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Early innate phagocytic response to *Mycobacterium bovis* BCG

Master thesis

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Statutory Declaration

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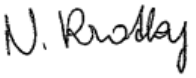

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Abstract

Tuberculosis is an infectious disease which is caused by the bacterium called *Mycobacterium tuberculosis* and can develop anywhere in the body but occurs primarily as a pulmonary disease (affecting the lungs). Tuberculosis is one of the top causes of deaths worldwide and killed more people than any other pathogen in 2018. The development of effective vaccines is limited due to the incomplete understanding of protective immune responses to mycobacteria. There is only one vaccine available on the market to prevent Tuberculosis, BCG, but it does not reliably protect against pulmonary Tuberculosis in adults. The immunological mechanisms and interactions which lead to protection after BCG immunization are yet poorly understood.

Our laboratory wants to investigate the key elements of immune responses regarding to BCG vaccination, as well as how these are relevant in the first two weeks of mycobacterial infection in a mouse model. This project investigates the effect of mycobacterial vaccination on innate phagocyte subsets in the lungs of mice, and how this contributes to anti-mycobacterial immune responses upon subsequent infection.

Previous work published from our laboratory demonstrated that vaccination with BCG results in recruitment of macrophages to the murine lung. Alveolar macrophages in BCG-vaccinated mice were more abundant and had increased CD11b expression compared to alveolar macrophages in naïve mice. Vaccinated mice also had more interstitial macrophages expressing high levels of CD11b. These CD11b-expressing macrophages made up a larger proportion of the total mycobacteria-associated cells in vaccinated mice and compared to macrophages that do not express CD11b they seem to have enhanced anti-mycobacterial activity.

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Abbreviations

AMΦ	Alveolar Macrophages
APC	Allophycocyanin
BCG	Bacillus Calmette-Guérin
CFU	Colony Forming Units
cIMDM	Complete Iscove's Modified Dulbecco's Medium
DC	Dendritic Cell
dH_2O	Distilled Water
FACS	Fluorescence Activated Cell Sorting
FBS	Fetal Bovine Serum
FCS	Forward Scatter
FMO	Fluorescence-minus-one
FVS	Fixable Viability Stain
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factors
HIV	Human Immunodeficiency Virus
HygB	Hygromycin B
i.n.	Intranasal
i.p.	Intraperitoneal
IFN	Interferon
IL	Interleukin
IMDM	Iscove's Modified Dulbecco's Medium
IMΦs	Interstitial Macrophages
m.bovis	*Mycobacterium Bovis*
MDR-TB	Multi Drug-resistant Tuberculosis

MOI	Multiplicity of Infection
Mtb	*Mycobacterium tuberculosis*
MΦ	Macrophages
NK cells	Natural Killer Cells
NLRs	NOD-like receptors
OADC	Oleic acid Albumin Dextrose Catalase
PAMPs	pathogen-associated molecular patterns
PBS	Phosphate Buffered Saline
PC2	Physical Containment Level 2
PC3	Physical Containment Level 3
PRR	Pattern Recognition Receptor
RBC	Red Blood Cell
RD1	Region of Difference 1
SSC	Side Scatter
TB	Tuberculosis
TdT	TdTomato
Th1	T-helper-1
TLRs	Toll-like receptors
TNF	Tumour Necrosis Factor
TPBS	Tween Phosphate Buffered Saline
WHO	World Health Organization
XDR	Extensively Drug-Resistant Tuberculosis

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1 Introduction

Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis (Mtb).* The infection can develop anywhere in the body but occurs primarily as a pulmonary disease (affecting the lungs). This bacterium is one of the top causes of deaths worldwide, and killed more people in 2018 than any other pathogen (WHO, 2019). Worldwide, two billion people are infected with *Mtb*. The disease is the leading cause of deaths in people infected with human immunodeficiency virus (HIV), and is responsible for one-third of global antimicrobial resistance (WHO, 2019).

The only vaccine available against TB for humans is Bacille Calmette Guérin (BCG), which does not reliably protect against pulmonary TB in adults. Its efficacy, especially in adults, is highly variable because of genetic variation between BCG strains, host nutrition and genetics as well as environmental factors. Development of an improved, efficacious vaccine is crucial for protection of individuals at high risk of infection. Incomplete understanding of protective immune responses to mycobacteria have affected development of an effective vaccine (Narayanan, 2006).

1.1 Mycobacterium tuberculosis

Mtb is part of the *Mtb* complex, which comprises ten species of mycobacteria. These organisms share 99.9% similarity at the nucleotide leve,l but they have different phenotypes and pathogenicity. Some mycobacteria are exclusively human or rodent pathogens, and others in turn have a wide host spectrum. For example *Mycobacterium bovis* (*M. bovis)* mainly infects cattle, but has also the ability to cause TB in immune-compromised humans.

Mycobacteria are unusual compared to other bacteria. *Mtb* was first discovered in 1882 by Robert Koch. (Brosch et al., 2002). The *Mtb* cell surface has a waxy coating, due to the presence of mycolic acids which makes the bacteria impervious to Gram staining, meaning *Mtb* can either be Gram-positive or Gram-negative. The outer membrane and the mycolic acid coating form the cell wall, which constitutes an efficient permeability barrier in conjunction with the cell inner membrane. The waxy coating functions as a protective layer against antibiotics and the host's immune system (Becker & Sander, 2016).

The interaction between *Mtb* with the host's immune cells occurs by distinct virulence mechanisms. The cell wall contains glycolipids and lipoproteins which together with

secreted effector proteins act as pathogen-associated molecular patterns (PAMPs), that are recognized by host innate immune cells (Tientcheu et al., 2017). Interaction with innate immune cells causes *Mtb* to end up inside the host cells, especially macrophages. During infection *Mtb* uses a specialized secretion system, called ESX-1, to compromise the integrity of the vacuole membrane in which it is enclosed. Thus, the membrane gets damaged and brings the mycobacterial components into contact with the cytosol of the host cell which then leads to a series of innate immune responses, affecting the control of mycobacterial growth (Conrad et al., 2017).

Adaptive T-cell immunity is required to prevent clinical TB disease and to control *Mtb* infection. T-cells expressing CD4 contribute to killing *Mtb*, and a loss of CD4+ T cells increases the risk of getting TB in HIV patients (Sonnenberg et al., 2001). It is believed that the main function of CD4+ T cells is to make a Th1 response which includes the production of the messenger molecules interferon gamma (IFN-γ) and interleukin 2 (IL-2), which each cause activation of macrophages and proliferation of lymphocytes.

The production of IFN-γ by CD4+ cells plays an important role for protection against TB in mice (Fernando & Britton, 2006; Rodgers, Whitmore, & Walker, 2006). In humans it is similar, as the deficiency of IFN-γ receptor leads to an increased risk to get a *Mtb* infection (Fernando & Britton, 2006). CD4+ T cells are more important in acute infection whereas CD8+ T cells are responsible for the control of latent infection, but the exact mechanism of the protective response in the host is yet unclear (van Pinxteren, Cassidy, Smedegaard, Agger, & Andersen, 2000). Vaccination with BCG induces and keeps a Th1 T-cell population in the lung which can respond earlier against infection.

Some of the protective effects of BCG against TB are due to a different type of immune memory, called trained immunity. The innate and adaptive immune responses are influenced and trained from all the infections and vaccinations a human had in their life. The innate immune response acts fast and is non-specific to viruses or bacteria that it detects, whereas an organism’s adaptive immune response occurs to a specific pathogen. These different pathogens leave imprints on the immune system and affect future immune responses. There are three main populations of immune cells which are potential cellular effectors of trained innate immune memory: neutrophils, NK cells and monocytes/macrophages (Mihai G. Netea & van Crevel, 2014).

1.1.1 Pulmonary infection and disease

Infection with *Mtb* mainly affects the lungs. All age groups can be infected with TB but most disease (about 90%) is found in adults. Patients with HIV have a 19 times higher risk of getting ill with TB, and the risk also increases in individuals with a compromised immune system. People who have diabetes, smoke, consume alcohol or are undernourished have also a higher risk for development of active TB (WHO, 2019).

An individual's TB disease can be active and transmittable, or latent and non-transmittable. The infection is transmitted through the air via coughing, sneezing or spitting. Active TB is highly contagious and close contacts are easily infected (WHO, 2019). Common symptoms are coughing (with sputum or even with blood), chest pain, weakness fever, night sweats or weight loss. Untreated active TB can be fatal, it takes around two years until it leads to death or self-cure (Tiemersma, van der Werf, Borgdorff, Williams, & Nagelkerke, 2011). The mortality rate for untreated TB is very high.

One fourth of the world's population has latent TB, where bacteria are inside the body, but the person does not become ill. Latent infected hosts cannot spread the disease as the infection is encapsulated in structures called granulomas. Granulomas can keep the infection contained for large periods of time, from decades up to a lifetime. After infection there is a 5-15% lifetime risk for latent TB to develop into active TB (Raviglione & Sulis, 2016). Diagnosis of latent TB and preventive treatment with antibiotics are important to reduce the number of latent TB infections developing to an active state (Raviglione & Sulis, 2016).

1.1.2 Epidemiology

The mortality rate from TB has decreased over the years; in 2018, worldwide about 10 million people had active TB, 1.2 million HIV-negative people died from TB (a 27% reduction compared to 1.7 million deaths in 2000), and there were 251 000 deaths from TB among HIV positive people (a reduction of 60% compared to 620 000 in 2000) (WHO, 2019).

The incidence of TB varies between countries. Over 95% of TB cases and deaths are in developing countries, such as South Africa, India, China, Philippines and Nigeria. In 2018, the biggest proportion of TB cases were in South-East Asia (44%), followed by Africa (24%) and the Western Pacific (18%). In contrast, America and Europe had just 3% of the global cases (WHO, 2019). In 2016 there were about 300 new cases of TB in New Zealand, which would be 6.3 per 100 000 population (Animal Welfare Act, 1999)

1.1.3 Treatment

Effective diagnosis of the infection and effective antibiotic treatment are important to decrease mortality. The most common used diagnostic methods for TB are the tuberculin skin test, acid-fast stain, polymerase chain reaction and most developed countries now use the QuantiFERON-TB blood test (Brosch et al., 2002; Ponce de Leon et al., 2008; WHO, 2018).Without proper treatment 45% of HIV-negative people and nearly all HIV-positive patients will die after active TB infection (WHO, 2018).

Drug susceptible TB can be cured with chemotherapy. The treatment usually consists of a combination of four antibiotics: isoniazid, rifampicin, ethambutol and pyrazinamide taken over six months. Rifampicin is considered the most effective first-line drug. The reason for taking four different antibiotics is to minimize the risk of developing antibiotic drug resistance. For effectively killing *Mtb* the long antibiotic administration time of six months is essential as most antibiotics target processes in actively growing organisms, and *Mtb* is a slow growing organism (WHO, 2019).

1.1.4 Multidrug-resistance Tuberculosis

Some patients develop multidrug resistance due to poor availability of drugs, inconstant intake, incorrect prescription for the drug susceptibility of the infection, or premature cessation of therapy (WHO, 2018). Multidrug-resistant TB (MDR-TB) is defined as infection which does not respond to the two most important TB drugs, isoniazid and rifampicin. Second-line drugs are used for treatment of MDR-TB, but the options are limited and require extensive chemotherapy with expensive and toxic medicines for up to two years. In extreme cases MDR-TB can develop into extensively drug resistant TB (XDR-TB) where the bacteria are resistant to the first and second-line antibiotics. Cases of XDR-TB often leaves the patients without further treatment options. In low income countries drug resistant TB often does not receive effective treatment, and leads to death and further spread (WHO, 2019).

In 2018 about 1.8 million people developed rifampicin-resistant TB and 78% of these had MDR-TB. The treatment of MDR TB usually takes about 24 months with a recovery rate of 56% worldwide, which has a strong impact to public health and economic losses (Stagg et al., 2016). In addition, about 6.2% of drug-resistant TB cases had XDR-TB.

1.1.5 Impact of coronavirus disease on TB diagnosis and treatment

The compromised immune system of people infected with HIV not just increases the risk of developing TB but could also increase vulnerability to coronavirus disease (COVID-19). Health care workers have to be more careful when handling samples for TB testing as the symptoms of both diseases, TB and COVID-19, are very similar. Typical symptoms for COVID-19 and TB are coughing, fever and shortness of breath. Both diseases mainly affect the lungs and get transmitted via close contacts.

In Nigeria, there is a lack of resources to protect health workers performing TB diagnosis. Tuberculosis treatment clinics ideally continue operation during lockdown, but often people do not attend due to stay-at-home orders and dependence on public services for transportation. It is also possible to give sufficient doses to patients which would last them for months, but there was inadequate opportunity to restock drugs before the lockdowns started. This situation also occurs in other regions where TB incidence is high and health resources are lacking (Adepoju, 2020).

1.1.6 Prevention of Tuberculosis

It is essential to treat patients who have a latent TB infection to avoid progression to active TB. Children can be protected from TB, especially from severe forms such as tuberculous meningitis and military TB, with BCG vaccination. In children, BCG decreases the risk of getting the infection by 20% and the risk of developing active TB by nearly 60%. The vaccine is especially given to neonates in high risk countries (WHO, 2018).

1.2 BCG Vaccine

Prevention of *Mtb* transmission is important for managing rates of disease and to minimise morbidity and mortality. Even after successful treatment with antibiotic, TB can result in permanent damage to the lungs and other disabilities if other tissues have been affected, for example irreversible bone destruction (Hnizdo, Singh, & Churchyard, 2000).

There is only one vaccine available on the market to prevent TB. The BCG vaccine is a live, attenuated strain of *M. bovis* and was first introduced in 1921. Over three billion people have received a BCG vaccine which makes it the most widely used vaccine worldwide. Originally BCG was derived from virulent *M. bovis*, it was passaged until it was not virulent anymore and was then used as a vaccine against TB (Teo & Shingadia, 2005). Passaging

BCG in different laboratories led to genetic diversification into several distinct sub-strains (Liu, Tran, Leung, Alexander, & Zhu, 2009). The immunological mechanisms and interactions which lead to protection after BCG immunization are poorly understood.

The biggest difference between *Mtb* and BCG is the genomic region of difference 1 (RD1), which is absent in BCG and present in all *Mtb* strains (Lewis et al., 2003). In humans RD1 is an important pathogenesis island for virulence. Deletion of RD1 inactivates virulent genes, which leads to less effective infection. Due to a deletion of a chromosomal region, BCG lacks the ESX-1 secretion system. The loss of ESX-1 protein secretion system, which is present in *M. bovis*, is the most likely cause of loss of virulence for BCG (Mahairas, Sabo, Hickey, Singh, & Stover, 1996).

In general, BCG has a similar cell structure to *Mtb* with hydrophobic mycolic acids, glycolipids and peptidoglycans in the cell wall. Like *Mtb*, BCG stimulates various classes of pattern recognition receptors (PRRs) such as Toll-like receptors and NOD-like receptors (NLRs). The components of BCG and their immunogenicity are very similar to a mycobacterial pathogen, and thus provide the efficiency of the vaccine (Mihai G. Netea & van Crevel, 2014).

In general, babies and young children have a higher risk of developing active disease after infection with *Mtb*. The BCG vaccine gives children about 74% protection against all forms of TB including miliary TB and TB meningitis, which are more common in children (Hatherill, Tait, & McShane, 2016). In most of the cases the protection lasts approximately 10 to 15 years (Moliva, Turner, & Torrelles, 2015).

The efficacy of the protection of the vaccine in adolescence and adults ranges from 0-80% (Trunz, Fine, & Dye, 2006). The broad efficacy range of BCG vaccination has several reasons. Younger age and lack of exposure to environmental mycobacteria can improve the protection of the vaccine (Narayanan, 2006). Other factors behind varied efficacy can be the BCG strain variation, nutritional and genetic differences between populations, and environmental conditions such as temperature and poor cold-chain maintenance (Fine, Carneiro, Milstien, Clements, & World Health, 1999).

Incomplete understanding of what constitutes a protective immune response against TB makes the development of novel vaccines which reliably protect against pulmonary TB difficult (Andersen & Woodworth, 2014). Most TB vaccine efforts have focused on eliciting long term memory responses from T-cells, particularly Th1 cells (Steigler, Verrall, & Kirman, 2019). Th1 cells help to control mycobacterial infection and prevent active disease

(Andersen & Woodworth, 2014). But there is not enough evidence for a role of memory Th1 cells in vaccine-induced protection (Steigler et al., 2019).

A new vaccine which works in both children and adults is urgently needed, but the immune response of the host to *Mtb* is very complex and not completely understood (Khader et al., 2020). Various studies and research projects tried to develop a new vaccine which offers better protection against TB. A promising vaccine candidate at the most advanced stage of clinical trials was the *Mtb*-antigen-85A-expressing Vaccinia which induced robust Th1 responses (McShane, 2011). However, in clinical trials it failed to improve BCG-mediated protection against TB (Tameris et al., 2013). The successful design of new vaccines depends on unveiling the immune mechanisms underlying protection against TB. Elucidating the host immune responses to early mycobacterial infection will help the strategic design of an effective vaccine.

1.3 Host response to TB

The immune system is complex and dynamic. It is broadly divided into the innate and the adaptive immune system. The innate immune system has broad specificity towards pathogens and acts within minutes to days. Innate immune cells are spread throughout the body in the circulating blood stream and tissues, and they are the first cells to respond to infection. The adaptive immune system is very specific towards target organisms and is able to develop and acquire immunological memory for subsequent infections, but acts slower than the innate system, taking days to weeks to enhance a response (Delves & Roitt, 2000).

After inhalation, when the tubercle bacilli have reached the lung alveoli they get taken up by phagocytosis. Phagocytic innate immune cells sense microbes using pattern recognition receptors (PRRs), and destroy microbes using their cellular effector functions. Phagocytes therefore play a major role in innate immune defence against *Mtb*.

Host innate immune cells involved in TB infection include neutrophils, natural killer cells (NK cells), macrophages and dendritic cells (DCs), which each express different PRRs, including Toll-like receptors (TLRs) and nucleotide oligomerization domain-like receptors (NLRs) (Sia, Georgieva, & Rengarajan, 2015). These receptors play an important role in the identification and uptake of *Mtb*, and are involved in the induction of diverse innate immune defence-associated effector functions like inflammasome activation, autophagy, phagocytosis and apoptosis (Lerner, Borel, & Gutierrez, 2015).

Latent TB infection is caused by host immune cells that were triggered into a non-sterilizing immune response to *Mtb* infection, which then lead to an equilibrium state between the host and the pathogen via granuloma formation. If the host cells are not able to inhibit *Mtb* growth, it leads to granulomatous lesions with more necrotic macrophage death, increased inflammatory cell recruitment and hence can lead to active *Mtb* infection (Korb, Chuturgoon, & Moodley, 2016).

Diverse virulence mechanisms are involved in the evasion of the host immunity so that mycobacteria are able to survive and remain within host cells. These mechanisms include blockade of phagosome maturation, modulation of autophagy, and impairment of inflammasome activation and apoptosis, which also inhibits the development of the adaptive immune response during *Mtb* infection (Goldberg, Saini, & Porcelli, 2014).

For the development of better drugs and vaccines it is important to get a better understanding of the molecular mechanisms involved in the interactions of the host and *Mtb* to prevent and treat TB.

1.3.1 Macrophages

Macrophages play a major role during early and chronic *Mtb* infection as they are the primary cellular niche for *Mtb*. They produce inflammatory cytokines and chemokines leading the recruitment of neutrophils, DCs, B cells and T cells, and more macrophages to the granuloma (McClean & Tobin, 2016). There are several mechanisms how macrophages might eliminate *Mtb*, for example phagosome acidification and production of reactive oxygen and nitrogen species to kill bacilli, cytokines which activate phagocytes, as well as autophagy which assists turnover of vacuoles containing intracellular *Mtb* (Cadena, Flynn, & Fortune, 2016).

There are two main groups of macrophages in the lung distinguished by their function, location and their origin: alveolar macrophages (AMs) which reside in the alveolar spaces and interstitial macrophages (IMs) in the lung parenchymal tissue (Chakarov et al., 2019). During early infection the first cells to encounter *Mtb* are AMs, which have the ability to phagocytose *Mtb* and recruit other phagocytes (Cadena et al., 2016). *Mtb* pathogen-associated molecular patterns (PAMPs) are recognized via macrophage PRRs and thus downstream signalling pathways get activated, leading to distinct gene expression profiles in macrophages at different stages of infection (Killick et al., 2013).

Mtb produces virulence factors which enable the bacteria to avoid protective host responses and persist within macrophages. By preventing phagosome-lysosome fusion and acidification of the phagosome, *Mtb* can maintain an environment which permits mycobacterial growth (Upadhyay, Mittal, & Philips, 2018). Also, diverse secretion systems and proteins are produced from *Mtb* which then lyse phagosome membranes and enable access to the host cytosol. Once in the cytosol, *Mtb*-produced effector proteins interfere with cell signalling pathways to inhibit host cell apoptosis and prevent bacterial killing by autophagy. *Mtb* interferes with host cell death to create necrosis, enabling replication in the extracellular space and dissemination to uninfected host cells. The evasion of anti-bacterial host responses by these mechanisms enables *Mtb* to persist and cause chronic infections.

Conversely, macrophages can be stimulated to increase their ability to kill mycobacteria. Infiltrating CD4+ T-cells release cytokines, such as granulocyte-macrophage colony-stimulating factors (GM-CSF) and IFN-γ, which then activate macrophages leading to an increase in their ability to kill *Mtb* (MacMicking, Taylor, & McKinney, 2003). The anti-microbial functions of macrophages can also be enhanced after BCG vaccination by modifying the transcriptional profile of myeloid progenitor cells (Kaufmann et al., 2018), suggesting protection can occur in ways besides T-cell help.

The early stage of *Mtb* infection could include sterilizing immunity without the direct contribution of the adaptive immune system (Lerm & Netea, 2016). If prior exposure to BCG can train macrophages and other immune cells to function better before T-cell help develops, this would provide an alternative pathway to inducing protection against TB. The investigation of different macrophage subsets in early stages of pulmonary infection in the lungs of naïve and vaccinated mice could demonstrate which cell populations show more effective initial response against *Mtb* infection after BCG vaccination.

1.3.2 Neutrophils

Neutrophils are phagocytes that accumulate rapidly in the area of a new infection. Neutrophils are important during early *Mtb* infection as they are the first cells to infiltrate the lungs after mycobacteria invade and are key mediators of the innate immune response. A study demonstrated that one day after BCG infection, neutrophils are the predominant infected phagocytic subset in murine lungs (Ryder et al., 2019). Neutrophils are the most common immune cell type in the bronchoalveolar lavage and the sputum of patients with active pulmonary TB (Eum et al., 2010).

Recruitment of neutrophils to the lung as well as their pathologic roles are regulated by different chemokines, cytokines, alarmins and intrinsically expressed micro-ribonucleic acids (miRNAs) (Niazi et al., 2015). During respiratory burst, neutrophils produce large amounts of reactive species. Neutrophils also release granule contents including proteases such as collagenase and elastase, which can contribute to both bacterial and host cell damage. Subsequently neutrophils establish an accumulation of effector cells that can mediate both anti-mycobacterial activity and immunopathology during *Mtb* infection (Dallenga & Schaible, 2016). Neutrophils also influence the induction of adaptive immunity to mycobacteria and play an important role in granuloma cavitation during *Mtb* infection (Seiler et al., 2003)

Previous studies showed an inverse correlation between the number of neutrophils in the peripheral blood and development of pulmonary TB in the close contacts of active TB patients. Neutrophil depletion from whole blood worsened control of *Mtb* growth *in vitro* (Martineau et al., 2007). Macrophages are able to take up apoptotic neutrophils and purified neutrophil granules (both containing active antimicrobial peptides) which enhances inhibition of bacterial replication (Tan et al., 2006).

Furthermore, neutrophils also have the ability to enhance antigen presentation, promote strong adaptive responses to *Mtb* and increase DC migration to lymph nodes (Abadie et al., 2005). DCs play an essential role in *Mtb* antigen presentation and are important to bridge innate and adaptive immunity (Blomgran & Ernst, 2011).

1.3.3 Identification of various innate cell subsets

Phagocyte subsets in murine lungs can be separated from each other based on their difference in expression of various key cell surface proteins. Macrophages can be distinguished and separated from DCs based on the expression of the glycoprotein receptor F4/80, which are constitutively expressed on tissue-resident macrophages in mice (McKnight & Gordon, 1998). Further differentiation of lung macrophages is possible as AMΦ have a high CD11c expression compared to recruited IMΦ. Whereas, the CD11b expression in IMΦ is higher than in AMΦ (Misharin, Morales-Nebreda, Mutlu, Budinger, & Perlman, 2013; Srivastava, Ernst, & Desvignes, 2014).

Cell surface proteins enable to distinguish between different phagocyte subsets in the lungs of mice as they are able to act as markers for specific myeloid lineages. Multi-parameter flow cytometry allows to detect the different expression of these lineage markers. For the

identification of specific myeloid populations in naïve and vaccinated lung cells, monoclonal antibodies are used against these proteins. For this study canonical surface markers are used to identify cell populations as this enables further experimental analysis after cell sorting, compared to the use of intracellular proteins. Myeloid cell subset definitions which were used in this study are summarised in **Table 1**. They are based on cell surface protein expression.

Table 1: Myeloid Cell Subset Definitions

	Cell Surface Protein Expression				
Cell Subset	**Gr-1**	**Ly6G**	**F4/80**	**CD11c**	**CD11b**
Neutrophils	+	+	-	-	High
Gr1+ Myeloid/Monocytes	+	-	-	+/-	+
Gr1- Myeloid/Monocytes	-	-	-	+/-	+/-
Alveolar Macrophages	-	-	+	High	High/Low/-
Interstitial Macrophages	-	-	+	Int	High/Low/-
CD11c- Macrophages	-	-	+	-	High/Low/-
Dendritic cells	-	-	-	+	High/Low/-

1.4 Animal models

It is nearly impossible to study early immune response to mycobacterial infections in humans as TB cases are hard to diagnose before symptoms develop. Animal models play an important role for investigating the biology of TB and in general for basic and translational medicine, as well as in the development of drugs and vaccines. All the different animal models of TB infection possess significant differences in disease resistance and sensitivity. The infection method and the dose have an impact on the disease progression and the intervention outcome (Singh & Gupta, 2018).

Animal models have provided some understanding of the mechanisms of early *Mtb* infection and how different factors influence disease progression. Many different animal models have been used to study various aspects of mycobacterial infection and disease, the most common species are mice and guinea pigs, but also rabbits, rats and zebrafish models have been developed to study mycobacterial infections.

Non-human primates, such as rhesus macaques, show similar clinical signs and are able to form granuloma with the same physiology and morphology as humans. They are

important for mechanisms of disease research but it is not universally acceptable to use them for research (Orme & Roberts, 2001; Zhan, Tang, Sun, & Qin, 2017). Non-human primates would provide the closest animal model that is similar to human TB disease. But there are strict ethics regulations for the use of non-human primates for research and according to the Animal Welfare Act 1999 they are banned in New Zealand (Animal Welfare Act, 1999).

Guinea pigs are a useful model for TB research as they have high susceptibility to *Mtb*. They can easily develop active TB after an infection with just one or two bacilli (Orme & Roberts, 2001). The development of pulmonary TB in guinea pigs show a lot of similarities to humans and provides a good model for the development of new skin test reagents as they show very strong delayed-type hypersensitivity reactions (Orme & Roberts, 2001). Due to their sensitive immune response they are also a good model for anti-TB vaccine evaluation (Zhan et al., 2017).

Zebrafish are advantageous as a model for TB research as they are small, they breed very quickly and a large number of fish can be kept together (Bouz & Al Hasawi, 2018). Furthermore, they have a functioning immune system including macrophages, T- and B-cells which is comparable to many other vertebrates. Zebrafish have the ability to mimic the formation of granuloma-like lesions and can establish a chronic or an acute infection. Zebrafish models enable study of the genetics of mycobacterial disease in the host and in the pathogen (Prouty, Correa, Barker, Jagadeeswaran, & Klose, 2003).

1.4.1 Mouse model (*Mus musculus*)

The mouse model is the most widely used animal model. It is cost effective and there are a lot of immunological reagents available. Compared with rabbits and guinea pigs, they need a smaller area for living, and a larger number of animals can be housed together. It is easy to keep mice in a biologically secure manner which is important when studying infectious diseases.

Besides their tendency to not develop granulomatous lung lesions, mice have a similar immune response as humans (Orme & Roberts, 2001). Mice are easy to manipulate, and inbred, outbred and transgenic strains provide many tools to study the interaction between the pathogen and the different components of the immune response. With the help of mouse models it was possible to show how important the role of specific genes and cytokines are,

as well as the importance of different cell types like T-cells, monocytes and DCs in anti-mycobacterial immune responses (Cooper, 2014).

In general, mice are resistant to mycobacterial infection and they develop a strong immune response when infected with *Mtb*, but mice do not naturally catch TB (Kramnik & Beamer, 2016). The most reliable technique to mimic a pulmonary infection in mice is to infect them with liquid mycobacterial cultures by intranasal, intratracheal or aerosol inoculation (Orme & Roberts, 2001). After administering BCG subcutaneously, mice show a better protective immune response to challenge infection with pathogenic *Mtb* (Jung, Ryan, LaCourse, & North, 2005). Protection by BCG vaccination is further enhanced when the BCG is administrated intranasally (Derrick, Kolibab, Yang, & Morris, 2014).

Mice are commonly used as animal models for vaccine development. In this study C57BL/6 mice are used, as it is the most widely used strain for TB vaccine research. They also possess a better post-infection survival time compared to more susceptible hosts such as BALB/c mice, and have a well-characterised immune system which makes reagents such as antibodies and markers more available for identifying immune cell populations (Singh & Gupta, 2018). For this study, male mice of this strain were used as they are more susceptible to mycobacteria than females, which provides a better opportunity to stimulate the immune system as much as possible to generate an immune response which is enhanced by vaccination (Dibbern, Eggers, & Schneider, 2017)

1.4.2 *M. bovis* BCG for modelling *Mtb* infection

In this study BCG is used as an organism to model mycobacterial infections. *Mtb* is classified as a risk group 3 organism and requires at least physical containment level 3 (PC3) conditions when used in experimental studies, whereas BCG only requires PC2 facilities. BCG is an attenuated strain of *M. bovis,* including some crucial differences to *Mtb* and so does not perfectly model *Mtb* disease (Upadhyay et al., 2018).

In comparison to *Mtb* it can be possible that attenuation of BCG influences the eventual outcome of infection with regards to the extent of disease. Nevertheless, it has been shown that BCG cause comparable inflammatory responses in the lungs as *Mtb* with pervasive recruitment of lymphocytes, granulocytes and macrophages to the infections site (Quinn et al., 2006). BCG therefore provides an opportunity to investigate immune dynamics in mycobacterial infection particularly in early stages, where there are no notable differences in disease symptoms yet.

1.5 Project Aims

Previous published work from our laboratory showed that BCG vaccination results in the recruitment of macrophages to the murine lung (Ryder et al., 2019). Mice which were vaccinated with BCG had more abundant AMs with increased CD11b surface expression compared to AM in naïve mice. Vaccinated mice also showed more IMs expressing high levels of CD11b regardless of BCG exposure. It was demonstrated that in vaccinated mice, CD11b-expressing macrophages constitute a bigger proportion of the mycobacteria-associated cells, however it is unclear whether these macrophages would be better in killing mycobacteria than those from naïve mice.

Macrophages derived from lungs of vaccinated mice may have better anti-mycobacterial activity than the macrophages from the lungs of naïve mice. The aim of this project is to investigate whether there are differences in anti-mycobacterial activity between phenotypically distinct lung macrophage subsets in vaccinated and naïve mice. If these macrophages are more restrictive of mycobacterial growth in vaccinated hosts, this could represent a mechanism for BCG intrinsically enhancing the function of innate immune cells against mycobacterial infection.

1.5.1 Hypothesis

BCG vaccination prior to infection alters the recruitment of CD11b high cells to the lungs.

2 Material and Methods

All buffers and media were prepared under aseptic conditions and stored at 4°C until use.

2.1 Buffers and Media

2.1.1 Mammalian Cell and Tissue Culture Media

Complete, antibiotic-free media was prepared aseptically by adding 55 µM 2-Mercaptoethanol and 5% Fetal Bovine Serum (FBS, Gibco, Life Technologies) to Iscove's Modified Dulbecco's Medium (IMDM, Gibco, Life Technologies). Cells and animal tissues were transported, handled and cultured in this antibiotic-free, complete IMDM (cIMDM).

Lung digest buffer was prepared freshly on the same day of use by adding 2.4 mg/mL collagenase powder (Gibco, Life Technologies) and 0.12 mg/mL DNase (Roche Diagnostics, GmbH, Mannheim, Germany) to incomplete IMDM. The lung digest buffer was then filter-sterilised using a 0.22 µm Millex-GS syringe filter (Merck Millipore Ltd., Ireland).

2.1.2 Phosphate Buffered Saline (PBS)

One package of Dulbecco's PBS powder (Gibco, Life Technologies, Grand Island, NY, U.S.A.) was dissolved in 1 L of distilled water (dH_2O) by magnetic stirring, followed by the addition of 0.1 g CaCl powder to make PBS. The pH of the solution was balanced between 7.2 and 7.4 and was then filter-sterilised using a 0.22 µm filter.

2.1.3 Fluorescence activated cell sorting (FACS) buffer

FACS buffer was prepared by adding 0.5% FBS and 2 mM ethylenediaminetetraacetic acid (EDTA, Sigma-Aldrich, St. Louis, MO, U.S.A.) to sterile PBS under aseptic conditions.

2.1.4 Cell Lysing Buffer

To prepare Tween-dH_2O, 0.5% polysorbate 80 (Tween-80, Sigma Aldrich) was added to distilled water and got mixed with a magnetic stirrer. The solution was then filter-sterilised with a 0.22 µm filter.

2.1.5 Antibody Purification Buffers

The pH of all buffers was modified with hydrochloric acid (HCl) solution. Each buffer was filter sterilized with a 0.45 µm ReliaPrep Syringe filter (Ahlstrom, Germany) before used.

2.1.5.1 Antibody Binding Buffer

For the antibody binding buffer 20 mM sodium phosphate (Calbiochem, Darmstadt, Germany) was dissolved in distilled water and set to a pH of 7.

2.1.5.2 Antibody Elution Buffer

Glycine (Sigma-Aldrich) was dissolved to 0.1 M in distilled water and the pH was set to 2.7.

2.1.5.3 Antibody Neutralizing Buffer

Tris-HCl (Sigma-Aldrich) was dissolved to 1 M in distilled water and the pH was set to 9.

2.1.6 Bacterial Media

2.1.6.1 Tween-PBS (TPBS)

For the dilution of BCG, TPBS was prepared by mixing 0.05% Tween-80 with PBS, which then got filter-sterilised with a 0.22 µm filter.

2.1.6.2 Oleic acid Albumin Dextrose Catalase (OADC)

For the preparation of the OADC, 50 g of Bovine Serum Albumin Fraction V (Roche Diagnostics, GmbH) were dissolved in 950 mL distilled water with the help of a magnetic stirrer. After dissolving, 8.5 g NaCl (Merck & Co., Inc, Kenilworth, NJ, U.S.A.), 20 g D-glucose (Sigma-Aldrich) and 0.5 g oleic acid (Sigma-Aldrich) were added and dissolved by magnetic stirring at 30 °C. Once dissolved, 40 mg catalase (Sigma-Aldrich) was added and the solution got filter sterilised using a 0.22 µm filter. The bottle got wrapped in aluminium foil to protect the solution from light and was stored at 4 °C.

2.1.6.3 7H11 Agar Plates

Middlebrook 7H11 agar was prepared by adding 19 g BBL Seven H11 Agar Base (BD Biosciences, San Jose, CA, U.S.A.) and 5 mL glycerol (Sigma Aldrich) to 900 mL distilled

water. The solution got mixed by magnetic stirring until dissolved and then autoclaved at 121 °C for 10 minutes. After autoclaving it got cooled down to 50 °C in a water bath, followed by aseptically adding 100 mL of OADC and 50 mg/mL of Hygromycin B (HygB, Sigma-Aldrich). About 20-25 mL of the agar was then pipetted into sterile petri dishes, once dried they were stored bottom-up at 4 °C and wrapped in aluminium foil to protect from light.

For sterility control plates the agar was prepared with OADC but without HygB, they were incubated for one week at 37 °C with 5% CO_2 to check for contamination.

2.2 Antibody purification

For antibody receptor blocking, anti-FcγR II/III monoclonal antibodies were produced. Pre-culture, 2.4G2 cells were grown to log phase. At least 25 x 10^6 cells got then inoculated into a CELLine CL 1000 Bioreactor (Argos Technologies, Vernon Hills, IL, U.S.A.). After seeding, the bioreactor was incubated at 37 °C with 5% CO_2. The first cell harvest was seven days after inoculation, 3 mL of the cell suspension was mixed with fresh cIMDM and split back into the bioreactor. The rest of harvested cell suspension was twice centrifuged at 320 x G for 8 minutes at 4 °C. The supernatant was collected and stored at 4 °C. Consecutive harvests were performed every 3-7 days until approximately 150 mL of supernatant was collected.

Before purification, all supernatant was centrifuged at 2000 x G for 30 minutes at 4 °C and filter-sterilized using a 0.2 µm filter. First the Hi-Trap Protein G HP column (GE Healthcare, Uppsala, Sweden) was washed with 50 mL antibody binding buffer to clear out all ethanol residues in the column.

For the purification, 2.4G2 supernatant was applied at an approximate flow rate of 5 mL/min. The column was then washed with 25 mL antibody binding buffer until the effluent appeared clear again. For the elution of the antibodies 10 mL of antibody elution buffer was injected to the column. The antibodies were collected in sterile FACS tubes filled with 200 µL antibody neutralization buffer per 1 mL eluate to neutralize the basic pH of the elution buffer and stabilize the antibody structure. The protein content in each fraction was then measured on the spectrophotometer, NanoDrop One (Thermo Fisher Scientific, Carlsbad, CA, U.S.A.) with the setting IgG at 280 nm.

The fractions were then dialysed in Slide-A-Lyzer 7K dialysis cassette (Pierce Chemical Company, U.S.A.). Under gentle agitation the eluate was dialysed against 1 L of PBS

overnight at 4 °C, then the PBS was renewed and dialysed for another 4 hours. Dialysed antibodies were diluted with PBS to a concentration of 2 mg/mL, filter sterilized, aliquoted and stored at -80 °C until use.

2.3 Mycobacteria

2.3.1 BCG Pasteur

M. bovis BCG Pasteur (1173P2 strain) was used to infect mice. It had been previously cultured and titrated to 5 x 10^7 CFU/ml, the aliquots were stored at -80 °C. This strain was used to vaccinate mice.

2.3.2 BCG TdTomato

M. bovis BCG TdTomato (Tokyo strain) was modified to express pTEC27 plasmid which expresses the tandem dimeric fluorescent protein TdTomato. TdTomato gets excited at 554 nm and emits at 581 nm, and expression is selected by a resistance gene to hygromycin on the same plasmid. When grown on agar the protein gives BCG colonies a bright pink colour, allowing distinction between BCG Pasteur and TdTomato colonies.

BCG TdTomato had been previously cultured and titrated to 1 x 10^7 CFU/ml, the aliquots were stored -80 °C. This strain was used to infect sorted cells.

2.3.3 Preparation of BCG

The BCG strains were stored at -80 °C in cryotubes (Greiner Bio-one, Kremsmünster, Austria). They were thawed for 10 minutes at room temperature and then sonicated three times for 10 seconds using a Sonorex sonicator (Bandelin Electronics, Berlin, Germany). After each 10 seconds of sonicating the tubes got rested on ice for 60 seconds. At the end they got vortex-mixed to make sure the suspension is evenly distributed to avoid clumps of bacteria.

BCG Pasteur was diluted with sterile TPBS to 2.5 x 10^6 CFU/ml, so that 40 µL contained 1 x 10^5 CFU for intranasal administration in mice.

2.3.4 Plating

The FACS-sorted immune cell subsets infected with BCG TdTomato were lysed after the indicated incubation time using sterile 0.5% Tween-dH_2O. The cell lysates were then diluted 10-fold up to a dilution of 1:1000. The agar plates were divided into six parts, three 10 µL spots of each dilution and undiluted lysate got pipetted on each section in duplicates..

2.4 Mice

Inbred C57BL/6 mice were used for all experiments. They were bred and housed by the Microbiology Hercus Taieri Resource Unit (HTRU, Dunedin, New Zealand) under specific pathogen-free conditions (*Helicobacter spp*, minute virus, mouse hepatitis virus, norovirus, parvovirus, pinworm, rotavirus and Theiler's encephalomyelitis virus). At the start of all experiments mice were between 8-9 weeks old, in each individual experiments they were age-matched and male. All animal work was performed in a Class II Biological Safety Cabinet. The project's animal work approval was obtained from the University of Otago Animal Ethics Committee, and all manipulations were carried out according to the Animal Welfare Act (1999).

2.4.1 Mycobacterial infection

Mice were intranasally (i.n.) infected with BCG Pasteur, first they were anaesthetized by intraperitoneal (i.p.) injection of ketamine (87 mg/Kg, Phoenix Pharm Distributors Ltd., Auckland, New Zealand) and xylazine (2.6 mg/Kg, Phoenix Pharm Distributors Ltd.). Once sedation was achieved, mice were laid supine alongside next to each other and 40 µL of the bacterial suspension was pipetted gently in a constant stream onto the nostrils, which then got inhaled by the mice. After infection all mice were placed back in their cages and monitored until they were fully recovered from anaesthesia. The body weight of each mouse was checked for the following three days after infection or longer until mice reached a stable weight.

2.4.2 Euthanasia and Tissue Harvest

For tissue harvest, mice were euthanised by i.p. injection of Pentobarbitone (150 mg/Kg, Provet NZ Pty Ltd., Auckland, New Zealand). Death was confirmed by stopping breathing and absent toe pinch reflex. With 70% ethanol-sterilized dissection kits, the thoracic cavity was dissected and sterile PBS got injected into the right ventricle of the heart, which was

still beating, to perfuse and flush red blood cells (RBCs) from the lungs. The lungs were extracted and placed in cIMDM on ice for transporting until further use.

2.5 Mammalian Cells

All cell culture work was performed under aseptic conditions in a Class II Biological Safety Cabinet. Cell centrifugations were carried out at 320 x G for 5 minutes at 4 °C, unless stated otherwise.

2.5.1 Lung Suspensions

The flushed lungs were transferred with ethanol-sterilised forceps to 3 mL lung digest buffer and got then chopped into 3-4 mm fragments with sterile scissors. The fragments were then incubated at 37 °C with 5% CO_2 for one hour to digest structural tissue. After incubation a 70 µm nylon cell strainer (Falcon, Corning Inc., Corning, NY, U.S.A.) was pre-wetted with 5 mL cIMDM and the lung fragments were transferred with a sterile Pasteur pipette into the cell strainer. The tissue was gently mashed through the cell strainer using the flat end of a sterile tuberculin syringe and got then washed twice with 5 mL cIMDM to squeeze out remaining cells. The lung cells were centrifuged at 320 x G for 10 minutes at 4 °C. A 30 µm nylon cell strainer (Falcon, Corning Inc.) was pre-wetted with 1 mL of FACS buffer, the centrifuged pellets were resuspended in 500 µL FACS buffer and were then transferred into the cell strainer. This was repeated for remaining group of lung cells to yield two pooled tubes per group and in the end the strainer was rinsed again with 2 mL FACS buffer to maximise the cell yield.

A 50 µL aliquot of each pooled tube of lung cells was taken to enumerate cells by light microscopy, lung cells were kept on ice until count was finished. Aliquots of each lung cell sample were pooled in one tube and got distributed among fluorescence-minus-one (FMO) controls and unstained controls in sterile and capped FACS tubes (BD Biosciences) so that each control tube contained 10^6 cells. All FMO and unstained controls including the lung cell suspensions were centrifuged at 320 x G for 10 minutes at 4 °C in sterile FACS tubes. The pellets were resuspended in PBS for staining.

2.5.2 Post-sort Macrophages Infection and Culture

After the macrophage subsets were FACS-sorted, the sorted products were centrifuged and resuspended in 500 µL cIMDM. For cell counting a 10 µL aliquot was taken of each sample.

The resuspended samples were then transferred to a 48-well tissue culture plate and got infected with BCG TdTomato at a multiplicity of infection (MOI) of 5. The infected macrophage subsets were cultured at 37 °C with 5% CO_2 for one, two, three or four days before harvest for agar plating.

2.5.3 Cell Counting

For cell counting the aliquots of the single cell suspensions were diluted and mixed, by pipetting up and down, with 0.1% Trypan Blue (Gibco, Life Technologies) and 10 µL of this mixture was transferred to a haemocytometer (Boeckel & Co. GmbH, Hamburg, Germany). Live cells were counted across four grids to yield an average count, RBCs were excluded from the count. The average count got multiplied by the dilution factor and by 10^4 to get the total cell number per mL, multiplying this result by the original volume results in the total cell number.

2.6 Cell Preparation

2.6.1 Fc Receptor blocking and Viability Staining

Fc receptor block ensures that only antigen specific binding is observed. Anti-CD16/32 monoclonal antibodies (clone 2.4G2) were produced as described. Fixable Viability Stain 700 (BD Pharmingen, San Diego, CA, U.S.A.) was used to identify a live or dead cell in sort products and during analysis.

Fc receptor block and viability stain were diluted in PBS, all FMO control tubes received 100 µL, each sample tube received 500 µL of this mixture and got gently vortexed. After an incubation of 15 minutes in a closed ice box all samples were topped up with PBS to a total volume of 1 mL and were centrifuged at 320 x G for 10 minutes at 4 °C.

2.6.2 Magnetic Depletion of Lymphocytes

An antibody mixture was prepared, anti-CD3, CD5 and CD19 antibodies which are conjugated to allophycocyanin (APC) were diluted in FACS buffer. Sample tubes and FMO control tubes were resuspended in this mixture and were incubated for 15 minutes at room temperature in the dark to label lymphocyte antigens. After incubation all tubes were topped up with FACS buffer to a total volume of 1 mL and were centrifuged. FMO and

unstained tubes were resuspended in 1 mL FACS buffer and put on ice until after the magnetic depletion.

Only the lymphocytes of sample tubes which were used for sorting got magnetically depleted. Sample tubes were resuspended in FACS buffer so that every tube had a volume of 500 µL, APC Selection cocktail (STEMCELL Technologies Inc., Vancouver, Canada) was added at a concentration of 100 µL/mL to conjugate to APC^{pos} cells and was incubated for 15 minutes at room temperature. EasySep Rapidspheres (STEMCELL Technologies Inc.) were evenly mixed by vortexing for 30 seconds and got then added to the suspension at a concentration of 75 µL/mL. The samples were incubated for another 10 minutes at room temperature to bind bead to APC^{pos} cells and afterwards topped up with FACS buffer to a volume of 2.5 mL. The tubes were inserted into an EasySep magnet (STEMCELL Technologies Inc.) and were incubated for 5 minutes to bind APC^{pos} cells to tube wall. The magnet with the tube inserted was then picked up and got poured in one motion into a new sterile FACS tube. The magnetisation was repeated for another 7.5 and 4 minutes to pull out remaining APC^{pos} lymphocytes.

2.6.3 Antibody Staining

For sorting the different cell populations and further analysis, various monoclonal fluorophore-conjugated antibodies and dyes against cell surface proteins were used. Brin Ryder, a PhD student from our laboratory, developed a panel for the identification and sorting of live murine lung phagocytes, which is shown in **Table 2**. This fluorescent antibody panel was used in all experiments. The antibodies were titrated on lung cells to determine the optimal concentrations with maximal fluorophore brightness and minimal background stain.

Antibodies were aseptically diluted in FACS buffer to prepare a master mix and FMO mixes. After magnetic lymphocyte depletion the sample tubes were resuspended in 200 µL of master mix and the FMO tubes in 100 µL of the corresponding FMO mix. All tubes were then incubated for 30 minutes at 4 °C in the dark to stain cell surface markers. After incubation, tubes were topped up with FACS buffer to a final volume of 1 mL and were centrifuged two times to wash residual, unbound antibodies. All tubes were then resuspended in FACS buffer and stored on ice until needed for flow cytometric analysis.

Table 2: Panel for identification and sorting of live and murine lung phagocytes

Specificity	Conjugate/ Fluorophore	Clone	Manufacturer	Dilution	BD FACS Aria Optical Channel
FcγRII/III	N/A	2.4G2	In-house	1:100	N/A
Live/Dead	FVS700	N/A	BD Pharminogen	1:2000	R730/45
CD3	APC	145.2C11	BioLegend	1:400	R670/14
CD5	APC	53-7.3	BioLegend	1:100	R670/14
CD19	APC	6D5	BioLegend	1:400	R670/14
Ly6G	BV605	1A8	BioLegend	1:400	BV610/20
F4/80	BV421	BM8	BioLegend	1:100	BV450/50
CD11b	APC-Cy7	M1/70	BioLegend	1:400	R780/60
CD11c	PE-Cy7	N418	BioLegend	1:100	YG780/60
Gr-1	PerCP-Cy5.5	RB6-8C5	BioLegend	1:200	B710/50

2.7 Flow Cytometry and Fluorescence-activated Cell Sorting

2.7.1 Flow Cytometric Analysis

All cells were stained according to the antibody panel described in **Table 2** and were then analysed and sorted using a BD FACS Aria Fusion cell sorter with BD FACSDiva software (BD Biosciences). Afterwards the FCS files were exported and further analysed with an external software called FlowJo Version 10.7.1 for Windows (BD Biosciences).

2.7.2 Compensation

Spectral overlap must be considered when using fluorescent antibodies, compensation controls are used to avoid these overlaps. OneComp eBeads (Thermo-Fisher Scientific, Carlsbad, CA, U.S.A.) were used to make single-stained antibody controls. Voltages were set by using unstained cells from all mice pooled together in an experiment.

In a sterile FACS tube one drop of OneComp eBeads was mixed with 1 µL of each respective antibody and was then incubated for 15 minutes at room temperature in the dark.

All tubes were topped up with 1 mL FACS buffer, centrifuged at 400 x G for 4 minutes at 4 °C and resuspended in 300 µL FACS buffer.

For live/dead compensation, ethanol-killed cells were incubated for 15 minutes on ice in the dark, centrifuged at 400 x G for 4 minutes at 4 °C and resuspended in 100 µl PBS with 1 µL Live/Dead FVS700. All compensation tubes were kept on ice until they were needed again for flow cytometry.

2.7.3 Fluorescence-Activated Cell Sorting

After lymphocyte depletion and cell staining the cells were resuspended in 500 µL FACS buffer and were then sorted using a BD FACS Aria Fusion cell sorter. The sorted cell populations were collected in sterile FACS tubes which contained 1 mL cIMDM. The FMO samples were used to set all the gates with the aim of minimising contamination between the sorted populations. From each sort product between 250 and 1000 events were re-acquired to determine sort purity.

2.8 Statistical Analysis

Non-parametric tests were used to test statistical differences as the normality and the degree of variance were not uniformly distributed between naïve and vaccinated groups or across cell populations. Pairwise comparisons were performed using Mann-Whitney U-test to check for statistically significant differences. All statistical analysis and graphs were generated with GraphPad Prism version 8.4.3 for Windows (GraphPad Software Inc., San Diego, California U.S.A.).

3 Results

3.1 Identification of sorted macrophage subsets from murine lungs

After intranasal vaccination two months prior with 10^5 CFU of BCG Pasteur, CD11b-expressing alveolar and interstitial macrophages increase in frequency and number in murine lungs (Ryder et al., 2019). It was shown that 14 days after challenging with BCG TdTomato, CD11b-expressing macrophages are the predominant infected phagocyte subset in vaccinated mice. On the other hand, the predominant infected subset in naïve mice 14 days after BCG TdTomato alveolar macrophages express low levels of CD11b, as the CD11b high macrophage population is considerably smaller without vaccination. Hence CD11b high macrophages which arise after BCG exposure clearly have a role in anti-mycobacterial responses in vaccinated mice. However, it is still unclear whether these CD11b high AMΦ are functionally superior to the predominant macrophage subsets seen in naïve mice. It is therefore important to find out which function these cells have and whether their function gets altered by vaccination.

Different macrophage populations from naïve and BCG-vaccinated murine lungs were sorted by FACS to measure the capacity these macrophages have to kill mycobacteria. Based on the populations which take up mycobacteria in naïve versus vaccinated hosts, it was decided to sort alveolar macrophages with high expression of CD11b (CD11b high AMΦ) and low expression of CD11b (CD11b low AMΦ). Furthermore, it was decided to also sort CD11b high-expressing interstitial macrophages (CD11b high IMΦ) and CD11c negative macrophages expressing high levels of CD11b (CD11c-/CD11b high MΦ). All four populations were sorted from both naïve and vaccinated mice.

In all experiments age and sex matched C57BL/6 mice were intranasally vaccinated with 10^5 CFU of BCG Pasteur. The lungs from vaccinated and naïve mice were harvested four weeks after vaccination. Prior to cell sorting, lymphocytes which express CD3, CD5 and CD19 were magnetically depleted to increase the efficiency of the sort, and to reduce the contamination of lymphocytes in the sort products.

Before cell sorting it was noted that very few CD11c negative macrophages also expressed CD11b. Most of these macrophage subsets were negative for CD11b. The gating strategy

was then slightly changed to include CD11c negative and CD11b negative macrophages (CD11c-/CD11b- MΦ) instead of CD11c negative and CD11b high/positive macrophages.

For sorting different macrophage subsets, a gating strategy based on previous experimental data from our laboratory was used (**Figure 1**). This gating strategy was used in all experiments for naïve (**Figure 1A**) and vaccinated (**Figure *1*B**) mice, and remained unchanged across all experiments. The gate names of sorted populations are circled in red.

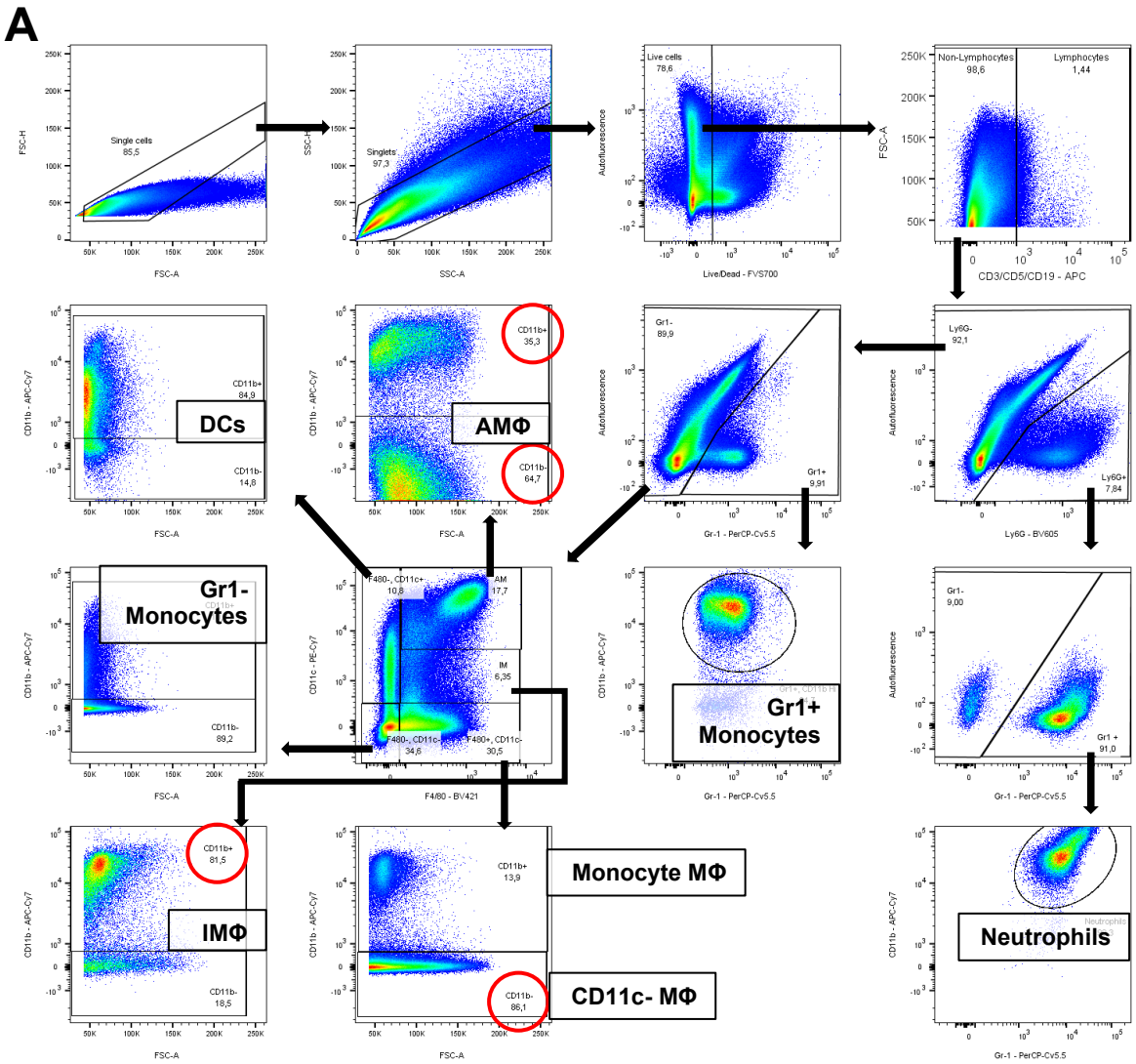
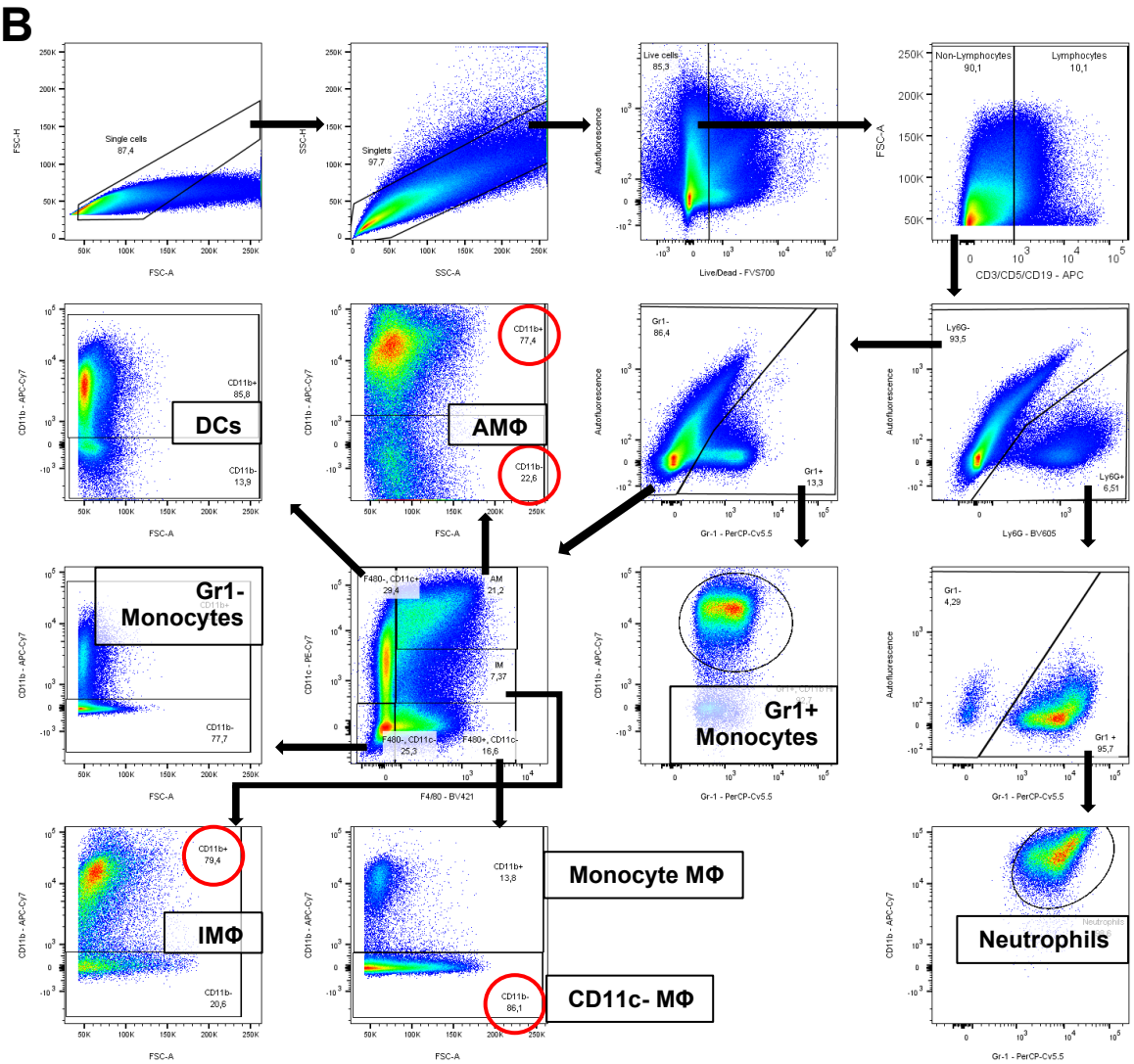


Figure 1: Flow cytometric gating strategy to identify and sort macrophage subsets from murine lungs.

Representative plots show the gating strategy to identify phagocyte subsets and sort macrophages from (A) naïve mice and (B) from vaccinated mice, four weeks after intranasal vaccination with 10^5 CFU BCG Pasteur. Surface protein expression for myeloid cell subset definitions can be found in **Table 1**. For each marker, gates were set with the help of unstained and fluorescence-minus-one controls. The macrophage populations which got sorted are circled in red.

AMΦ, alveolar macrophage. DC, dendritic cell. IMΦ, interstitial macrophage. +, positive. -, negative. The Figure is continued on the next page.

B


Single cells
87,4
Singlets
97,7
Live cells
85,3
Non-Lymphocytes
90,1
Lymphocytes
10,1
CD3/CD5/CD19 - APC
Ly6G-
93,5
Ly6G+
6,51
Gr1-
86,4
Gr1+
13,3
DCs
CD11b+
85,8
CD11b-
13,9
AMΦ
CD11b+
77,4
CD11b-
22,6
Gr1-
Monocytes
CD11b-
77,7
F480-, CD11c+
29,4
AM
21,2
IM
7,37
F480-, CD11c-
25,3
F480+, CD11c-
16,8
F4/80 - BV421
Gr1+
Monocytes
Gr1-
4,29
Gr1 +
95,7
IMΦ
CD11b+
79,4
CD11b-
20,6
CD11b+
13,8
Monocyte MΦ
CD11b-
86,1
CD11c- MΦ
Neutrophils
FSC-A
SSC-A
FSC-H
SSC-H
Live/Dead - FVS700
Autofluorescence
CD11b - APC-Cy7
CD11c - PE-Cy7
Gr-1 - PerCP-Cv5.5
Ly6G - BV605

3.2 BCG Vaccination Increases the Frequency of CD11b-Expressing Macrophages

Frequency of myeloid cell subsets in lungs from naïve and vaccinated mice were measured according to the gating strategy shown in **Figure 1** and the phagocyte subset definitions from **Table 1**, to determine how BCG vaccination changes the lung phagocyte compartment in the absence of infection. Four weeks after BCG vaccination all subset frequencies were determined among non-lymphocytes in all mice from four independent experiments, shown in **Figure 2**.

Intranasal BCG vaccination did not change the frequency of neutrophils, Gr1- monocytes or Gr1+ monocytes or interstitial macrophages four weeks after vaccination.

In general, AMΦ were more frequent in vaccinated mice compared to naïve mice. In naïve mice alveolar macrophages were mostly CD11b low. The increased frequency of CD11b high-expressing AMΦ in the lungs of vaccinated mice was consistent across all four independent experiments. CD11c- macrophages were more frequent in naïve mice than in vaccinated ones, correlating with the lower frequency of alveolar macrophages in these mice. CD11b expression on CD11c- macrophages was not altered by BCG vaccination, and most of them either did not express CD11b or expressed it at a low level.

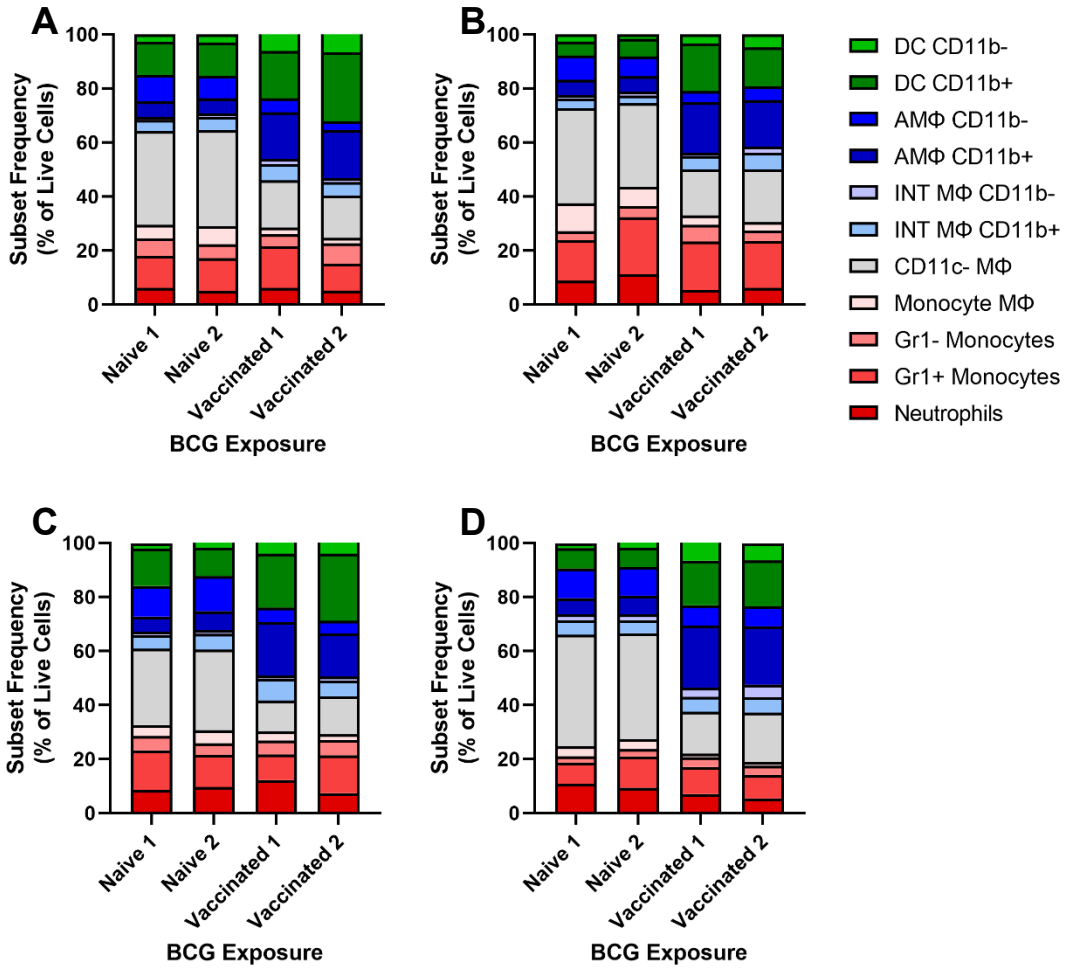


Figure 2: The frequency of CD11b-expressing lung macrophages is increased with prior BCG vaccination.

The lungs from either naïve or i.n. vaccinated mice, which received 10^5 CFU BCG Pasteur four weeks prior to harvesting, were harvested and processed. The frequency of phagocyte subsets from 4 independent experiments (A), (B), (C) and (D) are expressed as a percentage of live cells, based on the gating strategy shown in **Figure 1**. Each naïve bar represents a pool of 4 mice, and each vaccinated bar a pool of 2-3 mice.

3.3 Fluorescence-Activated Cell Sorting of Murine Lung Macrophages

The experimental procedure prior to cell sorting is shown in **Figure 3**. Age and sex-matched C57BL/6 mice were intranasally vaccinated with 10^5 CFU of BCG Pasteur. After four weeks, vaccinated and naïve mice were euthanized and the lungs were harvested, followed by surface staining and lymphocyte depletion. Lymphocytes which express CD3, CD5 or CD19 were magnetically depleted and gated out during sorting to increase the efficiency of the sort, and to reduce the contamination of the sort products. The lung macrophages were then sorted in four subsets, CD11b high AMΦ, CD11b low AMΦ, CD11b high IMΦ and CD11c-/CD11b- MΦ.

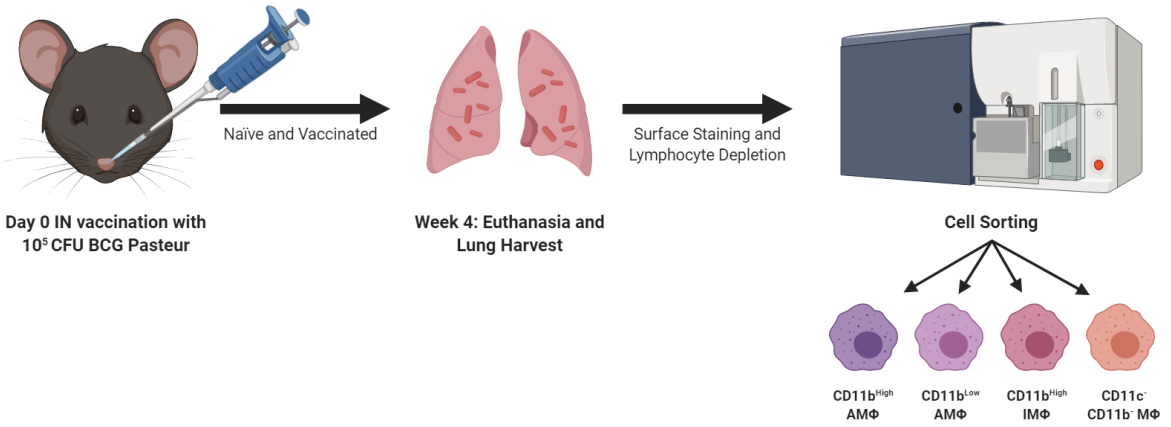


Figure 3: Experimental procedure to identify and sort phagocytes from murine lungs. Mice were either naïve or i.n. vaccinated with 10^5 CFU of BCG Pasteur. Lungs were harvested four weeks after vaccination from naïve and vaccinated mice, followed by surface staining, and CD3+/CD5+/CD19+ lymphocytes were magnetically depleted. Lung macrophages were then fluorescence-activated cell sorted in four subsets: CD11b high alveolar macrophages (AMΦ), CD11b low AMΦ, CD11b high interstitial macrophages (IMΦ) and CD11c-/CD11b- MΦ.

3.4 Purity Control of Sorted Lung Macrophage Populations

Because it is important that the sorted populations specifically contain the phenotypically distinct macrophage subsets, sorted samples were reacquired to check for purity. The same gating strategy as shown in **Figure 1** was used to assess the purity of sorted populations. Representative flow cytometry plots of each re-acquired sample from naïve mice are shown in **Figure 4A,** which illustrate sort products from the same group of mice and experiment. Representative plots from vaccinated mice can be seen in **Figure 4B.**

Purity of populations sorted from naïve and vaccinated mice are expressed as the percentage of total re-acquired events that fall within the final sort gate. All sorted populations except CD11c- MΦ were of high purity. In general, 70-90% of all CD11b high AMΦ sorted events and 75-95% of CD11b low AMΦ sorted events fell within the final sort gate, shown in **Figure 5A and B**. Sort products of IMΦ were between 65-90% pure, from both naïve and vaccinated mice, **Figure 5C**. Low purity in CD11c-/CD11b- MΦ was seen in nearly all experiments as lot of events of these subsets fell into the IMΦ gate, **Figure 5D**. Purity control of sorted products was done for each experiment.

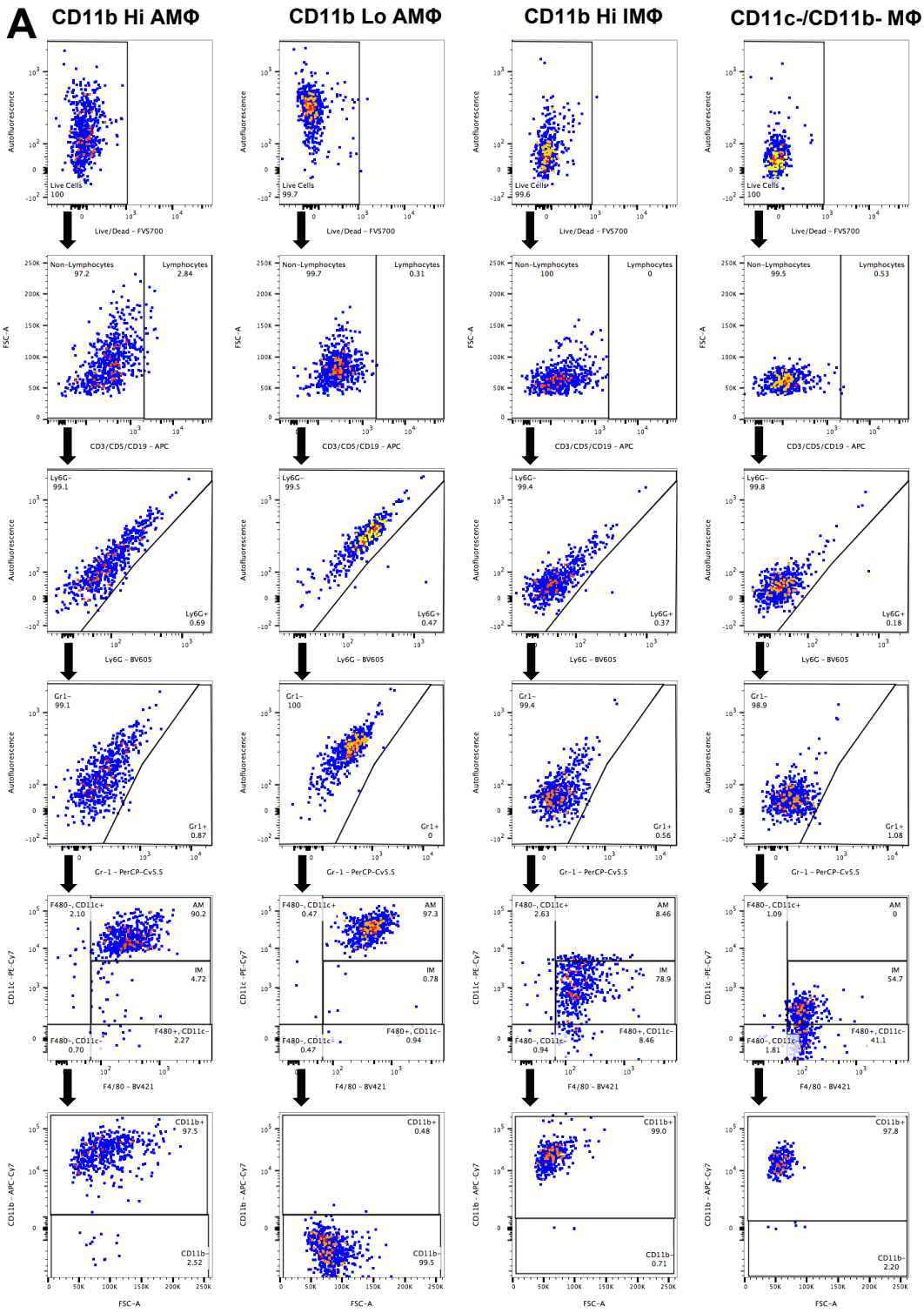
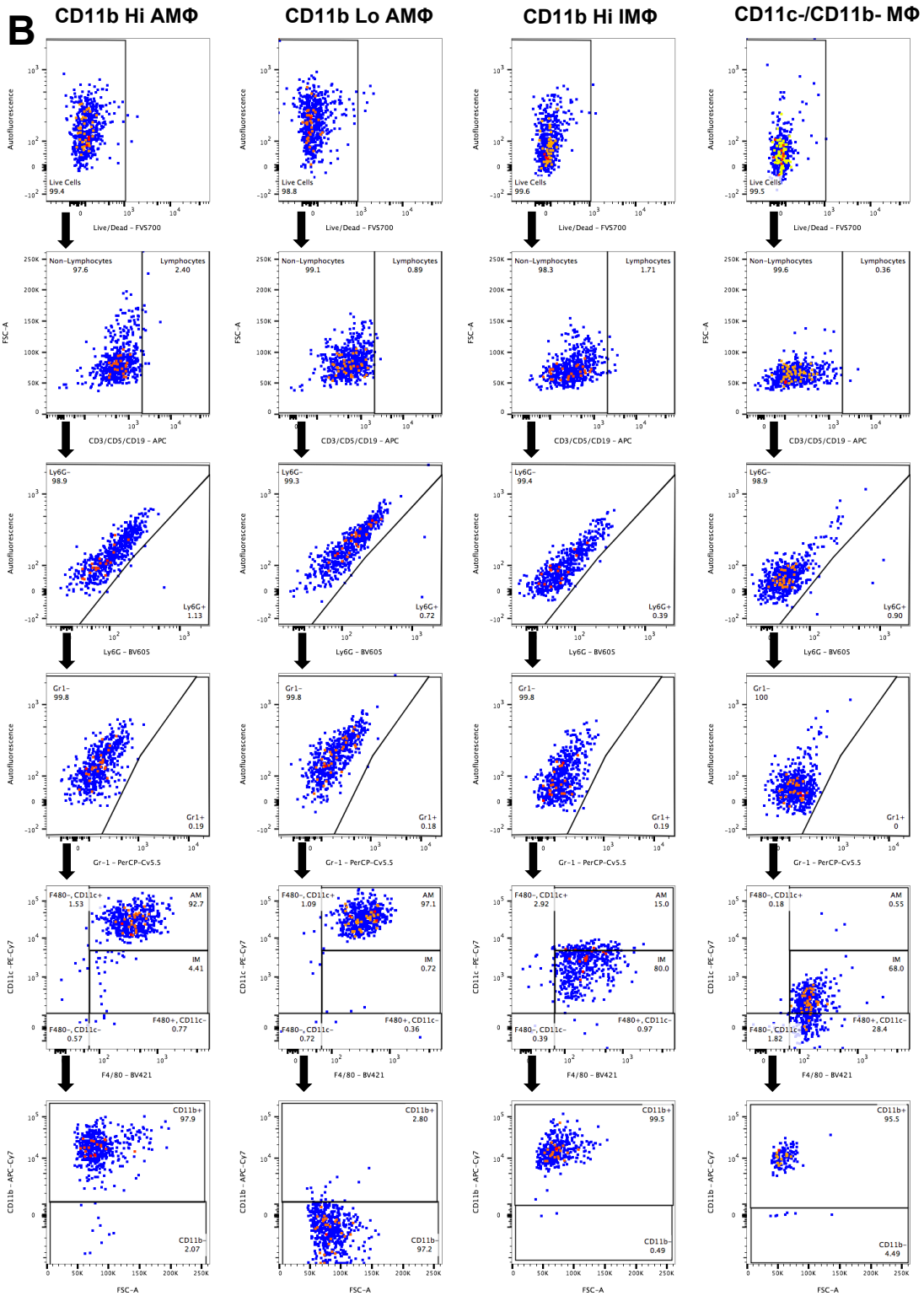


Figure 4: Purity control of re-acquired sort subsets.
Representative flow cytometry gating strategy from **(A)** naïve and **(B)** vaccinated mice with sorted CD11b low/- AMΦ, CD11b high IMΦ and CD11b-/CD11c- MΦ products. Figure continues on the next page.


B
CD11b Hi AMΦ
CD11b Lo AMΦ
CD11b Hi IMΦ
CD11c-/CD11b- MΦ
Live Cells
99.4
98.8
99.6
99.5
Live/Dead – FVS700
Non-Lymphocytes
97.6
99.1
98.3
99.6
Lymphocytes
2.40
0.89
1.71
0.36
CD3/CD5/CD19 – APC
Ly6G–
98.9
99.3
99.4
98.9
Ly6G+
1.13
0.72
0.39
0.90
Ly6G – BV605
Gr1–
99.8
99.8
99.8
100
Gr1+
0.19
0.18
0.19
0
Gr-1 – PerCP-Cv5.5
F480–, CD11c+
1.53
1.09
2.92
0.18
AM
92.7
97.1
15.0
0.55
IM
4.41
0.72
80.0
68.0
F480+, CD11c–
0.77
0.36
0.97
28.4
F480–, CD11c–
0.57
0.72
0.39
1.82
F4/80 – BV421
CD11b+
97.9
2.80
99.5
95.5
CD11b–
2.07
97.2
0.49
4.49
Autofluorescence
FSC-A
CD11c - PE-Cy7
CD11b - APC-Cy7

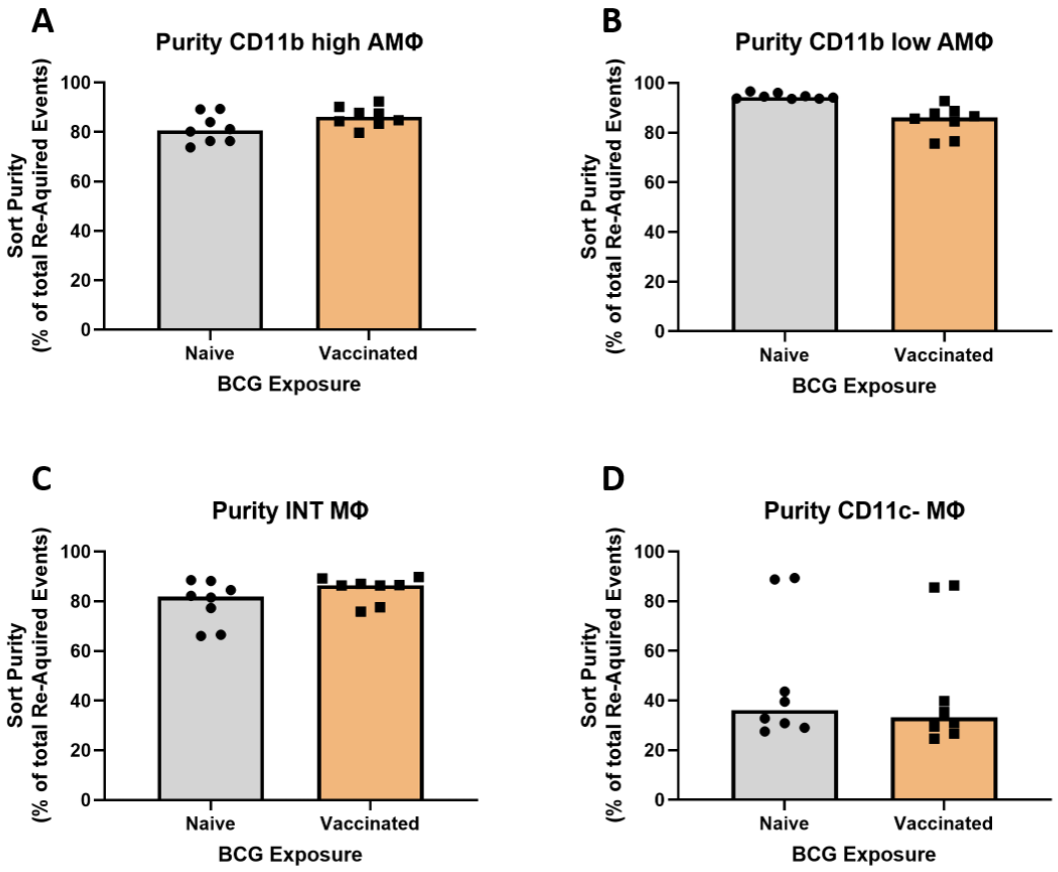


Figure 5: Purity plots of sorted macrophage populations.
Sorted samples were re-acquired and analysed with the same gating strategy used for sorting. Purity of samples sorted on **(A)** CD11b high AMΦ, **(B)** CD11b low/- AMΦ, **(C)** CD11b high IMΦ, **(D)** CD11c-/CD11b- MΦ from naïve and vaccinated mice are expressed as the percentage of total re-acquired events that fall within the final sort gate. Medians are plotted with values for each re-acquired and sorted population from four individual experiments.

3.5 Differences Between Sorted Macrophage Populations

The frequency of AMΦ was consistently larger in vaccinated mice and also expressed CD11b at higher levels compares to naïve mice. It was unclear whether these CD11b high macrophages had enhanced anti-mycobacterial activity compared to the macrophages seen in naïve mice as this may indicate a contribution to a protective immune response induced by BCG vaccination.

3.5.1 Experimental Procedure for BCG TdTomato Growth Inhibition Assay

It was hypothesised that the four sorted macrophage populations would inhibit mycobacterial growth with different abilities, and that this ability would be enhanced by prior BCG vaccination. For testing this hypothesis, lung macrophages from naïve and BCG-vaccinated mice (four weeks after vaccination) were sorted into four subsets: CD11b high and low/- AMΦ, CD11b high IMΦ and CD11c-/CD11b- MΦ. These sorted macrophage populations were enumerated by light microscopy and were then infected with BCG TdTomato at a MOI of 5.

Each experiment had a different incubation time in culture. In each experiment, cells were lysed after either 1, 2, 3 or 4 days incubation. Serial dilutions of the cell lysates were spot plated in duplicates on 7H11 TB agar containing 50 mg/mL HygB, as shown in **Figure 6**. HygB was used in agar plates to avoid contamination by BCG Pasteur in sorted macrophages from vaccinated mice.

Undiluted cell lysates of each macrophage population were plated on separate agar plates as a positive control to ensure BCG TdTomato is in the cells. For spot plating the cell lysates were diluted to a factor of 10^{-3}. Single colonies were already visible 1-2 weeks after plating and could be identified and enumerated within the diluted spots. Whereas colonies from undiluted lysates grew too large and fast to properly count.

BCG TdTomato CFU counts from diluted spots of cell lysates were divided by the number of enumerated cells in each sort product to calculate the viable CFU per infected macrophage, multiplying these numbers by 10,000 gives a CFU per 10,000 cells.

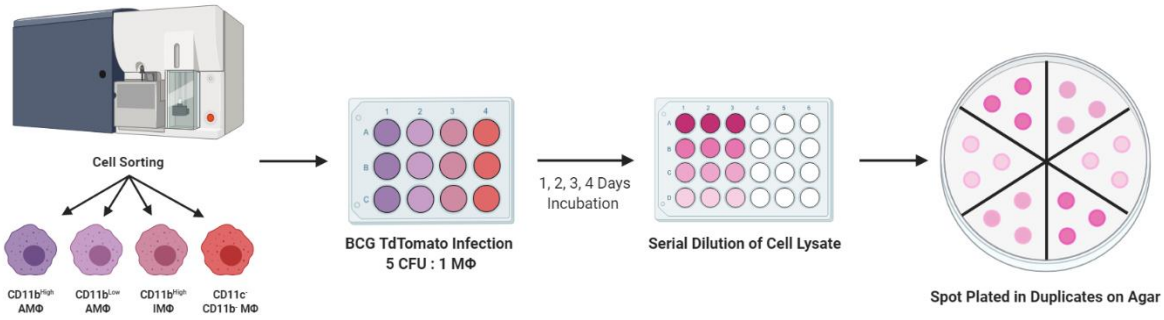


Figure 6: Experimental procedure for BCG TdTomato growth inhibition assay. Lungs were harvested and processed from naïve and vaccinated mice, four weeks after vaccination. Cells were sorted by gating for four different macrophage subsets: CD11b high AMΦ, CD11b low AMΦ, CD11b high IMΦ, and CD11c-/CD11b- MΦ. Sorted macrophages were enumerated by light microscopy and infected with BCG TdTomato at a multiplicity of infection (MOI) of 5. After 1, 2, 3 or 4 days of incubation in culture, cells were lysed and serial dilutions of cell lysates were spot plated in duplicates on 7H11 TB agar with 50 mg/mL hygromycin B (HygB). Individual pink colonies were enumerated after 1-2 weeks.

3.5.2 Growth Inhibition of BCG TdTomato by Sorted Macrophages

The anti-mycobacterial activity of lung macrophages was not consistently influenced by prior BCG vaccination in any of the four sorted subsets. In **Figure 7** and **Figure 8** the mycobacterial activity of all four subsets can be seen after 1, 2, 3 or 4 days of *in vitro* infection, where each day is an individual experiment. The naïve mouse population consists of eight mice which were split into two pools of four, whereas the other population is composed of five BCG vaccinated mice which was also split into two pools consisting of two and three mice each.

After one day of infection with TdTomato (**Figure 7A**) the macrophage subsets showed no difference in their ability to kill mycobacteria. In vaccinated mice, CD11c-/CD11b- MΦ exhibit a two-fold decrease in the number of viable mycobacteria due to BCG vaccination (**Figure 8D**).

Also after two days of infection (**Figure 7B**) no major difference in killing abilities were visible due to BCG vaccination. Naïve CD11b low AMΦs contained three-fold fewer CFU/10,000 cells compared to vaccinated cells, but there was no replicate for the naïve group at this timepoint. The variance of CD11c-/CD11b- MΦ is too high to clearly see a difference between the two groups.

Whereas three days after TdTomato infection, CD11b high AMΦ from vaccinated mice had a 18-fold better ability to control bacterial growth than these cells from naïve mice, shown in **Figure 7C** and **Figure 8A**.

On day 4 after infection, CD11b high AMΦ from both naïve and vaccinated mice begun to control bacterial growth. In the other macrophage populations, cells sorted from vaccinated mice contained greater numbers of viable CFU than naïve macrophages, shown in **Figure 7D**.

The number of bacteria was expected to decrease the longer the cells are incubated with BCG. **Figure 8** shows that CD11b low AMΦ and CD11c- MΦ subsets, the bacterial number per 10,000 cells decreased three days after infection but increased again after 4 days of infection, especially in vaccinated group. This is surprising, as it was expected that mycobacterial growth would decrease by this time, especially in macrophages from BCG vaccinated hosts. CD11b high AMΦ from vaccinated mice was the only population which continued to inhibit BCG TdTomato growth after four days of infection, shown in **Figure 8A**.

Other populations were more variable in their anti-mycobacterial activity. It can be said that CD11b high and low AMΦ were better in restricting the growth of BCG TdTomato than the other two subsets where it seemed to be constant rather than decreasing between 1-4 days after infection. In general, it can be said that CD11b high AMΦ are better because more of them exist after vaccination, and they were able to reduce the viability of BCG earlier by day 3 instead of by day 4 in naïve mice.

A Mann-Whitney U-test was performed to test the differences in anti-mycobacterial activity of each sorted macrophage subset between naive and vaccinated mice at each timepoint, but no statistically significant differences were observed for any population.

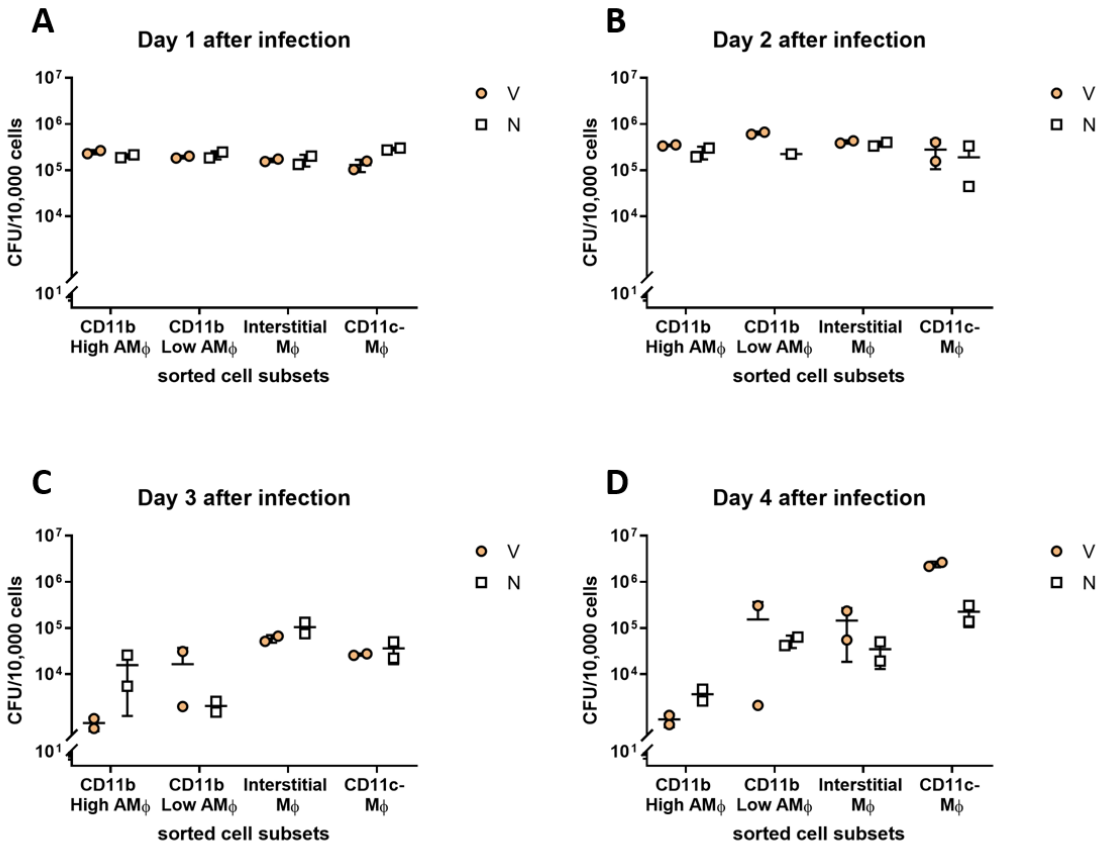


Figure 7: Anti-Mycobacterial Activity of Lung Macrophage Subsets after 1-4 days of BCG TdTomato infection

Sorted macrophage subsets from naïve and vaccinated mice were enumerated by light microscopy and were infected with BCG TdTomato at a MOI of 5. After **(A)** 1, **(B)** 2, **(C)** 3 or **(D)** 4 days after infection, cells were lysed and the lysates were plated on 7H11 TB agar with 50 mg/ml HygB. Mycobacterial survival is shown as colony forming units (CFU) per 10,000 infected macrophages, medians are plotted with individual values. Data represents n=1-2 from four independent experiments.

N, Naïve. V, Vaccinated

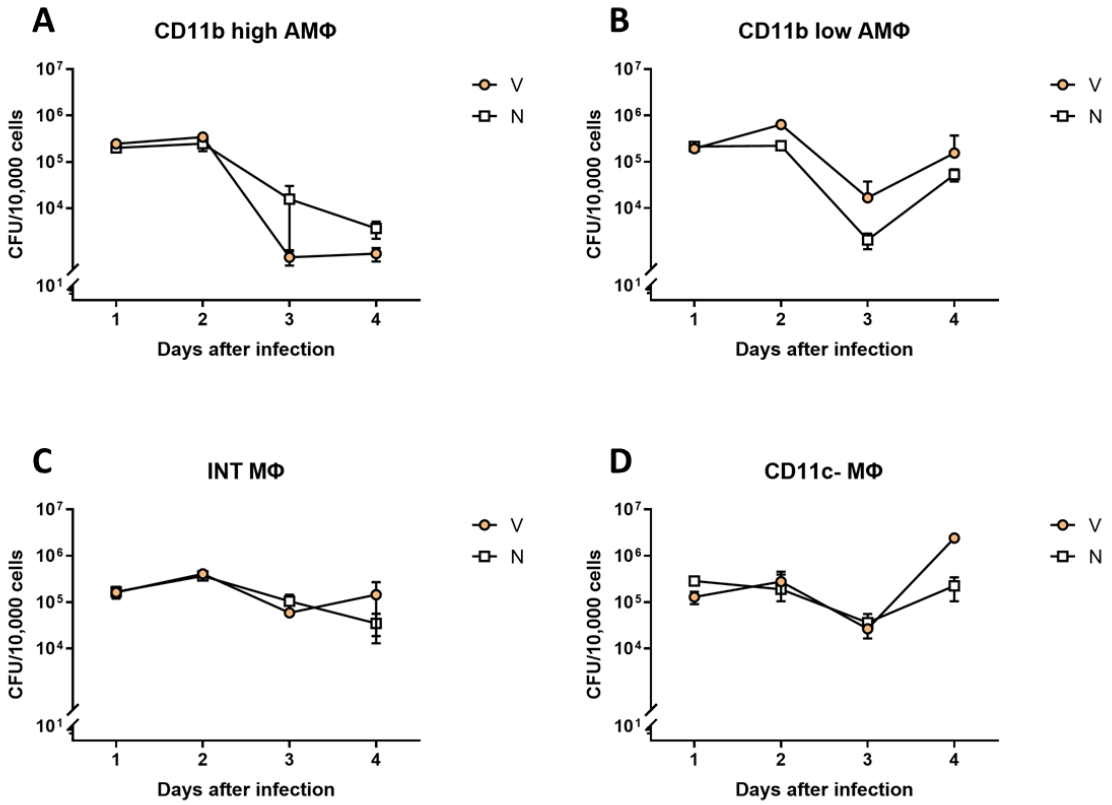


Figure 8: Anti-Mycobacterial Activity of Lung Macrophage Subsets
Sorted macrophage subsets from naïve and vaccinated mice were enumerated by light microscopy and were infected with BCG TdTomato at a MOI of 5. Bacterial growth after 1, 2, 3 and 4 days of infection in **(A)** CD11b high AMΦ, **(B)** CD11b low/- AMΦ, **(C)** CD11b high IMΦ and **(D)** CD11b-/CD11c- MΦ from naïve and vaccinated mice. Cells were lysed and lysates were plated on 7H11 TB agar with 50 mg/ml HygB. Mycobacterial survival is shown as colony forming units (CFU) per 10,000 infected macrophages, medians are plotted with individual values. Data represents n=1-2 from four independent experiments.
N, Naïve. V, Vaccinated.

4 Discussion

Previous work in our laboratory has shown Gr-1+ granulocytes are the major infected cells on the first day after infection with BCG, which is the opposite to popular belief that alveolar macrophages are the main cells to engulf mycobacteria (Ryder et al., 2019). After this first day, AMΦ and other MΦ subsets became the predominant infected cell subsets 1-2 weeks after infection. Among all infected cells, CD11b-expressing macrophages were more frequent in mice that had been vaccinated prior to challenge than in naïve mice. Whether the number of viable bacterial differs between each of these infected phagocytic subsets was unknown.

The aim of this project was to determine which of these previously described, phenotypically distinct lung macrophage subsets harbour more or less viable mycobacteria in the initial stage of infection, and how this is altered by prior vaccination. Flow cytometry was used to sort pure macrophage populations and sorted cells were infected with mycobacteria. The assay enabled direct assessment of the anti-mycobacterial activity of these sorted macrophage subsets and suggested that CD11b high-expressing AMΦ could have enhanced anti-mycobacterial activity in BCG vaccinated mice.

4.1 Anti-Mycobacterial Activity of Phenotypically Distinct Lung Macrophages

In this study, i.n. vaccination with BCG increased the frequency of AMΦ and IMΦ in murine lungs, as well as the frequency of CD11b-expressing AMΦ specifically. Besides the higher frequency of CD11b high macrophages in vaccinated mice compared to naïve mice, they could also be recovered in greater numbers. The gating strategy used to sort lung macrophages provides a high purity of the sorted products from all subsets except CD11c-/CD11b- MΦ. Low purity in CD11c-/CD11b- MΦ was seen in three out of four experiments as lot of events of these subsets fell into the IMΦ gate. However, this gating strategy is a viable way to obtain samples of the other macrophage subsets for future experiments.

In each experiment the cells were incubated for a different time frame, incubation was for 1, 2, 3 or four days. The number of sorted macrophage populations were not equal, in particular CD11c-/CD11b- MΦ were sorted in smaller numbers. The decreased number of sorted CD11c-/CD11b- MΦ influenced the accuracy of cell counting by light microscopy, therefore it cannot be said that the MOI was as consistent across all subsets. Differences in controlling mycobacterial growth could be due to over-infection of this rarer cell subset.

In previous experiments in our laboratory, this experiment was not performed with pooled macrophage populations from multiple mice which resulted in even less macrophage numbers. In future experiments this inaccuracy in cell enumeration could be overcome by sorting more CD11c-/CD11b- MΦ and taking extra care to ensure cells do not get lost during processing. This should increase the cell numbers and allow more accurate cell counting, which may reduce overestimation of cell numbers in sort products.

After three and four days of incubation with BCG TdTomato, CD11b high AMΦ showed enhanced anti-mycobacterial activity compared to CD11b low AMΦ, especially in vaccinated mice. CD11b low AMΦ from naïve mice seemed to have better abilities to control mycobacterial growth than vaccinated ones, still the variance between these two groups was too high to say it accurately. In general, it has to be mentioned that the experiments with each different time point has to be repeated to see whether macrophage subsets truly differ in their phagocytic activity.

Non-parametric tests were used to test statistical differences, pairwise comparisons were done using a Mann-Whitney U-test. Unfortunately, no significant differences are

seen as there is were only 1-2 replicates at each point, making the power to detect differences too low.

It was also seen that macrophages seemed to die after four days of culture with BCG TdTomato in previous experiments. In this study cells did not seem to be dead after four days incubation, still the *in vitro* growth assays described in this study needs further optimisation. For example, it would be advantageous to have greater numbers of replicates and repeated experiments at each timepoint, to tell better if differences in viable CFU are consistent. It would be good to count the cells before they get lysed to know how many dead and live cells exist in each cell population, as phagocytes dying by programmed death can correlate with killing bacteria.

If CD11b high AMΦ have enhanced anti-mycobacterial activity compared to CD11b low AMΦ, it would be of great interest to find out where the origin of these macrophages is, as well as to investigate which mechanisms are responsible for their altered anti-mycobacterial drive. Targeting these protective macrophage populations could potentially lead to the development of a new vaccine or a new immunotherapeutic strategy against TB.

4.2 Changed Lung Macrophage Populations After Vaccination

A measurable difference between AMΦ and IMΦ is their CD11b and CD11c expression. AMΦ are typically CD11c high with little to no expression of CD11b, whereas IMΦ express little CD11c with high CD11b expression (Byrne, Mathie, Gregory, & Lloyd, 2015; Srivastava et al., 2014). Most AMΦ in naïve mice are CD11b low and only a few AMΦ express high levels of CD11b. On the other hand, in vaccinated mice most AMΦ express high levels of CD11b. The reason for that is yet unclear, but it is unlikely that they derive from their CD11b low counterparts, as these were found in similar numbers from both naïve and vaccinated mice.

In viral infection AMΦ can develop into a memory subset which is maintained independently of peripheral monocyte contribution. These macrophages also show higher cytokine production compared to naïve AMΦ (Yao et al., 2018). As AMΦ are self-sustained in that model, the high number of CD11b high expressed AMΦ in BCG vaccinated mice could be due to the increasing expansion of the pre-existing CD11b high AMΦ after exposure to mycobacteria.

IMΦ originate from peripheral monocytes which were recruited to the lungs. The number of these cells can highly increase during pulmonary infections (Srivastava et al., 2014). Recruited monocytes from the blood originally stay in the interstitium but migrate to the alveolar space later on and remain there (Landsman & Jung, 2007). One month after *Mtb* infection in mice, the CD11c high/CD11b low AMΦ population does not include adoptively transferred monocytes. However, donor monocytes were present in CD11c low/CD11b high IMΦ population, showing at least some lung MΦ can include peripheral derived cells (Norris & Ernst, 2018).

During pulmonary infection with mycobacteria AMΦ are clearly altered. Typically, they indicate low expression of CD11b at steady-state and predominantly turn to CD11b high AMΦ after infection. On the other side, IMΦ do not change their high CD11b expression. Interestingly, both CD11b-expressing AMΦ and monocyte-derived IMΦ, increase in number after vaccination. This could be the reason that CD11b high expressing AMΦ population increases after vaccination.

It is likely that the high number of CD11b high expressing AMΦ originate from recruited IMΦ, which have migrated to the alveolar space and add to CD11b high AMΦ. These CD11b high AMΦ may play a role in a protective anti-mycobacterial response. It would be of great interest whether the AMΦ population is expanded in the lungs by other types of vaccination, as well as it would be interesting to determine the contribution from circulating monocytes.

4.3 Trained Lung Macrophages

Innate immune cells including macrophages can remain in an altered functional state after encountering pathogenic organisms. This state enhances subsequent non-specific responses against pathogens. The ensuing “trained” phenotype is mediated through modifications to the epigenome, which in turn typically enhances expression of pro-inflammatory genes (Hoeksema & de Winther, 2016; M. G. Netea et al., 2016). BCG is a potent inducer of trained immunity through the process of epigenetic modification which provides protection against a wide range of infections (Mihai G. Netea & van Crevel, 2014).

Early clearance of mycobacterial infection is defined as the elimination of infection before it becomes established, before an adaptive immune response develops (Verrall, Netea, Alisjahbana, Hill, & van Crevel, 2014). It is probable that trained immunity induced by BCG is a reason why early clearance of mycobacterial infections happens (Lerm & Netea, 2016).

Compared to untrained cells, macrophages which originate from BCG-trained hematopoietic progenitor cells have the ability to improve anti-mycobacterial activity. These trained macrophages also showed to have increased gene expression in response to IFN-γ and also other inflammatory cytokines, as well as in production of IFN-γ, IL-1β and TNF-α (Kaufmann et al., 2018).

BCG exposure clearly affects the macrophage population in the lung, as the number of CD11b high-expressing macrophages consistently increased after BCG vaccination. If lung macrophages get trained by vaccination with BCG, it would be expected that they show enhanced anti-mycobacterial activity compared to lung macrophages from naïve mice. In the data presented here, these macrophages were able to reduce the number of viable mycobacteria a day earlier in BCG vaccinated mice.

However, in this study the anti-mycobacterial activity was not statistically significantly different between naïve and vaccinated mice in any of the macrophage subsets. In another study it was shown that compared to naïve mice the predominant macrophage populations from vaccinated mice may have upregulated production of important cytokines, like TNF-α, IL-6 and IL-β, which are a part of anti-mycobacterial responses (Weiss & Schaible, 2015). This suggests that in addition to looking for changes in the frequency and number of macrophages, it would be worth measuring whether they produce different amounts of cytokines as well. In combination, and with more sensitive assays to detect differences in numbers, these factors may demonstrate whether BCG trains the lung macrophages to provide early clearance after mycobacterial infection.

5 Summary

Tuberculosis is an infectious disease which is caused by the bacterium called *Mycobacterium tuberculosis* and can develop anywhere in the body but occurs primarily as a pulmonary disease. Worldwide Tuberculosis is one of the top causes of deaths and kills more people than any other single pathogen. It is the leading cause of deaths in HIV infected people and responsible for one-third of global antimicrobial resistance.

Incomplete understanding of protective immune responses to mycobacteria complicate the further development of an effective vaccine. Currently, there is only one vaccine available to prevent Tuberculosis, BCG. But this vaccine does not reliably protect against pulmonary Tuberculosis in adults. The immunological mechanisms and interactions which lead to protection after BCG immunization are yet poorly understood.

Our laboratory wants to investigate the key elements of immune responses to BCG vaccination and how these are relevant in the first two weeks of mycobacterial infection in a mouse model. The aim of this project was to determine which of the previously described, phenotypically distinct lung macrophage subsets harbour viable mycobacteria in the initial stage of infection and how this is altered by prior vaccination

Previous work published from our laboratory demonstrated that vaccination with BCG results in recruitment of macrophages to the murine lung. Alveolar and interstitial macrophages became the predominant infected cell subsets after one week of infection. Among all infected cells, CD11b-expressing macrophages were more frequent in vaccinated mice than in naïve mice. These CD11b-expressing macrophages seem to have enhanced anti-mycobacterial activity compared to macrophages that do not express CD11b.

6 Zusammenfassung

Tuberkulose ist eine Infektionskrankheit, die durch das Bakterium *Mycobacterium tuberculosis* verursacht wird und sich überall im Körper entwickeln kann, jedoch hauptsächlich als Lungenerkrankung auftritt. Tuberkulose ist eine der häufigsten Todesursachen weltweit und tötet mehr Menschen als jeder andere Krankheitserreger. Weltweit sind zwei Milliarden Menschen mit *Mycobacterium tuberculosis* infiziert. Bei HIV infizierten Menschen ist es die häufigste Todesursache und ist für ein Drittel der weltweiten Antibiotikaresistenz verantwortlich.

Ein unvollständiges Verständnis der schützenden Immunantworten auf Mykobakterien schränkt die Entwicklung eines wirksamen Impfstoffs ein. Zur Vorbeugung von Tuberkulose gibt es nur einen Impfstoff, BCG, der jedoch keinen zuverlässigen Schutz vor Lungentuberkulose bei Erwachsenen bietet. Die immunologischen Mechanismen und Wechselwirkungen, die nach einer BCG-Immunisierung zum Schutz führen, sind noch wenig bekannt.

Unser Labor möchte die Schlüsselelemente der Immunantwort auf die BCG-Impfung herausfinden und auch wie diese in den ersten zwei Wochen der mykobakteriellen Infektion in einem Mausmodell relevant sind. Dieses Projekt untersucht die Wirkung der mykobakteriellen Impfung auf angeborene Phagozyten-Untergruppen in der Lunge von Mäusen und wie dies zu anti-mykobakteriellen Immunantworten bei nachfolgender Infektion beiträgt.

Frühere Arbeiten aus unserem Labor haben gezeigt, dass die Impfung mit BCG zur Rekrutierung von Makrophagen in der murinen Lunge führt. Alveoläre und interstitielle Makrophagen waren eine Woche nach Infektion die größte Untergruppe der infizierten Zellen. Unter diesen infizierten Zellen waren CD11b-exprimierende Makrophagen bei geimpften Mäusen häufiger als bei naiven Mäusen. Im Vergleich zu Makrophagen, die kein CD11b exprimieren, weisen CD11b-exprimierenden Makrophagen eine erhöhte antimykobakterielle Aktivität auf.

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