MUCOSAL AdHu5Ag85A VACCINE-INDUCED B CELL IMMUNITY AGAINST TUBERCULOSIS

THE ROLE OF B CELLS IN RESPIRATORY MUCOSAL AdHu5Ag85A VACCINE-INDUCED IMMUNITY AGAINST TUBERCULOSIS

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Descriptive Note

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Abstract

Background: *Mycobacterium tuberculosis* (*M.tb*) is the causative pathogen of tuberculosis (TB) that is currently the leading cause of death from a bacterial infection. The only licensed vaccine against tuberculosis is the Bacille Calmette-Guérin (BCG) vaccine which fails to effectively protect against adult pulmonary TB. For this reason, efforts have focused on developing novel vaccine platforms to boost BCG-induced immunity. In this regard, a human adenovirus serotype 5 expressing the mycobacterial secreted antigen Ag85A (AdHu5Ag85A) vaccine developed in our lab has been evaluated in a phase I clinical trial. It was shown to boost and elicit lasting T cell responses in BCG-vaccinated volunteers. Although inducing a strong type I T cell mediated immunity by this vaccine is crucial for protection, the role of vaccine-induced B cell responses in protecting against *M.tb* infection remains poorly understood. Recent studies have shown B cells to contribute to protection by modulating T cell immunity during intracellular bacterial infections. Therefore, we hypothesize that AdHu5Ag85A vaccination also induces antigen-specific B cell responses and such B cells have an immunomodulatory effect on vaccine-induced T cell immunity. **Methods**: We compared antigen specific T cell and antibody responses in wild type (WT) Balb/c mice with B cell deficient ($Jh^{-/-}$) mice after intranasal AdHu5Ag85A vaccination. **Results:** Interim data has demonstrated that AdHu5Ag85A induces antigen specific antibodies. In the absence of B cells, AdHu5Ag85A vaccination resulted in reduced Ag85A $CD8^+$ T cells within the BAL compared to WT. Importantly, this correlated with impaired protection against pulmonary *M.tb* infection. The study is ongoing to examine the mechanisms by which B cells modulate these T cell responses. **Conclusion:** These data suggest that B cells are required for optimal protective T cell responses following AdHu5Ag85A respiratory mucosal vaccination against *M.tb* infection.

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List of Abbreviations and Symbols

ACK	Ammonium-Chloride-Potassium
AdHu5Ag85A	Adenovirus Human Serotype 5 Antigen 85A
AM	Alveolar macrophage
APC	Antigen presenting cell
BAL	Bronchoaveolar lavage
BALT	Bronchus-associated lymphoid tissue
BCG	Bacille Calmette-Guérin
CAR	Coxsackie- and adenovirus receptor
CFP-10	Culture filtrate protein–10
CFU	Colony forming units
cRPMI	Complete RPMI
DC	Dendritic Cells
ELISA	Enzyme linked immunosorbant assay
ESAT-6	Early secretory antigenic target–6
FDC	Follicular dendritic cell
h	Hours
i.n.	Intranasal
i.t.	Intratracheal
i.v.	Intravenous
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin

Jh$^{-/-}$ KO	B cell knockout mice (*Igh-J^{tm1Dhu}* N?+N2)
LAM	lipoarabinomannan
LN	Lymph node
M.tb	*Mycobacterium tuberculosis*
MHC	Major histocompatibility complex
MOI	Multiplicity of infection
NO	Nitric oxide
ns	Not significant
ON	Ontario
PBS	Phosphate buffer solution
PFU	Plaque forming units
pIgR	Polymeric immunoglobulin receptor
PRR	Pattern recognition receptors
SEM	Standard error of mean
SUP	Supernatant
TB	Tuberculosis
Th	T helper
TLR	Toll like receptor
TNF	Tumour necrosis factor
Wt	Wild type
α-CD20	Anti-CD20 mouse antibody

1.0 CHAPTER ONE. INTRODUCTION

Mycobacterium tuberculosis is an ancient bacterium dating as far back as 3 million years [1]. Throughout this time, *M.tb* has evolved many mechanisms to evade the host's immune system [1]. Tuberculosis (TB) is a contagious disease and is contracted through aerosol droplets (from coughing or sneezing) of an infected individual [2]. Only 30% of the individuals exposed to *M.tb* become infected and of those 10% go on to develop the active disease [2]. This leaves 70% of exposed individuals (most of which reside in hyper-endemic regions) that immediately clear the infection by the innate immune system [2]. Today 8.6 million new cases of TB still arise each year, with approximately one third of the world's population latently infected [3]. It accounts for 1.3 million deaths annually, making *M.tb* the largest cause of mortality by a bacterial pathogen [3]. The majority of *M.tb* infections occur within the developing world, accounting for about 95% of all cases due to a higher prevalence of co-infections such as HIV and poor health care resources [3]. The lack of a vaccine that effectively controls infection, as well as the decline in development of new antibiotics has led to the emergence of multi-, extensively, and totally-drug resistant strains of *M.tb* [3]. Due to such impracticalities of using antibiotics, the need to develop better vaccines is imperative.

1.1 Diagnostic tests for tuberculosis

Currently there are several approved tests for persons suspected of having an *M.tb* infection. They include the Mantoux tuberculin skin test (TST), chest radiograph, Interferon-gamma release assays (IGRAs) and smear microscopy (acid-fast bacilli stain) [4, 5]. A negative test result however, does not exclude the diagnosis of TB disease or latent infection. Culturing *M.tb* is the gold-standard for diagnosis of TB. *M.tb* infection can also be directly detected via nucleic acid amplification tests [4, 5]. However, currently there are no approved tests for measuring *M.tb* specific antibodies due to wide variation (31-100%) and sensitivity (0-100%) of these tests [4]. The WHO strongly advises against the use of serological commercial tests available due to the wide variety of anti-TB antibodies used and high false positive and false negatives [5, 6]. The difficultly of designing such serological tests stems from the fact that TB infection produces a wide variety of antibodies and there is no single antibody biomarker for TB due to the huge heterogeneity of antibody responses. However, a few patterns in the antibody responses at the different phases of TB infection have been described [4, 7]. For example, ESTAT-6 specific antibodies have been found in individuals at higher risk of TB reactivation [4]. Moreover, anti-38-kDa antigen antibodies have been associated with those with a high bacterial burden and advanced TB [4]. A systematic understanding of the antibody response to *M.tb* still needs to be developed. Increased antibody responses may be an indication of disease progression due to higher antigen loads. This large variation in antibody responses seen in humans during disease is due to a number of

factors such as strain variation, the growth state of the bacteria, the stage and severity of the disease, presence of cross-reactive antibodies, host genetics etc. [4]. Therefore, further investigations are required for developing a reliable antibody based test for TB.

1.2 Innate and adaptive immune responses to *M.tb*

As mentioned, pulmonary tuberculosis is contracted through aerosol droplets of an infected individual [2]. Once the bacilli enter the respiratory tract, there is a period of postponed growth for 3 days after which they take an average of 28 hours to replicate [8]. Subsequently, *M.tb* is taken up by the resident alveolar macrophages (AMs) in the airway lumen, which make up 95% of the airway luminal cells, thus making them a perfect target for infection [9]. The phagocytosis of the bacterium by AMs is facilitated by receptor-mediated phagocytosis [2]. Specifically, complement receptors C3 & C4, CD14, mannose binding receptors and surfactant molecules are involved in phagocytosis [2]. Various pattern recognition receptors (PRRs) recognize *M. tuberculosis* components such as phosphatidylinositol mannosides (PIMs), 19-kDa lipoprotein, lipoarabinomannan (LAM), and trehalose dimycolate (TDM) [1]. The PRRs most commonly implicated in *M.tb* recognition include Toll Like Receptor (TLR)- 2, 4 and 9 as well as NOD2 [1]. PRR signalling occurs through the TLR adaptor molecule MyD88 which activates the NF-κB transcription factor to produce pro-inflammatory cytokines such as TNF-α, IL-1ß, IL-12 and IL-6 [1]. The common conception in the field is that IL-12 plays a critical role in initiating Th1 differentiation and IFN-γ production via T cells [10]. IFN-γ can then

activate macrophages to produce nitric oxide (NO), an important host defense molecule against intracellular bacteria [10].

The chemokine receptor CCR7 is then upregulated on infected dendritic cells (DCs) through the production of IL-12p40 by these antigen presenting cells (APCs) [2]. This initiates the migration of activated DCs to the mediastinal lymph nodes where the homing chemokines CCL19 and CCL21 are present [2]. This process takes around 8-9 days after which naive T cells are primed by APCs (macrophages and DCs) and begin to proliferate within the lymph nodes [11]. Around 18-21 days post-infection, effector T cells migrate back to the initial site of infection, the lungs [11]. This delay in APC migration and T cell priming allows the bacterium to replicate to approximately 5 logs by 20 days post-infection and establish a foothold within the lungs [8, 12].

1.3 Current vaccines and vaccination strategies

Currently, the Bacille Calmette-Guérin (BCG) is the only licensed vaccine against TB with over 3 billion people vaccinated worldwide [13]. Although BCG confers protection against severe or disseminated forms of childhood TB, it has shown varying protective efficacy (0-80%; region dependent) against pulmonary forms of the disease. The ineffectiveness of parenteral BCG vaccination has been attributed to a multitude of factors such as waning vaccine immunity with age (10-15 years), varying doses used, the age of vaccination, genetic differences in strains used and exposure to environmental

mycobacterium [13]. For this reason, in the past 10 years, vaccination efforts have focused on developing heterologous booster vaccines. Replacing the BCG platform would be impractical due in part to the ethical issues of withholding the BCG vaccine [13]. Compelling evidence has suggested that effective booster vaccines for BCG should be heterologous to BCG such as recombinant protein based (+adjuvant), mycobacterial based and/or viral vector based systems [13]. Examples of booster vaccines currently in the pipeline include H56 +IC31, recombinant BCG over expressing *M.tb* antigens (VPM1002), *Mycobacterium vaccae*, MVAAg85A, Ad35 expressing multiple immunodominant *M.tb* antigens and AdHu5Ag85A [14].

The human adenovirus serotype 5 (AdHu5) expressing the mycobacterial secreted antigen Ag85A (AdHu5Ag85A) is an example of viral vector-based booster vaccine, and has been developed in our lab. The first component of the vaccine is a double-stranded DNA adenovirus that has a natural affinity toward the respiratory epithelium and is known to cause cold-like symptoms [15, 16]. Specifically, Ad has a wide tropism for many cell types through binding of the coxsackie adenovirus receptor (CAR) [17]. Human adenovirus type 5 a genetically engineered replication-deficient adenovirus due to deletion of the E1 and E3 regions, was originally utilized as a vector for gene therapy due to its tissue-specific tropism and high gene transfer efficacy [18]. Strong vector-specific immune responses however abolished the viability of adenoviruses as platforms for gene therapy. Such robust immune responses coupled with the good safety profile of Ad5 prompted its use as vaccine vectors [15, 16]. Moreover, the adenovirus vector was

designed to encode a secreted version of *M.tb* immunodominant antigen Ag85A [16]. This antigen is a mycolyl transferase responsible for the incorporation of mycolic acids into the cell wall of *M.tb* as well as the synthesis of cord factor [19]. The antigen was also chosen for its high immunogenicity capable of inducing robust T cell and humoral responses [19]. Furthermore, our lab has provided evidence demonstrating that AdHu5Ag85A is highly protective against *M.tb* infection as both a stand-alone and booster vaccine [13, 20]. Specifically, $CD8^+$ T cells have been repeatedly shown as the primary immune cells involved in providing this protection [21-23]. We have also provided evidence that suggests $CD4^+$ T cells as inconsequential to generating a protective AdHu5Ag85A-specific $CD8^+$ T cell response [23]. Of particular note, this vaccine has been evaluated in a Phase I clinical trial and has demonstrated its ability to induce a long lasting T cell mediated response in BCG vaccinees as well as in BCG naïve subjects [24].

1.4 Influence of routes of vaccine delivery and localization of cell-mediated immunity

The mucosal immune system is highly compartmentalized in comparison to the systemic immune system. Recently, the important link between the route or site of infection and route of vaccination has been brought into attention [16, 20, 25, 26]. Specifically, an overwhelming amount of evidence points towards the presence of airway luminal T cells in addition to lung parenchymal T cells at the time of infection to

effectively confer protection against *M.tb* [16, 20, 25, 26]. The current anti-TB vaccine BCG, is given subcutaneously (parenteral) even though *M.tb* infection occurs through the respiratory mucosal route. Intranasal vaccination of BCG however is impractical due to safety concerns of using a live bacterium that could cause chronic immunopathology in the lung [15]. It has been well established that parenteral BCG vaccination does not result in the recruitment of T cells to the airway lumen where *M.tb* first resides and proliferates, but instead it causes them to populate the lung interstitium [16, 20, 25, 26]. Furthermore, BCG vaccination only accelerates T cell recruitment into the lungs by 4-5 days following pulmonary exposure to *M.tb*, allowing about 14 days for the bacterium to proliferate [25].

A comparison of vaccinating through the intramuscular (i.m.) versus intranasal (i.n.) route using AdHu5Ag85A showed that i.m. vaccination primarily results in effector T cells being drawn into the spleen and lung interstitium through the pulmonary circulation, with very few T cells penetrating into the airway lumen (Figure A) [26]. In contrast, i.n. AdHu5Ag85A vaccination recruited a significant population of effector T cells into both the airway lumen and lung interstitium [26]. This effectively increased the level of protection against *M.tb* infection as both a stand-alone and booster vaccine [26]. In fact, i.n. vaccination with AdHu5Ag85A alone even surpassed the protective effects of the BCG vaccine [26]. Focus has been placed on designing vaccines that recruit antigen specific T cells into the airway lumen, and have demonstrated to be effective in protecting against pulmonary *M.tb*. Our data supports the idea that the route of vaccination

determines the geographical location of antigen-specific T cells which in turn determines the immune outcome against pulmonary *M.tb* infection.

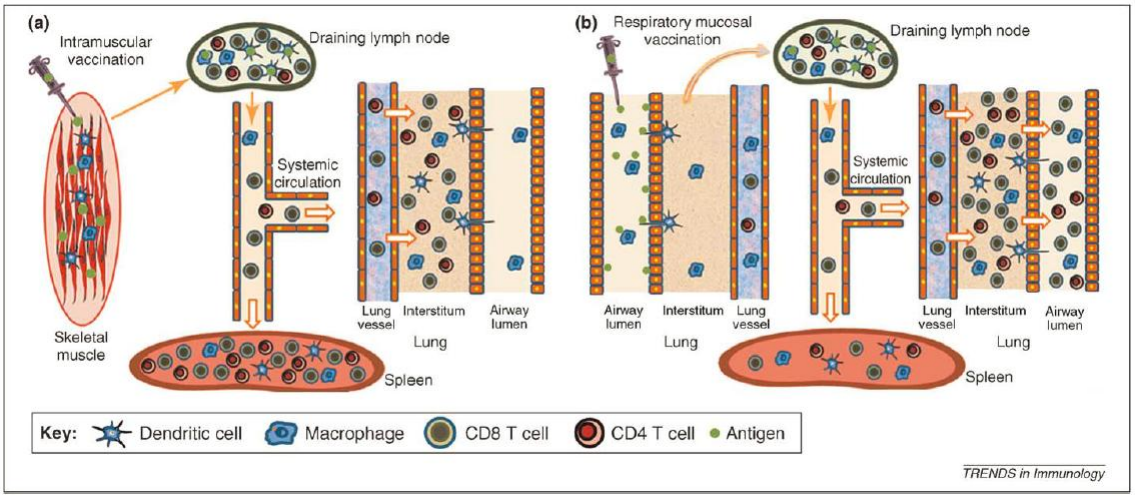

Figure A. Routes of vaccination influence T cell localization and protective efficacy [26]

1.5 Importance of B cells in *M.tb* infection

Mycobacterium tuberculosis has evolved to avoid antibody-mediated immunity by primarily residing and growing within macrophages. For this reason, B cells have been largely ignored in the field of *M.tb* and TB vaccine research due to the classical belief that T cell mediated immunity is crucial to providing protection against intracellular pathogens, while B cells play a limited role. However, there are exceptions to this phenomenon as antibodies have been found to play an important role during the extracellular phase of infection of intracellular pathogens [27, 28]. Correlations have also

been found between the dissipation of protective maternal antibodies in children and peak childhood TB cases [7, 29-31]. Furthermore, past attempts at utilizing serum therapy for treating *M.tb* yielded contradicting results due to the lack of appropriate controls and proper documentation as reviewed by Glatmann-Freedman and Casadevall [32]. However, recently there has been a growing body of evidence signifying the roles of B cells in protection against intracellular pathogens such as *Chlamydia trachomatis, Leishmania major, Salmonella typhimurium* and *Mycobacterium tuberculosis* [33-36]. These studies provide compelling evidence to revisit the role of B cells in TB immunity.

B cells consist of 10% of the total lung lymphocyte population [37]. They can be subdivided into three subsets, each unique in their functionality. These subsets include conventional B2 cells, B1 cells and marginal zone B cells [38]. Conventional B2 cells are responsible for producing class-switched antibodies with the help of T cells and can be further divided based on their cytokine production. B2 cells arise within the bone marrow and are able to differentiate into memory B cells or plasma cells with the ability to produce high affinity antibody. In contrast, B1 cells are rare innate-like B cells (comprising 5% of the B cell population) that first develop in the fetus and have a limited capacity to undergo somatic hypermutation [39]. They primarily produce large amounts of natural antibodies (IgM) and are found mainly in the peritoneal and pleural cavities [38, 39]. These B1 cells have limited diversity and memory and produce antibodies against T cell independent (carbohydrate) antigens and are part of innate immunity against encapsulated bacteria [38, 39]. Marginal zone B cells are non-migratory B cells

found within the spleen and are comparable to B1 cells in that they also produce mostly IgM and recognize carbohydrate antigens [38, 39]. In the context of *M.tb*, the focus has been on the role of B2 cells in protection due to their ability to produce class-switched high affinity antibodies and lead to the formation of memory B cells.

Until recently, B cells had been disregarded as an important component of anti-TB immunity. Inconsistent results in the primary *M.tb* challenge model have also been seen when studying B cell deficient mice, possibly due to the varying doses used, route of infection, strain of *M.tb* used and the type of transgenic B cell KO mouse used. In one report, no differences were observed in bacterial burden after aerosol challenge with CDC1551 *M.tb* strain [40], while another study reported higher CFU using high dose intravenous H_{37}Rv challenge [41]. This variation is a consequence of using different *M.tb* strains of varying virulence, different routes of infection and doses, as well as the use of different B cell KO mice.

In both mice and non-primate models, B cells have been shown to be in close contact with T cells. Upon infection with *M.tb*, B cells infiltrate and organize around the periphery of the granulomas. It is well known that T cells are a large cellular component of granulomas, B cells have also been reported to constitute about 1-10% of the lymphocytes within these aggregates [42]. While B cells have been found to be an integral part of the normal formation of granulomas, B cell deficient mice are still able to develop granulomatous structures. There are however some conflicting results regarding

how the absence of these cells influences inflammation within these granulomas [40, 43]. Differences seen in these studies may be a result of using acute verses chronic models of *M.tb* infection as well as different strains of mice.

B cells may also have a role in regulating neutrophils upon *M.tb* infection. One study highlights the role of B cells in down-regulating early neutrophil migration to the site of inflammation and reducing the effectiveness of BCG vaccination in mice [44]. The accumulation of neutrophils in B cell deficient mice is an observation supported by others [43, 45]. Neutrophils are one of the first responders upon *M.tb* infection where they have been shown to phagocytose *M.tb* and undergo apoptosis. Their role in TB immunity however, remains controversial due to conflicting reports [36, 46, 47]. Aberrant or excessive neutrophils in *M.tb* infection can be deleterious to the host's tissue integrity [47]. Since neutrophils, T cells and B cells are the cells that influence granuloma formation, it is not surprising that in the B cell KO model,there is a deregulation in anti-TB immunity [36, 46, 47].

1.6 Antibody-mediated immunity against *M.tb*

Recently, several attempts have been made to investigate the potential role of antibody-mediated immunity against *M.tb*. Antibodies produced against both polysaccharide and protein antigens from *M.tb* have shown to have protective effects. Although various studies have demonstrated that certain antibodies can be protective

against *M.tb*, the mechanisms by which they mediate this protection remain unclear. Pre-existing antibodies against *M.tb* may neutralize the bacteria to prevent infection, increase phagocytosis, or enhance antibody-dependent cell cytotoxity [29, 48]. B cells can also modulate macrophage functionality by antibody-mediated phagocytosis. Once opsonised, the coated bacterium is taken up through Fc receptor binding by macrophages, enhancing intracellular killing [29]. Of particular note, one study using LAM-specific human serum from BCG vaccinated individuals was shown to enhance phagocytosis, internalization, and killing by APCs with enhanced $CD8^+$ T cell responses [49]. In addition, mice lacking polymeric immunoglobulin receptor (pIgR) and activating FcγR have been found to be highly susceptible to *M.tb* infection [29, 50]. Passive IgA treatment of mice has also been shown to rescue mice from *M.tb* induced death [51]. Antibody mediated phagocytosis has been shown to increase intracellular Ca^{2+} concentrations promoting phagosome-lysosome fusion and intracellular killing.

1.7 Role of B cells as antigen presenting cells

Thus far, B cells have been discussed primarily for their antibody producing capabilities. However their roles extend to antigen presentation as well as cytokine production [52]. It is well established that dendritic cells are the principal antigen presenting cells (APCs) and are imperative during the initial T cell priming stages. Yet B cells have also been found to be competent APCs [53, 54]. B cells circulate between the blood and lymph nodes and home to secondary lymphoid tissues. B cells express

lymphotoxin-α/β that is responsible for normal lymphoid organogenesis and development of follicular dendritic cells (FDCs) [38]. FDCs are a major source of CXCL13 chemokine which helps recruit B cells (CXCR5) into the lymphoid follicles [55]. They also provide B cells with the survival signals via the secretion of BAFF [55]. The close network between FDCs and B cells maintains the lymphoid architecture and is believed to provide B cells with antigen [55].

One theory is that B cells are important in modulating secondary T cell responses for the reason that they require T cell help to activate their antigen presenting capabilities [54]. This is achieved through the ligation of CD40 on B cells and CD40L on T cells, as well as through the production of cytokines such as IL-4, IL-5, IL-6, and IL-10 [37]. It has been observed that B cells become even more important for antigen presentation when there are low levels of antigen. B cells may also have a role in transporting bacteria or viruses from the sites of infection to the lymph nodes. In one such case B cells were documented to be superior to DCs in transporting viral antigen to lymphoid tissue as they were able to avoid CTL killing [56]. These B cells did not become infected and present antigen but instead were able to bind viral particles on the surface [56]. Another study reported that B cells were responsible for promoting dissemination of anthrax spores by transporting them from the lungs into the draining lymph nodes [57].

1.8 Role of B cells in regulating T cell responses

It has been firmly recognized that $CD4^+$ T cells are important drivers of humoral immunity by providing help via co-simulation and cytokines to drive B cell activation and class-switched antibody production [52, 53]. Moreover, $CD4^+$ T cells can polarize B cells to also take on a similar cytokine producing phenotype [52]. For example Th1 cytokines such as IFN-γ can activate B cells to take on a Be1 IFN-γ^+ phenotype. However, the reverse scenario of B cells driving T cell activation and proliferation is not widely accepted nor studied, particularly for $CD8^+$ T cells [52, 53]. Many attempts using B cell deficient mice have yielded conflicting results. This is in part due to the inherent developmental and structural defects exhibited in B cell knockout mice such as altered dendritic cell populations, micro-architecture defects in the spleen and reduced T cell numbers in the spleen, all of which can influence normal T cell responses [38]. Thus, it is imperative to consolidate any observations seen in these mice in conjunction with other techniques such as B cell depleting antibodies and adoptive B cell transfer. The potential role of B cells in regulating T cell immunity has recently received increasing attention due to the use of B cell depleting antibodies in clinical trials to treat T cell mediated autoimmune diseases [53]. In particular, Rituximab (anti-CD20) is a clinically licensed B cell depleting antibody therapy to treat certain autoimmune diseases such as rheumatoid arthritis [53]. Such therapy has been shown to ameliorate the pathology seen as a result of aberrant $CD4^+$ T cells responses [53, 58]. This suggests that B cells are in some way regulating T cell responses. Although very uncommon, mycobacterial infections have

been reported following the administration of this drug [59]. Moreover, several studies using B cell deficient mice have demonstrated the ability of B cells to modulate T cell responses to infection [33, 36, 49, 60, 61]. One such study reported a 95% reduction in antigen specific $CD4^+$ T cells in B cell deficient mice post-infection with lymphocytic choriomeningitis virus (LCMV) [60]. In another study, both B cell deficient mice and antibody B cell depleted mice displayed compromised bacterial control as a result of increased neutrophilia in the lungs, which resulted in reduced BCG-induced Th1 responses [36].

1.9 Influence of B Cells in TB vaccine models

Most of the successful licensed vaccines on the market for various diseases are potent inducers of humoral immunity. Antibody based vaccines are still not being considered due to the lack of understanding of the efficacy of B cell mediated immune responses against intracellular pathogens. The BCG vaccine currently in use is a T cell mediated vaccine that only attenuates disease progression. Therefore the need to develop vaccines that elicit both arms of the adaptive immune response has become increasingly important. Further understanding mucosal AdHu5Ag85A vaccine-induced B cell and antibody responses and their potential roles in protection in *M.tb* will help develop effective respiratory mucosal vaccination strategies.

1.10 Rationale, Hypothesis, Objectives

Although mucosal AdHu5Ag85A vaccination has been proven to induce strong type I T cell mediated immunity and is crucial for protection against *M.tb*, the role of vaccine-induced B cell responses in protecting against *M.tb* infection remains poorly understood and understudied. Many studies focusing on the role of B cells in primary *M.tb* infection have yielded contradicting results. However, in light of recent studies, the role of B cells in host defense against *M.tb* is being re-evaluated. Latest publications have shown B cells to provide protection by modulating T cell immunity during intracellular bacterial infections including *M.tb*. Therefore, we hypothesize that AdHu5Ag85A vaccination also induces antigen-specific B cell responses and such B cells have an immunomodulatory effect on vaccine-induced T cell immunity that are protective against *M.tb*. My objectives include establishing the level of B cell responses induced by mucosal AdHu5Ag85A vaccination. Second, to understand the role of B cell responses in modulating mucosal AdHu5Ag85A induced T cell immunity. Thirdly, establish whether mucosal AdHu5Ag85A-induced B cells provide protection against pulmonary *M.tb* infection. Lastly, determine the mechanism by which mucosal AdHu5Ag85A-induced B cells modulate AdHu5Ag85A induced T cell immunity.

2.0 CHAPTER TWO. EXPERIMENTAL METHODS

Mice. Age and sex matched 6-8 wk old female Balb/c and B cell knockout mice (*Igh-J*^{tm1Dhu} N?+N2) were purchased from Taconic [62]. Mice were housed in specific pathogen free facility at McMaster University in accordance with the animal research ethics board.

Vaccination with AdHu5Ag85A. Mice were vaccinated through the intranasal route with recombinant replication deficient Ad-based TB vaccine (AdHu5Ag85A). Development of the vaccine has been previously described [16]. Each mouse received a dose of $5x10^7$ PFU in 25 µl of PBS. After a duration of 4 weeks the animal was sacrificed.

***M.tb* preparation and challenge**. *M.tb* bacilli preparation was performed as previously described [63]. Briefly, bacteria was grown in Difco 7H10 Middlebrook media supplemented with OADC Middlebrook enrichment (Invitrogen Life Technologies; Burlington, ON, Canada) and 0.002% glycerol and 0.05% Tween-80 for 10-15 days and stored at -70°C until required. Before use, the bacteria was washed twice with PBS containing 0.05% Tween 80 and passed through a 27-G needle 10 times to disperse clumps. All experiments displaying T cell and B cell analysis were carried out using the attenuated strain of *M.tb* H_{37}Ra (ATCC25177) due to experimental restrictions. Mice were challenged with *M.tb* H_{37}Ra intratracheally (i.t.) with a dose of 0.5 $x10^6$ colony

forming units (CFU) *M.tb* in 40µl of PBS/mouse. Experiments displayed bacterial burden/CFU data were carried out using the virulent strain *M.tb* H_{37}Rv (ATCC27294). The difference between the two strains is a deletion in the RD-1 region in the H37Ra strain which encompasses virulence factors such as ESAT-6 and CFP-10. Mice were challenged intranasally (i.n.) with a dose of 10,000 CFU *M.tb* in 25µl of PBS/mouse.

Colony formation assay. The level of bacterial burden was determined by plating serial dilutions of the lung homogenates and left to incubate at 37°C for 21 days. Colonies were counted and calculated and displayed as log10 CFU per organ.

B cell depletion. B cells were depleted using 5D2 anti-mouse CD20 antibody (α-CD20) (Genentech Inc.; California, U.S.). Mice were given intraperitoneal injection at an initial dose of 250 µg/mouse two days prior to vaccination and subsequently 200 µg/mouse every other week for maintenance of B cell depletion.

Anti-Ag85 antibody ELISA. 96 well flat-bottom immuno plates (Thermo Fisher Scientific) were coated with 0.01 µg/µL Ag85 antigen and left overnight at 4°C. Plates were washed with dilution buffer and samples were then added at various dilutions and incubated for 1 hour at 37°C. Plates were washed again and anti-mouse IgG2a- Biotin conjugated (Southern Biotechnology Associates Inc., USA) and anti-mouse IgA-biotin conjugated antibodies (Sigma-Aldrich, Germany) were added at a dilution of 1:500 for 1 hour at 37°C. Plates were again washed and strepavidin-alkaline phosphatase was added

and incubated for 30 min at 37°C. Plates were washed and substrate was added (p-nitrophenyl phosphate tablet in diethanolamine) incubated at room temp for 15-30 min. Lastly 2N NaOH stop solution was added and plates were read on spectrophotometer at 405 nm.

Bronchoalveolar lavage and lung mononuclear cell isolation. Bronchoalveolar lavage (BAL) was carried out on lungs to collect airway luminal cells as well as the conventional BAL supernatants[16]. The lavage was carried out by first washing the lungs with 250 μL of PBS, followed by 200 μL (considered conventional BAL). The lungs were then lavaged 3 times with 300 μL each (exhaustive BAL). The cells from the conventional and exhaustive BAL were pooled, while only the conventional BAL SUPs were kept for cytokine assays. Following lavage, the lungs were perfused through the right ventricle with Hanks to remove cells from the pulmonary vasculature.

Lung processing. Mononuclear cells were isolated from lung tissue as previously described [21]. Briefly, lungs were cut into pieces and put in 150 U/ml collagenase type I (Sigma-Aldrich, St. Louis, MO) for 1h at 37°C. Then the lungs were passed through 40-μm basket filters and red blood cell lysis was preformed with ACK lysis buffer. Cell pellets were resuspended in complete RPMI medium (RPMI 1640 with 10% FBS, 1% penicillin-streptomycin, and 1% L-glutamine).

Cytokine measurements in BAL supernatants. BAL fluids (supernatants) were collected and stored at -20°C until use. The level of IL-10 was measured by ELISA (R&D Systems, Burlington, ON, Canada).

Cell culture, tetramer staining, intracellular cytokine staining and flow cytometry. Approximately 2 million mononuclear cells from BAL, lung and spleen were cultured for 5 hours in the presence of Golgi plug (5 μg/mL; BD Pharmingen, Mississauga, ON, Canada) with or without Ag85A specific CD8 (MPVGGQSSF) T cell peptide at a concentration of 1 μg per well for 5-6 hours. Cells were first incubated for 15 minutes on ice with the CD16/CD32 Fc block antibody (BD Pharmingen). The cells were then immunostained using a tetramer specific for Ag85A CD8 T cell peptide bound to Balb/c MHC I allele H-2L^{d} and PE fluorochrome (MHC Tetramer Laboratory of Baylor College of Medicine; Houston, Texas) for 1 hour at room temperature and then washed. Extracellular staining was then carried out with monoclonal antibodies, washed, permeabilized and stained intracellularly. The following fluorochrome-conjugated monoclonal antibodies were used: CD3-v450, CD8a-PE-Cy7, CD4-APC-Cy7 and IFN-γ-APC (BD Pharmingen). B cells were stained with B220-PE and CD19-PerCP Cy5.5 (BD Pharmingen). Flow staining was also carried out for APCs [See **Figure 11**]**.** for gating strategy] with the following colours: CD45-APC-Cy7, CD11c-APC, CD11b- Pe-Cy7 (BD Pharmingen), Gr-1-Pacific Orange (Invitrogen Life Technologies), MHC II- Alexa Fluor 700 (ebioscience Inc., San Diego, Canada).

***Ex vivo M.tb* killing.** Naive wild-type $CD11c^{+}CD11b^{+/-}$ and $CD11c^{-}CD11b^{+}$ phagocytic cells were purified (50,000 cells per well) from the lungs using MACS CD11c and CD11b beads (Miltenyi Biotec; Auburn, Canada) according to the manufacturer's instructions. B cells were purified (0.2×10^6 cells per well) from naive wild-type or 3 week post-AdHu5Ag85A vaccinated mouse lungs using the EasyStep™ Mouse Pan-B Cell Isolation Kit (StemCell Technologies Inc.; Vancouver, BC, Canada). These cells were plated either together or alone on a 96-well plate in the presence or absence of *M.tb* $H_{37}Ra$ (multiplicity of infection 1:5) co-cultured with recombinant mouse IFN-γ (0.4 ng/mL) (R&D Systems). The plates were incubated at 37°C and 5% CO_2 for 24h, 48h, and 72h at which point the supernatants were collected for future cytokine measurements and NO assays.

The antimicrobial abilities of wild-type and $Jh^{-/-}$ KO $CD11c^{+}CD11b^{+/-}$ and $CD11c^{-}CD11b^{+}$ phagocytic cells (2.0×10^5 cells/well) will be evaluated by infecting these cells with $H_{37}Ra$ *M.tb* at MOI of 1:1. The cells will be cultured on a 96 well U-bottom plate in cRPMI containing only penicillin in the presence of serum (50 µl/mL). Plates will then be incubated at 37°C and 5% CO_2. Any remaining extracellular bacteria after 4h will be removed by spinning the plate for 10 minutes at 1400 rpm. Fresh media will then be added and further incubated for 12h, 24h, and 36h. After each time points, the cells will be lysed with water and intracellular bacteria counts determined.

Nitric oxide (NO) assay. The release of NO was measured from $CD11c^+$ purified cells by measuring nitrite levels (the end product of NO) [63]. Samples were diluted at various concentrations with Griess reagent (Sigma-Aldrich) absorbance was measured at 540 nm. The final concentration was calculated using a standard curve of sodium nitrite concentrations (0-100 μmol 1^{-1}).

B cell adoptive transfer. B cells were purified from naive wild-type spleens and were then adoptively transferred (10^7 cells resuspended in 200 μL PBS) intravenously (i.v.) through the tail vein of each $Jh^{-/-}$ KO mouse 2 days prior to vaccination.

Lung histology. Lungs were fixed in 10% formalin and kept in 5 ml of 10% formalin for at least 72h. Tissue sections were stained with hematoxylin and eosin (H&E) for histological examination.

Data analysis. All statistical analyses were performed using Graphpad Prism Software. A two-tailed Student's t-test was used for pair-wise comparisons while a one-way ANOVA using Tukey's post hoc test was carried when comparing multiple groups. A two-way ANOVA was used when comparing multiple groups with different conditions. Asterisk marks (*, **, ***) displayed on graphs indicate the level of significance ($P<0.05$, $P<0.01$, $P<0.001$ respectively).

3.0 CHAPTER THREE. RESULTS

3.1 Respiratory mucosal AdHu5Ag85A vaccination induces Ag85A-specific antibody responses within the airways and in circulation

Previous work has revealed that the BCG vaccine elicits antibody responses in humans and mice and that these humoral responses promote macrophage killing and improved T cell responses *in vitro* [49, 64]. Moreover, intranasal vaccination with Ag85 complex proteins has been shown to induce IgG2a and IgA responses in the nasal secretions [65]. While respiratory mucosal AdHu5Ag85A-induced T cell responses have previously been well studied, the vaccine-induced B cell responses remain poorly understood. Therefore, we wanted to characterize the kinetics of antigen specific B cell secreted antibody responses post mucosal AdHu5Ag85A vaccination. Antigen specific T cell responses post-AdHu5Ag85A intranasal vaccination peaked at 14 days post-vaccination[13, 16, 21]. Since it is well established that T cell help is required for B cell activation and isotype switching, we predict that the peak antigen specific B cell responses will occur following peak T cell responses. BAL sups and serum samples were taken from Wt mice at 0, 3, 7, 14 and 28 days post-vaccination. As expected, the peak in antigen specific IgA and IgG2a antibodies occurred at 28 days post-vaccination in the BAL and serum **(Figure 1)**. This is consistent with previous findings in Giri *et al.* where they examined antibody responses to Ag85 complex antigens [65]. Thus, we can conclude that AdHu5Ag85A does induce antigen specific B cell responses. As previous publications have highlighted the

importance of having *M.tb* specific antibodies prior to infection, the fact that AdHu5Ag85A also induces B cell responses in the airways is potentially another way this vaccine provides protection against *M.tb*. [49, 51].

3.2 Lack of B cells leads to reduced T cell responses within the draining lymph nodes and airways following respiratory mucosal AdHu5Ag85A vaccination

Igietseme *et al*. among others, have highlighted the roles of antibodies in regulating T cell responses against intracellular infection [49, 66, 67]. It has been well established by our lab that the presence of IFN-γ producing $CD8^+$ T cells in the airway is an important determinant of protection elicited by the AdHu5Ag85A vaccine [16, 23, 24]. To understand the role of B cells in vaccine-induced T cell immunity, we next employed B cell-deficient mice and determined whether the absence of B cells and antibody responses would affect mucosal AdHu5Ag85A induced T cell immunity. To this end, both Wt and $Jh^{-/-}$ KO mice were vaccinated intranasally with AdHu5Ag85A and antigen specific (tet^+) as well as IFN-γ producing $CD8^+$ T cell responses were evaluated 4 weeks post-vaccination. It was found that the $Jh^{-/-}$ KO mice had considerably reduced numbers of tet^+ and IFN-γ producing $CD8^+$ T cells in the BAL when compared to Wt mice **(Figure 2A and 2C)**. However, no significant differences seen within the lungs (**Figure 2B and 2D**), This is significant since we have previously established the importance of airway luminal T cells against pulmonary *M.tb* infection [26].

It is unclear why these $Jh^{-/-}$ KO mice have decreased T cell responses in the airway. However, it has been documented previously that B cell deficient mice have significantly higher levels of the IL-10 production upon *M.tb* infection [35, 43]. Given the immunosuppressive effects of IL-10 on anti-TB immunity, we were curious as to whether it was present at higher levels in the lung of KO mice and was responsible for suppressing the antigen specific T cell responses seen within these mice in the BAL. However, no significant differences were seen in IL-10 levels between the Wt and $Jh^{-/-}$ KO mice (**Figure 3**).

Another possibility is that this reduction in airway T cell responses in vaccinated $Jh^{-/-}$ KO mice was seen as a result of delayed T cell priming. Therefore, we measured antigen specific T cell responses at 0, 3, 7, and 14 days post-vaccination within the mediastinal lymph nodes in order to compare the kinetics of T cell priming in Wt and $Jh^{-/-}$ mice. There was significantly lower antigen specific T cells within the draining lymph nodes of the $Jh^{-/-}$ KO mice when compared to Wt mice beginning at day 7 **(Figure 4)**. These data suggest that AdHu5Ag85A -induced B cells do play an important role in mediating protective T cell responses.

3.3 B cell depletion in WT mice did not alter T cell responses following respiratory mucosal AdHu5Ag85A vaccination

Anti-CD20 also known as Rituximab is a licensed therapeutic used to treat autoimmune diseases such as rheumatoid arthritis. Use of this drug has been shown to substantially reduce T cell-mediated immunity [58]. Thus, in order to further confirm our findings from using $Jh^{-/-}$ KO mice, we employed the use of anti-mouse CD20 antibody also known as Rituximab. CD20 is a surface marker present on B cells, appearing first on pre-B cells until the memory stage (absent on plasma cells) and is believed to be involved in B cell activation, growth and calcium flux [38]. While this antibody has been shown to be effective at depleting circulating antibodies, it is less efficient at depleting germinal center B cells and marginal zone B cells [38]. We sought to first determine the ability of this antibody to deplete B cell responses in AdHu5Ag85A vaccinated Wt mice. The extent of B cell depletion was characterized in the lung, spleen and blood at various time points and was found to effectively deplete 98% of B cells in the lung and blood and 60% of B cells within the spleen **(Figure 5)**. Due to the fact that anti-CD20 does not deplete antibody producing plasma cells, this antibody was used to deplete B cells prior to vaccination to prevent the production of AdHu5Ag85A specific antibody responses [38]. However, after 4 weeks post-vaccination, we found that B cell depleted animals had no differences in the level of antigen specific or IFN-γ producing $CD8^+$ T cells compared to Wt mice in the BAL or Lungs **(Figure 6)**. Thus, within our hands, depletion of B cells has no affect on

the generation of antigen specific T cell responses following respiratory mucosal AdHu5Ag85A vaccination.

3.4 Lack of B cells leads to impaired control of M.tb infection in AdHu5Ag85A-vaccinated mice

Several studies employing the use of B cell/antibody deficient animals have shown these animals to be significantly more susceptible to primary *M.tb* infection in unvaccinated animals when compared to wild type mice [36, 43, 49]. However, only one group has investigated the potential role of B cells in mediating protection in the context of a vaccine model *in vivo*, specifically the BCG vaccine model [36]. Since we have demonstrated significantly abrogated AdHu5Ag85A specific T cell immunity in a B cell deficient ($Jh^{-/-}$ KO) model, we next examined if this would translate to reduced protection against *M.tb* in these animals. Due to the variable results seen in *M.tb* infected B cell knockout mice, we had chosen to first examine protection in unvaccinated Wt and $Jh^{-/-}$ KO in order to compare our results with what has previously been documented. To this end, naïve Wt mice and $Jh^{-/-}$ KO mice were both challenged with $H_{37}Rv$ i.n. for a 2 week period. At this time mice were sacrificed and bacterial burden was enumerated using CFU. The $Jh^{-/-}$ KO mice were found to have ~0.8 log more bacterial burden when compared to Wt **(Figure 7).** This is in agreement with earlier reports of high bacterial burden seen in the B cell KO mice [41, 43].

Next, we wanted to evaluate whether vaccine efficacy would be abrogated in the absence of B cells. Wt mice and $Jh^{-/-}$ KO mice were both vaccinated with AdHu5Ag85A intranasally for a period of 4 weeks, upon which time they were challenged with *M.tb* $H_{37}Rv$ i.t. for 2 weeks. The vaccinated $Jh^{-/-}$ KO mice were shown to have a significantly higher bacterial burden in the lungs when compared to the vaccinated Wt mice **(Figure 7)**. In separate experiments, the Wt mice were depleted of B cells by using anti-CD20 antibodies 2 days prior to vaccination as previously described for **Figure 5** and B cell depletion was maintained throughout the course of the experiment. The vaccinated α-CD20-treated mice however, did not show any difference in CFU when compared to the vaccinated Wt mice **(Figure 7)**. This is in agreement with the T cell data presented in **Figure 6.** where no differences were seen between the Wt and $Jh^{-/-}$ KO mice. Together these data suggest that the complete lack of B cells as in genetic B cell-deficient animals is detrimental to the effective control of pulmonary *M.tb* growth.

3.5 Lack of B cells leads to enhanced granulomatous lesions and leukocytic responses to M.tb exposure in the lung of AdHu5Ag85A-vaccinated mice

Given the impaired anti-TB host defense seen in vaccinated $Jh^{-/-}$ KO mice, we next assessed histopathological changes in the lung of these animals. When comparing naive Wt and $Jh^{-/-}$ KO mice, there are irregular granuloma aggregates (**Figure 8a**), bronchial hyperplasia (**Figure 8b**), increased neutrophilic infiltrates (**Figure 8c),** increased cellular infiltrates within the lung parenchyma and epithelial sloughing (**Figure 8d**) within the

lungs of the $Jh^{-/-}$ KO mice. As expected the overall pathology seen in the lung, comparing with unvaccinated groups, was reduced in the vaccinated groups and corresponded nicely with the CFU data displayed in **Figure 7**. Nevertheless, the lung of vaccinated $Jh^{-/-}$ animals still had worse off immunopathology than the lung of vaccinated Wt animals (**Figure 8**). In keeping with the CFU data, no differences in pathology were seen between vaccinated Wt and α-CD20 depleted mice **(Figure 8)**.

To further investigate the nature of severe inflammation and mononuclear cell infiltrates seen within the lung of vaccinated and *M.tb*-challenged $Jh^{-/-}$ KO mice, we next examined the cell types in the lung by flow cytometric analysis. As expected, vaccinated $Jh^{-/-}$ KO mice trended towards greater numbers of antigen specific (tet^{+}) and IFN-γ^{+} $CD8^{+}$ T cells in both the BAL and lungs 2 weeks post *M.tb* challenge **(Figure 9)**. In addition, the $Jh^{-/-}$ KO mice displayed increased frequencies of neutrophils post-vaccination (**Figure 10**) which is in agreement with previous studies [36, 44]. These data indicate that in agreement with impaired protection seen within the B cell-deficient hosts, there is also heightened immunopathology and T cell responses in the lung.

4.0 CHAPTER FOUR. DISCUSSION

There is no doubt that T cell-mediated immunity is the major contributor to the protection against intracellular pathogens including *M.tb*. However, there are clear examples of intracellular infections such as *Cryptococcus neoformans* and *Chlamydia trachomatis*, where pre-exisitng anitbodies against suface antigens of these pathogens has been shown to play an important role in controlling the initial stage of infections [66, 68-70]. Furthermore, recent re-visit to the role of B cells and antibody immune responses in protection against pulmonary tuberculosis by many researchers has shown a potential role of B cells against pulmonary *M.tb* infection. Studies making use of B cell depleting antibodies, B cell knockout mice as well as B cell or serum transfer approachs have provided evidence that B cells and antibody immune responses play an important role in primary pulmonary *M.tb* infection [35, 36, 41]. B cells have been shown to modulate anti-tuberculosis immunity via regulating inflammation (i.e neutrophilic inflitration), granuloma formation, and antigen specific T cell responses [36, 43, 69]. While mounting evidence suggests a role of B cells and antibodies in host defense against primary pulmonary *M.tb* infection, the role of B cells in TB vaccine-induced protective immunity still remains poorly understood.

Adenovirus-vectored TB vaccine, AdHu5Ag85A developed in our laboratory has been shown to be a potent vaccine against pulmonary tuberculosis in a variety of animal models when delivered via respiratory mucosal route [16, 20, 21, 71]. There is no doubt that T cell-mediated immunity elicited by this vaccine plays a major role in protection.

However, whether this vaccine induces B cell and antibody immune responses and if so whether these immune components play any role in influencing the T cell-mediated immune responses and protection against TB remains unclear. In this study, we have investigated the role of B cells in AdHu5Ag85A respiratory mucosal vaccine mediated protection against *M.tb.* Our study has provided evidence that respiratory mucosal AdHu5Ag85A-induced B cell immunity plays a significant role in vaccine-mediated T cell protection against *M.tb* infection. However more work is required to elucidate the potential mechanism.

Although, mounting evidence has suggested an important role of anti-*M.tb* antibodies in providing protection upon primary infection [48, 49, 51, 69, 72], none of these studies have looked at the potential of a respiratory mucosal vaccine in eliciting protective antibody responses against *M.tb*. Therefore, it must be fully elucidated whether it is the B cells influencing protective T cell responses or whether the B cell secreted antibodies are contributing to protection. We have found that repiratory mucosal vaccination with AdHu5Ag85A does induce antigen specific IgG2a and IgA antibodies in the lung and the circulation (**Figure 1**). To test whether these AdHu5Ag85A induced specific antibodies could enhance the killing of *M.tb* by phagocytic cells, we investigated the effects of pre-treating *M.tb* with serum taken from a 4 week AdHu5Ag85A vaccinated animal. We found that *ex vivo* culture of naive APCs infected with *M.tb* H_{37}Ra pretreated with AdHu5Ag85A immune serum showed higher NO production at 48h and 72h when compared to those cultured with naive serum (data not shown). This is consistent with

what has been previously published on the protective roles of anti-*M.tb* specific antibodies [48, 49, 51, 72]. However, whether they are protective *in vivo* remains to be answered. To determine this, serum will be transferred from a vaccinated wild type mouse into a naive $Jh^{-/-}$ KO mice to see whether these B cell deficient mice will demonstrate enhanced protection after infection. In such experiments, serum will be transferred via tail vein intravenous (i.v.) injection 2 days before challenge and maintained every 3 days for the course of the infection.

We have previously demonstrated the importance of airway luminal T cells in providing protection against pulmonary *M.tb* infection [2, 16, 21, 26, 73]. Specifically, we have seen repiratory mucsoal AdHu5Ag85A vaccination to elicit robust $CD8^+$ T cell response within the airway luminal space and is a correlate of better bacterial control within the lungs. In the current study we have shown that B cell deficiency attenuated AdHu5Ag85A-induced Th1 responses, specifically in the airway lumen (**Figure 2**). Recruitment of Ag-specific T cells to the respiratory mucosa requires specific signals. MIP-1α and IP-10 chemokine gradient has been shown to play an important role in recruting Ag-specific T cells into the airway lumen [2]. Furthermore, IP-10 production by macrophages was shown to be mediated by IL-6 produced by B cells [74]. Thus, we speculate that in the absence of B cells there is inadequate chemotactic signals for T cells to migrate into the airway lumen. Attenuation of vaccine-induced Th1 responses in the absence of B cells is not specific to AdHu5Ag85A respiratory mucosal vaccination. A previous study examining the role of B cells in regulating T cells responses induced by

intraderrmal BCG vaccination has also shown reduced BCG specific T cell responses in B cell deficient mice [36]. Such attenution in vaccine-induced Th1 responses was attributed to increases in neutrophilia in the B cell deficient mice which led to impaired DC migration into the lymph nodes, thus hindering T cell priming [36]. There are reports of neutrophils influencing the timing and development of antigen specific T cell responses [44, 47]. We have also observed increased neutrophils after vaccination, a possible mechanism for reduced T cell responses seen within our model (**Figure 10**). To further investigate the role of B cells invaccine-induced T cell immunity in the absence of B cells, we adopted B cell depletion approach in wild type mice. This approach would potentially allow us to determine whether B cells regulate vaccine-induced T cells responses without the confounding factors that may exist in B cell-KO animals. As mentioned previously, a limitation of using B cell KO mice is the possible disruption of the architecture of lymphatic sites and the intricate DC and T and B cell cross-talk at the lymphatic site [38]. In our hands, B cell depletion in wild type did not significantly affect the vaccine-induced T cell responses **(Figure 6)**. One possible explanation for this is that the anti-CD20 antibody we used is unable to completely deplete germinal center B cells and marginal zone B cells [38]. Indeed, we found the antibody to only deplete 60% of the B cells within the spleen **(Figure 5)**. Although not examined in the current study, we expect the same to be true in other lymphoid tissues such as the draining lymph nodes. Thus, failure of the B cell depleting antibody to effectively deplete all B cells within the lymphoid tissues may have attributed to the unimpaired vaccine-induced protective T cell immunity in wild type mice. Based on these data and to further consolidate the data

obtained by using B cell KO animals, an experiment has been set up where B cells were adoptively transferred intravenously from naive Wt mice into $Jh^{-/-}$ KO mice. These mice along with naive Wt and $Jh^{-/-}$ KO mice were vaccinated with AdHu5Ag85A for 4 weeks and challenged with H_{37}Rv *M.tb*. Upon 2 weeks post-challenge, the lung bacterial burden will be assessed.

The current study has for the first time demonstrated that in the absence of B cells, antigen-specific T cells are desruglated in a repiratory mucosal vaccine model. However, the mechanisms by which B cells shape up the vaccine-induced T cell immunity remains to be fully elucidated. We speculate that in the absence of B cells inflammatory responses may be desregulated resulting in impairment of APC functionality, T cell activation and recruitment of T cells to the airway lumen. Beginning to understand the mechanisms, further experiments are underway. Specifically, in order to assess if macrophage functionality is hindered as a result of the absence of B cells, an experiment has been set up where $CD11c^{+}$ and $CD11b^{+}$ APCs will be purified from 4 week vaccinated Wt and $Jh^{-/-}$ KO mice and will be infected with H_{37}Ra *M.tb*. The CFU will then be assessed at different time points to determine their killing capacity.

In agreement with previous reports, naïve $Jh^{-/-}$ KO mice were more susceptible to primary *M.tb* infection than their wild type counterparts (39). Furthermore, consistent with attenuation of AdHu5Ag85A-induced T cell responses, particularly in the airway lumen, in $Jh^{-/-}$ KO, upon exposure to pulmonary *M.tb* infection these mice were less

protected compared to AdHu5Ag85A vaccinated wild type counterparts **(Figure 7)**. Such impaired protection in $Jh^{-/-}$ KO mice correlated with severe immunopathology in the lung. Massive influx of mononuclear cells into the lung has attributed to immunopathology as indicated by histopathological analysis **(Figure 8)**. Quantification of antigen specific T cell responses post-*M.tb* challenge by flow cytometry shown a trend towards increased numbers of tet^{+} and IFN-γ^{+} $CD8^{+}$ T cells in these $Jh^{-/-}$ KO mice both within the BAL and lung compartments **(Figure 9)**. At present it is not clear why increased T cells in the lungs of vaccinated $Jh^{-/-}$ KO mice upon *M.tb* infection failed to translate to improved protection. As discussed above, increased inflammatory responses may represent a compensatory mechanism for B cell-deficient hosts to make up for impaired innate and T cell functionalities. Furthermore, it is also possible that the timing of T cell arrival at the lung is suboptimal. In this regard, we have previously reported that despite hyperactive T cell responses in the lung of TNF-alpha KO mice upon *M.tb* infection, these mice succumbed to infection due to overproduction of IFN-γ and IL-2 by these T cells and severe lung injury (63).

Conclusions and significance

The current data shows that intranasal AdHu5Ag85A vaccination induces cellular as well as humoral immunity specific to Ag85A in wild type (Wt) mice. Dampened antigen specific T cell responses were seen in the B cell KO mice ($Jh^{-/-}$) upon AdHu5Ag85A vaccination with enhanced neutrophil frequencies. Upon infection, these B cell deficient mice show enhanced immunopathology and as well as impaired *M.tb* control in the lungs compared to wild type mice. Taken together, this data suggests that respiratory mucosal AdHu5Ag85A-induced B cell immunity plays a significant role in vaccine mediated T cell protection against *M.tb* infection. However, further experimental investigations are underway to unravel the potential mechanism.

Currently, the majority of successful licensed vaccines are potent inducers of antibody immunity while there are no effective licensed T cell immunity-based vaccines. The BCG vaccine currently in use today elicits a T cell-mediated immune response that only attenuates disease progression within children. The need to develop vaccines that elicit both arms of the adaptive immune response has become increasingly important. As mentioned earlier, current vaccines against *M.tb* have focused exclusively on T cell mediated protection but have neglected to fully examine B cell immunity. In addition, the protective role of B cell and antibody immunity has never been studied in the context of mucosal vaccination against *M.tb.* Therefore in order to optimize vaccination strategies it is critical to first understand B cell mediated immune responses against intracellular

pathogens in order to design vaccines that induce both cell-mediated and humoral immune responses against *M.tb.* These studies will also shed further light on the functions of B cells in general and their involvement in protecting against intracellular infections. There are still a number of questions remained to be answered but hopefully further investigation into these cells will allow us to best utilize their functions for enhancing anti-TB immunity.

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6.0 APPENDIX- Data Figures

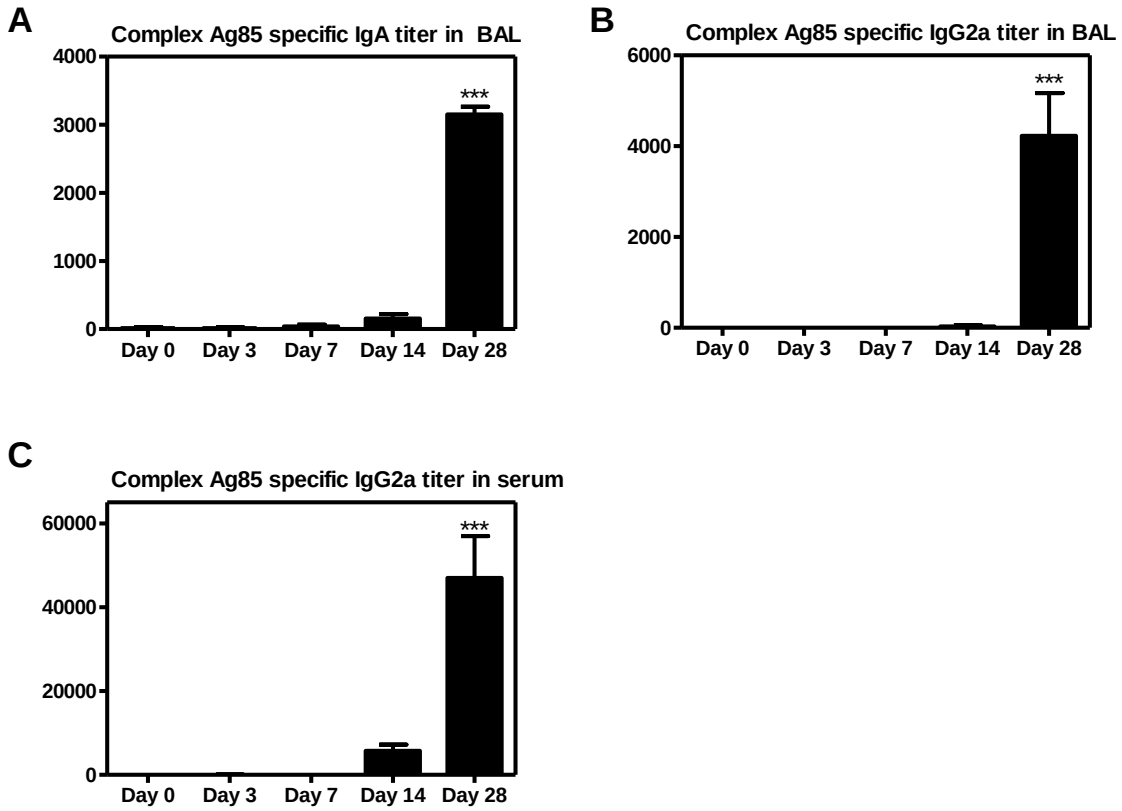

Figure 1. AdHu5Ag85A vaccination induces Ag85A-specific humoral immunity: ELISA was used to measure Ag85 specific **A)** IgA **B)** IgG2a antibody responses in the BAL of wild type (Wt) mice at various time points post-vaccination **C)** Ag85 specific IgG2a antibody titre in the serum of wild type at various time points post-vaccination. The data are expressed as mean ± SEM from three mice per group, representative of two independent experiments. ***, $P<0.001$ by one-way ANOVA.

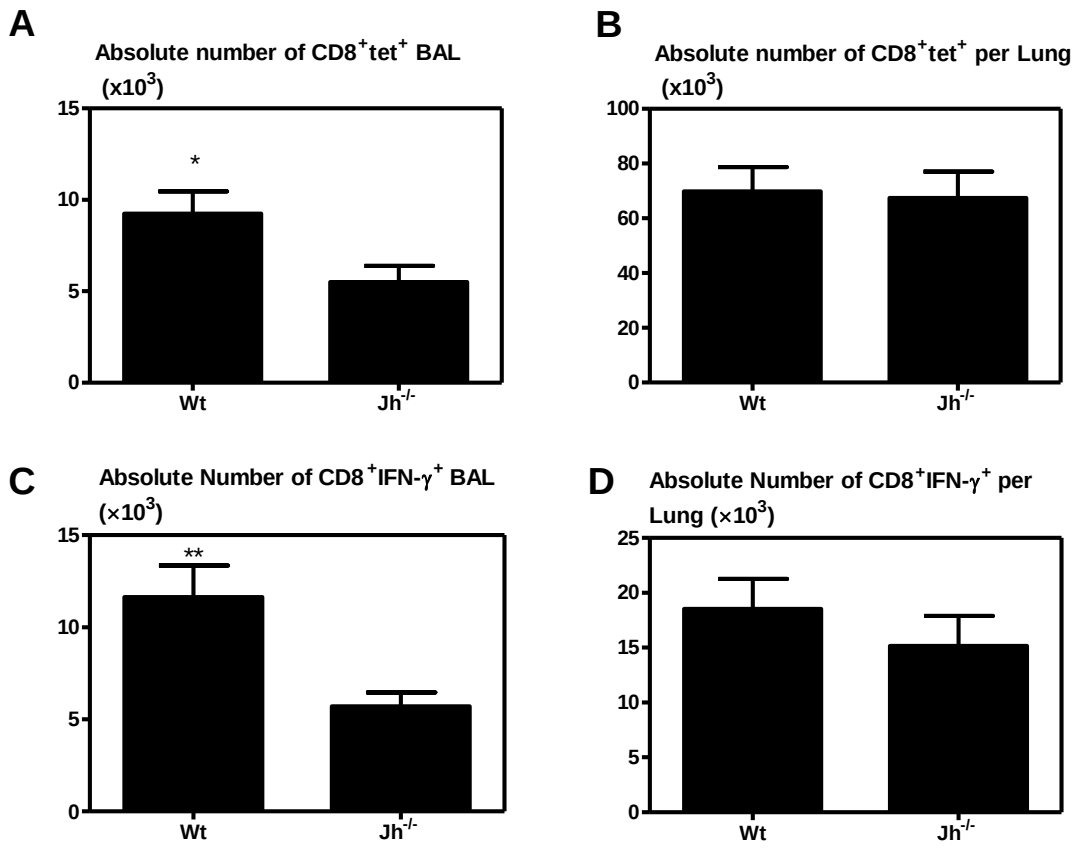

Figure 2. B cell KO (Jh $^{-/-}$) mice display reduced cellular responses in the BAL after AdHu5Ag85A vaccination. Ag85A antigen specific (tet^{+}) CD8^{+} T cells in the **A)** BAL and **B)** Lungs of wild type (Wt) and B cell KO (Jh$^{-/-}$) mice 4 weeks after AdHu5Ag85A vaccination. IFN-γ producing CD8^{+} T cells in **C)** BAL and **D)** Lungs. Data are expressed as mean ± SEM from three to four mice per group. The results displayed here are an average of five independent experiments. *, $P<0.05$; **, $P<0.01$ by t-test.

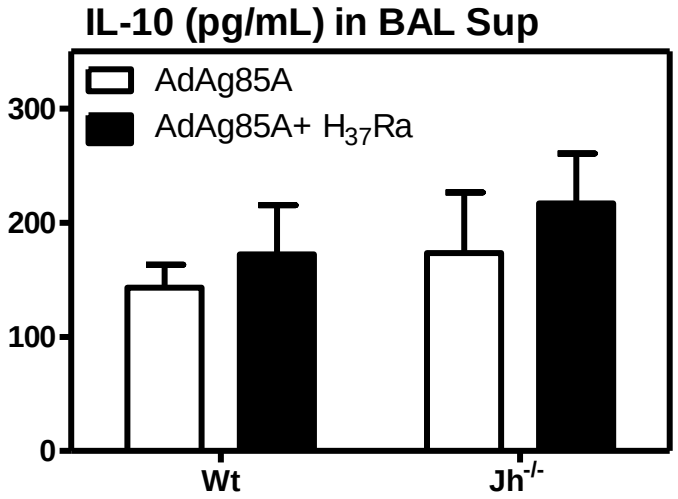

Figure 3. No significant difference in BAL IL-10 production between Wt and $Jh^{-/-}$ KO mice. Mice were vaccinated for 4 weeks with AdHu5Ag85A after which time they were given PBS or infected with H_{37}Ra *M.tb*. The data are expressed as mean ± SEM from four mice per group, representative of two independent experiments. No statisticantly significant difference was observed between the Wt and $Jh^{-/-}$ KO group.

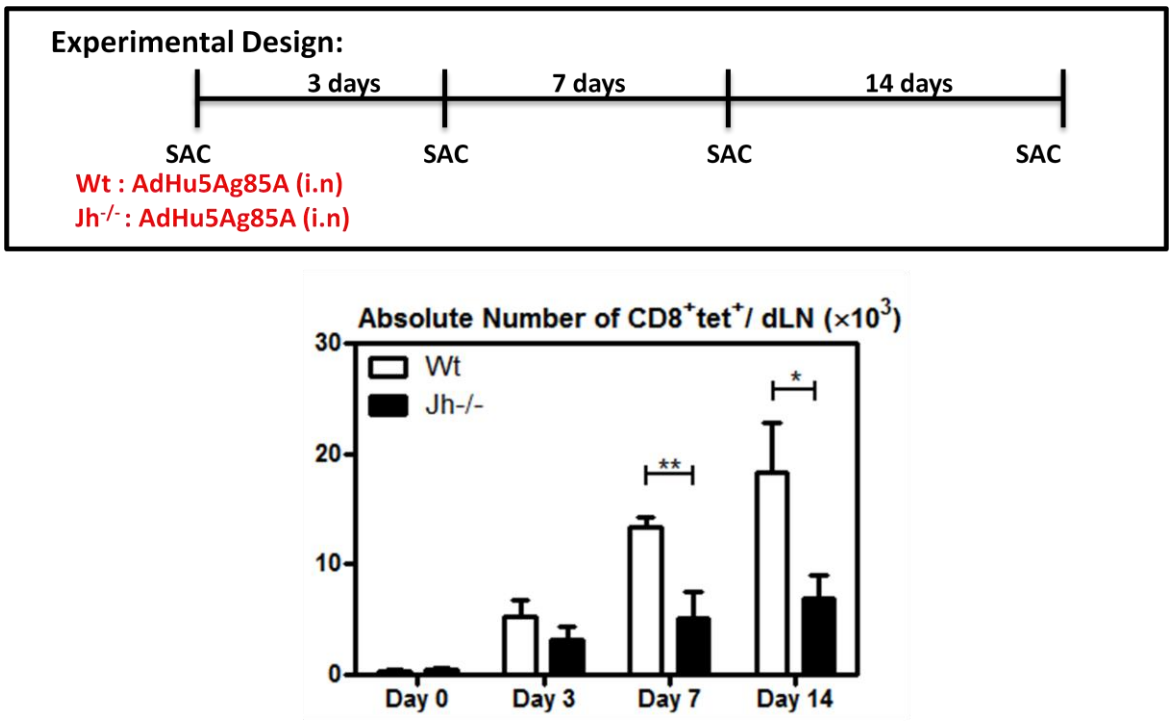

Figure 4. AdHu5Ag85A vaccinated $Jh^{-/-}$ KO mice display reduced antigen specific $CD8^+$ T cell responses in the mediastinal lymph node. Wt and $Jh^{-/-}$ KO mice were vaccinated for different time points at which point their the mediastinal lymph node were harvested. The data are expressed as mean ± SEM from three mice per group, representative of one experiment. *, P<0.05; **, P<0.01 by two-way ANOVA.

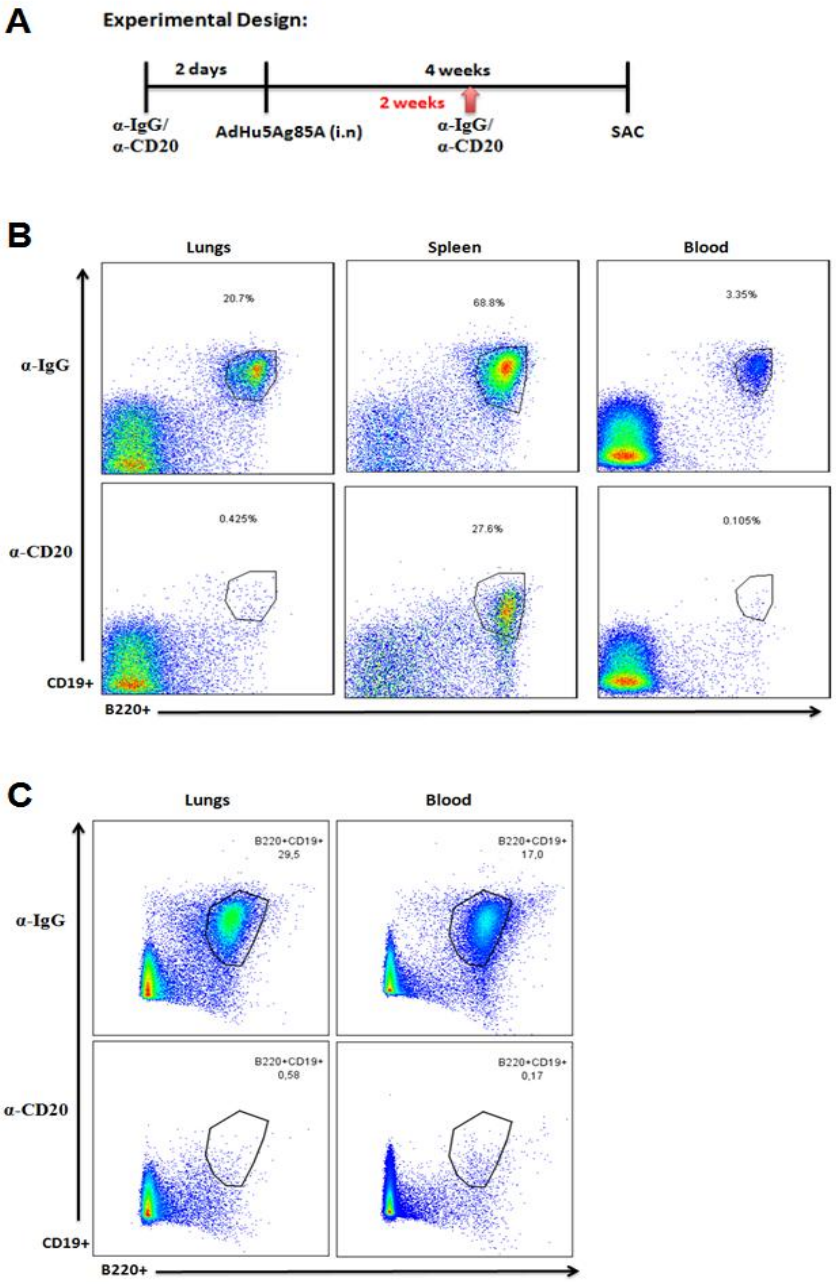

Figure 5. Verification of B cell depletion A) Experimental schema Balb/c mice were treated with an isotype control antibody IgG or α-CD20 antibody and flow cytometry was used to verify B cell depletion using $CD19^{+}B220^{+}$ gating. **B)** Two days after the first dose of depleting antibody **C)** Four weeks post- vaccination.

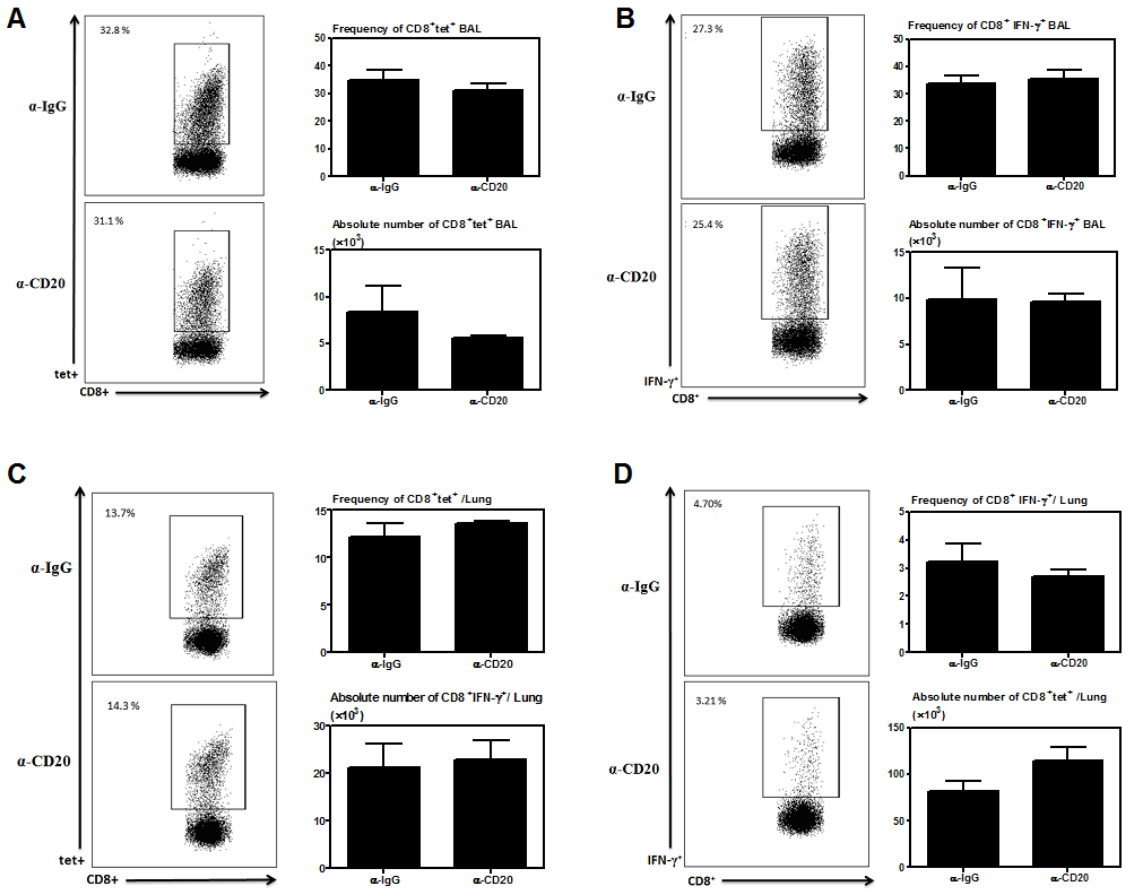

Figure 6. B cell depletion has no effect on antigen specific T cell responses induced by AdHu5Ag85A. Wild type (Wt) and α-CD20 depleted mice 4 weeks after intransal AdHu5Ag85A vaccination **A)** Ag85A antigen specific (tet^+) $CD8^+$ T cells in the lungs **B)** IFN-γ producing $CD8^+$ T cells in the BAL **C)** Ag85A antigen specific (tet^+) $CD8^+$ T cells in the BAL **D)** IFN-γ producing $CD8^+$ T cells in the Lungs. The data are expressed as mean ± SEM from four mice per group, representative of two independent experiments. No statistically significant difference was observed between the α-IgG and α-CD20 groups.

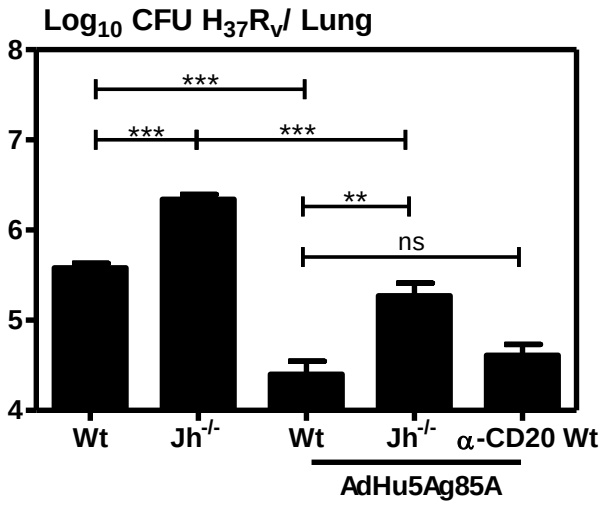

Figure 7. B cell KO ($Jh^{-/-}$) mice show reduced ability to control *M.tb* infection. *M.tb* burden in the lung of wild type and B cell KO ($Jh^{-/-}$) mice 2 weeks after *M.tb* challenge. The data are expressed as mean ± SEM from five mice per group, representative of two independent experiments. *, P<0.05; **, P<0.01; ***, P<0.001; (ns) not statistically signficiant by one-way ANOVA.

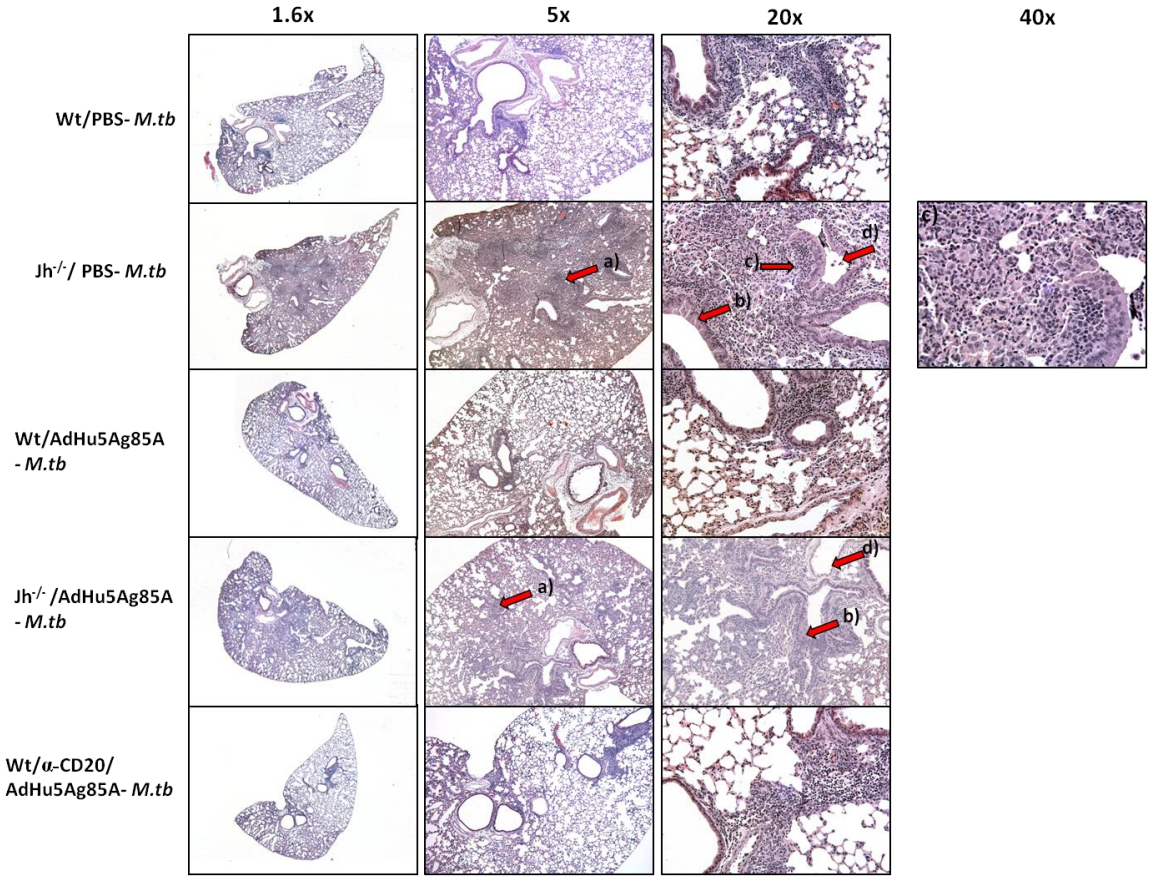

Figure 8. Histolopathological changes were assessed by H & E staining in the lungs of *M.tb* infected Wt and $Jh^{-/-}$ KO mice (n=5) either 2 weeks post-$H_{37}Rv$ challenge or 4 weeks post-AdHu5Ag85A vaccination and 2 weeks challenge **a)** granuloma formation **b)** bronchoepithelial hyperplasia **c)** neutrophilia **d)** intrabronchial inflammation. Note: Due to certain restrictions within the Level 3 facility, the lungs were not inflated with 10% formalin, only submerged in formalin.

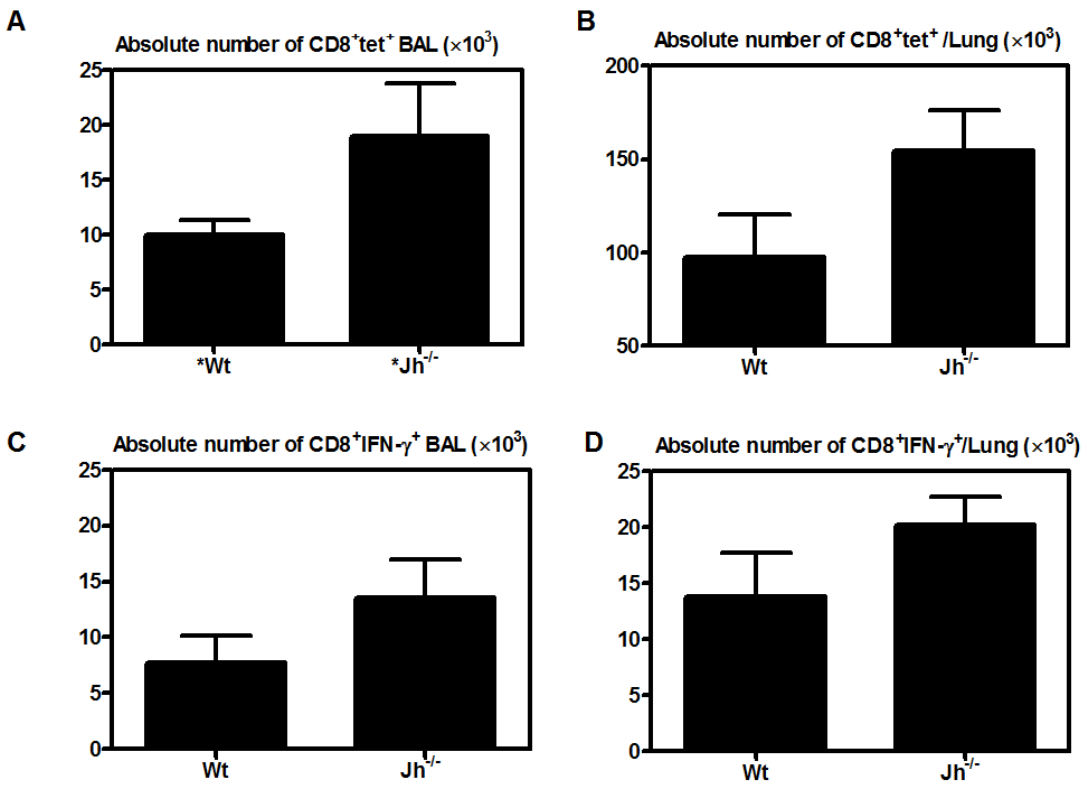

Figure 9. B cell KO ($Jh^{-/-}$) mice display increased cellular responses 4 weeks post-AdHu5Ag85A vaccination and 2 weeks post-*M.tb* H_{37}Ra challenge. Ag85A antigen specific (tet^{+}) $CD8^{+}$ T cells in the **A)** BAL and **B)** Lungs of wild type (Wt) and B cell KO ($Jh^{-/-}$) mice 4 weeks after AdHu5Ag85A vaccination. IFN-γ producing $CD8^{+}$ T cells in **C)** BAL and **D)** Lungs. The data are expressed as mean ± SEM from four mice per group, representative of one experiment. Not statistically significant.

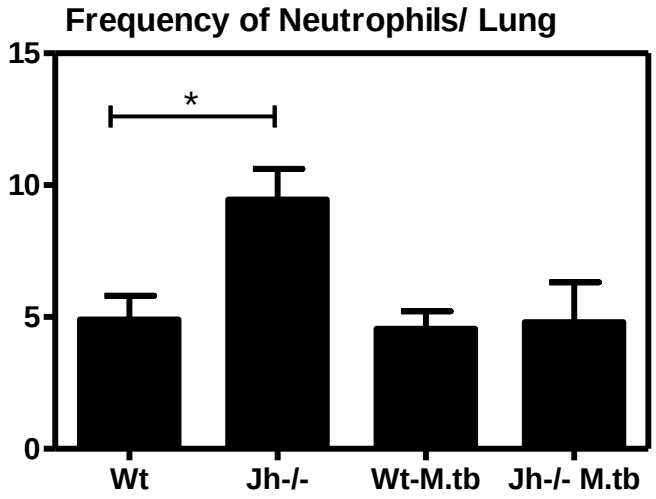

Figure 10. B cell KO ($Jh^{-/-}$) mice show increased neutrophil frequencies four weeks post intransal AdHu5Ag85A vaccination. No statistically signicant difference was seen between the Wt and $Jh^{-/-}$ KO mice that were AdHu5Ag85A vaccinated (four week period) and challenged with *M.tb* H37Ra (2 week period). The data are expressed as mean ± SEM from four mice per group, representative of two independent experiments. *, $P<0.05$ by one way ANOVA.

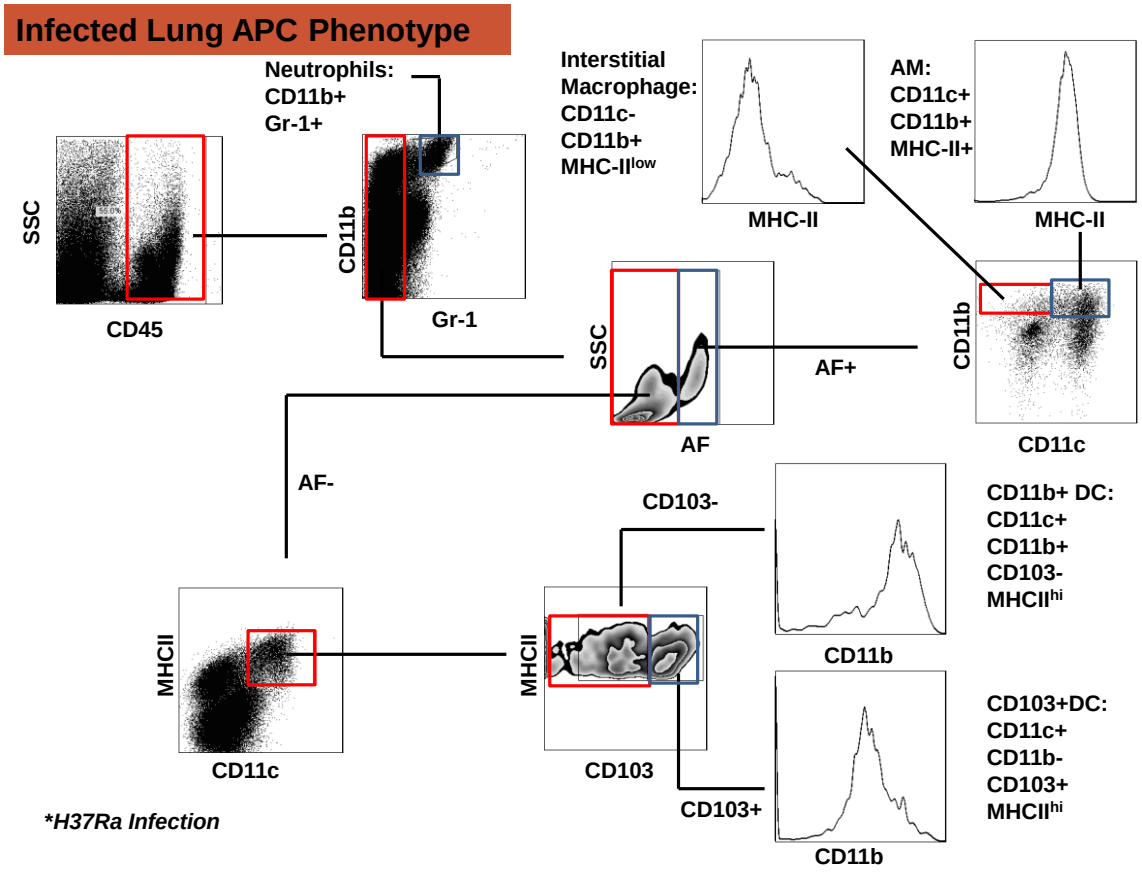

Figure 11. APC Flow gating strategy. AF=Autofluorescence, SSC= Side scatter