Investigating the role of memory alveolar macrophages in early innate immune control of pulmonary tuberculosis

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Title: Investigating the role of memory alveolar macrophages in early innate immune control of pulmonary tuberculosis

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Lay abstract:

Tuberculosis (TB) is a pulmonary disease responsible for 10 million new clinical cases and more than 1 million deaths annually. Over one quarter of the world is believed to be infected with TB. This is despite the near-universal administration of bacille Calmette-Guérin, a preventative TB vaccine, and an effective, but lengthy antibiotic treatment. With antibiotic resistance on the rise, developing a protective vaccine against TB is more important than ever. Tuberculosis has confounded researchers for decades and has thus escaped development of a more effective vaccine. One of the ways to improve a TB vaccine would be to inhale it, to have local effects at the main site of TB infection in the lung. We found that by aerosolizing our vaccine, we can impact local immunity within the lung in a way that has never before been described, opening new avenues for TB research.

Abstract:

Mycobacterium tuberculosis (*M.tb*) is the causative agent of pulmonary tuberculosis (TB). Over 25% of the world's population is estimated to be infected with tuberculosis, yielding over 10 million new cases and over 1.5 million deaths in 2017 alone. This is all despite the near universal implementation of bacille Calmette-Guérin (BCG) vaccination across TB endemic areas. BCG is capable of preventing childhood disseminated forms of disease but fails to induce potent immunity within the lung. We expect this to in part play a role in the lack of protection against pulmonary TB. Our lab has developed a human adenovirus serotype 5 vaccine expressing the *M.tb* antigen Ag85A (AdHu5Ag85A). When delivered directly to the respiratory mucosa (RM), AdHu5Ag85A has proven to be safe and immunogenic, generating both $CD4^+$ and $CD8^+$ T cell responses within the lung. We have found that RM AdHu5Ag85A vaccination also generates a long-lasting population of memory macrophages in the airway and lung, that are primed to better control early *M.tb* infection. Importantly, this was a vaccination route-dependent mechanism. Memory macrophages express a trained innate immune phenotype as they express high levels of MHC II, are highly glycolytic, and produce more IL-12 upon re-stimulation. Importantly, utilization of an *in vivo* T cell depletion approach allowed us to show that these memory macrophages are capable of limiting early *M.tb* infection independent of T cells. Our findings indicate that RM vaccination may be advantageous to parenteral routes by not only drawing antigen specific T cells into immunologically restricted lung mucosa but also generating a memory macrophage population within the lung. Induction of memory macrophages within the airway helps overcome early innate immune suppression by *M.tb*.

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Abbreviation	Full terminology
AdHu5Ag85A	Human adenovirus serotype 5 expressing mycobacterial antigen 85A
Ag85A	Antigen 85A
AM	Alveolar macrophage
ANOVA	Analysis of variance
APC	Antigen presenting cell
AW	Airway
BAL	Bronchoalveolar lavage
BALF	Bronchoalveolar lavage fluid
BCG	Bacille Calmette-Guérin
CD	Cluster of differentiation
CFU	Colony forming units
DC	Dendritic cell
dpi	Days post-infection
ECAR	Extracellular acidification rate
IFN	Interferon
IGRA	Interferon-I release assay
IL	Interleukin
i.m.	Intramuscular
IM	Interstitial macrophage
i.n.	Intranasal
iNOS	Inducible nitric oxide synthase
i.p.	Intraperitoneal
LPT	Lung parenchymal tissue
LV	Lung vasculature
MCP	Monocyte chemoattractant protein
MdM	Monocyte-derived macrophage
MFI	Media fluorescent intensity
MHC	Major histocompatibility complex
MLN	Mediastinal lymph node
MOI	Multiplicity of infection
M.tb	*Mycobacterium tuberculosis*
NO	Nitric oxide
RM	Respiratory mucosa
ROS	Reactive oxygen species
SD	Standard deviation
SEM	Standard error of the mean
TB	Tuberculosis
Th1	T helper 1
TII	Trained innate immunity
T_{RM}	Tissue resident memory T cells
TST	Tuberculin skin test

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1.0 – Introduction

1.1 – Epidemiology of tuberculosis disease

Mycobacterium tuberculosis (*M.tb*), the causative agent of tuberculosis (TB), is an intracellular pathogen that continues to cause a staggering number of deaths worldwide despite decades of use of the bacille Calmette-Guérin (BCG) vaccine and antibiotics. Over one-quarter of the global population is latently infected with *M.tb*, and thus is at risk of developing active disease at some point in their lifetime. There were over 10 million new cases of TB in 2017, with two-thirds of the infectious burden from just 8 countries: India, China, Indonesia, the Philippines, Pakistan, Nigeria, Bangladesh, and South Africa. TB was determined to be the cause of 1.3 million deaths in 2017 alone, plus an additional 300 000 deaths amongst people living with human-immunodeficiency virus (HIV)/acquired immune deficiency syndrome (AIDS)[1]. These astounding numbers make *M.tb* the leading cause of death due to a single infectious disease. The severity of this epidemic is compounded by the emerging prevalence of drug-resistant TB, with 30% of resistant cases coming from the population dense countries such as India and China. The duration and complexity of antibiotic treatment regimens required to treat TB, often involving 3 or more drugs and lasting upwards of a year, stresses the need for new efficacious vaccines to be developed.

1.1.1 – Current tuberculosis control measures

Current measures in place to control TB disease include the prophylactic vaccine, BCG, as well as a lengthy antibiotic cocktail consisting of rifampicin, isoniazid, ethambutol, and pyrazinamide. BCG has been administered across TB-endemic regions for over half a century but has ultimately failed to curb the spread of pulmonary TB. After BCG was implemented into the world's vaccine program, there were drastic reductions in childhood mortality, including miliary and meningeal TB. However, the ineffectiveness of BCG towards specifically pulmonary TB led researchers to believe that this is partially due to its inability to generate anti-TB responses within

the lung tissue. BCG is also contraindicated in HIV-exposed individuals, as it is a live organism-based vaccine. As such, BCG is capable of replicating if not appropriately controlled and can cause systemic infections in individuals with defects in their adaptive immune system[2]. The efficacy of BCG also wanes at latitudes closer to the equator, likely due to tolerization towards mycobacterial antigens as the prevalence of environmental mycobacteria increases, or shifts towards Th2/Th17 immunity due to parasitic coinfection[3–5]. Lastly, BCG re-vaccination to boost original responses fails to enhance protection, potentially due to these same pre-existing responses blocking bacterial replication and thus the immune reactivity to the vaccine[6–8]. Thus there are many aspects of BCG that can be improved upon with development of novel vaccination strategies.

Since BCG has failed to prevent pulmonary TB in adults across the world, one of the major remaining treatment options is the use of antibiotics. The WHO recommends a 6-month treatment regimen for treating standard TB disease. The first two months consist of a 4-drug cocktail (rifampicin, isoniazid, pyrazinamide, and ethambutol), which is then simplified to a 2-drug cocktail (rifampicin and isoniazid) for the remaining four months[1]. Regiment length is of major importance to the sterilizing properties of the treatment as it needs sufficient time to penetrate the granuloma and eliminate all populations of bacilli. Though cure rates for antibiotic treatment remain high, there are several roadblocks to treatment fulfillment that centre around full compliance for the duration of the extended treatment. Failing to adhere to the entire regimen is a major contributor to disease relapse and is the driving cause of drug-resistant disease. Roughly 5% of new TB cases are resistant to at least 1 of the 4 first-line antibiotics, further complicating and lengthening treatment[1]. Although recent developments in diagnostics (e.g. MTB/RIF GeneXpert, Cepheid Inc.) have accelerated the diagnosis of resistant strains, access to and side-effects from second line antibiotics become another major hurdle in disease treatment. Other than a relative lack of efficacy when

compared to first line antibiotics, secondary drugs can exacerbate other treatment-associated issues such as hepatotoxicity and ototoxicity. According to the WHO, only 25% of individuals diagnosed with multi-drug resistant TB had access to efficacious treatment, and of those, only half were successfully treated[1]. These numbers decrease exponentially for individuals infected with extremely drug resistant and totally drug resistant strains. Repurposing existing drugs as anti-TB treatment options is particularly emphasized to accelerate the antibiotic-development pipeline, as clinical safety has already been proven. Nonetheless, both preventative BCG vaccination and antibiotic treatment after infection with TB have a wide variety of faults which must be addressed if we are to further restrain this threat to global health.

1.2 – Primary pulmonary *M.tb* infection

Transmission of *M.tb* primarily occurs through inhalation of aerosolized droplets containing as few as 1-3 bacilli, generated by the coughing or sneezing of infected individuals. These droplets are deposited in the alveolar space, where pulmonary *M.tb* infection is established. Upon exposure to *M.tb*, 75-80% of individuals do become productively infected, but only 5-10% will ever proceed to a state of active TB. This is dependent on several factors, mainly revolving around their ability to launch an adaptive immune response. Following arrival of adaptive immune cells to the airway (AW), bacterial burden in the lung will plateau as immune pressures increase. It is here where 70-75% of people will control disease before proceeding to a state of latency. The remaining 20-25% of infected individuals appear to not launch adaptive responses against *M.tb* despite exposure, indicating that they may be "early clearers", controlling infection solely via innate mechanisms (**Diagram 1**). Thus, understanding the cellular and molecular mechanisms behind this clearance may be a crucial avenue of research. As innate cells within the AW are the

initial point of contact between the host and the infecting bacilli, these early events may dictate disease progression and their modulation via vaccination may be key to curtailing infection[9].

Diagram 1

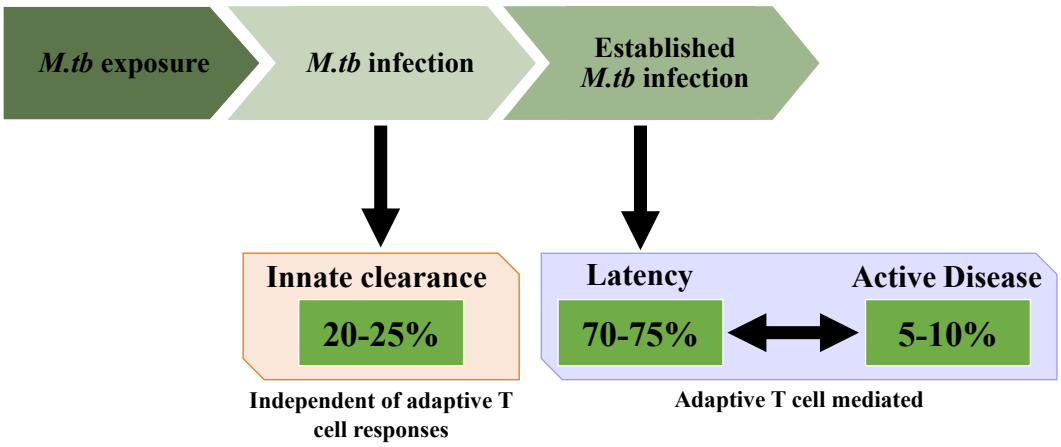

Diagram 1: Possible outcomes following *M.tb* exposure in BCG vaccinated individuals.

1.2.1 – Early innate immune responses to *M.tb* infection

Alveolar macrophages (AM) are a major innate immune cell type within the uninflamed airway and are widely considered to be the first cell infected by *M.tb*. In fact, within the first 8 days following aerosol exposure, almost all bacilli are internalized within AM, dropping to around 50% by 14 days post-infection (dpi)[10,11]. This is unsurprising given their highly phagocytic nature, and abundance in the airways. Several receptors are involved in uptake and recognition of *M.tb* bacilli, toll-like receptors (TLR) 1,2,4,6,8,9, Nod-like receptors (NOD2), c-type lectin receptors (mannose receptor – cluster of differentiation- (CD)-206, DC-specific intracellular adhesion molecule-3 grabbing non-integrin - DC-SIGN), and intracellular DNA sensors (cyclic GMP-AMP synthase – cGAS and stimulator of interferon genes - STING)[12–15]. Once internalized, the bacilli actively halt

phagolysosomal maturation, leading to bacterial escape and successful infection. Alveolar macrophages, DC, and inflammatory monocytes are likely the vehicles by which *M.tb* transports itself out of the airways and into the lung interstitium and lymphatics. Based on work performed in the zebrafish model of *Mycobacterium marinum* infection, mycobacteria soon encounter inflammatory monocytes that are preferentially recruited to the site of infection via the CCL2 (MCP-1)-CCR2 chemokine axis[16]. The permissiveness of monocytes to bacterial growth is dependent on mycobacteria concealing their pathogen-associated molecular patterns (PAMPs) with phthiocerol dimycocerosate lipids. These mask bacterial PAMPs, preventing the activation of the TLR-MyD88 pathway in infected phagocytes[17]. Inflammatory monocytes ($Ly6C^+/CD11b^+$) recruited in this analogous model of infection are permissive to mycobacterial growth as they express less inducible nitric oxide synthase 2 (iNOS2), and obtain mycobacteria directly from infected tissue-resident macrophages through a cell-cell fusion event reminiscent of trogocytosis[17]. Traditionally this has led researchers to characterize monocytes as more permissive to infection, but recent work in a more relevant mouse model suggests that they may instead be more bactericidal[11,16,18]. Following phagocytosis by AW phagocytes, virulent *M.tb* subverts maturation and acidification of the phagolysosome due to its unique cell wall and other effector molecules. Such factors, alongside the slow replication time of the bacterium, and other passive and active immunosuppressive mechanisms, lead to a dearth of available antigens. This delays initiation of the inflammatory response such that DC loaded with *M.tb* antigens do not arrive in the draining lymph node to prime naive T cells until 7-10 days post-infection[19–21].

Diagram 2

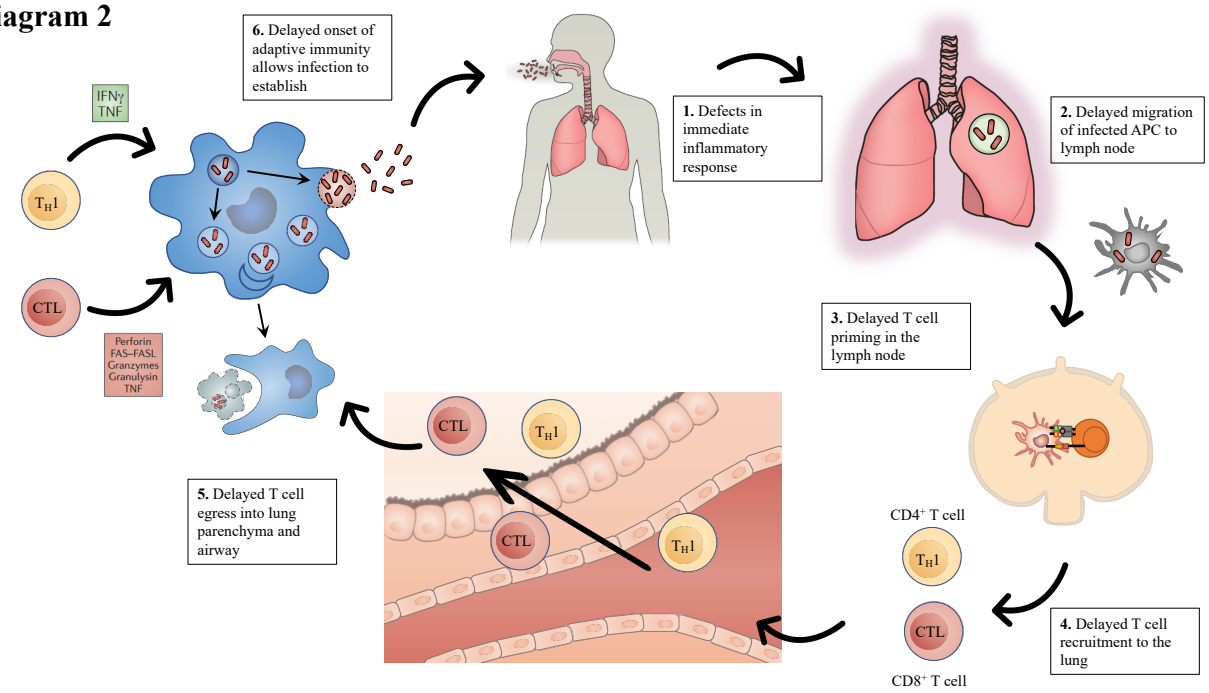

Diagram 2: Anti-TB immunity is delayed due to *M.tb* immunosuppression.

1.2.2 – Delayed antigen presentation and T cell priming

It is essential to note that the induction of T-helper type 1 (Th1) responses associated with mycobacterial control are regulated by effective initiation of the innate immune response. There are two major subsets of antigen-loaded conventional DC that migrate to the mediastinal lymph nodes (MLN) following pulmonary *M.tb* infection. $CD11b^+$ DC secrete large amounts of interleukin (IL)-12, promoting naive T cell proliferation and differentiation into Th1 cells. Infected $CD103^+$ DC harbour a higher bacterial burden owing to their position interdigitating between the alveolar epithelium, and secrete large amounts of IL-10[22]. IL-10 dampens the proinflammatory effect of the $CD11b^+$ DC, further slowing Th1 response initiation. Together, these migratory DC subsets orchestrate the initiation of the adaptive response within the MLN, and represent yet another mechanism of delayed Th1 onset caused by *M.tb* infection. The *M.tb*-specific Th1 cells generated in the MLN do not arrive at the lung until 18-21 days following infection. This leaves

open a window of uncontrolled mycobacterial growth contributing to the inability of the immune system to clear the infection[22].

1.2.3 – Delayed homing of T cell responses in the lung

Upon arrival at the lung, antigen-specific Th1 cells are further retarded by tightly regulated entry into the delicate lung architecture, becoming trapped within the pulmonary vasculature. Unable to clear the now expanded population of bacilli, a granuloma begins to form at the foci of infection. The persistent stimulation caused by *M.tb* causes the infected macrophages at the heart of the infection to undergo many physiological changes, including formation of multinucleated giant cells, foamy macrophages filled with lipids, and a transformation into epithelioid cells that interdigitate with one another and wall off the infection. Infected macrophages at the centre of the nascent granuloma will begin to undergo necrosis, and other inflammatory cells like neutrophils, monocytes, DC, natural-killer (NK) cells, T cells, and B cells will begin to arrive and play their own roles in shaping the infection. It is believed that the most critical cytokine responsible for structured granuloma formation is tumour necrosis factor-α (TNF-α), given its critical role in regulating macrophage trafficking and leukocyte recruitment[23]. Despite being a host conservation strategy, mycobacteria have evolved to manipulate the granuloma to facilitate bacterial proliferation and dissemination[24].

The delayed onset of the adaptive immune response allows *M.tb* to establish a foothold in restricted lung compartments that is difficult for natural immunity to overcome. Activated antigen-specific $CD8^+$ T cells subsequently are involved in controlling chronic infection. They are highly effective at mycobacterial control through cytokine secretion and direct cytotoxic activity, inducing apoptosis of infected cells[20]. Most vaccination methods currently in development produce mainly

$CD4^+$ T cell-mediated immunity and fail to significantly accelerate T cell responses in the lung upon subsequent exposure to *M.tb*. This is likely once again due to their inability to overcome the tight immunological restriction of the delicate lung architecture to maintain gas exchange.

1.3 – Parenteral BCG vaccination-induced immune responses

Bacille Calmette-Guérin, an attenuated form of *Mycobacterium bovis*, remains the only clinically approved TB vaccine to-date. Developed more than 90 years ago, this vaccine is administered intradermally shortly after birth in TB-endemic countries. Through serial-passaging, BCG lost its region of difference 1, encoding the mycobacterial virulence factors involved in the ESAT-6 secretion system. Despite its protective effects against childhood miliary TB, BCG's protective efficacy against adult pulmonary TB highly varies[3,25–27]. Assuming the highest attributed efficacy of BCG in prevention of TB disease, it is still only proven to protect against childhood disseminated TB. Though it is essential to not understate the critical role this vaccine plays in protecting against non-pulmonary forms of the disease, as morbidity and mortality of TB disease is extremely high in children under the age of 5; the incomplete immunity provided towards pulmonary TB wanes by adolescence and cannot be boosted by repeated vaccination[6–8]. Thus, most cases of transmissible, pulmonary TB occur in adolescents and young adults within the first 2-3 years following *M.tb* infection, with minimal residual protection offered from BCG.

1.3.1 – Failure to generate potent T cell responses in the lung and airway

As a live-organism based vaccine, BCG is capable of driving immune responses against numerous mycobacterial antigens, and expresses bacterial products that act as a natural adjuvant[28]. These by-products, as well as the ability of BCG to persist in the body after vaccination allow for effective generation of systemic anti-mycobacterial immunity. Ultimately this leads to marked

decreases in childhood mortality across heavily burdened areas. Due to its intradermal route of administration, BCG is phagocytosed by Langerhans cells and other subcutaneous macrophages. How these peripheral phagocytes recognize, phagocytose, and finally process mycobacterial antigens may differ from how AM recognize *M.tb* upon pulmonary infection. Thus, downstream processes immediately after exposure may also differ and launch a response that will not be sterilizing within the confines of the lung. T cell responses generated by BCG are dominated by $CD4^+$ effector memory T cells (T_{EM}), with suboptimal central memory T cell (T_{CM}) establishment in both mice and humans[29]. Though in humans who are exposed to environmental non-tuberculous mycobacteria, these pools of T_{CM} may be constantly depleted through differentiation into effector T cells[28,30]. BCG does elicit large amounts of antigen-specific T cells that reactivate quickly upon *M.tb* challenge and these are thought to be central to protection against disseminated disease.

Yet the mechanisms behind BCG's lack of efficacy towards pulmonary TB disease are yet to be wholly unraveled. Several observations have been made thus far, including that parenteral BCG vaccination fails to accelerate recruitment of vaccine-induced systemic $CD4^+$ T cells into the lung after *M.tb* infection[31]. It was found that following pulmonary *M.tb* infection in mice, subcutaneous BCG vaccination only accelerated T cell recruitment to the lung by 4-5 days when compared to a naïve control group[31]. Thus a 10-day period remained for *M.tb* to replicate unchecked by cellular immunity. The efficacy of BCG against pulmonary *M.tb* infection has been improved upon in experimental models of mucosal BCG vaccination, which further decreases bacterial burden compared to subcutaneous administration[32–34]. These studies highlighted a deficit in the current vaccination programme, and provides proof of concept warranting future investigation into mucosal vaccination strategies. Further reasons as to why BCG induced immunity may be suboptimal, are that virulence factors encoded in the missing region of difference

1 are present in virulent strains of *M.tb*. Many of these proteins play a key role in immunosuppression and may be enough to overcome any vaccine-derived mechanisms of protection via phagosomal escape. Since BCG is homologous to *M.tb*, its antigens are processed in a similar manner, and similarly generate a $CD4^+$ T cell dominated response. This we know is non-protective based on the natural immune responses against *M.tb* after infection. Therefore, we must focus on designing vaccine strategies that generate responses distinct from natural immunity. Vaccine efficacy is measured by the ability to elicit antigen-specific adaptive responses. Recent discoveries have placed particular emphasis on the early events immediately following pulmonary *M.tb* infection, stressing the critical role of events in the alveolar space towards shaping the immune response to come. The failure of BCG to decrease *M.tb* burden within the first two weeks following infection has long been attributed to its inability to accelerate T cell responses in the lung. The lack of efficacy specifically towards pulmonary disease may not just represent a failure of generating adaptive responses within the lung, but also an inability to modulate the pulmonary innate immune system. Due to its parenteral administration, BCG generates effective systemic responses but offers limited pulmonary responses. This may be due to ineffective bacterial dissemination into restricted lung mucosal tissues and therefore a lack of local inflammation to draw in adaptive immune cells or contact innate cells.

In mouse models of BCG vaccination, a 10-day window of unchecked *M.tb* growth within the lung remains, with comparable pulmonary mycobacterial burdens between BCG-vaccinated and control mice within this early timeframe[27,35]. However, recent imaging techniques show that these mice lack protection despite the presence of vaccine-derived T cells in the lungs of mice prophylactically immunized with BCG[36]. Previous work demonstrated that this was a serious flaw with BCG leading to inadequate protection against pulmonary TB. Delahaye *et al*. recently found

that T cells generated from BCG vaccination were indeed brought into both the lung vasculature (LV) and lung parenchymal tissue (LPT) of vaccinated mice[36]. However, it was upon subsequent *M.tb* challenge that these T cells failed to localize to the infected phagocytes until 2 weeks post-infection. This hints that to improve upon BCG, there may be more to it than just simply drawing T cells into the lung. The presence of these T cells in the lung, but not localized to infected phagocytes, leads to earlier control of *M.tb* replication, but not clearance of infection. Why these T cells do not co-localize with infected macrophages prior to this despite their presence in the lung remains to be elucidated. This indicates that a lack of T cell recruitment to the lung does not fully account for BCG's failure to protect against TB.

Recent studies in non-human primate models of aerosol BCG vaccination have shown improved protection against subsequent *M.tb* challenge merely by changing the route of administration as mentioned above[37]. Protection was primarily mediated through antigen-specific immunoglobulin (Ig)-A and T helper 17 (Th17) $CD4^+$ T cells, suggesting that BCG is capable of eliciting effective anti-*M.tb* responses but may be limited in part by route of administration[37]. This outlines a dire need for improved vaccination strategies to form more potent T cell responses, as well as to direct these responses to the lung mucosa. In addition, this study offers clear evidence to justify further investigation into mucosal vaccination strategies. In recent clinical trials, RM vaccination with viral-booster vaccines have shown particular promise at enhancing anti-TB T cell responses within the lung, and offer a promising avenue of development[38–41].

1.4 – New tuberculosis vaccine strategies

Due to the wide spectrum of disease that TB may present as, and the dynamics of the *M.tb* lifecycle within the host immune response, a true correlate of protection has yet to be ascertained.

This has led to a multi-pronged approach when it comes to developing vaccination strategies, as there are many stages of infection and thus immune responses to consider. The result is a burgeoning pipeline including several types of vaccines in development: prophylactic (to be used as a BCG-booster/replacement or as a standalone vaccine and given pre-infection), post-exposure (to prevent the progression to active disease after infection), and therapeutic (used in adjunct, or sequential to antibiotic intervention to prevent recurrence, or shorten the duration of the antibiotic treatment itself). An important factor to be considered when designing TB vaccines is the pre-existing immune responses generated by BCG. Though these offer T cell responses against a wide breadth of mycobacterial antigens, the pre-existing immunity to BCG has been suspected to limit the efficacy and durability of BCG-based boost vaccinations[42]. It is this initial early exposure to BCG, or other environmental non-tuberculous mycobacteria that may shape the response to vaccination with other live attenuated/whole mycobacterial vaccines, or BCG revaccination. Indeed, it is documented that BCG efficacy wanes at latitudes closer to the equator, correlating with childhood exposure to mycobacterial spp[7]. Due to its near universal administration in TB-endemic areas, extensive safety profile, and non-specific effect on childhood mortality, it is unlikely to ever be removed from early-life vaccination platforms. BCG elicits cellular immunity against many mycobacterial antigens, and is effective at preventing meningeal and miliary childhood TB. Therefore, an effective approach to improve upon BCG vaccine design instead would be to boost these responses, rather than entirely replace the vaccine. Effective BCG-booster vaccines should not only expand and stimulate the pre-existing anti-mycobacterial immune repertoire, but also fill the gap in BCG-derived immunity. An example of where novel TB vaccines could stand to improve upon BCG vaccine strategy would be directing protective immunity to the lung and airway, an area in which BCG is currently deficient.

1.4.1 – Current novel vaccine platforms

Over the last several decades TB vaccination strategies have been evolving as we begin to unravel more knowledge behind the immune mechanisms associated with control, or lack thereof, in *M.tb* infection. Many vaccines in development were designed to improve upon the natural immune responses following primary pulmonary *M.tb* infection. However, many of the correlates of protection drawn from these immune responses are inherently flawed, and the resulting vaccines have therefore yielded disappointing results. For example, individuals with genetic polymorphisms in the IL-12/interferon (IFN)-γ/Signal transducer and activator of transcription 1 (STAT1) pathway (deemed the Mendelian Susceptibility to Mycobacterial Disease), or individuals with HIV/AIDS, show an inability to control *M.tb* infection. From this we have extrapolated that $CD4^+$ T cells and IFN-γ are critical to protection, leading to an overemphatic focus on Th1 cells in vaccine design. Extrapolating a correlate of protection from cases where bacterial control is lost does not necessarily align these immune responses with those that would be sufficient for preventative or sterilizing immunity[43]. This may partially explain why one of the most advanced vaccines in the pipeline, a parenterally administered modified vaccinia Ankara (MVA) viral-vectored vaccine expressing antigen 85A (MVA85A) failed to provide protection over BCG-priming alone in clinical trials despite generating robust T cell responses[40,41]. Vaccinating 4-6 month old infants that previously received BCG with MVA85A failed to prevent either infection, or progression to active TB disease over placebo[40,41]. However, there has been a recent success with the adjuvanted subunit vaccine M72/$ASO1_E$. M72/$AS01_E$ consists of a fusion protein of the *M.tb* antigens Mtb32A and Mtb39A with the $AS01_E$ adjuvant, and provided 54% protection against the progression to active disease in HIV-negative adults with latent TB infection[44]. However, it is important to note that this protection was mainly observed in young males, and protection was not as efficacious in other demographics[44]. Importantly, this also proves that it is possible to generate potent immune

responses in individuals previously exposed to BCG. While these results are promising and are unique as they are in a vaccine platform designed to prevent reactivation, concerns have been raised over the use of the $AS01_E$ adjuvant. Despite playing an essential role in activating the innate immune system in response to M72, this proprietary adjuvant formulation is also included in the RTS malaria vaccine, and the recombinant zoster vaccine, both of which are already clinically approved. Thus the resources needed to develop this adjuvant may instead be subverted towards these other vaccine formulations as M72 may not be considered a priority. Another flaw inherent to both MVA85A and subunit vaccine formulations is that they both preferentially induce $CD4^+$ T cell responses in humans. This is analogous to the natural immunity generated from pulmonary *M.tb* infection that we know is not protective, and is not desirable for HIV^+ populations which are one of the most at risk groups for developing TB. These vaccines also only express 1-2 antigens and therefore cover a small breadth of the *M.tb* lifecycle. In a unique model of successful cytomegalovirus-vectored TB vaccination expressing 6 to 9 *M.tb* antigens, Hansen *et al.* found that the sterilizing protection produced in around half the macaques correlated more with a gene signature indicative of neutrophil degranulation and innate immune effector function rather than the magnitude of the polyfunctional T_{EM} response[45]. However, the vaccine failed to boost responses generated from the initial BCG priming, and the exact mechanism behind the protection remains to be dissected.

A promising vaccine in development designed to replace, rather than supplement traditional BCG is VPM1002. This recombinant BCG strain expresses the *Listeria monocytogenes* listeriolysin, but not urease C, and aims to be safer, more immunogenic, and longer lasting than traditional BCG[28,46]. This aids in phagolysosomal escape of BCG, more closely mimicking *M.tb* infection, and facilitates the cross-priming of $CD8^+$ T cells by exposing mycobacterial antigens to

major histocompatibility complex (MHC) I presentation machinery. Moving away from prophylactic BCG replacements and boosters, other TB vaccines in development are those designed to function therapeutically and enhance the efficacy of antibiotics. These include both RUTI and *Mycobacterium vaccae* inactivated whole-cell based vaccines, both of which utilize mixtures of mycobacterial products to generate immunity against a broader range of antigens. All of these vaccines in development have their merits as well as their flaws, but whether or not they are able to generate efficacious "unnatural immunity" within the lung remains to be fully evaluated in humans.

1.4.2 – Adenoviral-vectored TB vaccines

There are as many as a dozen TB vaccine candidates in clinical development, but adenoviral vectors in particular have consistently shown good efficacy and safety records. Adenoviruses are desirable candidates for vaccine development due to their genetic malleability, safety profiles, and mucosal tissue tropism[47]. One of the vaccines, a replication deficient human adenovirus serotype-5 that expresses the immunodominant *M.tb* antigen, Ag85A (AdHu5Ag85A) was developed in the Xing lab here at McMaster[48]. When given parenterally, akin to BCG, AdHu5Ag85A generates mainly antigen-specific $CD8^+$ T cells in the spleen and LV of mice, neither of which restrict *M.tb* growth within the respiratory mucosa (RM) to the extent of BCG-derived T cells. However, when administered intranasally (i.n), AdHu5Ag85A elicits long-lasting antigen-specific $CD8^+$ T cell responses within the LPT and AW that offer a similar or even better level of protection to that offered by BCG[48]. Parenteral BCG vaccination shows somewhat delayed efficacy against *M.tb*, as vaccine-induced T cells arrive to the lung by 10 days post-vaccination[49]. By delivering AdHu5Ag85A directly to the RM, we are directing the T cell responses to the lung and placing these antigen-specific T cells within the lung mucosa, where they can rapidly activate and protect

against *M.tb*[50]. Importantly, this adenoviral-vectored vaccine is efficacious both as a standalone vaccine, and as a booster to BCG[6]. Due to the resounding successes of the pre-clinical immunogenicity and safety studies in animal models, recently this vaccine has also undergone phase I trials in humans. AdHu5Ag85A induces robust polyfunctional $CD4^+$ and $CD8^+$ T cell responses in previously BCG-immunized humans when given parenterally[39]. This vaccine generates a more balanced $CD4^+/CD8^+$ T cell response in non-human primates and humans, but nonetheless provides significant protection against mycobacterial dissemination and pulmonary *M.tb* infection[6,39,51]. During ongoing clinical testing, AdHu5Ag85A has also proven to be safe and immunogenic when given via the aerosol route in humans, generating a large antigen-specific T cell population in the human AW (Xing lab, unpublished data). This accentuates that not only do the potency of T cell responses contribute to protection, but their geography within the lung also plays an essential role. Ultimately this reinforces initial observations from animal models, and places further emphasis on RM vaccination strategies as a research priority in the TB vaccine development pipeline.

Further advancements have led to expanded vector options that circumvent existing immunity towards the human adenovirus backbone, as neutralizing antibody titres against AdHu5 virus are often high in people living in most TB-endemic regions. A chimpanzee adenovirus serotype-68 (AdCh68) vector has been utilized in vaccines for rabies, HIV, and now TB[52]. When modified to express the same *M.tb* antigen, Ag85A (AdCh68Ag85A), it has been even more immunogenic in mice than AdHu5Ag85A[47,53,54]. A recent analysis of RM vaccination with AdCh68Ag85A demonstrated unprecedented efficacy as a therapeutic vaccine[55]. Unlike most TB vaccines in development which are prophylactic in nature and designed to be given as a booster to BCG, AdCh68Ag85A was also given therapeutically post-infection in adjunct with an antibiotic

cocktail[55]. This not only generated potent antigen-specific $CD8^+$ T cells in the lung, but also decreased mycobacterial rebound upon premature cessation of the antibiotics, a common cause of therapeutic default. Therefore, adenoviral-vectored vaccines in development for prophylactic functions are also capable therapeutics which may help reduce the duration of antibiotic treatment needed. Shorter treatment duration is a pillar of helping reduce global TB burden and would also help curb the rise of drug-resistant strains[55]. Vector development has been further advanced by an expanding the repertoire of *M.tb* antigens that can be expressed. As *M.tb* is a complex organism that is capable of long-term persistence within its host, it also has a highly dynamic infectious cycle, with antigenic expression varying depending on the current immunological pressures. Thus, depending on the stage of the *M.tb* lifecycle, certain vaccine-targeting antigens may or may not be expressed on infected host cells, negating vaccine-derived immune responses regardless of their potency. A majority of TB vaccine platforms in development target a small group of immunodominant antigens. This includes Ag85 complex antigens (which are mainly produced during acute infection), ESAT-6, CFP-10, and TB10.4 (which are expressed during the acute and chronic phases of infection)[56,57]. As adenoviral vectors offer genomic flexibility for expression of multiple foreign genes, novel adenoviral vaccine formulations may aim to include latency associated antigens from the *M.tb* DosR regulon, or resuscitation promoting factors such as RpfB[56,57]. This will expand the breadth of protection offered from vaccine-induced T cells to encompass more of the bacterial lifecycle, and fill gaps in the immunity provided by BCG[58].

1.4.3 – Respiratory mucosal vaccination

Mucosally delivered vaccines are well documented to offer enhanced protection relative to parenteral vaccination by inducing a robust pathogen-specific adaptive immune response within the mucosal sites of pathogen entry. For respiratory pathogens such as *M.tb*, this involves

generating effector and memory T cell responses elicited explicitly within the lung. Parenteral vaccination methods often fail to accomplish this because most of the antigen-specific T cells become trapped in the lung vasculature (LV). The LV is considered accessible to T cells under uninflamed conditions. This is directly contrasted by immunologically restricted mucosal sites such as the LPT and AW that are only accessible upon local inflammation. Current knowledge suggests that the presence of an antigen-specific T cell population in the lung preceding, or immediately following, *M.tb* infection is crucial for control of pulmonary TB disease[31,48,59,60]. New vaccination strategies must therefore aim to yield an *M.tb*-specific T cell population in the lung before infection, or shortly after infection. A key principle behind RM immunization is that it generates a long-lasting population of polyfunctional T cells within the LPT and AW, fulfilling this need. RM vaccine-derived antigen-specific T cells are believed to be licensed to enter the restricted lung mucosa by expression of homing molecules that likely begin to appear during priming by activated DC in the MLN. Homing molecules such as chemokine receptors and integrins allow antigen-specific T cells to return to the site of inflammation. Some likely candidates that may play partial, or redundant roles include the chemokine receptors CXCR3, CXCR6, and CCR5, as well as the integrin LFA-1 (a heterodimer consisting of CD11a and CD18)[61,62]. However, when dealing with inflammatory responses within the lung, a delicate balance must be struck between protective and excessive inflammation which may lead to lung immunopathology. One way to safely elicit an optimal inflammatory response within the lung that protects against pulmonary *M.tb* infection is to utilize replication-deficient adenoviral-vectored vaccines expressing one or more *M.tb* antigens. By co-evolving with humans for thousands of years, *M.tb* has perfected the art of crafting an immune response permissive to bacterial growth. Now we must turn to non-traditional vaccine strategies and focus on generating unnatural immune responses to subvert non-protective natural immunity[60,63].

1.5 – "Unnatural" immune responses to *M.tb* infection

1.5.1 – Tissue resident memory T cells

Respiratory mucosal adenoviral-based vaccination with AdHu5Ag85A is effective due to its ability to induce a local inflammatory response and create an antigenic depot in the lung, drawing *M.tb* antigen-specific T cells to the site of *M.tb* entry and maintaining memory T cells via specific antigen-dependent proliferation. Single mucosal vaccination with AdHu5Ag85A induces both antigen-specific $CD4^+$ and $CD8^+$ T cell responses that differ geographically from those induced when administrated parenterally[48]. Such T cells, particularly $CD8^+$ T cells, offer much enhanced protection against pulmonary *M.tb* infection, especially when used as a booster to BCG[19]. The localization of antigen-specific T cells within lung mucosal tissues is dependent on the mucosal route of immunization. Importantly, respiratory mucosal vaccination with replication-deficient AdHu5Ag85A also promotes the generation of antigen-specific tissue resident memory T cells (T_{RM}) that are retained in the lung upon contraction of the effector T cell responses[64]. Given that the lung mucosa is the immunological battleground upon *M.tb* infection, generating T cell responses that are already primed and situated within the lung is a logical approach to design an effective vaccine. Previous work defined these antigen-specific $CD8^+$ T_{RM} by expression of mucosa-specific surface integrins such as VLA-1 ($\alpha_1\beta_1$) and CD103 ($\alpha_E\beta_7$)[64]. A recent study in a similar prime-pull model of adenoviral-vectored TB vaccination shows that parenterally primed antigen-specific T cells can be pulled into restricted lung mucosa, becoming T_{RM} cells (Haddadi *et al.*, unpublished). However, this process is dependent on both the antigen being used to pull T cells into the lung being specific to the T cells, and $CD4^+$ T cell-derived TNF-α (Haddadi *et al.*, unpublished). Thus, RM vaccination with adenoviral-vectored TB vaccines generates a long-

lasting, polyfunctional T_{RM} cells in restricted lung compartments. Despite T cells being the most investigated aspect of anti-TB immunity, much of their functionality is dependent on how these T cells interact with infected macrophages, and if they enable them to kill intracellular *M.tb*. It is these innate immune cell populations which become directly infected by *M.tb* bacilli, that remain underappreciated in the scope of anti-TB immunity, and may hold the key towards future advancements in TB vaccine design. The effects of RM vaccination on T cell responses and localization within the airways have been well established, but the impact on the local innate immune system, and the role it plays in protection are only beginning to be unraveled.

1.5.2 – Antibody responses against *M.tb* infection

Canonical vaccination strategies of successful preventative vaccines rely on the generation of antigen-specific plasma cells and memory B cells that secrete antibodies to opsonize or neutralize a pathogen/virulence factor, minimizing the clinical manifestation of infection. However, the role of B cells and antibodies in TB infection and disease remains controversial, as increasing opsonization may actually lead to an increase in phagocytosis and thus infectivity. Inconsistent protection in animal models further muddies the waters, as serum transfer experiments from vaccinated animals failed to protect the recipients[42]. In contrast, animals deficient in B cells or antibody secretion present with higher bacterial burden and were more susceptible to infection[65]. Recently, there has been growing appreciation for the role of antibodies in anti-TB immunity, placing emphasis on antigen-specificity, antibody subtypes, fragment-crystallizable (Fc) glycosylation patterns, and innate effector function. Each antibody subtype exerts some functionality through the binding of Fc-receptors on innate cells like neutrophils, NK cells, or macrophages. The downstream processes triggered within each of these cell types is affected by the glycosylation pattern of the antibody Fc-region. Differential antibody glycosylation patterns

may reflect differential B cell priming/germinal centre interactions, yielding downstream effects on Fc-receptor engagement and thus the type of innate immune effectors activated. Using a systems serology approach to characterize 70 attributes of *M.tb* antibody responses, it was found that individuals with LTBI possessed antibodies better able to bind Fc-γ-receptor type III (FcγRIII) on NK cells and drive cytolysis of infected cells through antibody-dependent cellular cytotoxicity (ADCC)[66]. These people better resisted *M.tb* infection, with increased phagolysosomal fusion, inflammasome activation, and pyroptosis pathway enrichment described in infected macrophages[66]. Since their immune response against *M.tb* is characterized by high levels of CD154, and some combination of antigen-specific IgM/IgG/IgA, they remain persistently IFN-γ release assay (IGRA)-negative regardless of infection status. These so called "resisters" are able to fend off disease without $CD4^+$ T cell derived IFN-γ, due to enhanced innate mechanisms of protection mediated through antibodies. Interferon-γ responses against the *M.tb* antigens ESAT-6 and CFP-10 are absent despite the presence of antigen-specific, class-switched antibodies against them. Identifying responses against these particular antigens is essential to differentiate responses induced by BCG vaccination versus those generated from *M.tb* exposure. "Resisters" do possess antibodies specific to these *M.tb* proteins, indicative of exposure and demonstrating that "resisters" are not simply IGRA-negative because they remain unexposed to *M.tb*. Ultimately this emphasizes a glaring hole in the current gold-standard of TB diagnostics, that has for years failed to wholly capture the immunological spectrum of disease. This finding demonstrates that our understanding of anti-TB immunity remains incomplete, and that IFN-γ production is not the sole measure of adaptive engagement.

1.5.3 – Trained innate immunity

Traditional vaccine efficacy is based on the dogma of adaptive memory, and the generation of antigen-specific B and T cells. These adaptive memory cells are antigen-specific, long-lived and can provide accelerated and exaggerated responses upon re-exposure to the same antigen. Antigen-specific B and T cells will proliferate and contract following vaccination, leaving behind an expanded memory repertoire responsible for the heightened secondary response. This has proven to be an extremely effective strategy when combating infectious agents with highly immunogenic and conserved domains. In fact, this strategy has allowed for the global eradication of smallpox virus (excluding repositories in tightly-regulated government laboratories). Interestingly, *M.tb* does have highly conserved epitopes allowing for generation of large populations of antigen-specific adaptive cells from vaccines such as BCG or MVA85A, but they remain unable to clear or prevent pulmonary disease due to a number of mechanisms previously described. Recent developments in TB vaccine design have shifted focus away from non-sterilizing responses analogous to immunity generated after primary pulmonary *M.tb* infection or parenteral BCG vaccination. Instead, the goal is to generate "unnatural immunity", a type of the immune responses sufficiently different enough from known non-sterilizing ones. This could entail generating T_{RM} responses positioned at the site of *M.tb* entry balanced against multi-stage *M.tb* antigens, induction of anti-*M.tb* cell wall antibodies, and trained innate immunity (TII) via induction of innate immune memory[60]. This last phenomenon of TII has been well described in the context of BCG vaccination in humans, and has recently come to the foreground of TB research.

Individuals such as healthcare practitioners or sailors have been documented to remain tuberculin skin-test (TST) and IGRA-negative despite prolonged exposure to infected individuals. Roughly 10% of BCG-positive individuals exposed to *M.tb* are thus implied to either generate

immunity through IFN-γ-independent mechanisms as outlined above, or clear bacilli prior to initiation of an adaptive immune response[67,68]. Admittedly IGRA and TST are inherently incomplete measures of anti-TB immune responses, but this does not rule out "early clearance" of *M.tb* by the innate immune system. BCG has been shown to induce a state of memory in innate immune populations that are dogmatically neglected when characterizing vaccine-induced responses. Trained innate immunity is defined by a reset homeostatic state of innate immune cells leading to subsequent enhanced non-specific responsiveness (**Diagram 3**)[69–71]. It is believed that part of the reduction in overall childhood mortality observed after BCG vaccine rollout is due to TII, as the reductions seen cannot be accounted for by eliminating childhood TB disease alone[72–74]. Unlike other clinically approved vaccines, BCG has questionable efficacy against the lung disease that it is intended to prevent, but due to its non-specific effects on child mortality and its protective effects on disseminated TB, it is unlikely to ever be entirely replaced. Yet the door remains open to augment BCG-derived responses, particularly those within the lung, and the most effective way to do this is believed to be through RM vaccination[75].

Diagram 3

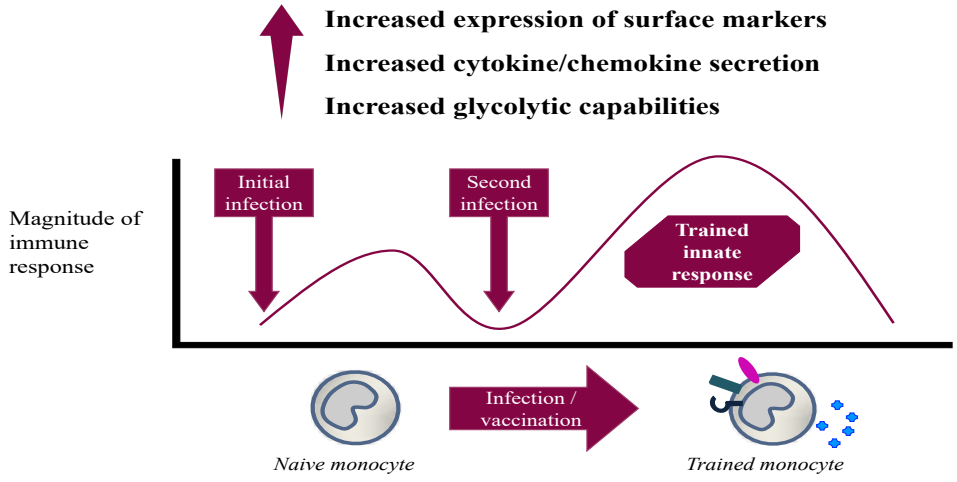

Diagram 3: Trained innate immunity describes memory-like characteristics of innate immune cells.

Thus far, we have summarized the benefits of TII in the form of innate immune memory, but we have neglected to acknowledge the other side of the coin. The innate immune memory, depending on the nature of inducing agents, may act in the other direction, in ways detrimental to health via innate immune-mediated autoimmunity or increased susceptibility to infection through innate immune tolerance. Therefore when investigating TII, these possibilities should be considered[76–78]. Both of these are two sides of the same coin that is TII, but each will have vastly different consequences during infection depending upon which side the coin lands. Innate immune memory of NK cells, monocytes, and macrophages plays an important role in immunity against subsequent infection with heterologous pathogens[79–82]. However, following infection with influenza virus, a large part of the mortality and morbidity comes from immediate successive infection with bacterial pathogens such as *S. pneumoniae* coinciding with viral clearance. Indeed, this was found to be likely caused by innate dysfunction of AM mediated through IFN-γ during influenza infection. These AM developed a state of innate immune tolerance, opening the door for an opportunistic secondary pathogen such as *S. pneumoniae*. Prior exposure of macrophages to the mouse-adapted influenza A/PR8/34 (H1N1) led to decreased IL-1β and TNF-α secretion upon bacterial challenge[75]. Interestingly, this anti-inflammatory response was also mediated by T cell secretion of IFN-γ, similar to what was observed in Yao *et al.*, but to the opposite effect[69]. In this analogous study, a transient pulmonary adenoviral infection recruited $CD8^+$ T cells into the AW of mice, where through contact and IFN-γ-dependent mechanisms they primed AM to readily recruit neutrophils leading to enhanced protection against *S. pneumoniae* challenge. The stark contrast in the outcome of these two respiratory viral infection scenarios despite the similarities in mechanism highlights how much they remain to be discovered in regards to TII, not just in the scope of infection, but also vaccination.

1.5.4 – Molecular mechanisms of innate immune memory

The key to the phenotypic plasticity of TII is the nature of the underlying molecular mechanisms; reversible phenomena associated with chromatin and metabolic remodeling. Innate immune memory or tolerance appear to be reversible given the right conditions, as LPS-tolerized monocytes were once again made reactive by stimulation with β-glucan[83]. In the case of BCG, innate immune memory has been characterized on circulating monocytes, and their haematopoietic stem cell progenitors[80,84]. The latter is essential to pass down innate memory characteristics from generation to generation of short-lived cells such as monocytes. Epigenetic changes like the acetylation (-Ac) of histone 3 at lysine 27 (H3K27), and tri-methylation (-3Me) of H3K4 in the promoters of immunoregulatory genes are the driving forces of innate immune memory[85]. These modifications are associated with local chromatin remodeling, facilitating access to transcriptional machinery. Upon removal of the training stimulus, H_3K27-Ac was gradually lost, while H_3K4-3Me was stable over time, and thus is implicated in the long term epigenetic changes of immune genes leading to non-specific memory[83,86,87]. For as long as three months following BCG vaccination, circulating monocytes demonstrate significantly increased H_3K4 trimethylation at promoters for genes encoding TLR4, and classical trained innate immune cytokines like IL-1β, and TNF-α[80]. Long-term epigenetic modifications such as these in monocytes and monocyte-progenitors allow BCG-imprinted monocytes to secrete more IL-1β, correlating with reduced yellow fever viremia following BCG vaccination in humans, and protection of severe-combined immunodeficient (SCID) mice from lethal *Candida albicans* challenge[80,88]. This mechanism of non-specific BCG-derived protection is generated and sustained wholly independent of adaptive immunity[80]. Heterologous protection provided by BCG-imprinted monocytes may in part explain the reductions in non-tuberculosis associated deaths seen following vaccine rollout[72–74]. Due to its non-specific

protective effects on child mortality and specific protective effects on disseminated forms of TB, BCG is unlikely to ever be replaced, but the door remains open to augment BCG-derived responses, particularly those within the lung at the primary site of *M.tb* infection.

A second central pillar of TII is a metabolic reprogramming, namely a shift in the steady state from basal oxidative phosphorylation to one that is highly glycolytic, deemed the Warburg effect[89]. This event is analogous to an effector state that is attained by highly activated macrophages and monocytes, and abrogation of this event by administering rapamycin prevents the onset of trained innate immunity[89]. In the steady state, AM rely on fatty acid uptake and β-oxidation as their principal metabolic pathway and are thus amenable to *M.tb* proliferation. Once phagocytosed by AW phagocytes, intracellular *M.tb* is known to access and utilize free fatty acids and cholesterol stores[90]. Having access to an abundance of free lipids is critical to efficient *M.tb* replication due to the energy intensive process of building its complex cell wall. Heterogeneity of lung macrophage populations is a notable contributor to the outcome of *M.tb* infection, partially through nutritional immunity[11]. Thus if the basal metabolic state of a cell once centred around fatty-acid oxidation, or oxidative phosphorylation, can be switched to a more glycolysis-based, effector-like metabolism, it may offer a potential avenue of nutritional restriction within the macrophage. Interstitial macrophages (IM) are believed to be monocyte-derived in the steady state, unlike their fetal-liver derived alveolar counterparts. These distinct ontogenies are reflected in the activation state of the macrophages upon stimulation. Prior research has shown that IM secrete IL-10 upon stimulation with bacterial CpG DNA, protective towards allergic inflammation, but might be detrimental in the scope of TB[91]. Inflammatory monocytes are recruited to the lung following *M.tb* infection and have been documented to be both more permissive and more bactericidal to *M.tb*[11,17]. This is likely due to inconsistencies in flow cytometry markers/gating strategies used to differentiate these

populations, the model of infection, the maturation state of the recruited monocyte, and through the receptor by which it first interacts *M.tb* bacilli. Recruited Ly6C$^+$ monocytes differentiate into IM which remain highly glycolytic, and actively produce more IL-1β, TNF-α and iNOS than their alveolar counterparts upon infection with *M.tb*[11]. Importantly, this supports the idea that these monocyte-derived macrophages are proinflammatory in the scope of *M.tb* infection. This is crucial to counteract anti-inflammatory type I interferon production, hallmark of infection with highly virulent strains of *M.tb* like Beijing 4334 or HN878[92]. Within the recruited IM population there were several subpopulations with differential expression of MHC II, the mannose-receptor CD206, and CCR2, all having different transcriptional profiles[11]. Subpopulations of macrophages from distinct lineages can divergently respond to the same cytokines (likely due to epigenetic control), but whether or not this holds true for *M.tb* infection remains to be elucidated[11,43,93]. Ultimately this work highlights the distinct profiles and metabolic activities of separate macrophage populations, the role metabolism plays in bacterial control, as well as the heterogeneity and diversity of subpopulations within these populations as they respond to *M.tb* infection.

Another branch of nutritional immunity is brought to light with the variable expression of iNOS in different macrophage populations, revolving around arginine utilization. Differences in expression of nitric oxide synthase relative to arginase in M1-like versus M2-like macrophages within the lung outline a metabolically privileged niche correlating with granuloma progression[94]. This finding was further reinforced with transcriptomic profiling of infected lung tissue[95]. Central to these differences in cellular metabolism is the role of the Dectin-1-Akt/mammalian target of rapamycin (mTOR)/hypoxia-inducible-factor-1α (HIF-1α) pathway, which is also modulated in trained innate immunity[87,89,96]. Stimulating human monocytes with β-glucan, induces changes in genes associated with glucose metabolism, and is a driver of the Warburg effect in trained

monocytes as outlined above[89]. There are several other metabolic pathways documented to be altered in trained innate immunity, and thus may have an effect on anti-TB immunity. Along with changes in glycolysis, changes in pathways involved in glutaminolysis and cholesterol metabolism have also been described[97,98]. As outlined above, changes in cellular metabolism can alter the ease at which *M.tb* can thrive within phagocytes. These changes to metabolism on a cellular level are part of the mechanism linking epigenetic changes to enhanced cytokine responses and thus, the defence-ready state observed in trained innate immunity. Utilizing vaccination to alter metabolic pathways by training innate immune cells may make formerly permissive cells more restrictive to *M.tb* and provides a mechanism of innate mycobacterial control unique to TII.

1.5.6 – Trained innate immunity in the airway following respiratory mucosal vaccination

Initial interactions between *M.tb*, and the airway phagocytes encountered largely shape the immune response that follows. Alveolar macrophages (AM) do have bactericidal mechanisms through which they can kill *M.tb*. In traditional methods of primary pulmonary *M.tb* infection, bacterial killing does not begin until the infiltration of antigen-specific T cells into the lung, 18-28 days following the initial infection. These effector $CD4^+$ and $CD8^+$ T cells can directly kill infected macrophages through Fas-FasL, and also produce IFN-γ and TNF-α to synergistically activate them to make iNOS2. Anti-mycobacterial pathways from macrophages infected with *M.tb* revolve around forming reactive nitrogen and oxygen intermediates toxic to *M.tb* bacilli such as nitric oxide (NO). These cytokines also promote phagosome-lysosome fusion to prevent bacterial escape through virulence factors like ESAT-6. The end goal is to make the environment within the phagocyte inhospitable to bacterial replication and survival[99]. By priming AW macrophages via RM vaccination, these macrophages may become more bactericidal and produce more NO upon infection. Contrary to the parenteral route of administration, and thus systemic training effects seen

following subcutaneous BCG vaccination, RM AdHu5Ag85A induces innate immune memory within the lung mucosa. AM are long-lived compared to circulating monocytes, and undergo *in situ* proliferation independent of monocytes in the steady state[100]. In human lung transplant recipients, 3-4 years post lung transplant, 85-90% of the AM remain of donor-origin[101,102]. Respiratory mucosal AdHu5Ag85A vaccination primes effector $CD8^+$ T cells in the MLN that home to the AW between 5- and 7-days post-vaccination[69]. Upon the arrival of $CD8^+$ T cells to the AW, they secrete IFN-γ and interact with AM via their T cell receptor to impart a highly activated, memory phenotype characterized by increased MCH II expression, cytokine/chemokine secreting ability, and glycolytic metabolism (**Diagram 4**)[69]. These "memory" AM are critical to trained innate responses against bacterial challenge with *Streptococcus pneumoniae*, launching an expedited and heightened response with the chemokines KC and MIP-2 to enhance neutrophilia. This describes a novel paradigm of immunological memory formation opposite to traditional dogma, where adaptive immunity licenses trained memory innate immunity of AM[69]. If innate immune cells can also be targeted via vaccination, there will be many novel targets to be considered in vaccine design. Recently, there has been increasing discussion around characterizing innate responses as a correlate of immune protection following BCG vaccination. However, parenteral BCG vaccination has failed to protect against pulmonary TB regardless of systemic innate immune contributions. Thus, the ability of respiratory mucosal vaccination with AdHu5Ag85A to induce memory AM and trained innate immunity against extracellular bacterial species such as *S. pneumoniae*, offers new hope that such vaccine strategy may be exploited to enhance anti-TB innate immunity in the airway before T cell immunity appears in the lung. This is still an emerging new area of research and the question regarding whether targeting alveolar macrophages via RM vaccination is able to enhance early restriction of *M.tb* growth remains to be investigated[60].

Diagram 4

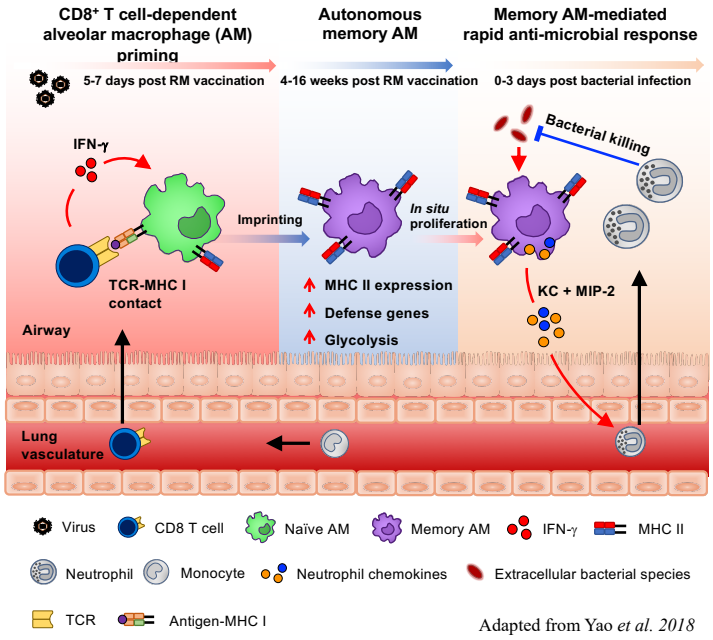

Diagram 4: Respiratory mucosal vaccination with AdHu5Ag85A generates memory alveolar macrophages that clear heterologous bacterial infection (adapted from Yao *et al.*)[69].

1.6 – Significance:

Current vaccination strategies against tuberculosis have failed to effectively quell pulmonary TB infection rates. This is likely due to their parenteral administration, and their focus on inducing immune responses that mimic immunity caused by natural infection. To address these deficiencies, recent research has focussed on the generation of "unnatural" immune responses to force *M.tb* into situations less amenable to its growth. One such way to modify adaptive immunity is to administer viral-vectored vaccines such as AdHu5Ag85A directly to the respiratory tract. Unlike parenteral vaccination strategies, this draws antigen-specific T cells into the airway, and establishes tissue resident memory T cells ready to respond upon *M.tb* exposure. These T cells have also recently been described to induce trained innate immunity via memory AM, the first such case of adaptive-innate long-term imprinting. Memory AM have demonstrated increased protection

against infection with *S. pneumoniae*, but whether or not this increased state of innate activation can also increase protection against *M.tb* remains to be investigated. We believe that memory AM can better contain *M.tb* via overcoming innate suppression imposed by infection during the hours and days immediately following infection, and that they can do so independent of pulmonary vaccine-derived T cell immunity. If proven true, it will shed new light on the mechanism of mucosal vaccine-induced anti-TB immunity, and support the concept of respiratory mucosal vaccine strategies for broad immune protective benefits.

1.7 – Overall rationale, hypothesis, and specific aims:

Current vaccination strategies against TB fail because they attempt to augment states of immunity associated with control, not clearance. Yao *et al.* have recently described the induction of TII in the peripheral mucosal tissues following infection with a viral-vectored TB vaccine[69,103]. Recent advances in understanding the changes behind the cellular processes that occur in TII have opened up an avenue for atypical vaccine development by targeting innate populations at mucosal sites. This is particularly attractive in the scope of TB vaccine development as macrophages are the main host of *M.tb* during the early stages of infection and are speculated to play a major role in mycobacterial control prior to the induction of adaptive immunity. Modulation of mucosal innate populations have proven to enhance their functionality against heterologous bacterial infections, but it remains to be seen if modulating resident mucosal innate cell populations like AM can effectively enhance early protection against *M.tb*.

Respiratory mucosal vaccination with AdHu5Ag85A generates effector $CD8^+$ T cell responses that return to the lung and AW. Here they interact with AM, and induce a state of mucosal innate immune memory that protects against infection with heterologous bacteria *in vivo* by

increasing the magnitude of the neutrophilic response[69]. However, whether or not trained AM launch an enhanced innate immune response that can enhance protection during early pulmonary *M.tb* infection remains to be determined (**Diagram 5**). Preliminary data suggests that memory macrophages can reduce bacillary burden in the lung within the first 7 days following *M.tb* infection. If states of mucosal TII are effective at increasing early protection against diseases like TB, they may prove to be a helpful target when considering readouts of vaccine efficacy. This is especially true when looking at tuberculosis vaccination strategies currently in the developmental pipeline such as respiratory mucosal AdHu5Ag85A vaccination. Amplifying innate immune responses against tuberculosis, such as early mycobactericidal activity in memory AM could promote early clearance in the lung without the need to establish sterilizing adaptive immunity.

Central Question: Whether or not RM vaccine-induced memory alveolar macrophages contribute to anti-TB immunity during early *M.tb* infection.

Hypothesis: We hypothesize that RM, but not parenteral, vaccination with AdHu5Ag85A induces memory alveolar macrophages to enhance early T cell-independent control of pulmonary *M.tb* infection.

Aim 1: Examine T cell-independent innate immune protection in RM vaccinated hosts in early stages of pulmonary *M.tb* infection.

Aim 2: Evaluate the immune properties of RM vaccination-induced memory alveolar macrophages.

Aim 3: Determine the role of RM vaccination-induced memory alveolar macrophages in early anti-TB immunity.

Diagram 5

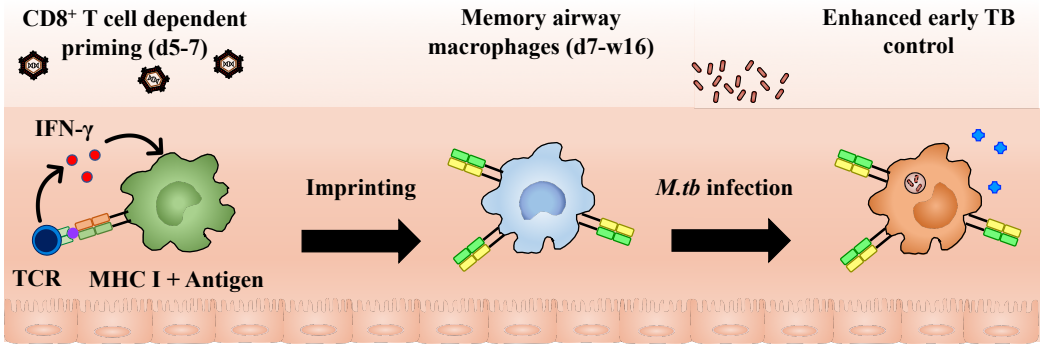

Diagram 5: Respiratory mucosal AdHu5Ag85A vaccination imprints alveolar macrophages to increase early *M.tb* control.

2.0 – Materials and methods

Mice

Wild-type female BALB/c mice were purchased from Charles River Laboratories (Saint Constant, QC, Canada) or the Jackson Laboratory (Bar Harbor, ME, USA). All mice were 6-8 week of age upon arrival. Mice were housed in either a specific pathogen-free level B, or a biosafety level 3 facility at McMaster University, Hamilton, ON, Canada. All experiments were carried out in accordance with the institutional guidelines from the Animal Research and Ethics Board.

Vaccination with an adenoviral vector TB vaccine

Vaccination was elicited by using a recombinant human serotype 5 adenovirus expressing an *M.tb* protein, AdHu5Ag85A[39]. The production and utilization of these viruses were previously described[69,104,105]. Virus was prepared in phosphate buffered saline (PBS) and used at $5x10^7$ PFU per mouse. Mice were vaccinated either intranasally with 25µL, or intramuscularly with 200µL of virus preparation.

Mycobacterium tuberculosis *infection*

The *M.tb* H37Rv strain was prepared for infection and delivered as previously described[21,31,52]. Briefly, *M.tb* was grown in 7H9 media supplemented with Middlebrook oleic acid-albumin-dextrose-catalase enrichment, 0.002% glycerol, and 0.05% Tween-80 for 10-15 days, aliquoted, and stored in -70˚C until use. Prior to use, *M.tb* was washed twice with PBS containing 0.05% Tween-20, after which it was passed through a 27-gauge syringe to ensure single cell suspension, after which the bacteria were delivered. Mice were infected with $1x10^4$ CFU *M.tb* intranasally. Bacterial burden was assessed at each experimental endpoint by plating serial dilutions of lung homogenate, bronchoalveolar lavage fluid, or bronchoalveolar lavage cells in triplicates onto Middlebrook 7H10 plates and incubated at 37˚C for 21-28 days before enumeration.

In vivo *depletion of T cells and MCP-1*

At 4, 8, or 12 weeks post-vaccination, mice were injected intraperitoneally with 200µg of anti-CD4 (clone GK1.5), and anti-CD8 (clone 2.43) or an IgG isotype control from Sigma-Aldrich (St. Louis, MO, USA). A second 100µg dose was administered intraperitoneally after 2 days, and this was repeated every 7 days as needed to maintain depletion as previously published[69]. Depletion was verified using flow cytometry on the LSR II FACSDiva software from BD Biosciences (San Jose, CA, USA). At 27-days post vaccination, mice were injected intraperitoneally with 200µL of anti-mouse MCP-1 rabbit sera or normal rabbit sera as a control. Further doses were administered every 2 days as previously published to maintain the depletion[106]. Rabbit sera was generously provided by Dr. Steven Kunkel.

Bronchoalveolar lavage and lung mononuclear cell isolation

Mice were euthanized by exsanguination. Cells from bronchoalveolar lavage and lung tissue were isolated as previously described (Jeyanathan *et al*., 2010, 2017; Yao *et al*., 2017, 2018)[61,69,104,105]. Briefly, following exhaustive bronchoalveolar lavage, lungs were cut into small pieces and digested with 150 U collagenase type 1 (ThermoFisher Scientific, Waltham, MA, USA) at 37˚C in an agitating incubator. A single-cell suspension was obtained by crushing the digested tissue through a 100 mm basket filter (BD Biosciences, San Jose, CA). Isolated cells were resuspended in either complete RPMI 1640 medium (RPMI 1640 supplemented with 10% FBS and 1% L-glutamine, with or without 1% penicillin/streptomycin) for *ex vivo* culture or in PBS for adoptive transfer or flow cytometry staining. Cells were counted in Turks Blood Dilution Fluid (RICCA Chemical, Arlington, TX, USA) and counted under microscope. Alternatively, cells were counted

automatically by a Sceptre 2.0 Cell Counter and Software Pro (Millipore Sigma, Etobicoke, ON, Canada).

Cell surface immunostaining

Cell immunostaining and flow cytometry were performed as previously described[61,69,104,105]. Briefly, mononuclear cells from bronchoalveolar lavage and the lung were plated in U-bottom 96-well plates at a concentration of $2x10^7$ cells/mL in PBS. Following staining with Aqua dead cell staining kit (ThermoFisher Scientific Waltham, MA, USA) at room temperature for 30 min, cells were washed and blocked with anti-CD16/CD32(clone 2.4G2) in 0.5% bovine serum albumin-PBS for 15 min on ice and then stained with fluorochrome-labeled mAbs for 30 min on ice. Fluorochrome-labeled monoclonal antibodies used for staining pulmonary myeloid cells were anti-CD45-APC-Cy7 (clone 30-F11), anti-CD11b-PE-Cy7 (clone M1/70), anti-CD11c-APC (clone HL3), anti-MHC II-Alexa Flour 700 (clone M5/114.15.2; eBioscience, ThermoFisher Scientific Waltham, MA, USA), anti-CD3-V450 (clone 17A2), anti-CD45R (B220)-V450 (clone RA3-6B2), anti-Ly6C-Biotin (clone HK1.4; Biolegend, San Diego, CA, USA), Streptavidin-Qdot800 (Invitrogen), anti-CD24-BV650 (clone M1/69), anti-CD64-PE (clone X54-5/7.1; Biolegend, San Diego, CA, USA), anti-Ly6G-BV605 (clone 1A8), and anti-Siglec-F-PECF594 (clone E50-2440). Stained cells were then fixed and permeabilized with BD Cytofix/Cytoperm followed by incubation in 1x BD Perm/Wash buffer (BD Biosciences, San Jose, CA, USA). Unless otherwise indicated, all monoclonal antibodies and reagents were purchased from BD Biosciences (San Jose, CA, USA). Immunostained cells were processed according to the BD Biosciences instructions for flow cytometry and run on a BD LSR II or BD Fortessa flow cytometer. Data were analyzed using FlowJo software (version 10.1; Tree Star, Ashland, OR, USA).

Purification of alveolar macrophages and lung CD11b$^+$/CD11c$^+$ antigen presenting cells

To isolate alveolar macrophages, single cell suspension of BAL was labeled with CD3ε microbeads (Miltenyi Biotech, Auburn, CA, USA) according to the manufacturer's instructions. Purified CD3$^-$ alveolar macrophages, were used for *ex vivo* culture experiments including bacterial stimulation. Separately, single cell suspension of the lung was labeled with either CD11b microbeads and/or CD11c microbeads (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions. Purified cells were then used for *ex vivo* culture.

Mycobacterial killing assay

Cells were isolated from naive, intranasal AdHu5Ag85A immunized mice, or intranasal vaccinated, T cell depleted mice at 4 weeks post vaccination. At this time, CD11c$^+$/CD11b$^+$ cells were isolated from the lungs as described from lung homogenate, or CD3$^-$ cells were isolated from bronchoalveolar lavage as described above. Cells were plated at a density of 1-2x10^5 cells/well in a 96 well plate and rested for 2 hours at 37˚C. After which the cells were infected with either *M.tb* H37Rv or BCG DsRed at a multiplicity of infection of 1:1 or 10:1 respectively. Four hours post-infection, extracellular bacteria were removed, and bacterial burden was then assessed at 4, 24 and 48 hours using a CFU assay or flow cytometry. For *in vivo* killing assays, mice were vaccinated or left naïve as described above, before being infected with 100 000 CFU *M.tb* H37Rv for 1, 3, or 7 days prior to sacrifice. Mice were then sacrificed, BAL was performed and lungs were homogenized as previously described. BAL was then centrifuged to pellet AW cells to separate them from acellular bacilli. BAL cells were then lysed in dH_2O and plated to enumerate burden separate from the BALF, and separate from the lung homogenate.

Cytokine and chemokine measurement

Cytokine and chemokine levels within lung homogenates, culture supernatants, or bronchoalveolar lavage fluid were determined using TNF-α, or IL-12 p40 ELISA kits (R&D Systems, Burlington, ON, Canada) according to the manufacturer's instructions. ELISA plates were read on a spectrophotometer at 450 nm. Chemokines including MIP-1α, IP-10, MCP-1 and RANTES were quantified by using MCYTOMAG-70K mouse chemokine and cytokine detection kit (Millipore Sigma, Etobicoke, ON) according to the manufacturer's instructions. Plates were read on a MagPix reader (ThermoFisher Scientific, Waltham, MA, USA) by using xPONENT software (ThermoFisher Scientific, Waltham, MA, USA). Concentrations of chemokines were calculated based on serial dilutions of standards by using spline curve fitting in GraphPad Prism software (Version 7, GraphPad Software, La Jolla, CA, USA).

Metabolic assay of alveolar macrophages

Real-time cell metabolism of alveolar macrophages was determined by using the Seahorse XF Glycolysis Stress Test Kit (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's instructions[69]. Alveolar macrophages were obtained from the BAL through negative selection by using CD3ε microbeads to remove T cells (Miltenyi Biotec, Auburn, CA, USA). Purified alveolar macrophages were seeded onto a 24-well microplate (Agilent Technologies, Santa Clara, CA, USA) at a density of $2x10^5$ cells/well. The plate was incubated for 2h in a 37˚C 5% CO_2 cell culture incubator and washed twice with culture media to further remove non-adhering cells. Alveolar macrophages were then incubated overnight in complete Roswell-park memorial institute (RPMI) 1640 supplemented with 10mM HEPES, 0.5mM sodium-pyruvate, 55μM 2-mercaptoethanol, 0.1mM non-essential amino acids in a 37˚C 5% CO_2 cell culture incubator. On the following day, alveolar macrophages were washed twice and cultured with

Seahorse XF base medium supplemented with 2mM L-glutamine (Agilent Technologies, Santa Clara, CA, USA) for the duration of the assay. Extracellular Acidification Rate (ECAR) was assessed by using a Seahorse XFe24 Analyzer (Agilent Technologies, Santa Clara, CA, USA). Glycolysis was represented by ECAR after the addition of 10mM glucose. Glycolytic capacity was represented by maximum ECAR following the addition of 1μM oligomycin. Data were analyzed using Wave Desktop software version 2.6 (Agilent Technologies, Santa Clara, CA, USA) and normalized to protein via Bradford assay.

Statistical analysis

Asterisks in the Figures indicate the level of statistical significance (*$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$) as determined using a two-tailed unpaired Student's T-test, one-way ANOVA or two-way ANOVA with a Tukey's post-hoc analysis. Tests were performed using GraphPad Prism software (La Jolla, CA, USA). Data are expressed as mean ± SEM unless otherwise stated.

3.0 – Results

3.1 – Respiratory mucosal, but not parenteral, vaccination controls the bacterial load in the lung in early stages of pulmonary *M.tb* infection

Mounting evidence suggests that respiratory mucosal infections are able to permanently modulate the lung and airway, establishing immunological memory attributed to both the adaptive and innate immune systems. Generation of innate immunological memory is supported by observations that previous lung infection can alter the immunity and pathology upon subsequent infections despite being antigenically distinct. Recently, we demonstrated that respiratory mucosal adenoviral-vectored TB vaccination alters the outcome of successive *S. pneumoniae* infection via induction of memory alveolar macrophages[69]. Based on these observations, we set out to investigate whether RM vaccination can also contribute to innate host defense during the early stages of *M.tb* infection. As AM have recently been described to be a sole cellular target of *M.tb* within the first week following infection, it has yet to be investigated if RM AdHu5Ag85A vaccination can provide early protection against *M.tb*[10,11]. We hypothesized that if the lung innate immune system is indeed modulated by prior RM vaccination, it would translate to increased control of pulmonary *M.tb* burden during the early stages of infection. To begin the investigation, mice were either vaccinated RM (**i.n.**) or parenterally (**i.m.**) for 4 weeks, or were left unimmunized (**naïve**). All mice were then infected with *M.tb*H37Rv and sacrificed 7 days post-infection (dpi) (**Figure 1A**). Lungs were assayed for bacterial load by colony forming units (CFU) (**Figure 1A**). We found that RM AdHu5Ag85A vaccination significantly reduced pulmonary *M.tb* burden in the lung compared to parenteral, and naïve animals as early as 7 dpi (**Figure 1B**). Mice that were vaccinated via the parenteral route (**i.m.**) failed to reduce bacterial burden within the lung (**Figure 1B**), once again highlighting the importance of RM vaccination on AdHu5Ag85A efficacy.

3.2 - Respiratory mucosal vaccination controls the bacterial load in the lung in early stages of pulmonary *M.tb* infection in a T cell-independent manner

Having demonstrated improved protection in early stages of infection by RM, but not parenteral, vaccination, we would like to address the potential mechanisms by which RM vaccination works. Previous studies from our group and others have appraised RM AdHu5Ag85A vaccine-mediated protection at 4 weeks after *M.tb* infection. This protection has been attributed to the presence of antigen-specific T cells in the respiratory mucosa of RM vaccinated hosts[50]. However, recent work by Yao *et al.* shows a profoundly altered lung innate immune environment following respiratory mucosal AdHu5Ag85A vaccination[69]. Therefore, we first determined if the lung equipped with vaccine-altered innate immunity would be better protected in early stages of pulmonary infection, in the absence of T cell immunity. To this end, mice were left unvaccinated (**Naïve**), or RM vaccinated **(i.n.**) for 4 weeks, and were depleted of both $CD4^+$ and $CD8^+$ T cells before infection, which was maintained until the time of sacrifice (**Naïve/ΔT, i.n./ΔT**). Control mice were also either unimmunized or RM vaccinated (**i.n.**) for 4 weeks but their T cell populations remained untouched (**Figure 2A**). Consistent with earlier observations (**Figure 1**), RM vaccination led to better control of bacterial burden when compared to naïve mice (**Naïve, i.n.**) at 7dpi (**Figure 2B**). Interestingly, depletion of T cells during *M.tb* infection did not negatively affect the enhanced bacterial control in the lungs of RM-vaccinated mice (**i.n./ΔT**) compared to T cell-depleted naïve counterparts (**Naïve/ΔT**). This reduction in bacterial burden was also comparable to the T cell replete controls (**i.n.**) (**Figure 2B**).

We next sought to determine whether RM vaccination could provide enhanced protection even beyond 7d post-*M.tb* infection in the absence of T cells. This was accomplished using the same experimental setup described in Figure 2A, but animals were instead sacrificed at 14 days of

infection (**Figure 2C**). Though the bacterial load increased in the lungs of all mice compared to 7 dpi, RM vaccination continued to control the bacterial burden in the lung independent of T cells (**i.n./ΔT**) (**Figure 2D**).

Since our recent study has shown a long-lasting training effect by RM vaccination on lung innate immune system, we next determined whether long after RM AdHu5Ag85A vaccination, the lung still remained protected in early stages of *M.tb* infection, independent of T cell immunity. To this end, mice were RM-vaccinated and their T cells depleted at 8 weeks (**Figure 3A**) or 12 weeks following RM vaccination prior to *M.tb* infection (**Figure 3C**). Lung bacterial burden was assessed at 7 dpi. Regardless of the duration of the vaccination period prior to infection, RM vaccination remained effective in enhancing early protection in the absence of T cells (**Figure 3B/D**). Taken together our data suggest that RM vaccination enhances immune protection in early stages of pulmonary *M.tb* infection and such enhanced protection is independent of T cell immunity.

3.3 – Induction of memory alveolar macrophages by respiratory mucosal, but not parenteral, vaccination

Thus far, we have demonstrated that RM, but not parenteral, vaccination offers early immune protection against pulmonary TB and RM vaccine-enhanced protection is independent of T cell immunity. To further investigate its cellular mechanisms, we focused on examining the potential role of AM. We have recently shown RM AdHu5Ag85A vaccination to induce a long-lived memory AM population within the AW, capable of protecting the lung against subsequent *S. pneumonia*e infection[69]. Therefore, we hypothesized that RM, but not parenteral, vaccination would alter lung innate immune environment by inducing memory AM. Thus, mice were either RM or parenteral vaccinated with AdHu5Ag85A, or left un-vaccinated as naïve control. At 4 weeks

post-vaccination, airway mononuclear cells were then harvested via bronchoalveolar lavage and assessed for markers of innate immune memory (**Figure 4A**). A comprehensive flow cytometry gating strategy was used to differentiate alveolar macrophage populations[69,107,108]. In our previously published study, bona fide AM are reliably differentiated from other macrophage populations by using a panel of monoclonal antibodies. AM are defined as $CD11b^-$, $Ly6G^-$, $CD11c^+$, $CD64^+$, $SiglecF^+$, $Ly6C^-$, while the lung interstitial macrophages (IM) are $CD11b^+$, $Ly6C^-$, $SiglecF^-$, and the monocyte-derived macrophages (MdM) are $CD11b^+$, $Ly6C^+$, $SiglecF^-$ (**Supplemental figure 1**). In accordance with the memory AM phenotype defined by Yao *et al.*, we observed an MHC II^{HI} phenotype on AM in RM vaccinated mice (**i.n.**) but not on those AM in parenterally vaccinated (**i.m.**) or naïve animals (**naïve**) (**Figure 4B/C**)[69].

In addition to MHC II expression, we examined other features associated with memory AM[69]. Mice were set up as per Figure 4A, and BAL was performed to isolate AW mononuclear cells. Lavage cells were depleted of $CD3^+$ cells and then cultured *ex vivo* in minimal media. A seahorse assay was performed, and lactic acid production mediated pH changes in media were used as a proxy of glycolytic rate. Alveolar macrophages from RM vaccinated mice (**i.n.**) displayed markedly increased glycolytic metabolism, indicative of the Warburg effect, a hallmark of trained innate immunity (**Figure 4D**). This phenomenon was once again absent in AM of naïve and intramuscularly vaccinated (**i.m.**) mice, which mirrored each other, as both had lower extracellular acidification rates than AM from RM vaccinated mice (**Figure 4D**).

We also examined cytokine production capacity of lung $CD11c^+/CD11b^+$ cells infected *ex vivo* with *M.tb*. In agreement with Figure 4B/C, we found that pulmonary macrophages isolated from RM vaccinated animals (**i.n.**) produced significantly higher levels of IL-12p40 than their

naïve or parenteral-vaccinated counterparts (**i.m.**) (**Figure 4E**). All together, the above data suggest that RM, but not parenteral, vaccination induces a population of MHC II^{HI} memory alveolar macrophages that are highly glycolytic and produce more IL-12 upon mycobacterial infection.

In separate experiments, we further examined the cytokine response of memory AM to pro-inflammatory stimulation with LPS or *M.tb* related antigens. Mice were either RM vaccinated or left naïve as a control. After 4 weeks, alveolar macrophages were isolated via BAL. Bronchoalveolar lavage was depleted of $CD3^+$ cells and cultured *ex vivo* with either LPS, or *M.tb* whole cell lysate (WCL) (**Figure 5A**). Supernatant levels of cytokines were quantified 48 hours later by Luminex. Stimulation of memory AM from the lung of RM vaccinated animals with either LPS or WCL led to increased production of the pro-inflammatory cytokines IL-1β, IL-6, and MCP-1 over that by AM from naïve control animals (**Figures 5B/C/D**). These data further indicate the functionally activated state of memory AM in the lung following RM vaccination.

3.4 – Respiratory mucosal vaccination leads to differential innate cellular responses in the lung to pulmonary *M.tb* infection

Our data have thus far linked RM vaccine-induced memory AM (**Figures 4/5**) to enhanced protective innate immunity we have observed in early stages of pulmonary *M.tb* infection (**Figures 1-3**). To further examine the relationship of memory AM to innate immune protection against *M.tb* infection, we assessed innate cellular responses in the airway following *M.tb* challenge. We initially focused on examining the responses of AM, interstitial macrophages (IM), and monocyte-derived macrophages (MdM) as it is known that following pulmonary infection, inflammatory monocytes are recruited to the lung where they can differentiate into monocyte-derived macrophages (MdM) or interstitial macrophages (IM)[108]. To this end, mice were RM vaccinated with AdHu5Ag85A

(i.n.) for 4 weeks, or a control group was left unvaccinated (Naïve). Mice were then infected with *M.tb*H37Rv and sacrificed at 0, 1, 3, or 7 dpi (**Figure 6A**). Bronchoalveolar lavage was used to sample AW mononuclear cells and flow cytometry was performed to analyze changes in the three major macrophage populations using the gating strategy outlined in Supplemental figure 1. At 0 dpi (before infection), RM vaccinated mice (**i.n.**) had much smaller frequencies of AM within the AW due to the presence of other leukocyte subsets, compared to unvaccinated mice (**Figure 6B/C**). However, the IM, and MdM populations expanded more in RM vaccinated mice relative to naïve mice in the days shortly after *M.tb* infection, as more Ly6C$^+$ inflammatory monocytes and cells of their lineage were recruited to the AW (**Figure 6B/D/E**). At 7 dpi, these subsets of macrophages increased further in the AW of RM vaccinated mice.

We next assessed changes in macrophage recruitment in the lung tissue of these mice at the same time points. Mice were set up as per Figure 6 (**Figure 7A**). Similar to the profile seen in the AW, RM vaccinated lung tissue had smaller frequencies of AM throughout compared to unvaccinated controls (**Figure 7B/C**). However, the frequencies of IM and MdM did not appear to differ as much in the lung tissue as in the AW following *M.tb* infection between RM vaccinated and control groups (**Figure 7B/D/E**).

Since neutrophils are a canonical inflammatory cell readily recruited to the lung and AW following pulmonary *M.tb* infection[109], we also examined neutrophil responses both in the airway and lung tissue. Using a simplified flow cytometry gating strategy (**Supplemental figure 2**), we observed a basal neutrophil population in the AW of RM vaccinated mice (**i.n.**) at steady-state (**0 dpi**) that was absent in naive mice (**Supplemental figure 3A**). The frequency of this population in

the AW of RM vaccinated mice rapidly peaked at 1 dpi, and decreased again to basal levels by 7 dpi (**Supplemental figure 3B)**. This occurred much more rapidly than in the AW of mice that were not vaccinated, as neutrophil influx was delayed until 7 dpi in naïve mice (**Supplemental figure 3B**). Unlike in the AW, overall frequencies were consistent in the lung tissue of both groups throughout the infection (**Supplemental figure 3C/D**). The above findings together suggest that memory AM induced by RM vaccination lead to differential innate immune cellular responses in early stages of pulmonary *M.tb* infection.

3.5 - Airway macrophages of RM vaccinated animals remain highly activated in early phases of pulmonary *M.tb* infection

We have observed high MHC II expression being the hallmark of RM vaccine-induced memory AM before *M.tb* infection (**Figure 4B/C**). Upon infection, there was an increased IM population in the airway (**Figure 6D**). The IM may ultimately differentiate to be AM. The question remained whether the MHC II^{high} memory macrophage phenotype might sustain during *M.tb* infection. This is a particularly relevant question in the scope of virulent *M.tb* infection, as it is very immunosuppressive and may revert memory AM to a naïve or even a tolerized state. Thus, at 0, 1, 3, and 7 dpi we determined the MFI of MHC II on both AM and IM populations in the airway. We observed that memory AM induced by RM vaccination remained highly activated in the AW after *M.tb* infection, as the MFI of MHC II is significantly higher than that on naïve AM (**Figure 8A**). Furthermore, IM from RM vaccinated mice also expressed significantly higher MHC II levels than naïve IM both prior to and after *M.tb* infection (**Figure 8B**). These data suggest that the airway macrophages in RM vaccinated animals are able to remain to be in a highly activated state in early stages of pulmonary *M.tb* infection.

3.6 – Memory alveolar macrophages lead to enhanced control of *M.tb* in the airway during early stages of pulmonary *M.tb* infection

It has recently been shown that all of the *M.tb* bacilli reside within AM of the respiratory tract in the first 7-10 days of infection[10]. To investigate the direct role of memory AM in enhanced innate immune protection during early stages of pulmonary *M.tb* infection, we developed an *in vivo* airway luminal killing assay. Mice were either RM vaccinated or left un-vaccinated as naïve controls, and infected for 1, 3, or 7 dpi with virulent *M.tb* (**Figure 9A**). The whole lung and BAL were collected. The lung was homogenized and plated to determine overall pulmonary bacterial burden. Bronchoalveolar lavage cells were separated from the lavage fluid via centrifugation and then lysed. Cells recovered from the BAL were plated separately from the BAL fluid (BALF) to distinguish between free bacilli and intracellular burden in the AW. We found that there were significantly fewer free bacilli in the BALF of RM vaccinated mice at 7 dpi compared to the BALF of naïve mice (**Figure 9B**). Of importance, while at 1 dpi the AM (BAL cells) from both RM vaccinated and naïve animals contained similar numbers of *M.tb* bacilli (**Figure 9C**), the cells in RM vaccinated animals had a marked reduction in bacterial burden both at 3 and 7 dpi relative to naïve counterparts (**Figure 9C**). In comparison, bacterial burdens in lung tissues of both RM vaccinated and naïve animals at 1 and 3 dpi were similar, whereas they were significantly lower in the lung of RM vaccinated group at 7 dpi (**Figure 9D**).

Since the increased control of *M.tb* in infected AM shown in the above airway luminal system occurred in the presence of other immune cells in the respiratory tract, we next undertook an *ex vivo M.tb* killing assay. Thus, mice were either RM vaccinated for 4 weeks or left un-vaccinated as a naïve control. Harvested total BAL cells were subjected to CD3 column purification to remove T cells. The remaining macrophages were plated, and infected *ex vivo* with *M.tb* and

cultured for 4, 24, or 48 hrs (**Figure 10A**). Infected cells were then lysed and plated for CFU enumeration. At 4 hrs post-infection, the numbers of bacilli in AM were similar between RM vaccinated and naïve animals (**Figure 10B**), indicating similar infection rates in the both. Although no significant differences were observed at 24 hrs post-infection, the AM from RM vaccinated mice had significantly reduced *M.tb* burden relative to naïve macrophages (**Figure 10B**). The above data together indicate that RM vaccination-induced memory alveolar macrophages play an important role in effectively controlling *M.tb* replication in early stages of pulmonary infection.

3.7 – MCP-1 is not involved in memory alveolar macrophage-mediated innate immunity in early stages of pulmonary *M.tb* infection

We have seen increased IM and MdM in the airway of RM vaccinated animals (**Figure 6B/C**) and mycobacterial Ag-stimulated memory AM produced much more MCP-1 (**Figure 5D**), a chemokine with a major role in monocyte recruitment to the lung[106]. In an attempt to examine the potential molecular mechanisms underlying memory AM-mediated trained innate immunity, we next determined the role of MCP-1. Mice were RM vaccinated for 4 weeks or left un-vaccinated as a naïve control, and just prior to infection, MCP-1 was systemically neutralized *in vivo* using rabbit anti-sera (**Figure 11A**)[106]. We utilized our *in vivo* airway killing assay and separately analyzed mycobacterial burden in BALF, AW cells and lung tissue (**Figure 9**). Consistent with the 7 dpi data presented in Figure 9, compared to naïve controls, RM vaccinated animals had significantly lower numbers of bacilli in BALF, BAL cells, and lung tissue (**Figure 11B-D**). However, MCP-1 depletion failed to abrogate enhanced protection seen in all of these compartments (**Figure 11B-D**). These data suggest that MCP-1 or MCP-1-mediated recruitment of monocyte-derived macrophages to the airway does not play a role in memory AM-mediated trained innate immunity against pulmonary *M.tb* infection.

4.0 – Discussion

Mycobacterium tuberculosis continues to ravage human populations as it has done for millennia. In 2017 alone, there were over 10 million newly diagnosed cases of TB, and 1.3 million deaths, not including individuals coinfected with HIV. Altogether, TB is the leading cause of death from a single infectious agent worldwide. The WHO has identified TB as a priority target to relieve stress on the global healthcare system, and is aiming to curb much of the epidemic by 2035[1]. However, progress thus far has been agonizingly inefficient. Several approaches must be taken to combat global burden including the development of novel therapeutics, shortening the length of current pharmaceutical regimens, discovering novel biomarkers/diagnostic tools, and developing new vaccines. Existing pharmaceutical interventions consist of a multi-drug cocktail, and can last up to two years in duration. As one may expect from a therapy this intensive, many patients inevitably default due to inaccessibility to healthcare or severe adverse events. Ultimately incomplete courses of pharmacotherapy lead to disease relapse contributing to the emergence and inevitable spread of drug-resistant TB. The recent rollout of novel anti-bacterial drugs such as linezolid and bedaquiline are expected to have some impact on those currently infected, but resistant strains have already been isolated. Despite the efficacy of anti-tuberculosis drugs on disease cure rates, prescribing the correct drugs to infected individuals is heavily dependent on their diagnosis. Modern diagnostic methodologies can be sensitive and accurate, but some of the most widely implemented ones have major flaws in their applications. For example, sputum culture, microscopy, and polymerase-chain reaction technologies like GenXpert can all effectively diagnose *M.tb* but rely on the availability of free bacilli and thus are inappropriate to diagnose latent, asymptomatic disease. On the other hand, immunologic diagnosis based on TST or IGRA fail to distinguish between *M.tb* and other non-tuberculous mycobacteria, or non-IFN-γ based responders to infection, respectively[42,110,111]. Since diagnostic and pharmacotherapeutic strategies

remain thoroughly flawed, development of an efficacious prophylactic vaccine is expected to have an enormous impact on disease burden.

Current prophylactic TB vaccination involves intradermal administration of BCG shortly after birth. BCG initiates a self-limiting infection and generates adaptive immune responses against an array of mycobacterial antigens. This immunity generates protective systemic responses against disseminated TB, while its efficacy within the lung remains highly questionable, hence the current pulmonary TB epidemic. Systemic effects can likely be attributed to the ease at which a live bacterium may access the bloodstream and generate global T cell responses as it circulates through the tissues. An added effect is its ability to impact bone marrow progenitors, shifting them towards myelopoiesis and training circulating monocytes, although this has only thus far been proven in mice. Recently, Delahaye *et al*. have shown in mice that parenteral BCG vaccination draws T cells to the lung parenchyma, but why these lung T cells remain non-protective against pulmonary TB remains to be elucidated[36]. BCG does not induce immunity drastically different to the non-sterilizing immunity generated by primary *M.tb* infection, and its efficacy may be even further reduced by not eliciting said immunity in the immunologically restricted lung mucosa. Therefore, there are several areas in the existing BCG vaccination platform that can be improved upon with novel vaccination methods focused on generating "un-natural immunity".

Natural immunity generated against TB is generally effective at controlling the disease and thus a majority of infected individuals will only ever become latently infected. Once inhaled into the lung, *M.tb* has evolved to dictate the events that follow in a manner that allows it to establish an infectious foothold that will be nearly impossible to eliminate with natural immunity. Due to a number of mechanisms, both indirect (slow replication time of mycobacteria/ability to become

non-replicating, general resistance to reactive oxygen and nitrogen intermediates), and direct (ESX-1-dependent phagolysosomal escape, restriction of antigen presentation via MHC, delayed migration of infected DC to the MLN, and secretion of IL-10 from infected DC), *M.tb* thwarts adaptive immunity at every turn. It is based on these observations, as well as the increased mortality seen in HIV-positive individuals correlating with loss of $CD4^+$ T cells that have turned researchers towards using generation of potent Th1 immunity as a measure of vaccine efficacy. This paradigm has been the central focus of vaccine development thus far, but may have unintentionally led the field astray[99]. An elegant analogy was used by Dr. D.G. Russell in a recent review, where he states: "If you take a wheel off a car, it does not work very well. However, there is no guarantee that adding a fifth wheel to the car is going to improve its performance"[43]. This speaks to the lack of efficacy seen in one of the most highly-touted TB vaccines in the pipeline, MVA85A. Despite generating potent IFN-γ responses in a gauntlet of pre-clinical animal models, MVA85A failed to lead to increased protection against TB disease in several clinical trials[40,41]. Viral-vectored vaccines are proving to be one of the most effective ways of generating vaccine antigen-specific responses, particularly by generating large quantities of polyfunctional $CD4^+$ and $CD8^+$ T cells. But the choice of viral vector has proven to be just as important as the antigens selected. For example, both vesicular stomatitis virus (VSV) and AdHu35 are genetically malleable and proven to be safe to administer in humans, but both also generate large levels of type I IFN, and subsequently IL-10, dampening the innate system despite potent adaptive immune responses against vaccine antigens[104]. Slight modifications to the viral backbone such as those between serotypes, namely switching the backbone from an AdHu35-based vaccine to one based on AdHu5 drastically reduces the amount of type I IFN, and thus maintains an intact innate response. But viral-vectored vaccines that are naturally tropic to humans, face the threat of loss of infectivity due to cross-protective pre-existing immunity. Analogous to how BCG is less effective at equatorial latitudes where exposure

to environmental mycobacteria is more common, in areas like sub-Saharan Africa the cross-reactive antibodies against the AdHu5 backbone entirely abrogate any protective effects that would be seen in vaccinated individuals. Thus further decisions must be made when it comes to vector selection. An example is generating viral-vectored vaccines using viruses such as chimpanzee adenovirus serotype 68 (AdCh68) that are not naturally tropic to humans and do not generate type I IFN responses. Both AdHu5Ag85A and AdCh68Ag85A have undergone extensive bouts of pre-clinical testing for safety and immunogenicity, and have recently been expanded to incorporate more mycobacterial antigens into their genome to encompass a greater breath of the mycobacterial lifecycle. All of these advantages in viral-vectored vaccines are exploited to improve the current TB vaccine, BCG which fails to generate potent Th1 and $CD8^+$ T cell immunity in the lung after intradermal vaccination.

One way to modulate the location of protective immune responses is to alter the route of vaccine administration[60]. Currently, BCG is the only clinically approved TB vaccine, and it is given intradermally to infants shortly after birth. Parenteral BCG generates systemic immunity against mycobacteria outside of the lung, but the efficacy within the lung itself is questionable. This is likely another contributing mechanism as to why BCG fails to protect against pulmonary TB. Thus, an effective vaccine strategy needs to take into consideration both using which vector to deliver the antigen and via which route a vaccine is given. Shifting away from parenteral routes to more targeted platforms such as aerosolization will now draw adaptive immunity out of the vasculature and into restricted lung mucosal tissues. This allows us to subvert a mechanism of immune evasion utilized by virulent *M.tb*, namely the delayed return of Th1 cells to the lung parenchyma after priming that parenteral BCG fails to enhance. The adenoviral-vectored vaccine AdHu5Ag85A was designed to be given to the RM route and has been shown to be safe and immunogenic in all pre-

clinical evaluations. The concept of RM vaccination is that by causing a transient inflammatory reaction in inherently anti-inflammatory tissues such as the alveolar space, infiltrating immune cells such as T cells will readily enter and eventually take up residence upon contraction. This poises vaccine-derived immunity at the immunological battleground, ready to immediately respond upon exposure to a pathogen, in this case *M.tb*. Aerosolized AdHu5Ag85A vaccine is being delivered to the respiratory tract of healthy BCG-vaccinated human volunteers with demonstrated safety and immunogenicity in an ongoing clinical trial.

Thus far, it has been demonstrated that by utilizing novel vaccine platforms, we can generate potent anti-TB immunity relative to BCG. By altering the method of antigen delivery to viral-vectored vaccines, we can generate potent adaptive immune responses as well as avoid the immune dampening effects from exposure to non-tuberculous environmental mycobacteria. Furthermore, by utilizing RM vaccination, we are now able to draw these adaptive immune cells to the immunologically restricted AW. Vaccine design has centred around the generation of antigen-specific adaptive immune responses to measure efficacy. Recently, the non-specific protective innate effects after vaccination with live-attenuated or viral-vectored vaccines have come to light. It is estimated that between 20-25% of individuals exposed to *M.tb* rapidly clear the infection without the induction of adaptive immunity as measured by TST/IGRA[67,68]. This suggests that the innate immune system is capable of dealing with *M.tb* infection and opens a new avenue of approach when it comes to TB vaccine design. It is well documented that upon infection with virulent *M.tb*, AW phagocytes are actively suppressed[21,22]. This is the first event in a cascade orchestrated by *M.tb* to delay the onset of adaptive immunity and establish an infectious foothold within the lung. One way to overcome the initial immune suppression is to imprint AW macrophages prophylactically to enhance their response upon *M.tb* exposure. In systemic BCG

vaccination in humans, BCG has been found to train circulating monocytes and their progenitors to such an extent that BCG-vaccinated individuals experience protection against heterologous pathogens[80,84]. As monocytes are short-lived, it is essential that monocyte progenitors in the bone marrow are also imprinted as is the case with BCG. The epigenetic changes in these innate immune cells are long-lasting and persist in humans for at least several months post-vaccination. But it is likely that RM vaccination will be unable to affect bone marrow progenitors, and thus imprinting circulating monocytes may not be effective. In this regard, we may take advantage of the property of innate immune cell populations native to the lung mucosa. AM are fetal-liver derived, long-lived, and proliferate independent of monocyte-derived populations in the steady state. Such evidence indicates that lung macrophages may be trained through RM vaccination to enhance T cell-independent mycobacterial killing, and that this phenotype may persist. Trained memory alveolar macrophages have already been demonstrated in our model of RM AdHu5Ag85A vaccination[69]. Through a series of elegant experiments, Yao *et al.* proved that after RM adenoviral infection, $CD8^+$ T cell entry to the AW, resident macrophage populations acquired a defence-ready gene signature, increased MHC II expression and increased glycolytic metabolism, and could readily secrete neutrophil chemokines upon heterologous bacterial challenge[69]. Importantly, this mechanism of generating memory AM was independent of the *M.tb* antigen expressed in this vaccine, and instead was dependent on the self-limiting infection in the lung after RM vaccination. This proved to be effective against challenge with *S. pneumoniae*, but it remains unknown if these memory AW macrophages or trained innate immunity can better control *M.tb*, and re-shape the early responses to infection. Our current study set out to address this question.

We began by verifying that our model of TII was consistent with that previously documented. Indeed, we found that AM expressed increased MHC II and had increased glycolysis,

and were able to secrete more IL-12p40 upon re-stimulation. Importantly, these indicators of innate immune memory were wholly dependent on the route of vaccination, as parenteral AdHu5Ag85A vaccination failed to change the AW macrophage phenotype. This is in accordance with the $CD8^+$ T cell-dependent training of AM previously described in this model as parenteral vaccination does not draw antigen-specific $CD8^+$ T cells to the AW[69]. Although such T cells are involved in the jump start of induction of memory AM phenotype, we found that these T cells were disposable to restricting early mycobacterial growth within the first 2 weeks following infection. This suggests that RM vaccination induces important changes both in innate macrophages and adaptive T cells with the AW and lung tissue, which all play a role in host defense against pulmonary *M.tb* infection.

Through flow cytometric analysis, we carefully dissected the myeloid landscape of both AW and lung macrophages after RM vaccination. RM AdHu5Ag85A vaccination established a resident population of IM in the AW and lung. This is the first characterized generation of a distinct population of resident macrophages in restricted lung mucosa. By altering the innate profile of the lung prior to *M.tb* infection, there are a variety of phagocytes that may become infected and thus may lead to different infection outcomes. Pathogenesis of TB is associated with the influx of myeloid cells into the infected lung, but inconsistencies in naming and definitions (e.g. by flow cytometric markers) have complicated what is considered protective, and what is considered harmful. Monocytes and other monocyte-derived macrophage subsets like MdM and IM that are $Ly6C^+$ and $CD11b^-$ are associated with early protection, but may be detrimental as infection persists. Recruitment of $Ly6C^+$ monocytes is in a CCR2-MCP-1 dependent manner and provides fresh host cells in which *M.tb* may proliferate and disseminate. Recent evidence suggests that $Ly6C^+$, $CD11b^+$, Siglec-F^-, MHC II^{HI} IM are recruited to the murine lung in the first 2 weeks after *M.tb* infection[11]. These recruited IM were monocyte-derived, expressed more iNOS than resident

AM, and placed more stress on intracellular bacilli, preventing their replication[11]. In our study, we find that RM vaccination establishes a small IM population in the AW prior to infection, and thus the increased early decrease in bacterial burden may be due to eliminating the 2-week delay in monocyte-lineage macrophage recruitment. Importantly, this mechanism is also independent of T cells, reinforcing our protection phenotype. Establishing monocyte-derived memory macrophage populations in the AW also inherently decreases the proportion of highly susceptible AM. Respiratory mucosal vaccinated mice had accelerated recruitment of MdM after *M.tb* infection compared to naïve mice. By priming innate AW macrophages prior to *M.tb* infection, we potentially enhance their ability to recruit inflammatory monocytes despite early immune suppression. Infiltrating monocytes may downregulate Ly6C upon tissue entry and help sustain the resident IM population as it copes with bacterial insult. Indeed, we do believe the memory AM and IM populations in both the AW and lung tissue remain primed by RM vaccination after *M.tb* infection as they continue to express high levels of MHC II. Importantly, this proves that the phenotypic changes made to memory AW and lung macrophages are stable upon exposure to virulent *M.tb*, demonstrating that RM AdHu5Ag85A-induced TII is not easily abrogated by subsequent infection[103].

RM vaccinated mice showed a rapid, but transient neutrophilia that subsided by 3 dpi, just as the neutrophilic response was building in naïve mice. This could be indicative of neutrophils being rapidly drawn in to deal with the severe bacterial insult and contracting as infection is more rapidly controlled in RM vaccinated mice. The distinctive defence-ready state imparted on alveolar macrophages following RM AdHu5Ag85A vaccination may offer enhanced control of *M.tb* by mimicking the mechanism in Yao *et al*[69]. In this identical vaccination model, memory AM recruit a stronger and more rapid neutrophil response against heterologous bacterial challenge by secreting

more keratinocyte chemokine (KC) and macrophage inflammatory protein (MIP)-2[69]. Pulmonary neutrophil recruitment is traditionally thought of as detrimental, as their presence within the lung is associated with more severe disease, pulmonary damage, and a failed immune response[112,113]. However, during early infection they may serve a protective role through secretion of chemokines like IL-1β and TNF-α, antimicrobial peptides like the cathelicidin LL-37, or by generating their own reactive oxygen species through nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase and superoxides[114–117]. Neutrophils may also directly phagocytose *M.tb*, potentially through complement- or antibody-mediated mechanisms[118,119]. The extent to which neutrophils can control *M.tb* replication after uptake affects the cell death pathway of the cell similar to that in macrophages[67,120]. However, we can neither dismiss nor implicate their involvement in early anti-TB immunity as their functionality or bactericidal capabilities have yet to be assessed. This would be an interesting avenue of further research, as neutrophilia in response to increased KC and MIP-2 are the mechanism behind extracellular bacterial protection in this model of RM vaccination[69].

A majority of protective anti-TB immune responses characterized in the literature focus around Th1 cells and the cytokines they produce. Activation of the innate compartment by RM AdHu5Ag85A vaccination likely plays an important role in early reduction in bacterial burden against *M.tb* infection as it is shown to offer marked protection independent of vaccine-derived T cells. Thus we also sought to investigate the role of innate effector cytokines in memory macrophage-mediated protection against *M.tb*. *In vitro* stimulation of AW macrophages from RM vaccinated mice produced more IL-6, IL-1β, and MCP-1 than their naïve counterparts. However, we were unable to detect significant differences of any of these innate cytokines from *M.tb* infected lungs after RM vaccination. We have no evidence suggesting that any cytokines or chemokines in our extensive Luminex panel play any role in AW macrophage-mediated early control of *M.tb*. We

were unable to detect significant differences even when sampled from *in vitro* cultures with proven greater mycobacterial killing. This may suggest that these memory macrophages are able to better respond and handle *M.tb* without any of the cytokines in our panel. A majority of protective mechanisms against TB described thus far mainly involve Th1 cytokines as well as IL-12. However, in this novel model of memory macrophage generation, we may be looking at an unconventional mechanism of protection. This could be as simple as a cytokine not included in our panel (e.g. granulocyte-macrophage colony stimulating factor, GM-CSF), or it may be something more intrinsic within the macrophage such as increased anti-microbial peptide production, shifts in the prostaglandin-E_2/lipoxin-A_4 axis to modulate macrophage death, or metabolic restrictions[121,122]. Since TII has long been described to function via shifts in metabolic state towards a highly glycolytic effector metabolism, similar to that shared by both MdM and IM, this may limit the amount of free fatty acids and cholesterol available within the host cell needed for *M.tb* to replicate. To interrogate the role of metabolic changes in memory AM-mediated protection, the differences in metabolism such as the increased glycolysis seen in the seahorse assay could be targeted both *in vivo* and *in vitro* by adding a glycolysis inhibitor such as 2-deoxyglucose or fatty-acid oxidation inhibitor etomoxir similar to in Huang *et al*[11].

Finally, we observed that RM vaccination decreases not only total mycobacterial burden in the lung tissue, but also lowered the number of acellular bacilli recoverable from BAL fluids and within AW cells themselves. To our knowledge this is the first anyone has shown in a TB vaccination model that bacterial burden specifically within the AW can be lowered. This phenomenon held true even when MCP-1 was systemically depleted for the course of the infection. Therefore, MCP-1 does not play a role in the recruitment of bactericidal monocytes and MdM after *M.tb* infection or if it does, such recruited cells do not play a significant role in enhanced protection

within the AW. Since Ly6C^{+} inflammatory monocytes require CCR2/MCP-1 interactions to exit the vasculature and enter inflamed tissue, it is expected that it would indeed play a role. In order to further elucidate the distribution and potential killing of *M.tb* in AW macrophages, the use of a fluorescent reporter *M.tb* strain would be helpful. Fluorescent strains have been a tool refined over the past several years to include a replication-clock to indicate proliferation, a stress reporter expressed upon exposure to NO *in vivo*, or live-dead reporter strains[11,111].

In summary, we have identified a unique form of trained innate immunity, namely the establishment and priming of memory AW macrophages following RM AdHu5Ag85A vaccination. Importantly, these memory macrophages are able to control *M.tb* in the early stages of infection independent of T cells. We demonstrate a method by which innate cells within the AW that are the favourite host of *M.tb*, can be made less hospitable to bacterial replication. This priming within the AW has already proven to be protective upon subsequent challenge with heterologous extracellular bacteria, but now also has shown efficacy against TB. Our study reveals a potential mechanism of early innate clearance of *M.tb* bacilli generated by a respiratory mucosal vaccine strategy and provides the rationale for targeting resident innate immune populations within the lung as a strategy to enhance vaccine efficacy.

5.0 – References

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6.0 – Figures

Figure 1:

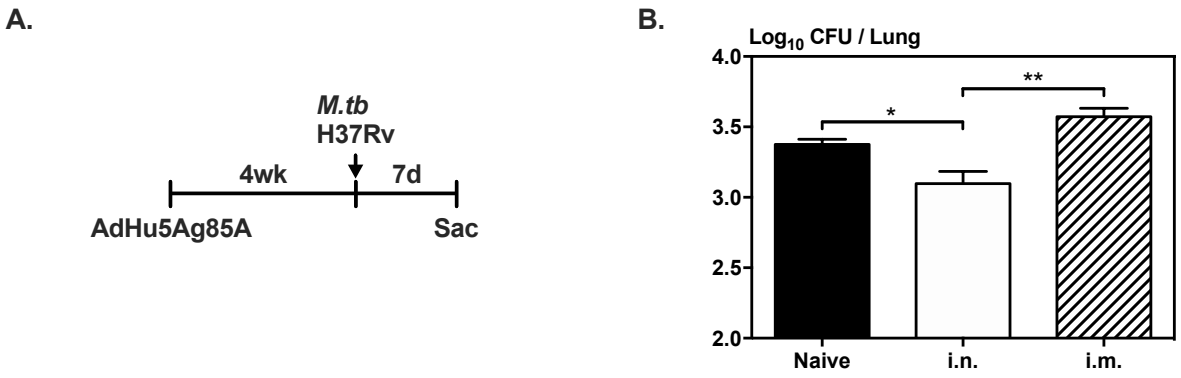


Figure 1: Respiratory mucosal, but not parenteral, vaccination with AdHu5Ag85A enhances protection in the early stages of pulmonary *M.tb* infection.

Figure 1: A) Experimental schema. Mice were immunized either i.n. or i.m. for 4 weeks, prior to *M.tb* infection. B) Bar graph depicting bacterial counts (CFU) in the lungs of naive, i.n., or i.m. immunized mice at 7 days post-*M.tb* infection. Data are expressed as mean±SEM of 5 mice/group from one experiment. * $P \leq 0.05$, ** $P \leq 0.01$, two-tailed unpaired Student's T test (95% CI).

Figure 2:

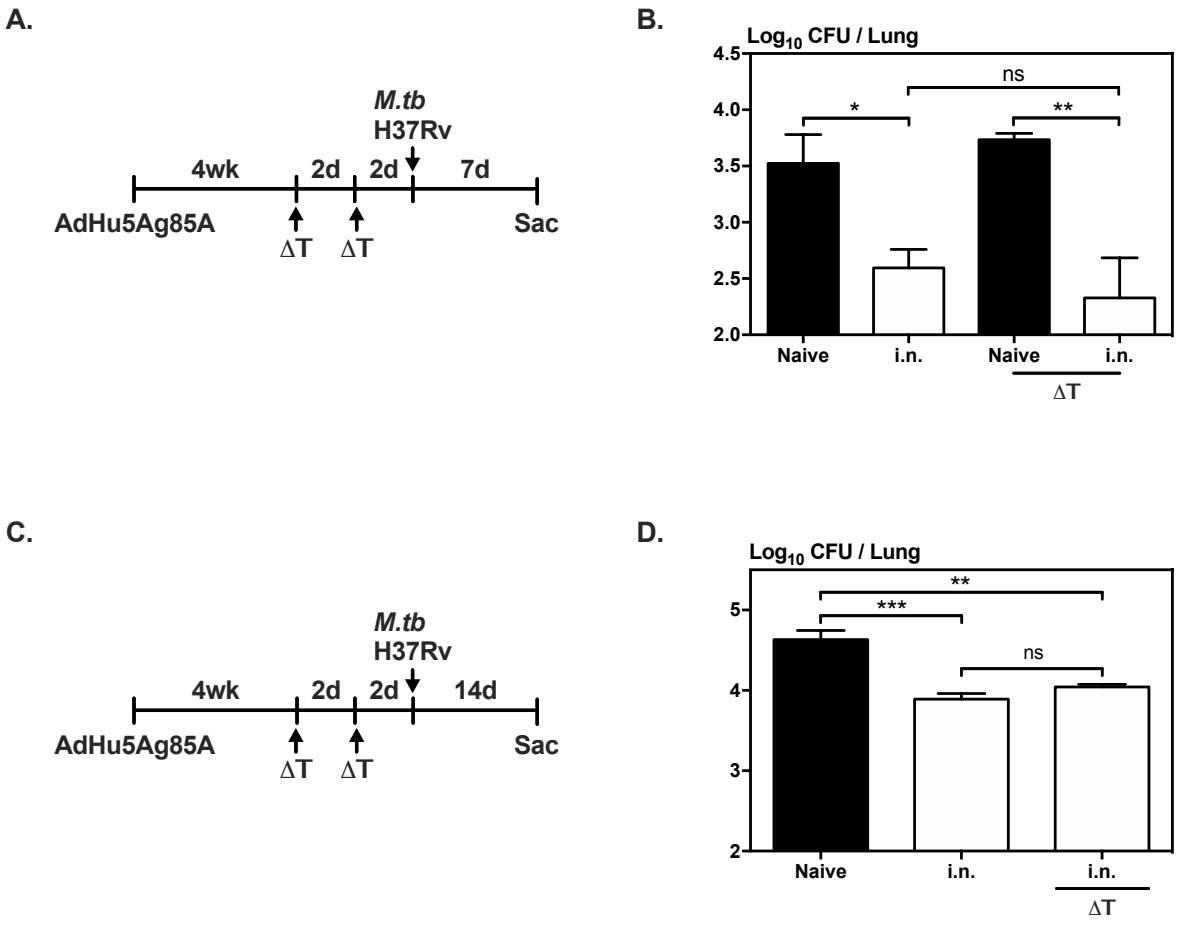


Figure 2: Respiratory mucosal vaccination with AdHu5Ag85A enhances protection in the early stages of pulmonary *M.tb* infection independent of T cells.

Figure 2: A) Experimental schema. Mice were immunized i.n. for 4 weeks before T cells were depleted (ΔT), and infected with *M.tb*. for 7 days. B) Bar graph depicting bacterial counts (colony-forming units, CFU) in the lung at 7 days post-*M.tb* infection from naive and i.n. immunized mice, with or without T cells. C) Experimental schema. Mice were immunized i.n. for 4 weeks before T cells were depleted, and infected with *M.tb*. for 14 days. D) Bar graph depicting bacterial counts (CFU) in the lung at 14 days post-*M.tb* infection from naive and i.n. immunized mice, with or without T cells. Data are

expressed as mean±SEM of 5 mice/group, representative of two independent experiments (B) or from one experiment (D). * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, two-tailed unpaired Student's T test (95% CI).

Figure 3:

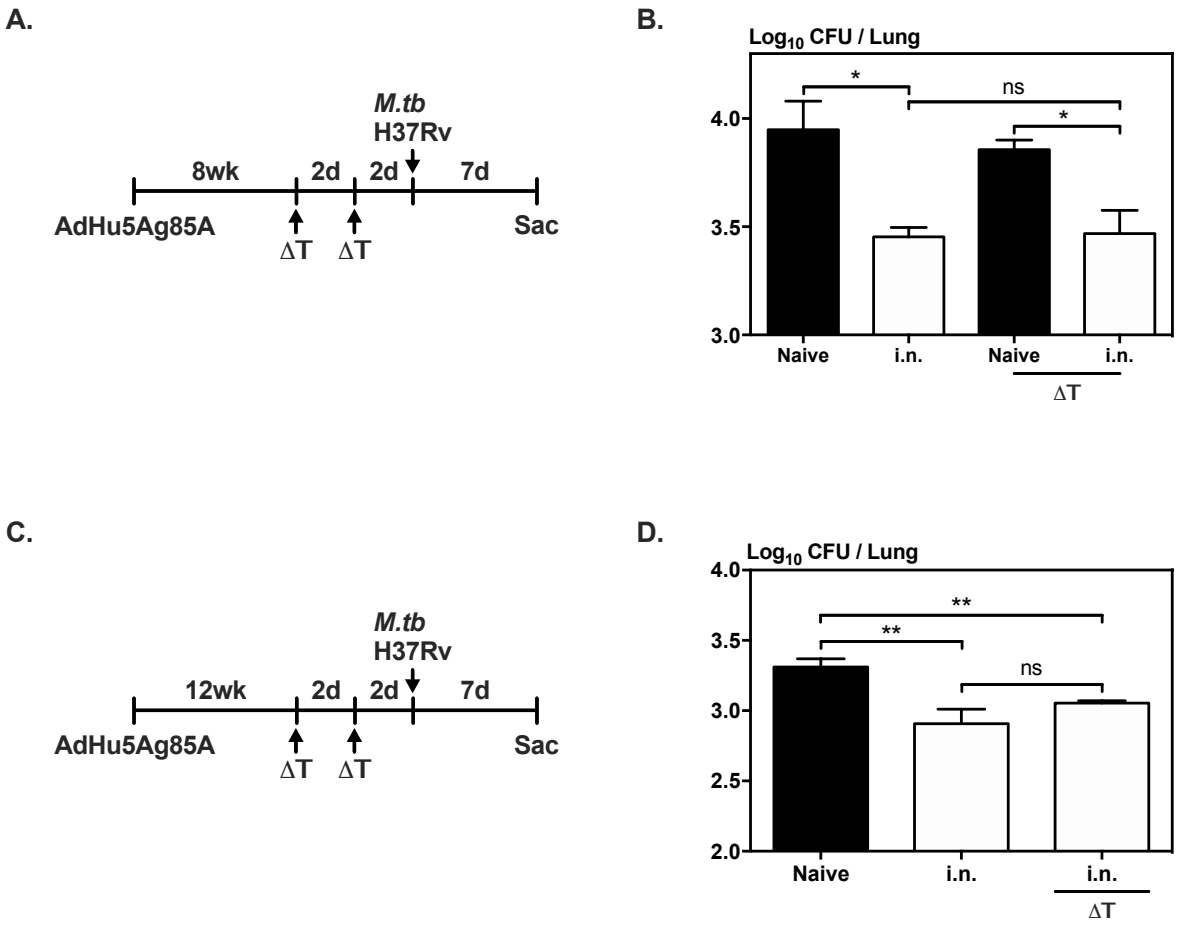


Figure 3: The lung remains protected long after respiratory mucosal AdHu5Ag85A vaccination in early stages of pulmonary *M.tb* infection independent of T cells.

Figure 3: A) Experimental schema. B) Bacterial counts (CFU) in the lungs of naive or 8 week i.n. vaccinated mice with or without T cells (ΔT) at 7 days post-*M.tb* infection. C) Experimental schema. D) Bacterial counts (CFU) in the lungs of naive or 12 week i.n. vaccinated mice with or without T cells at 1-week post-*M.tb* infection. Data are expressed as mean±SEM of 5 mice/group from one experiment. * $P \leq 0.05$, ** $P \leq 0.01$, two-tailed unpaired Student's T test (95% CI).

Figure 4:

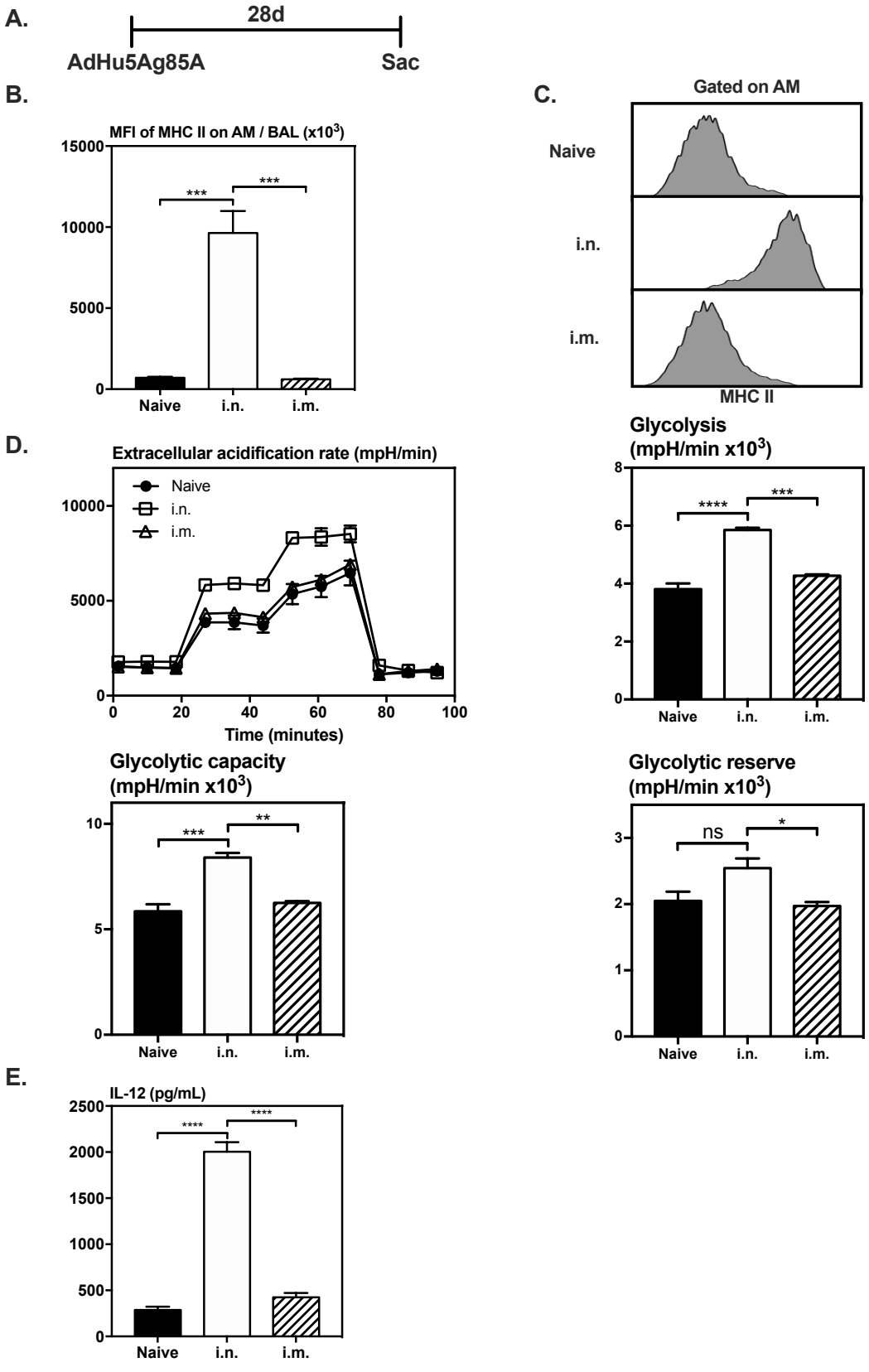

A.
28d
AdHu5Ag85A
Sac
B.
MFI of MHC II on AM / BAL (x10^3)
15000
10000
5000
0
***
***
Naive
i.n.
i.m.
C.
Gated on AM
Naive
i.n.
i.m.
MHC II
D.
Extracellular acidification rate (mpH/min)
Naive
i.n.
i.m.
10000
5000
0
0
20
40
60
80
100
Time (minutes)
Glycolysis
(mpH/min x10^3)
8
6
4
2
0
****
***
Naive
i.n.
i.m.
Glycolytic capacity
(mpH/min x10^3)
10
5
0
***
**
Naive
i.n.
i.m.
Glycolytic reserve
(mpH/min x10^3)
3
2
1
0
ns
*
Naive
i.n.
i.m.
E.
IL-12 (pg/mL)
2500
2000
1500
1000
500
0
****
****
Naive
i.n.
i.m.

Figure 4: Respiratory mucosal, but not parenteral, vaccination with AdHu5Ag85A induces memory alveolar macrophages.

Figure 4: A) Experimental schema. B) Bar graphs comparing median fluorescent intensity (MFI) of MHC II on alveolar macrophages from i.n. or i.m. vaccinated mice to naïve group. C) Histograms comparing major histocompatibility complex (MHC) II expression of alveolar macrophages at 4-weeks post-intranasal (i.n.) or intramuscular (i.m.) vaccination to naïve group. D) Real-time extracellular acidification rate (ECAR) of airway (AW) macrophages at 4 weeks post-vaccination (top left). Bar graphs showing glycolysis (top right), glycolytic capacity (bottom left), and glycolytic reserve (bottom right). E) Lung-derived $CD11c^+/CD11b^+$ cells from naive or i.n. vaccinated mice were infected *ex vivo* with *M.tb*. Bar graph showing IL-12p40 protein concentration in culture supernatants. Data are expressed as mean±SEM of 3 mice/group from one experiment. *** $P \leq 0.0005$, or **** $P \leq 0.0001$, two-tailed unpaired Student's T test (95% CI).

Figure 5:

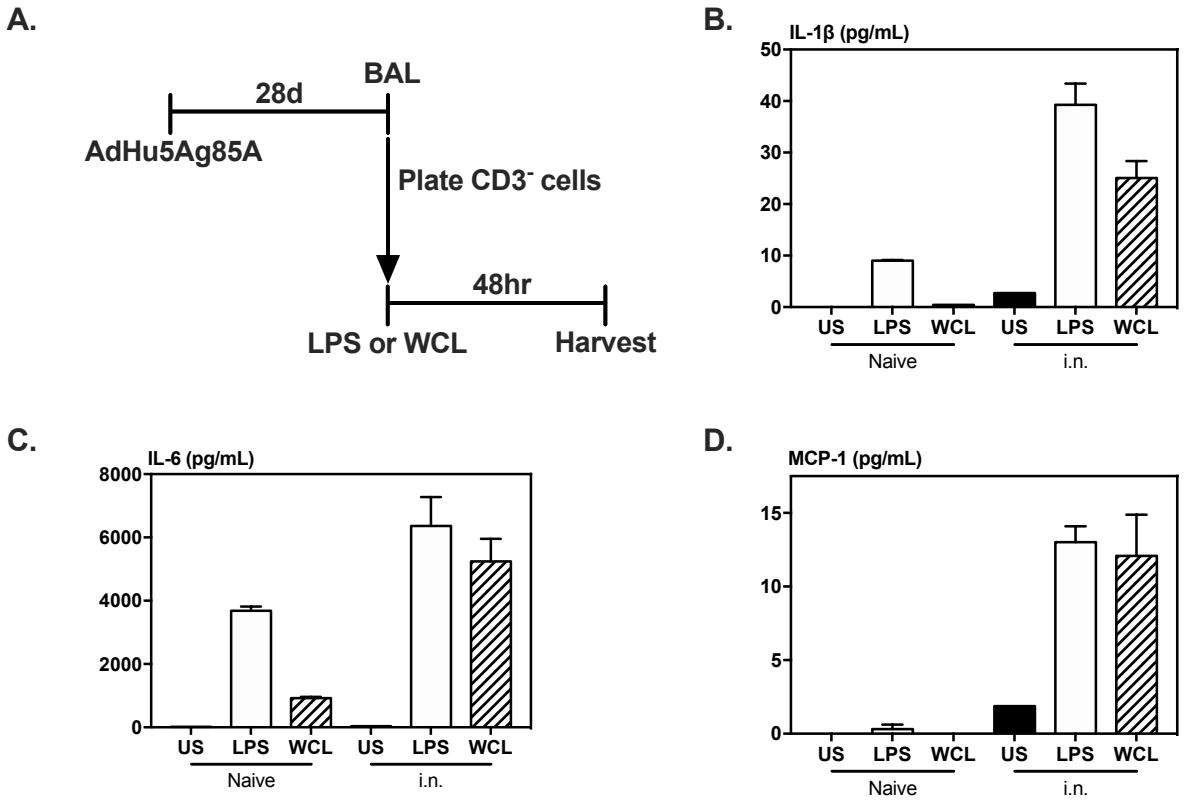


Figure 5: Memory alveolar macrophages have enhanced cytokine responses to stimulation by LPS or mycobacterial antigens.

Figure 5: A) Experimental schema. B) Bar graphs comparing the cytokine concentrations of IL-1β (B), IL-6 (C), or MCP-1 (D) in supernatants of $CD3^-$ bronchoalveolar lavage (BAL) cells after stimulation with lipopolysaccharide (LPS) or *M.tb* whole cell lysate (WCL) for 48 hours. Data are expressed as mean±SEM of 1-3 replicate wells/group from one experiment.

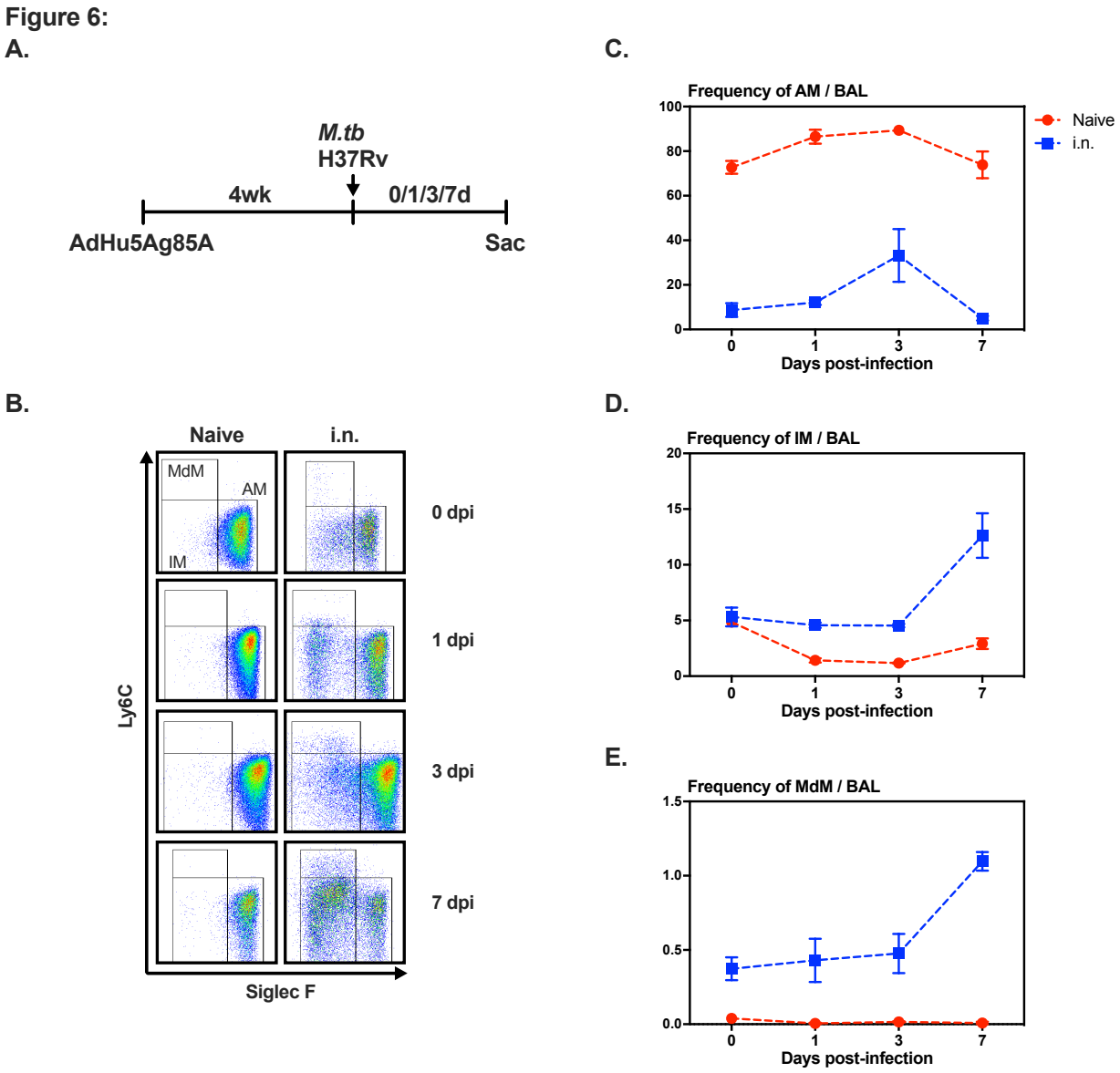


Figure 6: Respiratory mucosal vaccination leads to differential innate cellular responses in the airway to pulmonary *M.tb* infection.

Figure 6: A) Experimental schema. Mice were intranasally (i.n.) vaccinated for 4 weeks prior to *M.tb* infection and sacrificed at d0, d1, d3, and d7 post-infection. B) Representative dot plots of airway (AW) macrophage populations following *M.tb* infection in naive, and i.n. vaccinated mice. Line graph depicting frequencies of alveolar macrophages (AM) (C), interstitial macrophages (IM) (D), or

monocyte-derived (MdM) (E) in total live $CD45^+$ AW cells following *M.tb* infection in naive, and i.n. vaccinated mice. Data are expressed as mean±SEM of 3 mice/group from one experiment.

Figure 7:

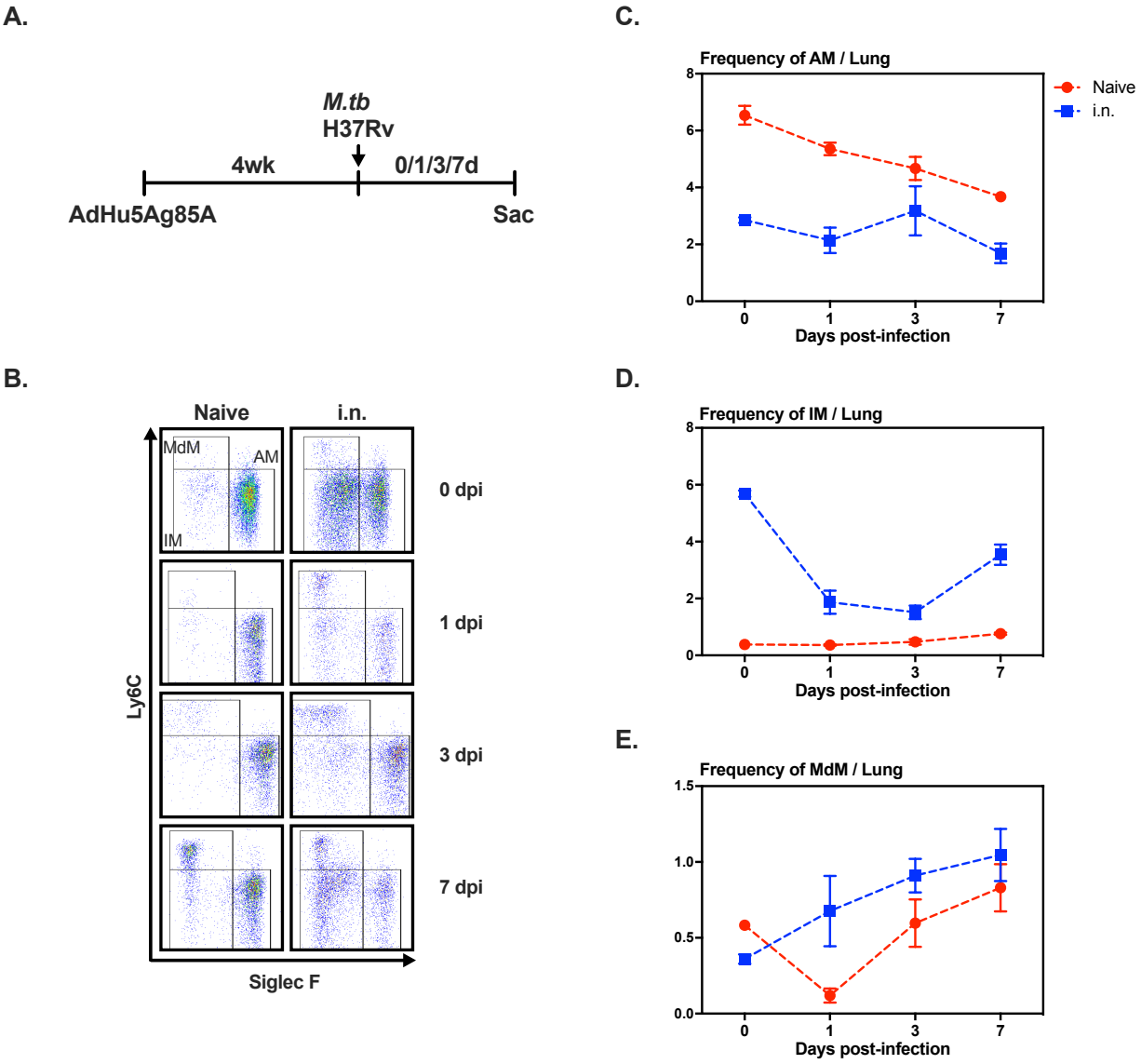


Figure 7: Respiratory mucosal vaccination leads to differential innate cellular responses in the lung tissue to pulmonary *M.tb* infection.

Figure 7: A) Experimental schema. Mice were intranasally (i.n.) vaccinated for 4 weeks prior to *M.tb* infection and sacrificed at d0, d1, d3, and d7 post-infection. B) Representative dot plots of lung macrophage populations following *M.tb* infection in naive, and i.n. vaccinated mice. Line graph depicting frequencies of alveolar macrophages (AM) (C), interstitial macrophages (IM) (D), or monocyte-derived macrophages (MdM) (E) in total live $CD45^{+}$ lung cells following *M.tb* infection in

naive, and i.n. vaccinated mice. Data are expressed as mean±SEM of 3 mice/group from one experiment.

Figure 8:

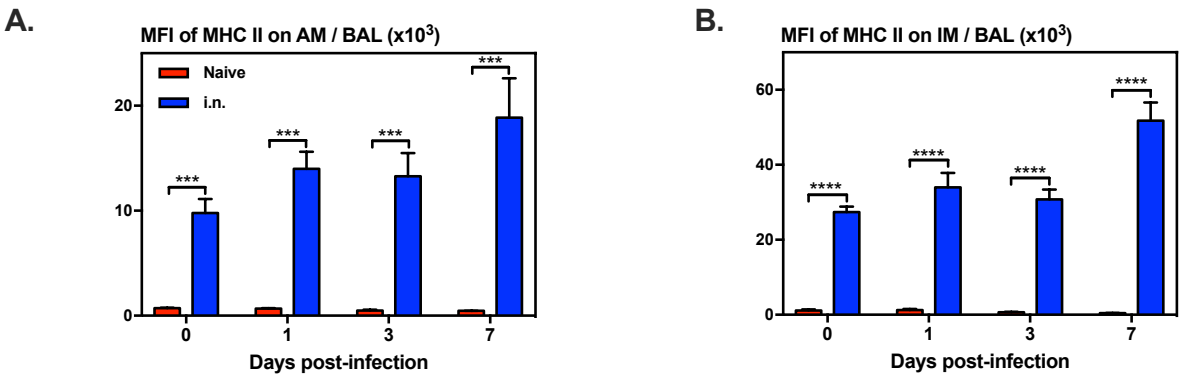


Figure 8: Airway macrophages of respiratory mucosal vaccinated animals remain highly activated in the early stages of pulmonary *M.tb* infection.

Figure 8: Mice were experimentally set up as per Figure 6A/7A. Bar graphs showing MHC II expression on airway alveolar macrophages (AM) (A) or interstitial macrophages (IM) (B) from naive, and i.n. vaccinated mice following *M.tb* infection. Data in are expressed as mean±SEM of median fluorescent intensity (MFI) of 3 mice/group from one experiment. ** $P \leq 0.005$, *** $P \leq 0.001$, **** $P \leq 0.0001$, two-tailed unpaired Student's T test (95% CI).

Figure 9:

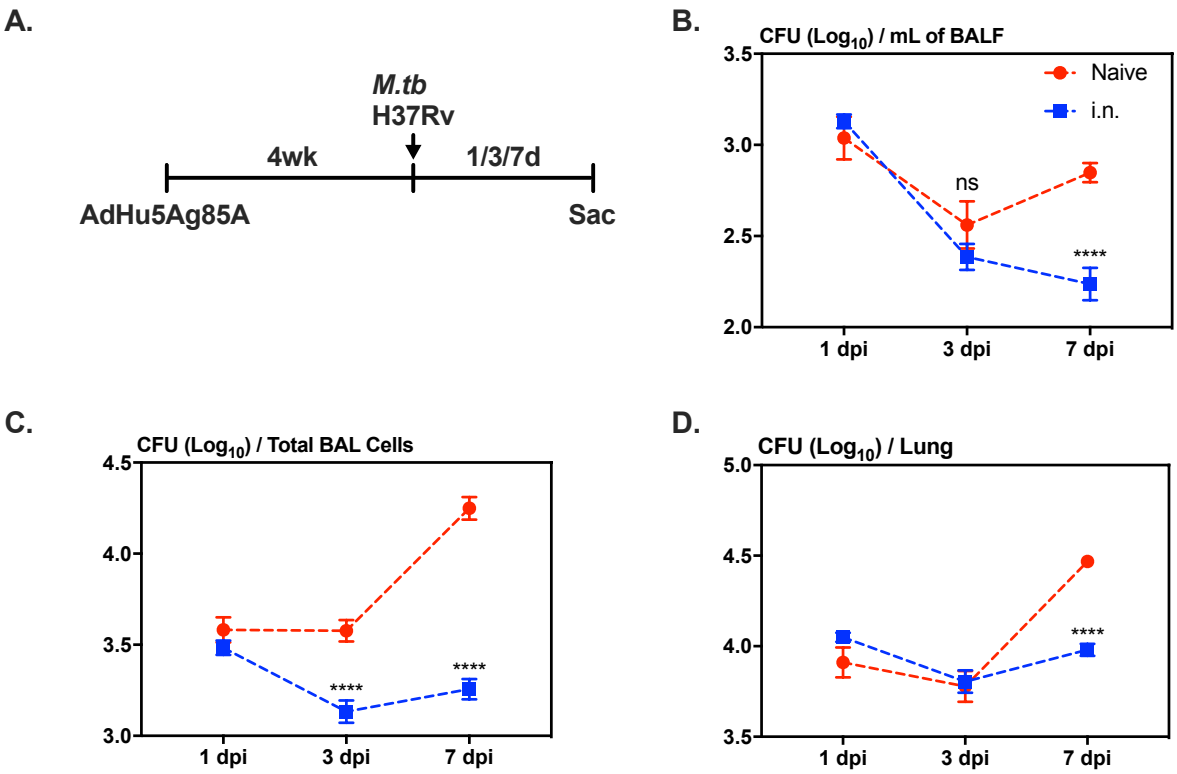


Figure 9: Memory alveolar macrophages lead to enhanced control of *M.tb* in the airway in the early stages of pulmonary infection.

Figure 9: A) Experimental schema. Mice were vaccinated i.n. for 4 weeks, prior to *M.tb* infection and sacrificed at d1, d3 and d7 post-infection. Line graph depicting bacterial counts (CFU) in the bronchoalveolar lavage fluid (BALF) (B), bronchoalveolar lavage cells (C) or lungs (D) of naive, or i.n. vaccinated mice at 1, 3, and 7 days post-*M.tb* infection. Data are expressed as mean±SEM of 5 mice/group from one experiment. * $P \leq 0.05$, ** $P \leq 0.01$, two-tailed unpaired Student's T test (95% CI).

Figure 10:

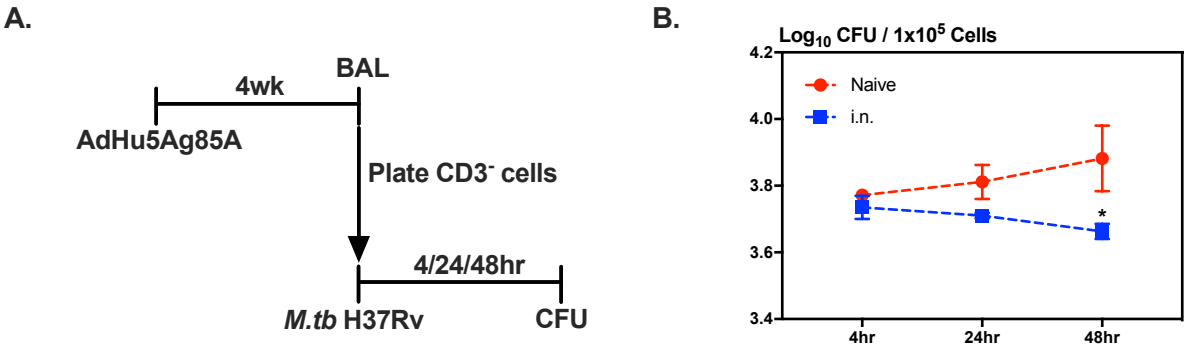


Figure 10: Memory alveolar macrophages enhance *M.tb* killing capacity in an *ex vivo* model.

Figure 10: A) Experimental schema. Mice were intranasally vaccinated for 4 weeks. Airway macrophages were collected by bronchoalveolar lavage and infected *ex vivo* with *M.tb.* B) Line graph depicting bacterial counts (CFU) after 4, 24, or 48 hours of infection. Data are expressed as mean±SEM of triplicate wells with the cells harvested from 5 mice/group from one experiment. * $P \leq 0.05$, two-tailed unpaired Student's T test (95% CI).

Figure 11:

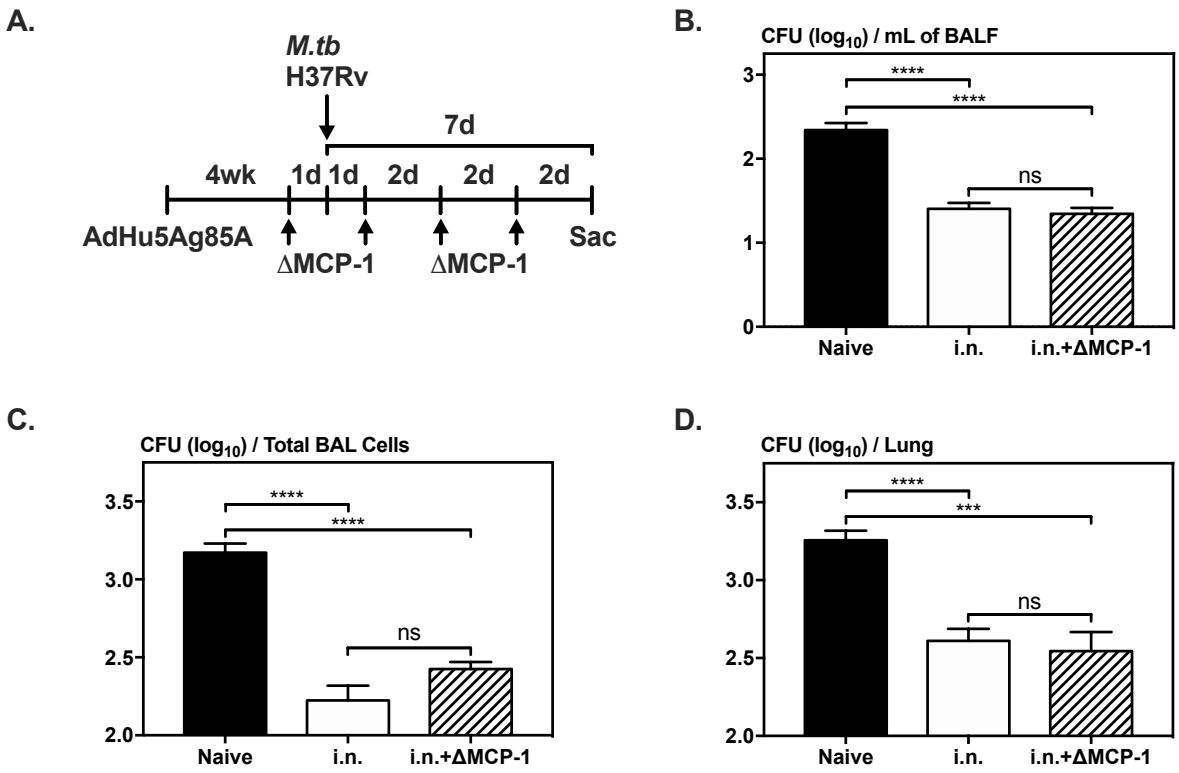
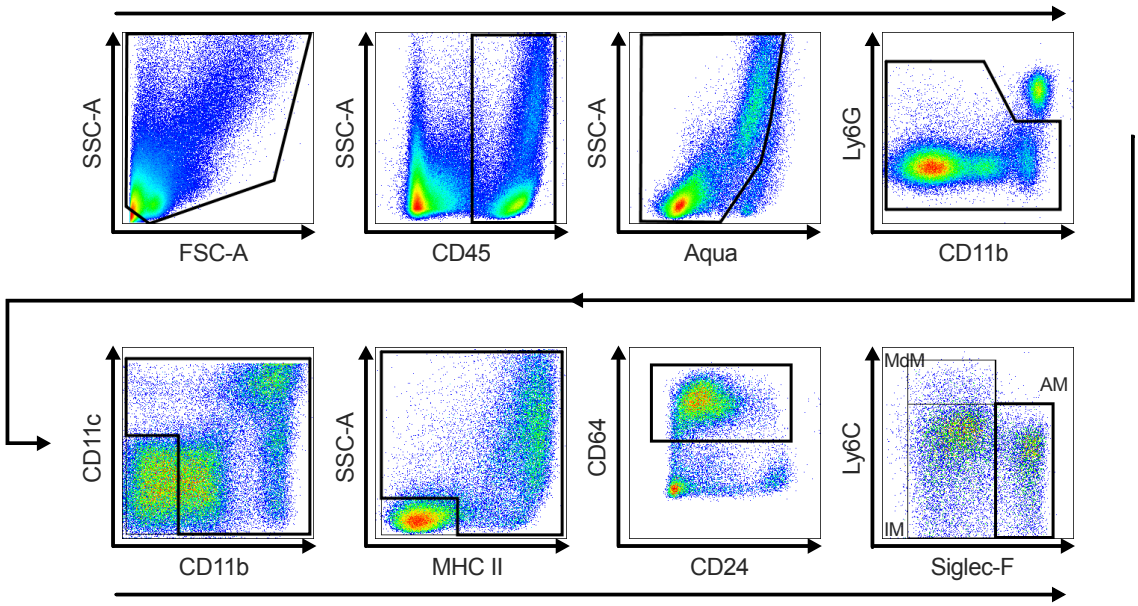


Figure 11: MCP-1 is not involved in memory alveolar macrophage-mediated innate immunity in early stages of pulmonary *M.tb* infection.

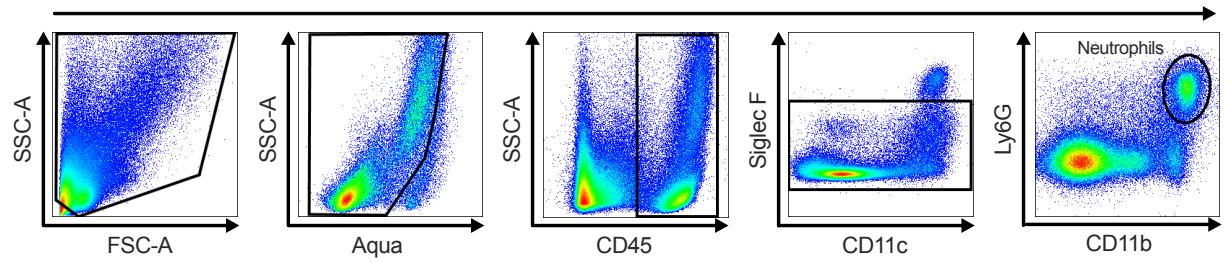
Figure 11: A) Experimental schema. Mice were intranasally vaccinated with AdHu5Ag85A for 4 weeks, at which point MCP-1 was systemically neutralized (ΔMCP-1) using rabbit sera prior to, and throughout infection. Bacterial burden was assessed in the bronchoalveolar lavage fluid (BALF) (B), bronchoalveolar lavage (BAL) cells (C), or lung homogenate (D) after 7 days post-infection. Data are expressed as mean±SEM of 5 mice/group from one experiment. **** $P \leq 0.0001$, two-tailed unpaired Student's T test (95% CI).

Supplemental figure 1:



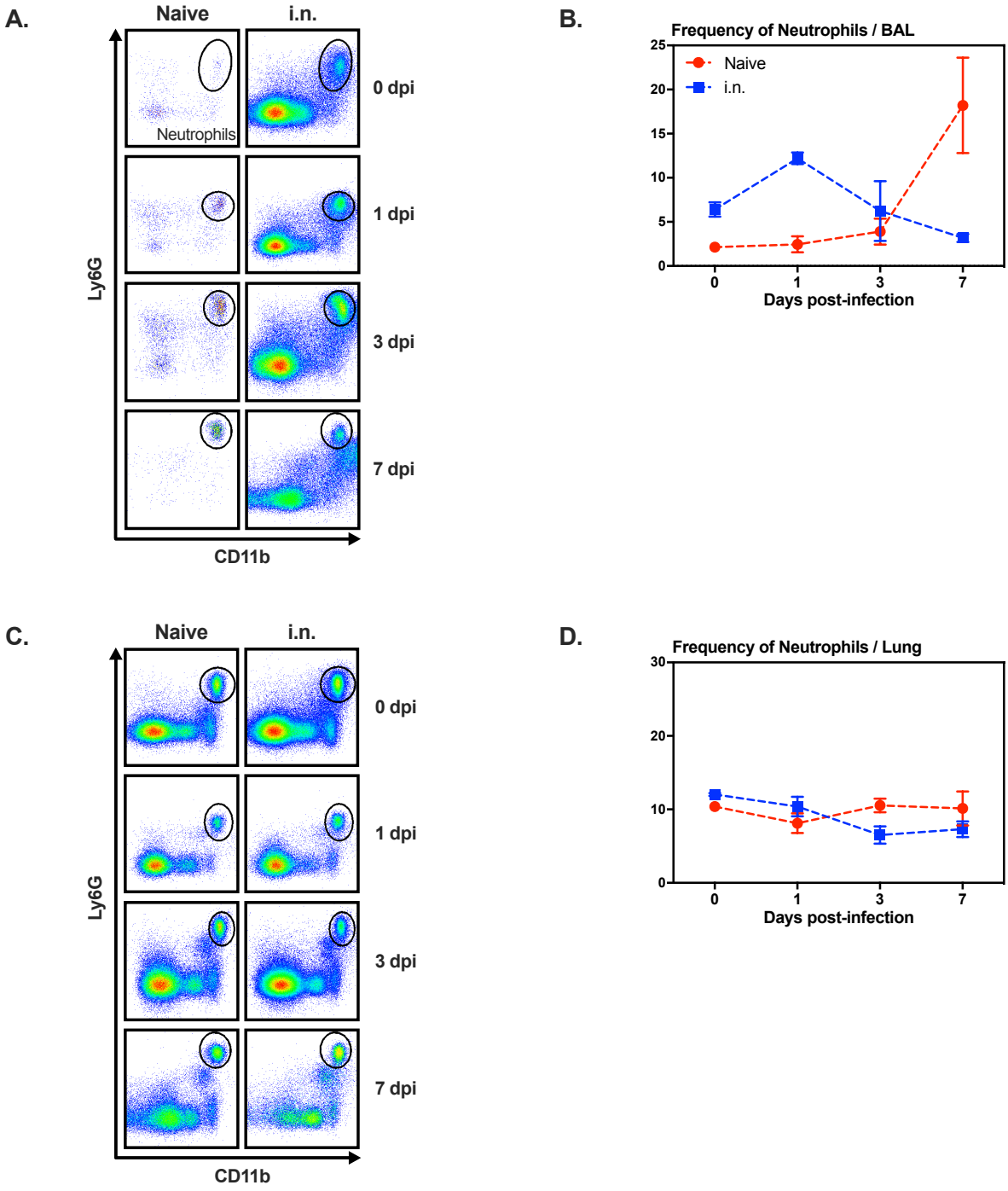
Supplemental figure 1: Gating strategy for discrimination of pulmonary macrophage populations. Example shown is the lung of a mouse at 4 weeks post-intranasal AdHu5Ag85A vaccination.

Supplemental figure 2:



Supplemental figure 2: Simplified gating strategy to identify neutrophils. Example shown is the lung of a mouse at 4 weeks post-intranasal AdHu5Ag85A vaccination.

Supplemental figure 3:



Supplemental figure 3: Memory alveolar macrophages accelerated neutrophilia in the airway during early pulmonary *M.tb* infection. Mice were experimentally set up as described in Figures 6A/7A.

Representative dot plots of neutrophils ($CD11b^+$, $Ly6G^+$) in the airway (AW) (A), and lung tissue (C), of mice following *M.tb* infection from naive, and i.n. vaccinated mice with or without T cells. Frequencies of live $CD45^+$ neutrophils in the airway (B) and lung (D). Data are expressed as mean±SEM of 3 mice/group from one experiment.