APCs. The cellular composition of APCs we detected in the brains of infected mice not only revealed increased numbers of microglia after *M. tuberculosis* or BCG intracerebral infection; but also, recruitment and expansion of macrophages and dendritic cells originating from the periphery. Brain APCs from infected animals displayed highly activated and mature phenotypes shown by increased numbers of these cells expressing MHCII (antigen-presenting molecule), co-stimulatory CD86 molecule, pro-inflammatory cytokines (IL-1β, IFNγ, TNFα, IL-6, IL-12) and anti-inflammatory (IL-10) cytokines compared to saline control mice. APCs from infected mice doubled or tripled when compared to controls, a finding similar to previous studies conducted in mice (Matyszak and Perry, 1995; Mazzolla et al., 2002; Lee et al., 2008 and 2009; Zucchi et al., 2012; Francisco et al., 2015). These three brain APC subpopulations are commonly present during CNS inflammation or mycobacterial infection of the CNS and perform distinct functions (Fischer et al., 2000; Lee et al., 2019; Hsieh et al., 2013; Yamasaki et al. 2014; Wlodarczyk et al., 2014; Francisco et al., 2015; Kim et al., 2016). Although these innate immune responders play important roles that can either be protective against *M. tuberculosis* or assist in causing tissue

damage (Natarajan et al., 2011; Cresswell et al., 2019), in our model they appeared to be protective. All three brain APCs presented with highly activated (MHCII+) phenotypes but the levels of CD86 expression, a co-stimulatory molecule that is required for enhancement of immune responses and increasing effectivity of T cell activation (Lenshow et al., 1996) greatly differed. More than 50% of recruited brain DCs expressed CD86 compared to 20% of brain infiltrating macrophages and 2,5% of microglia during mycobacterial infection of the CNS. According to our data, we suggest recruited brain DCs are the most effective APCs, followed by brain infiltrating macrophages and to lesser extent microglia during mycobacterial infection of the CNS. Our findings support the notion that DCs remain potent APCs in the CNS compared to microglia and macrophages based on higher MHCII and costimulatory molecule expression (Steinman and Cohn, 1973; Steinman, 1991; Ford et al., 1996; Liu, 2001; Hickman et al., 2002; Greter et al., 2015; Remington et al., 2007; Goverman, 2009; Anandasabapathy et al., 2011; Mundt et al., 2019). The resulting outcome of CNS-TB disease is majorly defined by the interaction of APCs with adaptive T cells, important key cell types in antimycobacterial immunity (Choi et al., 2018). Both animal models and humans need to initiate and maintain sufficient adaptive T cell immune responses to achieve *M. tuberculosis* control (Mogues et al., 2001; Ulrich's and Kaufmann, 2006; Lee et al., 2009; Philips and Ernst, 2012; Srivastava and Ernst, 2013; Hsu et al., 2017) and this can be established by dendritic cell-T cell interaction. Our study showed for the first time the preferential recruitment and expansion of mature conventional DCs (MHCII+, CD86+, inflammatory cytokine+) to the CLNs of mice after intracerebral infection with *M. tuberculosis* and BCG, respectively. Similar to recruited mature brain DCs, increased numbers of recruited CLN DCs during mycobacterial infection of the CNS also expressed CCR7, a migratory molecule that determines DC capacity to traffic antigens to CLNs (Schreiber et al., 2011, Clarkson et al., 2017). This is an important feature for enhancing DC functional capabilities that include the ability of DCs to prime and activate naïve T cells. Our data showed for the first time the kinetic recruitment and expansion of effector CD4+ T cells and CD8+ T cells to the CLNs after *M. tuberculosis* or BCG intracerebral infection, CLNs are a site for innate-adaptive crosstalk for CNS antigens (Phillips et al., 1997; de Vos et al., 2002; Clarkson et al., 2017; Louveau et al., 2018; Esposito et al., 2019). Our findings showed recruitment and expansion of effector CD4+ T cells and CD8+ T cells to the brain after *M. tuberculosis* H37Rv or BCG intracerebral infection of wild-type C57BL/6J mice compared to saline inoculated mice as previously observed in mice (Lee et al., 2009; Hernandez et al., 2016; Hsu et al., 2017). These previous studies associated the presence of these T cell subsets with protection against mycobacterial infection of the CNS. We found effector CD4+

T cells to be the predominant subset over CD8+ T cells being recruited to both the brain and CLNs during mycobacterial infection of the CNS suggesting a prominent induction of helper T cell phenotype which is protective against CNS-TB. These T cell immune responses appear to have contributed to the significant reduction in the brain bacterial burdens that we observed in our model because T cells promote T cell clearance. In our *in vivo* model, complete clearance of *M. tuberculosis* or BCG in the brain during mycobacterial infection of the CNS was not achieved. Our findings support previous studies conducted on pulmonary TB that showed *M. tuberculosis* infection is controlled by T cell responses, but *M. tuberculosis* persists in the host (human and animal) because it is never eliminated (Feldman et al., 1938; Srivastava et al., 2016). Taken together, our overall findings of increased numbers of activated brain APCs and T cells suggest the same conclusion of protection against mycobacterial infection of the CNS can be drawn regarding our study since we observed mycobacterial control in the brains of infected animals and 100% survival. We confirmed our DC and T cell *in vivo* findings during CNS-TB through the generation of an *in vitro* DC-TC coculture set up using sorted DCs from intracerebrally infected mice and naïve splenocytes. Our DC-TC coculture revealed a predominant Th1 phenotype that was regulated as evidenced by high percentage expression of IFNγ, TGF-β and IL-10 by both CD4+T cells and CD8+ T cells. Our coculture findings not only validate our *in vivo* findings but show for the first time the mechanism employed by *M. tuberculosis-infected* DCs to modulate T cell immune responses during CNS-TB. Although previous studies characterized these cells during CNS-TB in the brain, they did not fully characterize their functional profiles such as their wide cytokine signatures and cellular interactions. We then extensively examined the phenotypes and functional profiles of our CNS-TB target cells.

Similar cytokines can be secreted by different cell types. Increased numbers of the three brain APCs (microglia, macrophages, and DCs) detected in our study all expressed pro-inflammatory cytokines; IFNγ and IL-12 during the entire course of CNS-TB infection and these cytokines are extremely crucial for TB immunity in humans and mice (Newport et al., 1996; Jouanguy et al., 1996; de Jong et al., 1998; Altare et al., 1998; Lee et al., 2009). During CNS-TB, our data showed that increased numbers of microglia expressed the highest IL-12, followed by recruited brain DCs and the least numbers by brain infiltrating macrophages. Whereas, increased numbers of microglia and recruited brain DCs expressed equivalent IFNγ that was higher than brain infiltrating macrophages. Early proinflammatory responses against infection can be brought about by phagocytes producing IFNγ which activates phagocytes to

perform their effector functions (killing mycobacteria) and this production of IFNγ that is regulated by IL-12 plays a key defense role against intracellular *M. tuberculosis* infection (Schroder et al., 2004; Munder et al., 1998; Otani et al., 1999; Domingo-Gonzalenz et al., 2016; Nikitina et al., 2016). While IL-12 is important for Th1 protective responses in mice during TB (Flynn et al., 1995; Cooper et al., 1995) and IL-12 deficiency in mice leads to *M. tuberculosis* susceptibility (Khader et al., 2006; Holscher et al., 2001). Taken together, our data suggest that these cytokine responses contributed to the significantly decreased brain bacterial growth we observed in our murine model because it has been shown that for the maintenance of IFNγ production and continuous limitation of bacterial growth, prolonged production of IL-12 is required (Feng et al., 2005). The production of IL-12 by APCs in the CNS has the potential to determine the effectiveness and quality of intracerebral immune responses (Aloisi et al., 1997) and IL-12 produced by APCs during infection has been shown to polarizes T cells towards Th1 immune responses (D'Andrea et al., 1992; Heufler et al., 1996; Trinchieri, 1998; Gately et al., 1998; Frucht). We detected increased numbers of mature DCs that were recruited to the cervical lymph nodes during CNS-TB infection expressing IL-12 as compared to saline control mice. The crosstalk between innate and adaptive immune responses orchestrated by APCs and T cells occurs in the cervical lymph nodes (Esposito et al., 2019; Clarkson et al., 2017) and this places DCs as central orchestrators of specific T cell immune responses. In our study, IL-12 signaling by APCs was involved in the induction of the increased numbers of activated brain CD4+ T cells that expressed IFNγ during CNS-TB. Although brain APCs expressed IFNγ, we found increased numbers of activated CD4+ T cells recruited to the brain during CNS-TB to be the major expressing IFNγ. This was an expected result because T cells are one of main producers of IFNγ, a Th1 cytokine that helps control intracellular pathogens through activation of phagocytes such as macrophages (Flynn et al., 1993; Domingo-Gonzalenz et al., 2016). Another pro-inflammatory cytokine expressed by CD4+ T cells that assists with enhancing macrophage and microglia activation is TNFα (Grotzke and Lewinsohn, 2005; del Corral et al., 2009) and we found increased numbers of recruited brain CD4+ T cells expressing it. We suggest that recruited brain CD4+ T cells detected in our study contributed to decreased brain bacterial burdens during CNS-TB through activation of microglia/macrophage. In conclusion, our data suggest induction of *M. tuberculosis* specific Th1 immune response against CNS-TB based on IFNγ+CD4+T cells being Th1 specific immune cells that protect against tuberculosis (Orme et al., 1993; Flynn et al., 1993). These polyfunctional brain IFNγ+IL-10+TGFβ+CD4+ T cells we detected supported regulated immune responses. The presence of IFNγ testing in the

CSF is specific for diagnosing TBM (San-Juan et al., 2006) and our study identified specific cell type sources of this crucial anti-mycobacterial cytokine during CNS-TB. We propose the importance of the crosstalk between brain APCs and T cells to be facilitated by three signaling methods; MHCII, CD86, and proinflammatory cytokines (IL-12 and IFNγ) expression to be beneficial for protection against CNS-TB. TNFα was expressed by low increased numbers of recruited brain CD4+ T cells during mycobacterial infection of the CNS as compared to slightly higher numbers by brain infiltrating macrophages and DCs (equivalent). We found no differences in the number of microglia expressing TNFα post BCG or *M. tuberculosis* intracerebral infection in comparison to controls. Our *M. tuberculosis* findings add new knowledge to the field of CNS-TB while the BCG findings are in contrast to findings by Lee and colleagues (2009) who showed DCs produce little TNFα compared to microglia post-BCG CNS infection. Some previous studies showed that TNFα production facilitates the spreading of the bacteria in the brain when the BBB is breached leading to CNS pathogenicity caused by mycobacteria (Mastroianni et al., 1997; Tsenova et al., 1998 and 1999; Thwaites et al., 2003; Curto et al., 2004). While others showed TNFα also contributes to the containment of *M. tuberculosis* (Denis, 1991; Hirsch et al., 1994; Flynn et al., 1995) and work emanating from our research group showed that TNF produced by both myeloid cells and T cells is required for protection against CNS-TB (Francisco et al., 2015; Hsu et al., 2017). Lee and colleagues (2009) also showed that TNFα produced by microglia, macrophages, DCs, and CD4+ T cells post BCG i.c. infection contributed to protective immune responses suggesting the same conclusion can be drawn regarding our study. We found higher increased numbers of recruited brain CD4+ T cells (4 weeks post-infection) expressing IL-10, followed by recruited brain DCs then macrophages later (4-6 weeks) during mycobacterial infection of the CNS. Cytokine IL-10 is known to dampen immune responses and prevent Th1 immunity if secreted early during pulmonary tuberculosis resulting in poor disease outcomes (Lai et al., 2018). Our data suggest that the expression and timing of IL-10 responses were beneficial in regulating and preventing what could have been detrimental hyper Th1 immune responses in our murine CNS-TB model. We also detected increased numbers of cervical lymph node DCs expressing IL-10 but it was throughout infection. The same cell type can act as either pro-inflammatory to fight off infection or anti-inflammatory for resolution of inflammation (Lee et al., 2020). We found increased numbers of recruited brain DCs and brain infiltrating macrophages expressing IL-1β and IL-6 during mycobacterial infection of the CNS, which were equivalent while DCs in the CLNs expressed higher numbers. IL-1 and IL-6 that play a crucial role in helping with the

recruitment of cells to the site of infection during pulmonary TB infection and induce protection against TB (Ladel et al., 1997; Giacomini et al., 2001). IL-1β is important for promoting resistance to *M. tuberculosis* and IL-1β knockout (KO) mice showed that *M. tuberculosis* infection causes increased bacterial burdens and mortality indicating susceptibility (Juffermans et al., 2000; Sugawara et al., 2001; Fremond et al., 2007; Mayer-Barber et al., 2010). Even though IL-6 stimulates the production of IFNγ, levels that were detected in CSF of TBM patients it has been associated it with severe disease outcome (Saunders et al., 2000; Simmons et al., 2006). We speculate that IL-6 was proinflammatory and performed a positive function in our model. Taken together, these cytokine responses contributed to immunity in our CNS-TB model due to the recruitment of effector innate and adaptive immune cells we observed at the site of infection that led to due to decreased brain bacterial growth. Within the brain, higher numbers of recruited brain DCs and brain infiltrating macrophages expressed pro-inflammatory cytokines (IFNγ, TNFα, IL-1β and IL-6) and anti-inflammatory cytokine (IL-10) during mycobacterial infection of the CNS compared to control mice. Some of our cytokine signatures are similar to previous studies which showed that brain homogenates of *M. tuberculosis* or BCG mice presented with increased production of IL-6, TNFα , and IFNγ from intracerebral murine models (Mazzola et al., 2002; van Well et al., 2007; Francisco et al., 2015; Hsu et al., 2017). We also found that increased numbers of cervical lymph node DCs expressing pro-inflammatory cytokines (TNFα, IL-12 and IL-1β) and IL-10 compared to saline controls during mycobacterial infection of the CNS. Unlike previous studies, our study succeeded in identifying the specific cell types in the brain and CLNs that expressed these multiple pro-and-anti-inflammatory cytokine signatures during CNS-TB. Our CNS-TB murine model cytokine profiles resembled those of human disease that showed several proinflammatory cytokines (TNFα, IFNγ, IL-6 and IL-1β) and anti-inflammatory cytokine IL-10 is induced and elevated in TBM patients CSF (Donald et al., 1995; Misra et al., 2010; Visser et al., 2015). These cytokines are associated with persistent inflammation that is caused by CNS-TB in humans (Babu et al., 2008) and our study also showed persistent inflammation during mycobacterial infection of the CNS. In conclusion, our characterization of dendritic cell, microglial, macrophage, and T cell immune responses during mycobacterial infection in the CNS using our murine model demonstrated the induction of protective innate and adaptive immunity.

Macrophages are a crucial cell type required for the active killing of *M. tuberculosis* by employing various mechanisms such as the production of inducible nitric oxide synthase (Lee et al., 1993; Peterson et al., 1995; MacMicking et al., 1997; Flynn and Chan, 2001; Olin et al.,

2008). Activation and production of IFNγ by macrophages induced their killing function (Domingo-Gonzalenz et al., 2016). Microglia (brain-resident macrophages) play a crucial role in the pathogenesis of cerebral tuberculosis (Zucchi et al., 2012) and remain central regulators of neuroinflammation, a process required for wound healing, especially from bacterial infections (Peterson et al., 1995; Puliti et al., 1999; Rock et al., 2005; Prinz et al., 2011; Kim et al., 2016), while macrophages like microglia help in regulating CNS inflammation (Rock et al., 2004; Sevenich, 2018) thus making them key players during CNS-TB disease outcome. Microglia and brain infiltrating macrophages have also been detected in TB lesions of rabbit models of TBM (Tucker et al, 2016; Cresswell et al., 2019) and humans (Shams et al., 2003). The increased numbers of both activated microglia and brain infiltrating macrophages we detected after *M. tuberculosis* or BCG intracerebral infection like previous studies in mice (Matyszak and Perry, 1998; Lee et al., 2009; Zucchi et al., 2012; Francisco et al., 2015) were expected because microglia and macrophages have been implicated as cells preferentially targeted by *M. tuberculosis* (Peterson et al., 1995; Rock et al., 2005; Yang et al., 2007). However, we noted number of microglial cells only significantly increased later during BCG infection (4 weeks) compared to saline controls, a finding that is not congruent to observation reported by Matyszak and Perry (1995) who showed that response by microglia to BCG CNS infection occurs earlier from 1-2 weeks. This difference can be attributed to the fact that their study was qualitative and not quantitative like ours and they used heat-killed BCG, whereas we used live-attenuated BCG for i.c. infection. It was microglia from *M. tuberculosis* H37Rv i.c. infected mice that showed sustained expansion during CNS-TB and underwent a larger increase in cell numbers than those from BCG infected mice. This was an expected finding because not only did previous studies show that microglia are preferentially infected by virulent *M. tuberculosis* as compared to avirulent strains of mycobacteria but also showed that microglia internalize large amounts of *M. tuberculosis* (Peterson et al., 1995; Rock et al., 2005; Yang et al., 2005; van Well et al., 2007; Curto et al., 2004; Spanos et al., 2015). We also found the recruitment and expansion of brain infiltrating macrophages to be significantly higher in *M. tuberculosis* H37Rv infected mice than BCG infected mice. Taken together, this data showed virulence-driven immune responses by microglia and brain infiltrating macrophages during mycobacterial infection of the CNS. These increased numbers of microglia and brain infiltrating macrophages mainly displayed classically activated (M1) phenotype and all expressed MHCII, CD86, pro-inflammatory IFNγ and IL-12. These two proinflammatory cytokines support mycobacterial killing through phagocytosis and induction of protective Th1

immune responses. IFNγ is a key pro-inflammatory cytokine required by the host for antimycobacterial activity during *M. tuberculosis* disease. In conclusion, our data suggest that both activated macrophage types (microglia and brain infiltrating macrophages) contributed to inducing protective innate immune responses against mycobacterial infection of the CNS due to controlled brain bacterial growth we observed in our model after *M. tuberculosis* H37Rv and BCG i.c. infection, a macrophage feature that has been shown by Guirado and colleagues (2013).

In our mouse model, microglial activation was mainly virulence-dependent during mycobacterial infection of the CNS. Microglial activation is an important quality for host defense (Hanisch, 2002) and has been shown in cerebrospinal fluid (CSF) of mice intracerebrally infected with *M. bovis* BCG (Mazzolla et al., 2002) and rabbit models of pediatric TB meningitis (Tucker et al., 2016). Activated microglia phagocytose bacilli, get recruited to TB lesions and are involved in presenting antigens to T cells to regulate T cells (Kielian and Drew, 2005; Hernandez-Pando et al., 2010; Green et al., 2011; Zucchi et al., 2012; Tucker et al., 2016). Our data showed that microglia were activated (MHCII+) as a result of both brain injury (surgery inflicted) or mycobacterial infection, a finding similar to previous observations that showed activation of microglia in mice due to injury or as a result of BCG intracerebral infection (Lee et al., 2009; Chhor et al., 2017). However, the degree of activation resulting from non-pathogenic insult was small (0-3%) compared to most mycobacterial infections of the CNS (84-95%). We observed significantly high increases in the number of microglia expressing MHCII and low increases in CD86 expression, and upregulation of these two molecules allows microglia to not only act as APCs but also communicate with other immune cells (Taylor et al., 2005). Our data suggest the potential involvement of activated microglia in the crosstalk with T cells for mycobacterial control, however, their signaling through CD86 is not robust. Unlike BCG-infected mice, microglial activation in *M. tuberculosis-infected* mice was sustained throughout CNS-TB infection. Sustained microglial activation could lead to detrimental tissue damage (Hanisch, 2002), however, it appears to have not been the case in our 100% survival CNS-TB model rather we speculate microglia contributed to protection against *M. tuberculosis* because microglia are involved in regulating inflammation caused by pathogen replication (Perry and Teeling, 2013). We observed a significant decrease in the number of microglial cells at week 6 post saline inoculation which can be attributed to wound healing after inflammation as shown by Matyszak and Perry (1995). However, these authors used immunohistochemistry to show clearance of non-pathogenic

inflammation post intracerebral inoculation that was accompanied by BBB repair even though their clearance occurred earlier between weeks 3-4 post-inoculation. Our data suggest that the alternatively activated (M2) phenotype of microglia was not the major phenotype induced during CNS-TB due to the non-resolution of inflammation (Olin et al., 2015). Therefore; the classically activated (M1) phenotype of microglia seemed to be mainly induced, which results in the killing of mycobacteria and also the presentation of antigens to T cells antigens (MacMicking et al., 1997; Olin et al., 2015) and potentially contributed to decreased brain bacterial burdens. However, we cannot confidently comment on the crosstalk between T cells and microglia because we did not investigate their cellular interaction. Qin and colleagues (2015) showed that during CNS-TB, microglia take on M1 phenotype and to a lesser extent, the M2 phenotype after being exposed to medium cultured from macrophages that were challenged with mycobacteria (*Mycobacterium marinum*). Our data demonstrated the predominant M1 phenotype of microglial polarization through their cytokine signatures. The secretion of cytokines and chemokines is how microglia regulate immune responses against mycobacteria (Curto et al., 2004; Rock et al., 2004 and 2005). Microglia have been shown to secrete cytokines after *M. tuberculosis* infection (Peterson et al., 1995; Rock et al., 2005) and expression of cytokines by microglia is also one component of protective immunity against mycobacteria (Lee et al., 2009). We found increased numbers of microglia expressing IL-12 during mycobacterial infection of the CNS when compared to saline controls. This result was expected because microglia cells are suggested to be major producers of IL-12 in the CNS (Taoufik et al., 2001) and both human and mouse microglia have been shown to produce IL-12 *in vitro* following mycobacterial stimulation and also promote antigen presentation to T cells (Becher et al., 1996; Aloisi et al., 1997 & 1999; Puliti et al., 1999). IL-12 is important for the induction of Th1 immune responses in *vitro and in vivo* (Manetti et al., 1993; Hsieh et al., 1993; Afonso et al., 1994; Gately et al., 1994). Our data suggest that cytokine signaling by microglia may potentially have played a role in inducing Th1 immunity against CNS-TB infection. An increased number of microglia detected in our study from *M. tuberculosis* or BCG infected mice also expressed IFNγ during CNS-TB infection, a proinflammatory cytokine that activates phagocytes to perform their effector function (Domingo-Gonzalenz et al., 2016). Microglia polarization can be influenced by Th1 (IFNγ or TNFα) or Th2 (IL-4 or IL-13) cytokines (Durafourt et al., 2012). This microglial M1 polarization was potentially induced by increased numbers of recruited brain IFNγ+CD4+ T cells that we detected in our study during CNS-TB. It has been shown that T cell production of IFNγ s also induced by IL-12 (Trinchieri,

1995). IFNγ has also been shown to induce MHCII expression by microglia in the CNS (Sethna and Lampson et al., 1991), which we speculate might be the case in our study. Our *in vivo* data found no difference in the number of microglia expressing TNFα, IL-1β and IL-10 in *M. tuberculosis* or BCG i.c. infected mice compared to saline controls; an observation similar to Curto and colleagues (2004) who showed that *in vitro* infection of human microglia with *M. tuberculosis* inhibits the production of IL-1 and IL-10 and also inhibited TNFα production later during infection. Our study is in contrast with findings by Lee and colleagues (2009) who showed the production of TNFα by microglia to be increased post intracerebral infection with BCG. In conclusion, our data showed that the cytokine profiles of microglial cells favor protective innate immunity and might play a role in the induction of Th1 immune responses against CNS-TB in our model. We speculate that one of the mechanisms being utilized by activated microglia to kill mycobacteria can be the production of reactive oxygen species or reactive nitrogen species such as inducible nitric oxide synthetase (iNOS) which has been shown in murine models of *M. tuberculosis* infection but not by human microglia (Denis et al., 1991; Lee et al., 1993; Peterson et al., 1995; MacMicking et al., 1997; Flynn and Chan, 2001; Olin et al., 2008 and 2015). Taken together, this indicates that microglial activation played a role in the maintenance of balance between sufficient activation for protection and excessive activation that might have led to detrimental responses.

Our data suggest that the recruitment and expansion of macrophages to the brain during mycobacterial infection of the CNS is pathogen driven than caused by mild brain injury from surgery and that these macrophage responses could be specific to mycobacteria. We base our assumption on the fact that we detected significantly higher numbers of macrophages in infected mice with mycobacterial compared to negligible numbers in saline inoculated control mice. Brain infiltrating macrophages that were activated by mycobacterial infection during this study took on the M1phenotype and not the M2 phenotype as described by Gordon (2003). Our data showed increased numbers of brain infiltrating macrophages expressing many pro-inflammatory cytokines (IFNγ, TNFα, IL-1β, IL-6, and IL-12) and anti-inflammatory IL-10 during mycobacterial infection of the CNS. Four out of five of these multiple pro-inflammatory cytokines (TNFα, IL-1β , IL-6, and IL-12) are similar to ones produced by macrophages during PTB infection upon the interaction of macrophages with bacteria (Weiss and Schaible, 2015) which macrophages use to perform host protection function against *M. tuberculosis* (Domingo-Gonzalez et al., 2016). These similar cytokine profiles between lung macrophages and brain infiltrating macrophages suggest induction of protective immunity against

mycobacterial infection of the CNS. IFNγ and TNFα production is crucial for controlling *M. tuberculosis* infection and recruiting effector cells to the site of infection (Co et al., 2004; Allie et al., 2013; Segueni et al., 2016), suggesting macrophages not only assisted in controlling brain bacterial burdens but also played a role in recruiting effector T cells and mature dendritic cells to the brain during CNS-TB (O'Garra et al., 2013). IL-1β plays an important in anti-tubercular immune responses in macrophages (Mayer-Barber et al., 2010). We found increased numbers of brain infiltrating macrophages expressing IL-12 during CNS-TB and IL-12 produced by macrophages activates T cells to polarize towards a Th1 immune response that produces TNFα to enhance macrophage activation (Weiss and Schaible, 2015), and increased recruited brain CD4+ T cells we detected expressed TNFα. Our results suggest that these macrophages are involved in the induction of *M. tuberculosis-specific* T cell responses that resulted in the reduction of brain bacterial burdens. One mechanism employed by activated macrophages to kill bacteria after phagocytosis is phagosome maturation (Flynn and Chan, 2001; Behar et al., 2010; Mihret et al., 2012). Our data found increased numbers of brain infiltrating macrophages expressing IL-10 later (6 weeks) during CNS-TB infection. IL-10 is known to block macrophage activation and phagosome maturation which results in increased *M. tuberculosis* bacterial growth within the phagosome during PTB (O'Leary et al., 2011; Weiss and Schaible, 2015) and early production of IL-10 by macrophages has been shown to result in *M. tuberculosis* infection susceptibility in CBA mice (Beamer et al., 2008) but later psecretion of IL-10 by macrophages helps suppress T cell immune responses (Weiss and Schaible, 2015). Therefore, our data suggest that brain infiltrating macrophages expressing IL-10 contributed to the non-susceptibility of wild-type C57BL/6J mice thus favoring antimycobacterial activity that played a protective role against CNS-TB. Virulent *M. tuberculosis* H37Rv CNS infection resulted in significantly higher immune responses than BCG CNS infection, however, both resulted in immune responses that favor protection against CNS-TB.

There are limited studies of DCs during mycobacterial infection of the CNS due to controversies that surrounded their existence in a healthy CNS. We found low numbers of plasmacytoid DCs (pDCs) in the brains of naïve mice but no conventional DCs (cDCs), unlike previous studies that found both subsets in the CNS of naïve animals (Giles et al., 2018; Mundt et al., 2019). Our data support the existence of DCs in a healthy CNS like other previous studies (Steinman,1972; Serot et al., 1997; McMenamin et al., 1999; Karman et al., 2004; Lindquist et al., 2004; Anandasabapathy et al., 2011; D'Agostino et al., 2012; Giles et al., 2018; Mundt et

al., 2019). We detected low numbers of pDCs and cDCs in control animals due to non-pathogenic inflammation of saline inoculated mice which had no effect on DC numbers in the brain, which were equivalent to naïve mice. This suggests that DC recruitment to the brain resulting from non-pathogenic inflammation is negligible. When we investigated the immune effects of dendritic cells as outcomes of *M. tuberculosis* or BCG infection of the CNS, we found an accumulation of DCs that were recruited to the brain as previously shown by work originating from our research group during CNS-TB (Francisco et al., 2015) and others post BCG intracerebral infection (Matyszak and Perry, 1995; Mazzolla et al., 2002; Lee et al., 2008 and 2009). This can be attributed to the fact DCs increase in numbers during neuroinflammation (Ludewig et al., 2016). We observed increases in the number of DCs during infection resulting from *M. tuberculosis* or BCG intracerebral infection, but they were not significant kinetic increases as shown in previous studies (Lee et al., 2009; Francisco et al., 2015). Similar to microglia and brain infiltrating macrophages, DC recruitment was virulence driven as evidenced by a larger magnitude of immune responses in *M. tuberculosis* i.c. infected mice compared to BCG infected mice. Although the immunological roles of DCs in mice have been explored during BCG CNS infections and CNS-TB (Matyszak and Perry, 1996; Mazzolla et al., 2002; Lee et al., 2009; Francisco et al., 2015). However, their phenotypes have not been fully characterized. The type of DC subsets present during infection dictates the direction of the immune responses (Macri et al., 2018) which is what led us to investigate the cellular phenotype of DCs during mycobacterial infection of the CNS. We identified mature cDCs as the dominant DC subset preferentially recruited to the brain and CLNs after *tuberculosis* or BCG intracerebral infection as compared to pDCs. Our study showed a novel finding of recruitment and expansion of these DCs in CLNs during mycobacterial infection of the CNS. DCs transport *M. tuberculosis* to lymphoid organs because they possess a poor killing capacity for bacilli they phagocytose (Krishnan et al., 2010), and DCs drain CNS antigens into CLNs (Clarkson et al., 2017). Our data showed significant swelling of CLNs during mycobacterial infection of the CNS as compared to control animals and DC recruitment has been shown to cause lymph node swelling when immune responses are underway (Acton et al., 2014). Lymph nodes are the other extra-pulmonary site for TB infection, and this may suggest that these mice also developed lymph node tuberculosis called cervical tuberculosis lymphadenitis (CTL) (Taşbakan et al., 2010; Deveci et al., 2016) because lymph node TB frequently occurs (Maji et al., 2015). CTL has been suggested to be caused by hematogenous dissemination of pulmonary TB or direct exposure to bacteria (Kent et al.,1967; Yew et al., 1995; Powell et al., 1999). A contrasting study by Selimoğlu and colleagues (1995) suggested that mycobacterial spread that

causes CTL was non-pulmonary which may be the case in our study because our site of infection was the brain. However, it would require further investigation. We detected greater numbers of cervical lymph node DCs which could not be solely attributed to DCs draining from the brain. The CNS consists of the brain and spinal cord (Ousman and Kubes, 2012) so we speculate that drainage from the spinal cord's cerebrospinal fluid (CSF) contributed to these high numbers of DCs in the CLNs. During BCG CNS infection, Lee and colleagues (2008) showed dissemination of bacteria to the spinal cord post intracerebral infection and DCs that infiltrate the CSF drain into CLNs (Hatterer et al., 2006). Even though we detected low numbers of pDCs and greater numbers of cDCs during mycobacterial CNS infection, it does not imply that the role of pDCs is not important because it has been shown that different subsets of DCs (pDCs and myeloid DCs) can communicate and cooperate during pulmonary TB infection to direct T cell immune responses that are protective against TB (Pulendran et al., 2008; Lozza et al., 2014). Lozza and colleagues showed that pDCs enhanced CD4+ T cell proliferation that was induced by myeloid CD11c+ DCs that were infected with *M. tuberculosis*. Conventional DCs present CNS antigens to T cells (Mundt et al., 2019) suggesting the phenotype of DCs in our study presented *M. tuberculosis* or BCG antigens to T cells. Whereas, plasmacytoid DCs are susceptible to *M. tuberculosis* while cDCs are resistant but both DC subsets contribute to immunity against TB (Lozza et al., 2014). DCs have been implicated in immune-mediated CNS diseases (Fischer and Reichmann, 2001) and two subsets of DCs (myeloid and plasmacytoid) have been found in humans during neuroinflammatory diseases and shown to potentially contribute to local immune responses (Pashenkov et al., 2001). We speculate that in this study, pDCs and cDCs may have cooperated during mycobacterial infection of the CNS and played a role in immunity that was induced in our model in which all mice survived. In conclusion, we found cDCs to be the preferential phenotype recruited to the brain and CLNs during mycobacterial infection of the CNS, a cellular phenotype that favors the induction of adaptive T cell immune responses. DC maturation reflects the functionality of DCs and can be measured by the upregulation of MHCII, co-stimulatory molecules (CD80 and CD86), and chemokine receptor CCR7 which are all expressed by DCs (Azuma et al., 1993; Banchereau et al., 2000; Gunn, 2003; Sanchez-Sanchez et al., 2006). Our study showed recruitment of mature DCs (MHCII+, CD86+, inflammatory cytokine+) to the brain and CLNs during the entire course of mycobacterial infection of the CNS compared to controls. Previous studies also showed MHCII and CD86 expression of DCs during BCG and *M. tuberculosis* infection (Lee et al., 2009; Francisco et al., 2015). The crosstalk between innate and adaptive immune responses orchestrated by APCs

and T cells occurs in the cervical lymph nodes (Esposito et al., 2019; Clarkson et al., 2017) and this places DCs as central orchestrators of specific T cell immune responses because they form a bridge between innate and adaptive arm (Kleinnijenhuis et al., 2011; Sáenz et al., 2013 Our data showed that increased numbers of recruited brain DCs expressed CCR7 during mycobacterial infection of the CNS, which demonstrated their capability of trafficking mycobacterial antigens into CLNs similar to what was observed in previous mycobacterial and non-mycobacterial studies (Schreiber et al., 2011; Clarkson et al., 2017) and increased numbers of CCR7+DCs in the CLNs indicated the presence of mostly migratory and not resident DCs. Migration of DCs to lymph nodes also involves CCR7 upregulation and also interleukin (IL)-12p40-dependent mechanisms (Khader et al., 2006; Schreiber et al., 2011), both mechanisms appear to apply to our study because we also observed increased numbers of DCS expressing IL-12. It is known that during brain inflammation, DCs migrate out of the CNS into the CLNs to induce T cell immune responses that are targeted to the CNS (Kida et al., 1993; Carson et al., 1998; Fischer and Reichmann, 2001; Karman et al., 2004; Hatterer et al., 2006) and *in vivo* DCs have been shown to induce improved T cell immune responses as a result of their enhanced migration capabilities (Khan et al., 2016). CNS antigens drain into the cervical lymph nodes (Kida et al., 1993; Weller, 1998) because antigen presentation of neuroinflammatory diseases mainly occurs in CLNs (Phillips et al., 1997; de Vos et al., 2002). Although we mainly found mature DCs during mycobacterial infection of the CNS, *M. tuberculosis-infected* DCs have been shown to transfer antigens to bystander DCs in the lymph nodes during pulmonary TB for presentation to naïve T cells due to the non-efficiency of tuberculosis-infected DCs (Griffiths et al., 2016). In conclusion, we showed that during CNS-TB infection, mature DCs in the CLNs were capable of presenting *M. tuberculosis* or BCG antigens to naïve T cells and potentially activating them to induce antimycobacterial immunity at the site of infection (CNS). These findings align with our data which showed activated T cells at the site of infection (brain) and CLNs. DCs that phagocytose live *M. tuberculosis* is not destructive to the mycobacteria, but their maturation and cytokine production contribute to protective immune responses against TB (Ryan et al., 2011). We investigated the effect of *M. tuberculosis* and BCG on the functional profiles of DCs during infection of the CNS because the cytokine profiles of DCs during CNS-TB have not yet been completely elucidated. Our data showed increased numbers of recruited brain DCs and DCs from CLNs expressed increased pro-inflammatory (IL-1β, IL-6, IL-12, IFNγ and TNFα) and anti-inflammatory (IL-10) cytokines after *M. tuberculosis* or BCG i.c. infection as compared to saline controls. These cytokine profiles are involved in both innate

and adaptive antimycobacterial immune responses. Human DCs have been shown to respond to BCG vaccination by secreting pro-inflammatory cytokines (IL-1β, IL-6 and TNFα) and a lessor anti-inflammatory IL-10 cytokine (Lozza et al., 2014) which resembled some of our study findings. We found increased numbers of recruited brain DCs and CLN DCs that expressed IL-12 during CNS-TB but not during BCG intracerebral infection. DCs are one of the major producers of IL-12 and IL-12 secreted by DCs normally plays an important role in DC migration to the lymph nodes as well as signaling cells to polarize towards T-helper 1 (Th1) immune responses (Gee et al., 2009; Vignali and Kuchroo, 2012). Our findings suggest IL-12+DCs were capable of inducing protective Th1 immune responses against CNS-TB infection that we observed in our model evidenced by controlled brain bacterial burdens. We also found increased numbers of recruited brain DCs and DCs in the CLNs expressing IL-1β after *M. tuberculosis* or BCG intracerebral infection, a proinflammatory cytokine associated with resistance against PTB *in vitro* and *in vivo* (Mayer-Barber et al., 2010; Bourigault et al., 2013). We found IL-1β to be the main cytokine expressed by the highest numbers of DCs compared to other cytokines during mycobacterial infection of the CNS by both recruited brain DCs and DCs from CLNs. Even though increased numbers of brain infiltrating macrophages also expressed IL-1β during mycobacterial infection of the CNS, the numbers were 10-fold lower compared to DCs. Pathogen virulence is a factor for IL-1 pathway signaling because protection against intravenous BCG challenge in mice that lack IL-1β is not affected (Bourigault et al., 2013). Our data supports this phenomenon because the magnitude of IL-1β responses were virulence driven during mycobacterial infection of the CNS regardless of our study limitation of not using IL-1β deficient mice for validation. We found increased numbers of recruited brain DCs expressing TNFα after *M. tuberculosis* infection but not post BCG intracerebral infection. This finding is in contrast to Lee and colleagues (2009) who showed that brain DCs produce little TNFα post BCG intracerebral infection. We also found that increased numbers of CLN DCs expressed TNFα during mycobacterial infection of the CNS, a novel finding that has never been demonstrated during mycobacterial infection of the CNS. Increased numbers of recruited brain DCs expressed IL-6, numbers that were equivalent to those expressed by brain infiltrating macrophages. IL-6 is a proinflammatory cytokine known to block the activation/maturation of DCs and favor differentiation of Th2 immune responses and minimal production of IL-12 required for Th1 immune responses (Park et al., 2004; Krishnamurthy et al., 2007). However, IL-6 produced by DCs also helps with the recruitment of cells to the site of infection (Giacomini et al., 2001) that assists in inducing immunity. Our data suggest that DCs played

role in inducing both innate and adaptive immunity in our murine CNS-TB infection model because these proinflammatory cytokines are associated with an antimycobacterial activity. DCs also produces IL-10 during mycobacterial infection (Verreck et al., 2004; Jang et al., 2004). We found increased numbers of recruited brain DCs and DCs from the CLNs expressing IL-10 later during CNS-TB infection (6 weeks). While later secretion induces suppressive immune responses necessary for regulating inflammation. This suggests that IL-10+DCs contributed to preventing detrimental disease outcomes and regulating CNS inflammation during mycobacterial infection of the CNS. We noted increased numbers of DCs in the CLNs expressed IL-10 throughout mycobacterial CNS infection, suggesting unique features of recruited brain IL-10+DCs to help sustain CNS immune privilege during infection. The function of brain DCs has been said to look like that of splenic DCs and bone-marrow-derived DCs because of little IL-10 production and robust capabilities of Th1 polarization (Fischer and Reichman, 2001). Our data showed increased numbers of DCs that expressed IFNγ during mycobacterial infection of the CNS. This finding is met with a lot of controversies because it is not yet widely accepted that non-lymphoid cells like DCs produce IFNγ (Hart, 1997; Frucht et al., 2001). However, small amounts of IFNγare produced by DC subsets (cDCs and pDCs) from the spleen (Vremec et al., 2007) and IL-12 produced by bacterial infection causes DCs to produce IFNγ (Ohteki et al., 1999; Frucht et al., 2001). In conclusion, our data suggest that the proinflammatory and anti-inflammatory cytokine profiles of DCs during mycobacterial infection not only play a role in early innate immunity but also contribute to the duction of Th1 and Treg immune responses against CNS-TB infection.

We investigated the contribution of T cells to disease outcomes during mycobacterial infection of the CNS by characterizing their functional profiles because mechanisms employed by these cells to induce protection against CNS-TB have not been fully defined. It has been shown that during active pulmonary TB and extra-pulmonary TB (lymph node and disseminated TB), *M. tuberculosis-specific* T-helper 1 (Th1) CD4+ T cells get recruited to the site of infection and undergo expansion contributing to immunity (Barry et al., 2003; Cooper, 2009). Our data suggest that the same phenomenon occurred during mycobacterial infection of the CNS as evidenced by the predominant influx and expansion of effector (CD44+CD62L-) CD4+ T cells than CD8+ T cells to the brain and CLNs as compared to saline controls. Immunity observed in our model is similar to previous mice studies (Lee et al., 2009; Hsu et al., 2017) that showed CD4+ T cells and CD8+ T cells after CNS are critical for establishing tuberculosis control and ultimately contributing to immunity against CNS-TB infection. With

CLNs as the location of CNS antigen crosstalk for T cell activation. CD4+ T cells are important for TB control in humans and animals (Havlir et al., 1991; Tsukaguchi et al., 1995) and in humans, the predominance of classical T cells in the CSF has been associated with better survival in TBM patients (van Laarhoven et al., 2019) which correlates with our murine CNS-TB model. Our data found recruited brain CD4+ T cells from *M. tuberculosis* mice increased in numbers earlier (2-4 weeks) during CNS-TB and decreased later (4-6 weeks), while BCG CNS infected mice showed CD4+ T cell accumulation in the brain as infection progressed. This suggests the antigen-specific immune responses were not delayed in reaching the brain and it explained the significant decrease in bacterial loads that we observed from 2 to 4 weeks post intracerebral infection. This data also shows that T cell inflammatory responses are regulated after 4 weeks. Saline inoculated control mice presented with low numbers of both T cell subsets in the brain during non-pathogenic conditions that decreased at week 6 post-inoculation as previously observed by Hernandez-Pando and colleagues (2010), again attesting to T cell recruitment to the brain being mainly pathogen-driven during CNS infection. For T cells to undergo activation, proliferation, and differentiation to occur, the following signals are required; (1) presentation of antigens by APCs on MHCII molecules binding to T cell receptor (TCR) and (2) stimulation of co-stimulatory molecule CD80/86 on APCs that binds to CD28 ligand on T cells (3) changes in gene expression and (4) cytokine signaling (Liberman et al., 2003; Grotzke and Lewinsohn, 2005; Riha and Rudd, 2010; Philips and Ernst, 2012; Srivastava and Ernst., 2013; Wlodarczyk et al., 2014). *M. tuberculosis* antigens get presented to CD4+ T cells by host APCs through the MHCII molecules and induction of specific CD4+ T cell immune responses which are central and crucial for the protection of the host against TB (Repique et al., 2003; Grotzke and Lewinsohn, 2005). As we described earlier, all mature brain APCs (microglia, infiltrating macrophages, and dendritic cells) and cervical lymph node DCs detected in our study presented with functional phenotypes capable of presenting *M. tuberculosis* H37Rv and BCG antigens to CD4+ T cells in the CLNs, activating and differentiating T cells into Th1 immune responses during mycobacterial infection of the CNS. Previous studies using mice deficient in CD4+ T cells showed poor infection outcomes due to failure in controlling *M. tuberculosis* bacteria that resulted in increased bacterial growth, bacterial dissemination, and increased mortality (Mogues et al., 2001; Philips and Ernst, 2012). Effector CD4+ T cells that directly recognize *M. tuberculosis* infected cells play a major role in controlling bacterial burdens through cytokine signaling of infected cells (Srivastava and Ernst, 2013). Our data suggest that brain bacterial control observed in our CNS-TB infection model can be partly attributed to the presence of CD4+ T cells that signal activation of

microglia and macrophages leading to mycobacterial killing mechanisms by these innate cells. Additionally, recruited CD8+ T also contributed to bacterial control in the brain because CD8+ T cells contribute to protective immune responses against TB through the cytotoxic activity of killing mycobacteria by releasing granzymes and perforin (Srivastava and Ernst, 2013; Bini and Hernandez-Pando (2013). Th1 immune responses required for protection against pulmonary TB (Lande et al., 2003; Desvignes et al., 2012; Rai et al., 2016) are also required for bacterial control against CNS-TB infection. Lee and colleagues (2009) showed this phenomenon after BCG i.c. infection that resulted in dominant Th1 and Treg immune responses which were similar to the periphery. Th1 cells are crucial for immunity against tuberculosis with the development and maintenance of Th1 cells relying on the production of IL-12 and T-bet (Th1 transcription factor) for successful control of tuberculosis (Flynn and Chan, 2001; Khader et al., 2006; Gallegos et al., 2008; Cooper, 2009; Phillips and Ernst, 2012; Vignali and Kuchroo, 2012; Nunes-Alves et al., 2014; Wlodarczyk et al., 2014; Choi et al.,2017). Increased numbers of all three brain APCs expressed IL-12 during mycobacterial infection of the CNS suggesting promotion of Th1 immune responses in our model. T cell activation and T helper differentiation depend on the changes in gene expression which are regulated by transcription factors (Liberman et al., 2003), therefore, it led us to assess the transcription factor (TF) profile during mycobacterial infection of the CNS. We found increased numbers of both CD4+ T cells and CD8+ T cells expressing higher T-bet and compared to saline controls and other three TFs (GATA3, RORγT , and FoxP3) in the brain and CLNs after *M. tuberculosis* or BCG intracerebral infection. T-bet expression has been studied in other CNS disease models such as murine models of experimental autoimmune encephalomyelitis (Wang et al., 2014; McPherson et al., 2015) but not during CNS-TB infection. T-bet mainly is responsible for controlling the polarization and effector functions of CD4+ T cells to Th1 and increased T-bet expression has been observed in the CD4+ T cells of active PTB patients and is associated with immunity (Szabo et al., 2000; Yang et al., 2015). Whereas, mice deficient in T-bet have been shown to result in enhanced progression of *M. tuberculosis* infection evidenced by increased bacterial loads and drastically reduced IFNγ production by CD4+ T cells (Sullivan et al., 2005). We found increased high numbers of recruited brain CD4+ T cells from *M. tuberculosis-infected* CNS-TB mice that expressed FoxP3 and low to negligible increased numbers expressing FoxP3. This finding is in contrast to work by Lee and colleagues (2009), who found an increased accumulation of FoxP3+CD4+ T cells in the brain and spinal cord post BCG intracerebral infection even though experimental

time points measured differed from ours (3-5 weeks vs 2, 4-6 weeks). Our data showed for the first time FoxP3+CD4+ T cells in the brain during CNS-TB. FoxP3 is a regulatory T cell (Treg) TF that dampens protective inflammatory responses to decrease pathology damage (Lee et al., 2009). Tregs have been suggested to be activated by infected DCs in the lymph nodes during pulmonary infection and proliferating simultaneously with effector cells which they suppress at the site of infection (Suffia et al., 2006; Belkaid et al., 2008). The development of Tregs is controlled by T-bet and T-bet also controls Th1 immune responses that are directed by IFNγ (Koch et al., 2009). Taken together, our data suggest that Th1 immune responses induced in our CNS-TB model were regulated as evidenced by the presence of both T-bet+CD4+T cells and FoxP3+CD4+ T cells. Our data showed for the first time that recruited brain CD8+ T cells barely expressed FoxP3, GATA3, or RORγT after *M. tuberculosis* or BCG i.c. infection compared to the high numbers of CD8+ T cells that expressed T-bet. Our data suggest CD8+ T cells played a role in controlling *M. tuberculosis* CNS infection through reduction of bacterial burdens as has been shown in previous PTB studies in mice (Müller et al., 1987; Orme, 1987). Whereas, CLNs presented with increased numbers of CD4+ T cells and CD8+ T cells that expressed FoxP3 at week 4 after *M. tuberculosis* or BCG i.c. infection which explains the presence of FoxP3+CD4+ T cells in the brains of CNS-TB mice. GATA3, a Th2 TF was not expressed by either CD4+ T cells or CD8+ T cells after *M. tuberculosis* or BCG intracerebral infection in the brain or CLNs suggesting the absence of Th2 immune responses. While extremely low numbers of recruited CD4+ T cells and CD8+ T cells in the brain and CLNs expressed RORγT, a Th17 TF during mycobacterial infection of the CNS. Our CD4+ T cell findings for both the brain and CLNs are similar to what has been observed during pulmonary TB in mice by Lozza and colleagues (2014), who showed that gene expression displayed the upregulation of CD4+ T cell TF T-bet (TBX21), RORγT (RORC), FoxP3 and no detection of GATA3, a phenotype that was induced by activated and mature DCs. Regulation of specific and effective T cell immune responses (Th1 or Th2) to pathogens is key in preventing detrimental disease outcomes because non-regulation of specific immune responses can lead to tissue damage and chronic inflammation (Vigano et al., 2012). For sterilizing immunity against TB, effector CD4+ T cells that are capable of producing Th1 cytokines are desirable. We investigated the cytokine responses of recruited brain CD4+ T cells during CNS-TB by performing *in vitro* re-stimulations with *M. tuberculosis-specific* ESAT-6, non-specific positive control αCD3CD28, whole live *M. tuberculosis* (H37Rv), and only media on total brain cells harvested from mice that were intracerebrally infected with *M. tuberculosis* at week

4post-infection. The number of recruited brain CD4+ T cells was only increased in saline control mice that were re-stimulated *in vitro* with H37Rv but no other re-stimulants indicating infection of T cells were *in vitro* with *M. tuberculosis*. We found that increased numbers of recruited brain CD4+ T cells expressed higher Treg anti-inflammatory cytokines (TGFβ and IL-10), high Th1 proinflammatory cytokine (IFNγ) and low numbers of Th1 (TNFα), Th2 cytokine (IL-4) and Th17 cytokine (IL-17) during CNS-TB compared to saline controls. However, *in vitro* re-stimulants did not lead to enhancement of cytokine expression by recruited CD4+ T cells during CNS-TB suggesting that cytokine saturation capacity was already reached by *in vivo M. tuberculosis* H37Rv intracerebral infection. Our data showed that increased numbers of recruited brain CD4+ T cells highly expressed TGFβ and previous studies have shown elevated levels of TGFβ during pulmonary TB and in TB meningitis patients (Olobo et al., 2001; Kalita et al., 2017). TGFβ production inhibits the proliferation of intracerebral T cell immune responses in the CNS and its upregulation induces neuroprotective roles in CNS disorders (Streilein et al., 1992; Vivien and Ali, 2006). While IL-10 is produced in large amounts, mainly by regulatory T cells limits pathogenic immune responses (Redford et al., 2011) and our data showed brain APCs also expressing IL-10. Our data suggest that anti-inflammatory cytokines (TGFβ and IL-10) were key cytokines in regulating immune responses in the CNS. We found increased numbers of IFNγ+CD4+ T cells and TNFα+CD4+ T cells during CNS-TB and TBM patients present with increased levels of TNFα and IFNγ in the CSF (Mastroianni et al., 1997; Juan et al., 2006). IFNγ is mainly secreted by T cells and natural killer cells and helps control intracellular pathogens through the activation of phagocytes such as macrophages (Flynn et al., 1993; Domingo-Gonzalenz et al., 2016). Th1 immune responses control *M. tuberculosis* growth but do not eradicate bacteria (Grotzke and Lewinsohn, 2005). Our *in vivo* findings suggest that cytokine signaling by recruited CD4+ T cells is a potential mechanism that is employed during CNS-TB to induce regulated Th1 immunity in the brain through the expression. These Th1 proinflammatory cytokines, IFNγ and TNFα produced by CD4+T cells activate and enhances microglia and macrophages to perform their functions of killing mycobacteria and also helps with recruitment cells to the site of infection to form granulomas which help in containing mycobacteria and preventing bacterial dissemination (Grotzke and Lewinsohn, 2005; del Corral et al., 2009). Although dissemination from the brain to other organs was not prevented in our murine model and we did not identify granulomas in the brain, we also did not show increased kinetics in the number of microglial cells and brain infiltrating cells beyond two weeks during CNS-TB suggesting

they played a role in decreased brain bacterial burdens. Polyfunctional cytokine profiles of CD4+ T cells during TB infection have been extensively investigated for TB vaccines and production of three co-expressed pro-inflammatory cytokines (IFNγ, TNFα, and IL-2) has been shown by some studies to correlate with protective immunity (Tameris et al., 2013; Lewinsohn et al., 2017). Lee and colleagues (2009) showed the accumulation of IFNγ+IL-17+CD4+ T cells in the CNS during BCG intracerebral infection. However, the polyfunctional cytokine profiles of the brain CD4+ T cells during CNS-TB had not yet been elucidated. Our data showed for the first time the polyfunctional cytokine profiles of recruited brain CD4+ T cells as consisting of higher frequencies of Th1 and Treg cytokine combinations which were; 1) TGFβ + IFNγ+ and (2) TGFβ + IL-10+, (3) TGFβ + IFNγ +IL-10+. Cytokines; IL-2, IL-10, and TGFβ function to protect the CNS tissue from pathology damage caused by excessive inflammation during CNS-TB (Hendrix and Nitsch, 2007; Hernandez et al., 2010). In conclusion, we found recruited brain CD4+ T cells presented with inflammatory cytokine profiles of highly regulated Th1 phenotype during CNS-TB, therefore implying induction of immunity against CNS-TB with limited pathology damage that pertains to CNS immune privilege.

Several studies that targeted DCs for therapeutic intervention during pulmonary TB have reported mature DCs are potent activators of *M. tuberculosis-specific* CD4+ T cells and CD8+ T cells required for vaccine development and also demonstrated that increasing DC efficiency reduces the dosage of effective anti-tuberculosis drugs (Malowany et al., 2006; Khan et al., 2016; Griffiths et al., 2016; Choi et al., 2018). Dendritic cell-T cell interaction (DC-TC) is a completely unexplored research area during CNS-TB, which led us to investigate DC-TC interaction during CNS-TB to uncover mechanisms associated with the modulation of T cells by *M. tuberculosis-infected* DCs. Our study found no differences in the activation status of T cells (CD4+ and CD8+ T cells) as measured by percentage expression of CD44 molecule (T cell activation molecule). Experimental limitations prohibited us from measuring CD69 surface expression (T cell activation biomarker) that is related to tuberculosis infection in animal models (Andersen et al., 2000; Kauffman et al., 2018). Even though we did not observe changes in the activation status of *Mtuberculosis-infected* T cells, our results showed significant changes in cytokines expressed by coculture CD4+ and CD8+ T cells. To produce an effective and productive DC-T cell interaction, mature DCs (MHCII+, CD86+, cytokine+) must be capable of capturing, processing, and presenting antigens to stimulate naive T cells and DC numbers control antigen-specific T cell proliferation as well as induce regulatory T

cells (Cella et al., 1997; Banchereau and Steinman, 1998; Lanzavecchia and Sallusto, 2001; Jonuleit et al., 2001 Gee et al., 2002; Hopken et al., 2005; Lim et al., 2012). This was the case in our *in vivo* CNS-TB study where we demonstrated that increased numbers of DCs recruited to the CLNs after *M. tuberculosis* H37Rv intracerebral infection were mature (MHCII+, CD86+, CCR7+, and inflammatory cytokine+) and favored Th1 immune responses. Additionally, Lim and colleagues (2012) demonstrated that cytokine production by DC-T cell interaction is decreased when CD86/80 is inhibited, which we speculate was not the case in our study. However, we could not measure T cell proliferation (Ki67 expression) in our coculture which is promoted by DCs *in vitro* (Gee et al., 2002) due to experimental limitations. DCs that are activated by mycobacteria direct CD4+ T cells to either differentiate into Th1 or Th2 immune responses (Demangel and Britton, 2000), and for *M. tuberculosis* to be contained and controlled, CD4+ T cells and CD8+ T cells must produce IFNγ (Bozzano et al., 2014). We found increased percentages of both CD4+ T cells and CD8+ T cells in our H37Rv coculture expressing proinflammatory cytokine (IFNγ) and anti-inflammatory cytokines (TGFβ, IL-10, and IL-4). Our data suggest that DCs from our *in vivo* CNS-TB infected mice were successful in inducing T cells in the H37Rv coculture to express dominantly Th1 and Treg immune responses. Our study showed a novel mechanism that is employed by DCs to modulate T cell immune responses that are protective against CNS-TB and our *in vitro* H37Rv DC-TC coculture findings validate our *in vivo* CNS-TB findings which demonstrated induction of Th1 and Treg immune responses as evidenced by increased numbers of recruited brain TGFβ+IFNγ+IL-10+CD4+ T cells and increased lower numbers of IL-4+CD4+ T cells at week 4 after *M. tuberculosis* intracerebral infection. With regards to TFs, T-bet+CD4+ T cells and T-bet+CD8+ T cells aided in the promotion of Th1 immune responses during *in vivo* CNS-TB infection. In conclusion, our data demonstrated that the mechanism employed by DCs to prime and activate antigen-specific T cells during CNS-TB is through alteration of proinflammatory and anti-inflammatory cytokines. These T cell responses induce CNS-TB immunity as evidenced by decreased brain bacterial burdens and 100% survival rates of intracerebrally infected mice. Therefore, we propose that DCs are key target cells for therapeutic intervention against CNS-TB. We support the continued investigations on-going efforts of targeting DCs for tuberculosis vaccine development for PTB because it will also be beneficial for CNS-TB because recruited brain and CLNs DCs originated from the periphery.

CHAPTER 6: CONCLUSION

CNS-TB which commonly manifests as TBM remains a serious disease associated with high rates of disability and death, especially in under-developed and developing countries. There is limited understanding of cellular and molecular mechanisms associated with TBM pathogenesis and targets for improved therapeutics. Very few studies have focussed on basic TBM research, a field that requires attention and it led to the 2018 launch of a specific US National Institutes of Health (NIH) grant that focuses on advancing basic and translational research, following a meeting with CNS-TB researchers (Jain et al., 2018). Our study contributes to advancing this goal by providing new knowledge and mechanistic insights that can lead to potential targets for strategic therapeutic intervention. Animal models are important in conducting basic research because they provide the platform to investigate and understand disease mechanisms, which cannot be conducted in human studies. We were successful in reproducing and validating the *in vivo* murine model of CNS-TB infection with characteristics observed in previous animal studies. Although our mouse model mimics human TBM and is beneficial for studying acute inflammatory responses to TBM, we acknowledge our animal model limitations which includes the route of infection that does not resemble the natural disease development in humans and also the absence of clinical symptoms. Regardless of our model limitations, it proved beneficial in investigating cellular-mediated immune responses that occur during CNS-TB infection and helped us generate new knowledge that enabled us to understand host-pathogen interactions for host benefit.

Immune responses induced in the CNS by *M. tuberculosis* or BCG intracerebral infection do not completely resolve, a phenomenon similar to PTB as was evidenced by significant reductions in brain bacterial loads but not mycobacterial eradication. There is an influx of mature immune cells (macrophages, DCs, and T cells) to the brain and CLNs that accumulate as well as the expansion of mature microglia during mycobacterial infection of the CNS. Additionally, the cellular composition of these infected cells changes over-time during CNS infection, as observed in a previous study. There are some shared immunological responses among recruited brain APCs (including microglia) and between APCs and recruited T cells that lead to protective immune responses during CNS-TB. However, these cells are also distinct functional features that can be virulence driven since virulence was mostly a factor in the magnitude of immune responses (*M. tuberculosis* vs BCG) induced and also the type of immune responses during mycobacterial infection of the CNS. This suggests that innate APCs can perform similar functions and also individually unique effector functions that are involved

in inducing protective innate and adaptive immune responses against CNS-TB. This was evidenced by mycobacterial control in the brain that resulted in 100% survival of the animals.

Our findings showed that conventional DCs preferentially recruited to the brain and CLNs during mycobacterial infection of the CNS and that DCs appear to possess the superior capacity that favors the induction of Th1 immune responses as compared to other brain APCs. The polyfunctional cytokine signature of CD4+ T cells during CNS-TB that achieved immunity in our model is distinct from that of pulmonary TB. We showed the novel mechanism employed by DCs to orchestrate the induction of *M. tuberculosis-specific* T cells for immunity against CNS-TB. This mechanism was the modulation of CD4+ T cells and CD8+ T cells to express majorly a combination of anti-TB proinflammatory Th1 cytokines and anti-inflammatory Treg cytokines, resulting in regulated Th1 immunity. Unlike the pulmonary TB polyfunctional cytokine profile consisting of Th1 cytokines which have been shown by some studies to correlate with immunity, the polyfunctional cytokine during CNS-TB immunity mainly consists of Th1 and Treg cytokines. This CD4+ T cell polyfunctional phenotype is important in limiting pathology damage in the CNS, an immune-privileged site. In our model, mature DCs migrated to the CLNs during CNS-TB, which is crucial in overcoming the challenge of delayed antigen-specific Th1 responses that lead to poor *M. tuberculosis* control. Mature DCs that accumulated in the brain were recruited from the periphery suggesting novel therapeutic interventions that target DCs to induce protective immunity against pulmonary TB can also be beneficial for protection against CNS-TB. Recruitment of DCs to the brain can be an advantage in overcoming the current challenges with TBM treatment drugs such as failure to penetrate the BBB and help with modifications in PTB that can be optimized to also treat TBM. Although further investigations and studies are needed to validate DCs as potential target cells for CNS-TB immunity, we support the continuation of the very few limited research studies that target DC maturation stages/phenotypes/genes for tuberculosis vaccine development and host-directed therapy.

CHAPTER 7: FUTURE PERSPECTIVES

Although our CNS-TB murine model proved valuable in studying immune responses during CNS-TB, we acknowledge limitations and recommend the development of improved mouse models that will assist in the better extrapolation of research findings from mouse models to human disease. It is advisable to do a comparative study of cellular-mediated immune responses in the lungs and CNS/brain during CNS-TB because histology revealed that regardless of bacterial control in the brain, the lung bacterial burdens kept exponentially increasing regardless of lung granuloma formation. Our study was met with limitations due to experimental challenges, funding, time constraints, and topics beyond the scope of this PhD project. We, therefore, propose the following future experiments for further investigations that would strengthen and validate our findings.

It is important to visualize the changes in cell morphologies that occur after intracerebral infection with *M. tuberculosis* or BCG in the CNS and also view cellular interactions, especially in the case of DC-TC coculture. This can be achieved by using confocal microscopy and transgenic CD11c mice for DCs (CD11c-eYFP) to visualize DCs internalizing *M. tuberculosis* during CNS-TB, track the migration of DCs from the brain and other locations including the CLNs because we found exponential expansion of DCs in the CLNs that cannot be solely attributed to those draining from the brain. The crosstalk between DCs and T cells that occurs in draining CLNs can be visualized and compared to the crosstalk that has been suggested to occur in the brain because DCs and T cells have also been shown to interact in the perivascular space at the site of infection. We suggest measuring T cell proliferation in the DC-TC co-culture during CNS-TB which was a limitation in this study. Additionally, use of robust controls for DC-TC coculture such as LPS infected DCs, and microglia cultured with T cells, to validate DCs as are superior stimulators of protective antigen-specific T cells during CNS-TB.

Our study showed that the phenotype and functional profiles of resident microglia and immune cells (DCs, macrophages, and T cells) that infiltrated the brain played a role in immunity during mycobacterial infection of the CNS. However, we could not validate the exact contribution of each cell type to the overall disease outcome. Further experiments that quantify the concentrations of cytokines secreted by the brain APCs and CD4+ T cells during CNS-TB can confidently allocate key target cells that are major producers of cytokine responses involved in protective immune responses. The challenge is, total cell counts used in our study can sometimes not reveal the exact contribution of individual cell types. Quantification of the

cytokines using proteomic real-time PCR is recommended and can also reveal a broader spectrum of multiple cytokines/chemokines and other protein responses that occur during CNS-TB, including identification of molecules involved in mycobacteria killing mechanisms such as iNOS. Transcriptomic studies using RNA sequencing can assist in identifying differentially expressed genes in *M. tuberculosis-infected* DCs to identify key genes and signaling pathways that can be targeted for DC maturation to favor protective disease outcomes during CNS-TB. The omics findings can unravel signaling pathways that will not only provide an understanding of mechanisms and signaling pathways that can be targeted for CNS-TB therapeutic intervention but also help us uncover novel genes/proteins. Further experiments can include mice deficient of each cell type (knock-out mice) or antibody depletion of each cell type in the CNS-TB infection model. For DCs, CD11c-diphtheria toxin (CD11c-DTR) mouse model [good at depleting cDCs (van Blijswijk et al., 2013)] can be used and kinetics studied to include time-points earlier than two weeks for investigation of early antigen priming and activation of T cells by DCs because *M. tuberculosis* infection is known to impair DC function and delay antigen-specific T cell responses. Targeting DCs *in vivo* and *ex vivo* for TB treatment like it is currently being done for DC-based anti-cancer immunotherapy can be an advantage for positive disease outcome. DCs can be infused into the host for vaccination development and drug testing. Some of these suggested experiments are already underway and being conducted by other members in our research group.

In conclusion, we hope our initial findings that have added to the body of knowledge will be validated through further investigations of immune responses that occur during CNS-TB and lead to translational applications that will contribute to reducing morbidity and mortality associated with CNS-TB.

CHAPTER 8: REFERENCES

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CHAPTER 9: APPENDIX

Appendix A: General and Homogenation buffers

1) 10x Phosphate Buffered Solution

80g of PBS

2g KCI

14.4g Na_2HPO_4

and 2.4g KH_2PO_4 were dissolved in 900ml distilled water then pH adjusted to 7.4 before making it up to 1000ml solution. The solution was filter sterilized through a 0.45µm Millipore filters (Millipore Corporation, Bedford, USA) and stored at room temperature.

2) Saline Solution (0, 9%)

0.9g of NaCl was dissolved in 100ml distilled water and the solution was autoclaved at 121°C for 30 min then stored at room temperature.

3) Saline-Tween 80

0.9% NaCl was added to 0.04% Tween 80 and distilled water, then to made up to 1000ml. The solution was filter sterilized through a 0.45µm Millipore filters (Millipore Corporation, Bedford, USA) and stored at room temperature.

Appendix B: Histology Reagents and Preparations

1) Buffered 10% Formalin (Tissue fixative)

270ml formaldehyde (37% formaldehyde solution) in 730ml 1x PBS (pH 7.4). The solution was stored at room temperature.

Haematoxylin and Eosin (H & E) *Reference: Cullings 3rd edition page 214*

2) Mayers haemotoxylin:

Dissolve all the following reagents the same time;

2.1) Add 1 g haematoxylin (yellow) pH5-7.2

2.2) Add 50g ammonium alum (aluminium ammonium/potassium sulphate)

2.3) Add 0.2g sodium iodate

2.4) Add 50g chloral hydrate

Add above reagents to distilled water and filter solution through Whatmann filter paper before being stored at room temperature in the dark.

3) Eosin solution preparation

3.1) 150 ml of 1% Eosin yellow dissolved in distilled water and thymol added to prevent bacterial growth

3.2) 75 ml of 1% Phloxine in distilled water

Add 2 parts of 1% eosin + 1 part 1% phloxine + 225 ml distilled water and filter solution through Whatmann filter paper before being stored at room temperature.

4)Scotts Water Preparation

Add 2g Potassium bicarbonate 2g and 20g magnesium Sulfate to 1000ml distilled water.

Ziehl-Neelsen (ZN) staining

1) Carbol fuchsin:

6% Basic fuchsin in absolute alcohol (10 ml) was added to 5% carbolic acid (90 ml). The solution was filtered through Whatmann filter paper before being stored at room temperature.

2) Loeffers' Methylene blue:

2.1) Add 1% KOH to 99 ml of distilled water

2.2) Add 0.8% Methylene blue to 1000% alcohol

1ml of 1% KOH solution was added to 3ml of 0.08% to make up the solution, then filtered through Whatmann filter paper no.1. Solution was stored at room temperature.

Appendix C: Flow Cytometry reagents and buffers

1) 10% Sodium Azide

10g NaN_3 was dissolved into 100ml of distilled water. Then stored at 4°C until use.

2) FACS Buffer

0.1% BSA and 0.01% NaN_3 were added were added to 1 x PBS (pH 7.4). The solution was filter sterilized with a 0.45µm Millipore filter (Millipore Corporation, Bedford, USA). Then stored at 4°C.

3) FACS blocking buffer

Add 0.9375µl αFcyRIII (CD32/CD16c)

Add 3.75µl Normal rat serum (heat inactivated)

Add 3.75µl Normal mouse serum (heat inactivated) were diluted in
Add 141.56µl of FACS buffer.
After adding all then vortex and store at 4°C.

4) Fixation Buffer
Add 2% paraformaldehyde (PFA) in 1 x PBS
Dissolve 4g NaOH was in 100ml distilled water.
Dissolve 20g PFA in 600ml of 1 x PBS. Then adjust pH to pH 7.2 using concentrated HCl and make up to a volume of 1000ml. Sterilize the fix buffer through a 0.45µm Millipore filter (Millipore Corporation, Bedford, USA), then cover the bottle with aluminum foil and stored at 4°C.

5) Permeabilization buffer
0.1% Saponin
1mM $CaCl_2$
1mM $MgSO_4$
And 10mM HEPES were dissolved in 1x PBS that contained 0.05% NaN3 and 0.1% BSA. The solution was sterilised through a 0.45µm Millipore filter (Millipore Corporation, Bedford, USA) and stored at 4°C.

Appendix D: Re-stimulation assay reagents and stimulants

1) RPMI
RPMI powder was equilibrated at room temperature before being dissolved 200 ml of distilled water. 2g of $NAHCO_3$ was mixed with solution then pH was adjusted to 7.4 then made up to 1000 ml. RPMI media solution was sterilised through a 0.45µm millipore filter (Millipore Corporation, Bedford, USA) and stored at 4°C.

2) Complete re-stimulation buffer
150 ml RPMI media solution (pH 7.4) was added to 10 ml Fetal Calf Serum (FCS), then 2 ml L-glutamine (2 mM) and 200 ul penicillin-streptomycin antibiotic (10 ug/ml+ 10 ug/ml penicillin) and finally 200ul Beta-mercaptoethanol was added. Ph was adjusted to 7.4 and complete re-stimulation solution was sterilised through a 0.45µm millipore filter (Millipore Corporation, Bedford, USA) and stored at 4°C.
3) Stimulants

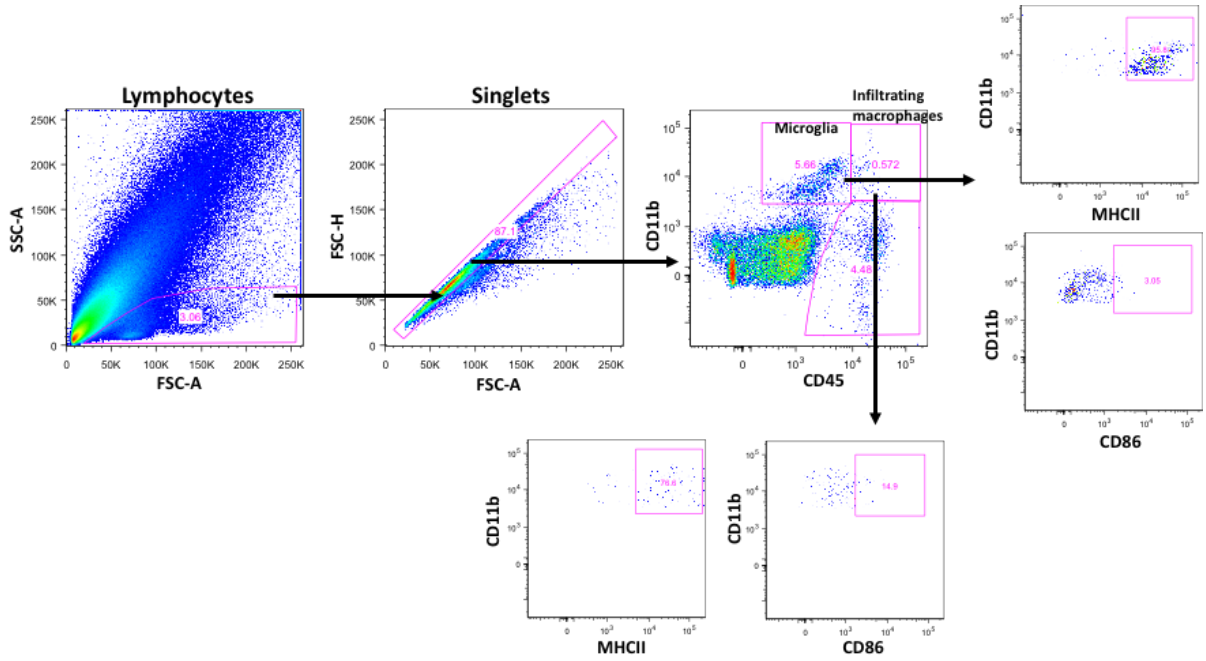
1) αCD3 and αCD28 cocktail in PBS (10 ug/ml each)

2) H37Rv in complete medium (MOI 2:1)

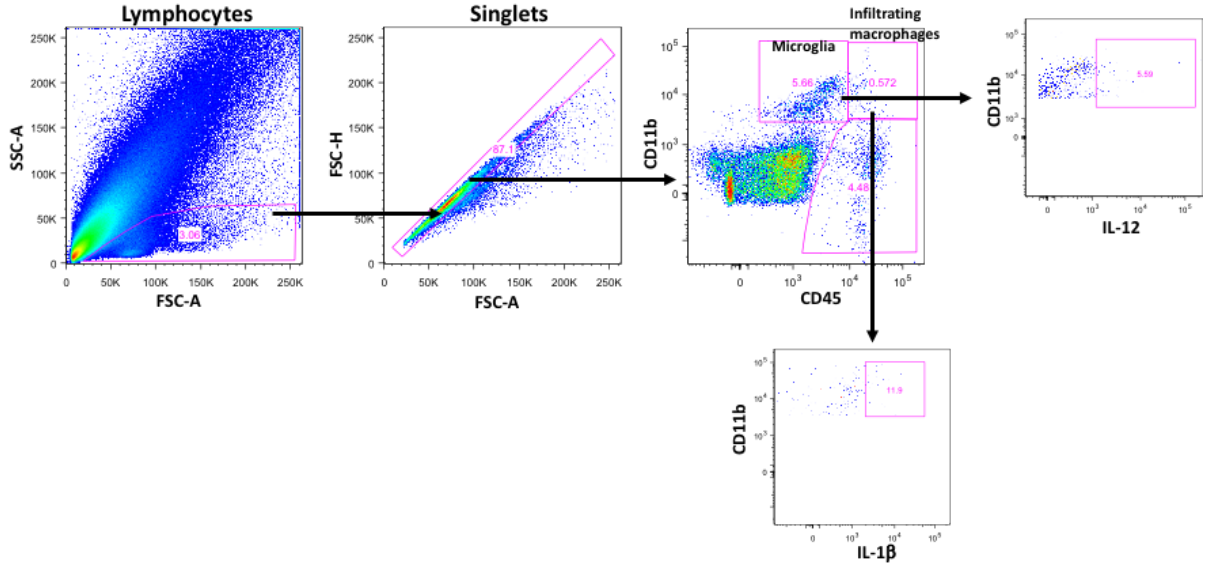
3) ESAT-6 in complete medium (10 ug/ml working solution)

4) No antigen (complete medium only)

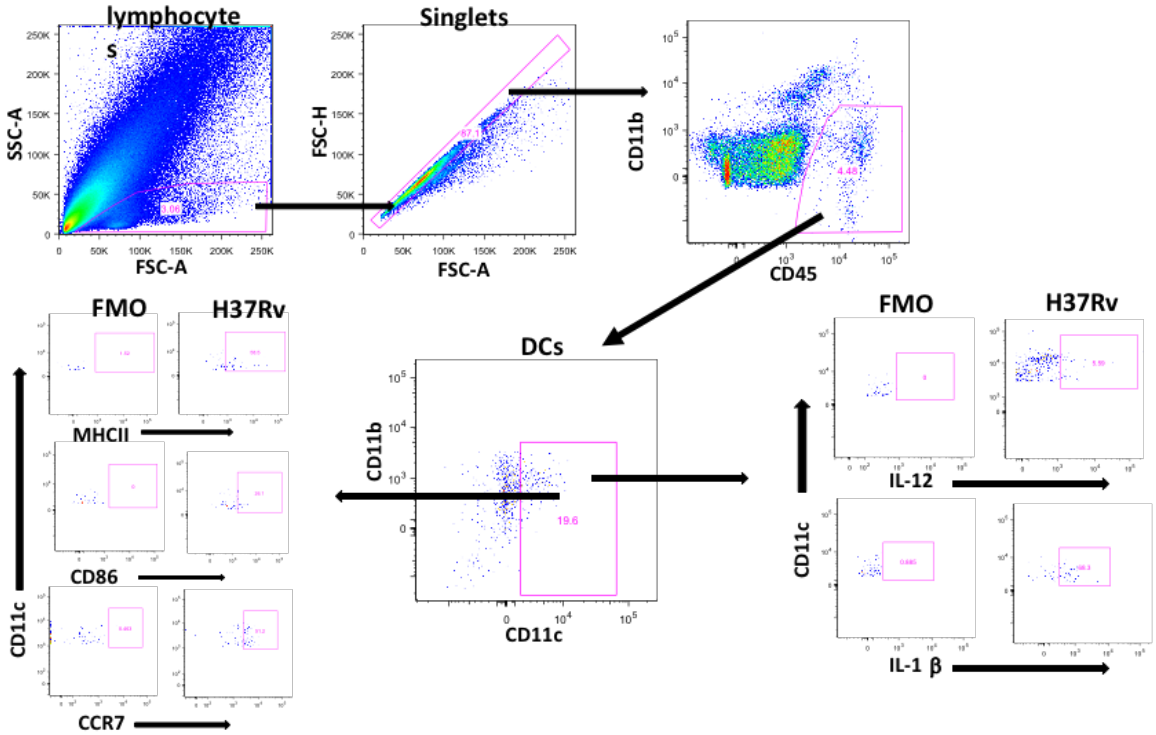
Appendix E: Representative flow cytometry gating strategies for MHCII+ and CD86+ microglia and brain infiltrating macrophages during mycobacterial infection of the CNS.



Appendix F: Representative flow cytometry gating strategies for cytokines expressed by microglia and brain infiltrating macrophages during mycobacterial infection of the CNS.



Appendix G: Representative flow cytometry gating strategies recruited brain DCs expressing MHCII, CD86, CCR7 and cytokines during mycobacterial infection of the CNS.



Appendix H: Representative flow cytometry gating strategy for DC-TC coculture showing gating of CD4+ and CD8+ T cells expressing CD44.