Development of chimpanzee adenovirus-vectored vaccine strategies against pulmonary tuberculosis

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Lay Abstract

The immense global tuberculosis (TB) burden highlights the shortcomings of current vaccination and antibiotic regimens. Novel prophylactic TB vaccines that can either boost or replace BCG entirely remains an active area of research. Additionally, the success of current antibiotic therapies against TB is hindered by their complexity and duration, with large percentages of patients failing to complete treatment.

Multi-armed approaches are required to properly and efficiently combat diseases. Besides prophylactic vaccines, development of therapeutic vaccine strategies as an adjunct to antibiotic treatment would represent another major step in TB control. To achieve such a goal, vaccines must consider the pathogen's life cycle, the immunological responses which they drive, and the populations in which they will ultimately be administered.

As such, the purpose of this dissertation is to utilize state-of-the-art molecular cloning techniques to construct novel chimpanzee adenovirus-vectored vaccines that provide prophylactic and therapeutic immunity against pulmonary TB. By considering different phases of the pathogen's life cycle, we aim to select a collection of antigens that are protective, regardless of disease state. Development of such platforms would lay and bolster the groundwork for improved vaccine strategies against TB.

Acknowledgements

Words simply cannot capture how immensely grateful I am for all the individuals whom I have met, interacted, and bonded with throughout my graduate studies. This journey has been more than just an academic opportunity of a lifetime; the skills that I have learned from my mentors, the experiences that I have lived, and the relationships I have made over the last 6 years only begin to capture what this experience has meant to me.

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Figures

Abbreviations

ABx……Antibiotics
AdCh68……Chimpanzee adenovirus serotype 68
AdHu5……Human adenovirus serotype 5
Ag85A……Antigen 85A
AM……Alveolar macrophage
ANOVA……Analysis of variance
APC……Antigen presenting cell
BAL……Bronchoalveolar Lavage
BALT……Bronchial Associated Lymphoid Tissue
BCG……Bacille Calmette-Guerin
CD……Cluster of differentiation
CFU……Colony forming unit
CTL……Cytotoxic T lymphocyte
DC……Dendritic cell
DNA……Deoxyribonucleic acid
EM……Environmental mycobacteria
FBS……Fetal bovine serum
GI……Gastrointestinal
I.M. ……Intramuscular
I.N. ……Intranasal
I.P. ……Intraperitoneal
IFN……Interferon
IL……Interleukin
KBP……Kilobase pairs
M.avium……Mycobacterium avium
M.marinum……Mycobacterium marinum
M.tb……Mycobacterium tuberculosis
manLAM……Mannose-capped lipoarabinimannan
MHC……Major histocompatibility complex
MVA……Modified Vaccinia Ankara
NALT……Nasal Associated Lymphoid Tissue
NHP……Nonhuman Primate
NK……Natural killer
NO……Nitric oxide
PBS……Phosphate buffered saline
RM……Respiratory mucosal
RNA……Ribonucleic acid
RNASeq……RNA Sequencing
RNI……Reactive nitrogen intermediates
RNS……Reactive nitrogen species
ROS……Reactive oxygen species
rpf……Resuscitation promoting factor
SD……Standard deviation
SEM……Standard error of mean
TB……Tuberculosis
Th1……T helper cell 1
TNFα……Tumor necrosis factor alpha
Treg……Regulatory T cell
T_{RM}……Tissue resident memory T cell
WHO……World Health Organization

Declaration of academic achievements

Chapter 3: Novel chimpanzee adenovirus-vectored respiratory mucosal tuberculosis vaccine: overcoming local anti-human adenovirus immunity for potent TB protection

*Jeyanathan M., *Thanthrige-Don N., ***Afkhami S**., Lai R., Damjanovic D., Zganiacz A., Feng X., Yao X-D., Rosenthal KL., Medina M Fe., Gauldie J., Ertl HC., and Xing Z. ***Mucosal Immunol*.** 2015; 8(6):1373-1387.

*Equal contribution

- M.J., N.T.D., S.A., and Z.X. conceived and designed the study.
- M.J., N.T.D., S.A., R.L., D.D., and A.Z. performed experiments.
- X.F., X-D.Y., K.L.R., J. G., and M.M.Fe constructed the adenoviral vector.
- H.C.E. provided key reagents.
- M.J., and S.A. analyzed data.
- M.J., S.A., and Z.X. wrote the manuscript.

Chapter 4: Single-dose mucosal immunotherapy with chimpanzee adenovirus-based vaccine accelerates TB disease control and limits its rebound following antibiotic cessation

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- S.A., M.J., and Z.X. conceived and designed the study.
- S.A. performed the majority of experiments with aide from R.L., M.R.D., M.V-S., A.Z., and Y.Y.
- S.A. analyzed data.
- S.A. wrote the manuscript with input from M.J., and Z.X.

Chapter 5: Markedly improved protection against established pulmonary tuberculosis by a multi-antigenic chimpanzee adenovirus-vectored vaccine targeting both acute and resuscitation antigens

Afkhami S., D'Agostino M.R., Vaseghi-Shanjani M., Lai R., Haddadi S., Imran A., Zganiacz A., Ertl C. Hildegund., Ottenhoff T., Mangalakumari J., and Xing Z. *To be submitted for publication*. 2019

- S.A., M.J., and Z.X. conceived and designed the study.
- SA performed the majority of experiments with aide from M.R.D., M.V-S., R.L., S.H., A.I., and A.Z.
- H.C.E., and T.O provided key reagents.
- S.A. analyzed data.
- S.A. wrote the manuscript with input from M.R.D., and Z.X.

Chapter 6: Spray dried human and chimpanzee adenoviral-vectored vaccines are thermally stable and immunogenic *in vivo*

Afkhami S.**, *LeCLair D.A., Haddadi S., Lai R., Toniolo S.P., Ertl H.C., Cranston E.D., Thompson M.R., and Xing Z. ***Vaccine. 2017;34 (22): 2916-2924.

*Equal contribution

- S.A., D.A.L., M.R.T., E.D.C., and Z.X. conceived and designed the study.
- S.A., and D.A.L. preformed the majority of experiments with aide from S.D., R.L., and S.P.T.
- S.A., and D.A.L. analyzed data.
- S.A., and D.A.L. wrote the manuscript with input from E.D.C., M.R.T., and Z.X.

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1.0 - Chapter 1 - Background

1.1 - Preface

Since its declaration as a global health emergency by the World Health Organization (WHO) in the 1990's, tuberculosis (TB) remains the leading cause of infectious-based death in the world[1]. In 2018, an estimated 10.4 million individuals developed disease, with 1.6 million ultimately succumbing to it. Milestones established by the WHO, such as the end TB initiative (expanded on in section 1.3), continue to fall short despite bolstered efforts by numerous health and regulatory organizations.

Additionally, newly emerging cases of drug-resistant TB have continued to rise, of which treatment failure remains staggered at approximately 50%. Such failure furthers the TB epidemic by feeding back into disease reactivation, and drug resistance[2].

These statistics scratch the surface as to the reasons why TB remains a global threat. As discussed in this dissertation, major innovative strategies are required to meet the end TB initiative, to which vaccines are a major component of.

1.2 - The clinical spectrum of TB disease and the host immune response

Classically, TB is characterised as a pulmonary disease. Tuberculosis however can manifest in nearly all organ systems and tissues[3]. As such, depending on the infected organ, disease etiology and respective treatment strategies vary. This section focuses on and dissects the pathological and host immune events that occur during pulmonary disease.

Tuberculosis pathogenesis is broadly defined by the formation of granulomatous foci[4]. In humans, this is moulded by complex host-mycobacterium interactions[4–6]. As such, to properly understand

TB pathogenesis requires a strong understanding of the host pulmonary physiology, and immunological / microbiological events that occur during infection.

1.2.1 - Early infection events and the innate immune response

Mycobacterium tuberculosis must bypass numerous intrinsic and immunological barriers within the respiratory mucosal tract in order to establish an infectious foothold[7]. **Figure 1** summarizes how these barriers protect against infection, and how *M.tb* exploits them to its advantage.

Infection occurs following inhalation of bacilli-laden droplets. Within the upper respiratory tract, droplets can be trapped within mucus secretions and subsequently drained innocuously into the gastrointestinal (GI) tract[7]. This occurs through drainage from the upper respiratory tract (nasal passages), or through the action of the mucociliary elevator within the trachea. Factors that dictate droplets deposition are believed to focus around droplet size. Droplets larger than 5μm are unable to reach the lower divisions of the respiratory tract and are more prone to be deposited in the upper respiratory mucosa[8,9]. Under certain circumstances, deposition of mycobacteria in the upper respiratory tract does not lead to clearance but rather may seed infection through bacterial uptake by microfold cells[10,11].

Droplets smaller than 5μm are more readily able to transverse into the lower airways, depositing into the luminal space of the alveoli. The alveolar space is physiologically designed to maximize gas exchange. As such, the environment is naturally tuned to be suppressed immunologically as to not interfere with this delicate process. This provides a favourable environment for infection. That being said, *M.tb* that do reach the alveoli encounter host soluble agents that possess anti-mycobacterial properties such as soluble antibodies, complement factors, and surfactant proteins.

Immune surveillance within the alveoli is executed by a population of alveolar macrophages (AM)[12]. These are the first immune cells to encounter, and subsequently become infected with *M.tb*. Although these cells are poised to maintain homeostasis and play roles in debris removal, they are capable of mounting protective immune responses against invading pathogens.

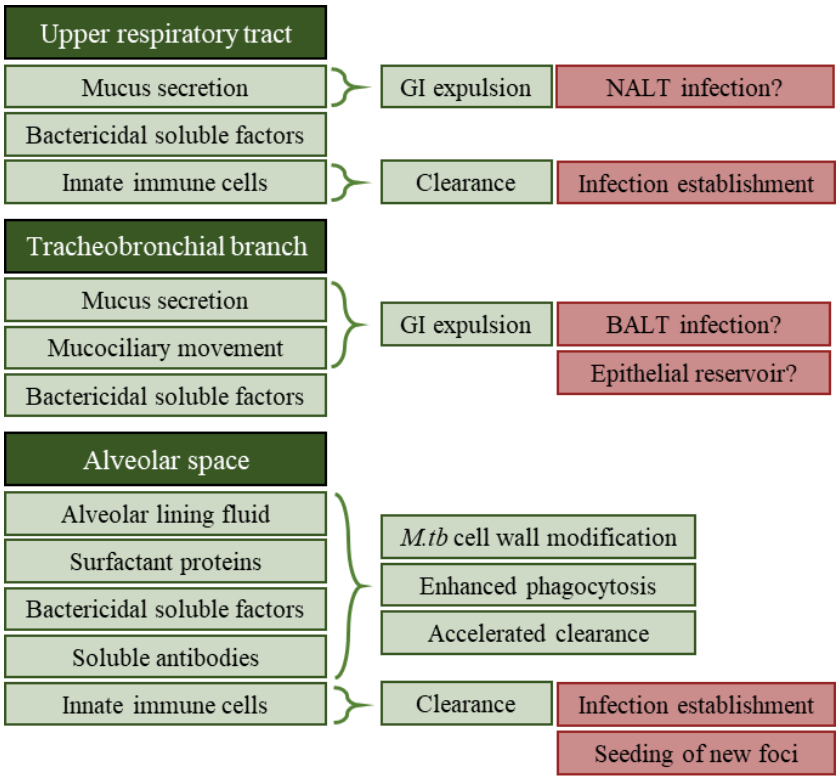


Figure 1. Anatomical and immunological barriers to *M.tb* infection. *Mycobacterium tuberculosis* (*M.tb*) must bypass physical and immunological barriers throughout the respiratory tract in order to establish infection. These barriers are largely successful in preventing infection; however they can also be exploited by *M.tb* to facilitate infection and persistence.

Following infection, AM attempt to eliminate *M.tb* through a series of processes[13,14]. These include (i) phagolysosome fusion and acidification, and/or generation of reactive oxygen (ROS) and nitrogen species (RNS) which limit mycobacterial access to nutrients and disrupts its metabolism, and (ii) exposure to antimicrobial peptides which disrupt the mycobacterial cell wall integrity. The effectiveness of such responses on their own is however limited as *M.tb* has evolved strategies to circumvent the host innate immune response. Such strategies focus on impairing the bactericidal

functionalities of macrophages. For example, inhibition of phagolysosome fusion, and impairment of inflammatory cytokine production are mediated in part by *M.tb* cell wall constituents mannose-capped lipoarabinomannan (manLAM), and phthioceral dimycocerosate (PDIM)[15–18]. Such events also can impair antigen presentation processes, thereby impact the adaptive responses. Collectively, such innate and subsequent adaptive immune suppressive events establish a niche for *M.tb* to grow.

Pro-inflammatory cytokines (primarily TNFα) secreted by infected AM trigger a cascade of leukocyte recruitment into the lung to aide in mycobacterial control[19–21]. Through a dynamic cycle of phagocytic cell recruitment, immune impairment, and failure to successfully clear infection, an organized structure of innate cells, known as the innate granuloma, is formed.

Imaging studies in the analogous model of zebrafish infection with *M.marinum* show that monocytes/macrophages recruited to the site of infection facilitates their exposure to, and infection with either extracellular mycobacteria or those encased within cellular apoptotic blebs/debris[22]. These macrophages express a highly motile phenotype, allowing them to become infected, and travel to other regions to seed secondary granulomas.

The central core of the nascent granuloma is largely composed of macrophages struggling to curb mycobacterial replication. Secretion of vascular growth factors promotes granuloma vascularization, thereby providing additional portals for innate, and eventual adaptive immune cell recruitment as well as bacterial dissemination[23–25].

The inability to control mycobacterial infection alongside the constant recruitment of immune cells eventually causes this central core to become apoptotic/necrotic[26]. The resulting cellular and bacterial milieu is phagocytosed by an outer ring of macrophages which eventually develop a lipid

engorged foamy phenotype. These events temporally precede, and continue alongside the induction of adaptive immune responses.

1.2.2 - Late infection events and the adaptive immune response

Adaptive immune elements only begin to furnish the lung three weeks following infection[27]. This contrasts with other bacterial infections such as with *Listeria monocytogenes*, where adaptive responses can be registered as early as 8 days post-infection[28]. This staggering delay is orchestrated by *M.tb* and occurs through a multitude of mechanisms including: (1) impairing the production of chemoattractant molecules that recruit antigen presenting cells (APC) such as dendritic cells (DC), (2) impairing the maturation and migration of DC to the draining lymph nodes (dLN), (3) differential recruitment of CD103+ DCs that impair T cell priming, and (4) modulation of lung natural regulatory T cells (Treg) to express immunosuppressive cytokines such as IL-10[18,29,30].

Collectively, the delay in the generation and recruitment of adaptive responses provide a temporal gap that allows *M.tb* to replicate relatively uncontrolled, establishing additional infectious foci and a solid foothold within the lung. As such, the recruitment of adaptive responses into the lung correlates with a plateau of infection, but not elimination of mycobacteria.

As *M.tb* is an intracellular, phagosomal pathogen, CD4 T cells represent the major subset of T cells involved in natural anti-TB immunity[27]. By recognizing infected macrophages through T cell receptor (TCR):MHC II interactions, CD4 T cells are capable of activating the bactericidal functionality of these cells by producing of IFNγ and TNFα[31,32]. These cells organize around the granuloma core, generating a lymphocytic cuff, particularly in infected human lungs, which provides intense immune stimulatory pressures to the infected core. *Mycobacterium tuberculosis* has also evolved mechanisms to bypass CD4 T cell immune pressures through neutralizing ROS and RNS induced by IFNγ and TNFα, or through phagosomal escape mediated by mycobacterial

virulence factors such as ESAT-6[33–35]. This exposes the now cytosolic mycobacterial antigens to MHC I machinery, thereby allowing CD8 T cell recognition. As such, CD8 T cell responses are also critical in anti-TB immunity through production of type 1 cytokines and direct killing of infected cells through cytolytic granules or through receptor mediated cytotoxicity[36–38]. Once again, the immunomodulatory capacity of *M.tb* allows for escape from endogenous CD8 T cell responses.

1.2.3 - Immune evasion and the shift to disease persistence

Infection with *M.tb* does not always lead to active disease. Rather, over 90% of immune competent individuals who become infected enter a state of latency[1]. Current dogma states that latent disease is associated with mycobacteria in a minimally replicative, quiescent form. Recent developments have shown that latency is a highly dynamic state involving constant interaction between the bacteria and the host[39,40].

Alongside host immune pressures, the nutrient barren and oxygen starved environment within the granuloma drives the transition away from actively replicating mycobacteria to a non-replicating, persistent state[41–43]. Although the molecular mechanisms of persistence remain to be fully elucidated, evidence suggests that shifts to fatty acid metabolism, and cell wall remodelling all play a role. Genetically, persistence is associated with significant increases in mRNA transcript stability, and up-regulation of latency-associated genes such as the ppGpp hyper-phosphorylated stringent response, and genes of the dosR regulon[44–46].

Establishment of persistence is not unique to just mycobacteria. Rather, numerous bacterial species such as *Micrococci* are capable of transitioning to a non-replicative state as a means to enhance its own survival[4748]. Given that the natural host of *M.tb* are humans however, persistence also has a role in its pathogenesis. Aside from shielding itself from the host immune response, this occult

bacterial population is phenotypically resistant to the major frontline anti-TB antibiotic regimens[49,50]. Since these drugs target bacterial cell wall formation, transcription, and metabolism, they only have bactericidal capabilities against actively replicating bacilli. Such processes are either ablated or significantly decelerated during persistence, significantly diminishing the efficacy of these drugs. This state of persistence can be maintained for the entirety of the host's natural life. Under certain conditions, *M.tb* can resuscitate, entering an actively replicating state.

1.2.4 - Mycobacterial resuscitation and disease reactivation

When the pressures that drive persistence are alleviated, mycobacteria can undergo phenotypic and metabolic changes that promote active replication. Resuscitation is mediated by the enzymatic action of a class of proteins known as resuscitation promoting factors (rpf). First discovered in *Micrococcus luteus*, genetic homologues of this protein class were also found within the genome of *Mycobacteria*. Specifically, five mycobacterial rpf proteins exist (rpfA/B/C/D/E)[49,51–53]. *In vitro* studies have shown that these enzymes function collectively in resuscitating non-replicating, persistent mycobacteria into an actively replicating state. Although the precise mechanisms of resuscitation are not fully known, microscopy studies show that rpf proteins co-localize with the cell membrane and at the junction during bacterial division, suggesting a role in cell wall remodelling during bacterial replication.

Pre-clinical studies have shown that deletion of *M.tb* rpf proteins impairs both mycobacterial growth during active disease and also ablates resuscitation from persistence[54–56]. This is in agreement with transcriptional studies which show that *rpf* expression is biphasic, expressed during the logarithmic growth phase, dropping during dormancy/latency, and exponentially increasing during resuscitation[57].

Akin to these pre-clinical findings, expression and immunological responses to rpf proteins are readily detectable in infected humans. Immunohistochemistry from pulmonary and lymphoid samples from individuals with active disease clearly show localization of rpf expression in, and around granulomatous lesions, particularly in areas of necrosis and/or caseation[51]. Immunologically, rpf-specific CD4 T cell responses are detectable in individuals with both active and latent disease. Longitudinal studies have shown proportionally higher expression of said responses in individuals who remain latent/subclinical, suggesting a role of these responses in maintaining latency[58].

1.3 - Towards the end TB initiative

1.3.1 – The pillars of the end TB initiative

The end TB initiative encompasses strategies to prevent new infections, complete treatment of current infections, and inhibit disease reactivation in latently infected individuals[1]. Current trends have clearly shown that the existing 2020 goal will not be met. As such, policies pertaining to this initiative have been modified in hopes of reaching the revised 2035 goal. Highlighted in **Figure 2** are the three pillars established by the WHO that form the foundational basis to reach this goal –

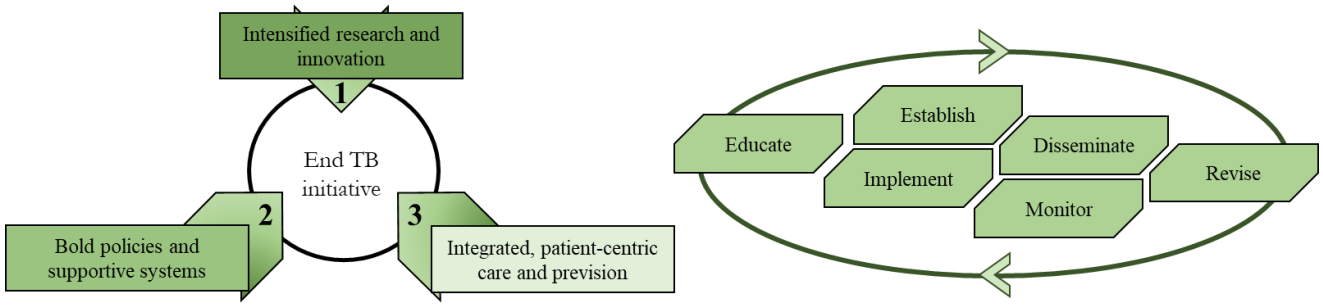


Figure 2. Foundational pillars and goals of the END TB initiative. Left panel; In order to achieve the END TB goal by 2035, there is a major requirement for (1) intensified research and innovation, (2) emboldened policies and systems to support these policies, and (3) improved patient-focused treatment. These are the foundational pillars of the END TB initiative, set by the WHO. Right panel; flow chart depicting the cyclical flow of information required to ensure new therapies, policies, etc. are properly being implemented, monitored, and revised when needed.

(1) research and innovation, (2) policy improvements and implementation, and (3) patient-centric healthcare. Intensified research and innovation will be the main pillar of focus in this dissertation.

1.3.2 – Grand Challenges in global health

The pillars of the end TB initiative fall under an umbrella of challenges set by the Bill and Melinda Gates Foundation (**Figure 3**). These challenges broadly encompass immunological, socioeconomic, and political issues. Solutions to said issues would have sweeping implications in improving global health in the developing world.

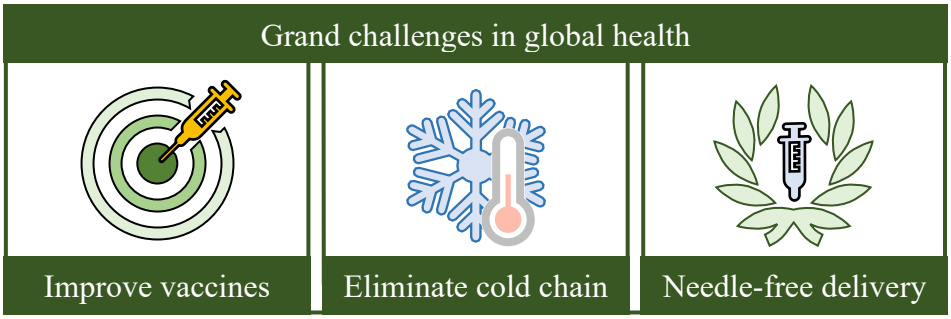


Figure 3. Grand challenges as set by the Bill and Melinda Gates Foundation. Major efforts are needed towards (1) improving the development of efficacious vaccines, (2), generate strategies that allow these vaccines to reach their target geographical location without reliance on cold chain, and (3) to be able to be deliver the vaccines in a needle free fashion.

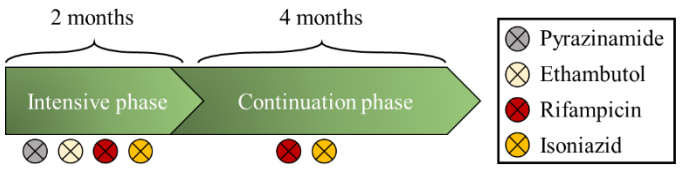
Solutions to these challenges are not only applicable for TB vaccines but for vaccines as a whole. In the context of TB vaccines, these challenges consist of: (1) developing an improved and efficacious vaccine, (2) eliminating dependency on refrigeration to maintain vaccine stability and activity, and (3) developing a strategy to eliminate the requirement of needles for vaccine delivery (see: https://grandchallenges.org/).

1.4 - TB antibiotic therapy and the clinical horizon of discovery

Given that individuals who suffer from untreated pulmonary TB on average infect 8-14 others, there is a dire need for bolstering early disease detection and treatment. Such goals once again centre around a foundational support of all the pillars depicted in **Figure 2**[1].

TB treatment is complex. As such, accurate and sensitive diagnostic tools are imperative for formulating appropriate antibiotic regimens. Although not a focus here, the advancements in TB diagnosis, in terms of sensitivity and global availability, have substantially approved. Systems like Gene Xpert represent one such technology, allowing for diagnosis of infection as well as antibiotic resistance in a matter of hours[1,59,60].

Figure 4 details a biphasic treatment regimen recommended by the WHO for treating drug-susceptible TB. This regimen requires 6 months of continued patient adherence to the therapy, which is divided into a 2-month intensive phase and a 4 month continuation phase[60]. The intensive phase is tailored to rapidly eliminate actively replicating bacilli, thereby eliminating the infectious nature of the disease. This includes a cocktail of rifampicin, isoniazid, ethambutol, and pyrazinamide. Given the heterogeneity in the mycobacterium population, the continuation phase is designed to be sterilizing in nature, eliminating all mycobacteria and preventing disease relapse. This includes a cocktail of rifampicin and isoniazid.



Antibiotic	Effect	Functionality
Pyrazinamide	Bactericidal	Impairs *M.tb* metabolism
Ethambutol	Bactericidal	Cell wall synthesis inhibitor
Rifampicin	Bactericidal	RNA synthesis inhibitor
Isoniazid	Bactericidal/static	Cell wall synthesis inhibitor

Figure 4. Antibiotic therapy for treatment of drug-susceptible TB. Left panel; Treatment of pulmonary TB requires 6 months of antibiotic therapy – a 2-month intensive phase of pyrazinamide, ethambutol, rifampicin, and isoniazid, and a 4-month continuation phase of rifampicin and isoniazid. Right panel; broad effects and targets of these antibiotics.

Although overall cure rates for drug-susceptible TB are upwards of 80%, a myriad of roadblocks continue to impede the complete success of these treatments (**Figure 5**). Of these, the treatment duration remains the main contributing factor[59,61,62]. Individuals who reside in TB endemic regions of the globe generally have poor patient-practitioner relations, which prevent dissemination of information regarding the importance of adherence in treatment success to the patients. As such, factors including, but not limited to, the adverse side effects associated with anti-TB therapy give rise to regimen changes, fragmented patient adherence, and ultimately lead to disease relapse and development of multi-drug resistant disease.

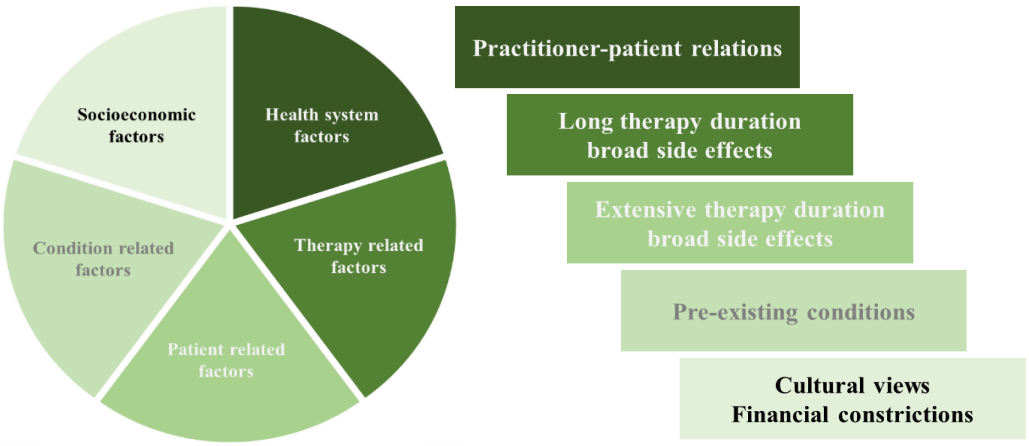


Figure 5. Factors contributing to fragmented patient adherence in anti-TB antibiotic therapy. Numerous factors contribute to individuals prematurely stopping antibiotic therapy. Pie graph (left) lists the major contributing factors, while boxes (right) provide an example.

Approximately 5% of new TB disease cases present with resistance to at least one of rifampicin and/or isoniazid. Of these, 10% show additional resistance against a multitude of second and third-line antibiotics. Treatment failure for cases of multi- and extensively-drug resistant disease exponentially increases, reaching as high as 80%[1,63]. The major contributing factor to such failure is treatment duration which exacerbates other treatment-related issues such as hepato/odo-toxicity, contributing to deteriorating quality of life. This highlights the need for developing strategies to accelerate successful treatment.

Although discovery of drugs that shorten therapy duration has been ongoing with multiple candidates reaching clinical evaluation, if history holds true, resistance to these new drugs will also inevitably develop[64]. Drug repurposing continues to be extensively investigated as well in improving treatment, with a focus on multidrug resistant disease. Drug classes such as oxazolidinones are showing surprising efficacy in a series of phase II clinical trials[65]. Regardless, the development of concurrent therapies that complement antibiotic regimens by enhancing bacterial clearance, thereby reducing the duration of antibiotic treatment serves as a means to alleviate the current roadblocks preventing successful treatment and cure.

1.5 - The current TB vaccine landscape

1.5.1 - Bacillus Calmette-Guerin

Bacillus Calmette-Guerin (BCG), an attenuated form of *Mycobacterium bovis*, remains the only clinically approved TB vaccine used to-date[1]. Although this near-century old vaccine has proven highly efficacious against severe, disseminated childhood forms of TB such as tuberculosis meningitis, it fails to confer protection against the adult pulmonary form of the disease. Elaborated on in section 1.6, such shortcomings are attributed to a variety of factors including: (1) impaired immunogenicity due to exposure to environmental mycobacteria, (2) immunization route and lack of immune responses at the respiratory mucosa, and (3) inadequate antigenic and immunogenic breadth. Furthermore, BCG has recently been deemed unsafe for use in HIV positive individuals, leaving this population extremely vulnerable to tuberculosis disease[66–70]. As such, there is an increasing need to find a suitable vaccine aimed at controlling this expanding epidemic. As BCG is still globally utilized and is deeply threaded into the WHO immunization program due to its efficacy in children, current research efforts put forth are in developing vaccines to boost pre-existing BCG immunity.

1.5.2 - Current TB vaccine pipeline

Given the sheer complexity of this disease, a "silver bullet" approach towards TB vaccine design is unlikely. Rather, effort must be put towards: (1) generating vaccines that target specific phases of the disease lifecycle (**Figure 6**), and (2) designing vaccines to drive "un-natural" immunological responses that do not mimic the non-sterilizing responses seen during natural infection[71–74]. As of

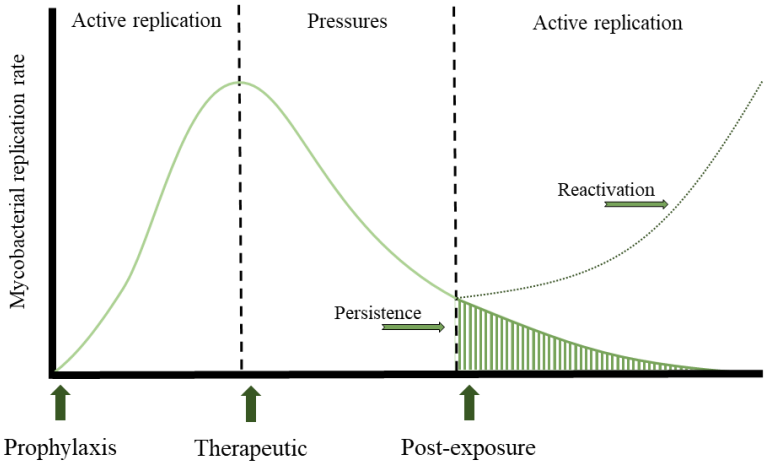


Figure 6. The *M.tb* life cycle and TB vaccination strategies. *Mycobacterium tuberculosis* has a complex life cycle. Initial infection is associated with rapid mycobacterial replication. Prophylactic vaccination strategies are designed as to prevent infection from occurring. Following establishment of infection, immunological and/or pharmacological pressures drive the bacteria into a non-replicating, persistent state. Therapeutic vaccination strategies are designed to be administered during active disease to accelerate bacterial clearance. Once mycobacteria are in a persistent state, they can maintain this for decades. Factors such as immune suppression can provide *M.tb* with a niche to resuscitate into an actively replicating state once again. Post-exposure vaccines are designed to be administered during latency to delay/prevent reactivation.

2018, there are over 12 TB vaccine candidates in the clinical pipeline. These are categorised by their intended purpose and include prophylactic, and therapeutic vaccination strategies.

1.5.3 - Prophylactic vaccination strategies

Most TB vaccines in pre-clinical or clinical development are designed to prevent initial infection. Three main formulations of prophylactic vaccines exist and include recombinant BCG strains,

subunit/adjuvant formulations, and viral-vectored platforms[75–78]. The former is designed to replace BCG while the latter two are designed to boost BCG immunity.

There is currently one recombinant BCG strains in clinical testing (VPM1002). This strain has been genetically modified to either express additional immunogenic antigens or drive stronger Th1-skewed immune responses. Although it has proven to be more immunogenic in humans, emerging safety concerns question the reasonability of this, and related platforms[75,76].

Subunit/adjuvant-based TB vaccines are designed to boost pre-existing BCG immunity and represent almost half of candidates in the clinical development pipeline. Such platforms are formulated to express multiple immune-dominant *M.tb* antigens alongside strong adjuvants which drive cell-mediated immune responses[75,76]. The majority of such subunit/adjuvant based-TB vaccines show excellent safety profiles and drive robust multifunctional T cell immune responses.

There are limitations to subunit vaccines such as the requirement of multiple administrations to induce strong immune responses, and the limited availability of adjuvants for respiratory mucosal delivery. Although, the results from a recent phase IIb trial with a subunit candidate M72 vaccine suggests the potential of this platform in preventing disease reactivation[79], with most of the protected being young males. Additionally, it is important to highlight that these candidates have been exclusively administered parenterally – as detailed below, immunization route is a critical consideration in TB vaccine design[74].

Viral-vectored vaccines are amongst the most promising candidates. Unfortunately, the most advanced candidate, a modified vaccinia Ankara expressing the *M.tb* immune dominant antigen 85a (MVA85A), failed to further prevent infection over BCG when administered parenterally in a recent phase IIb clinical trial[80]. Although a setback, results from such failed clinical trials have

provided invaluable information that is currently being utilized in designing new prophylactic TB vaccines. A recently published trial assessing respiratory mucosal delivery of MVA85A is a prominent example of such progresses[81].

Adenoviral-based TB vaccines represent the other major subset of viral-vectored TB vaccines, with human adenovirus serotype 5 (AdHu5) representing the most immunogenic serotype[82]. A large body of work from our lab has extensively characterised the prophylactic potential of an AdHu5-based TB vaccine expressing Ag85A (AdHu5Ag85A)[83–87]. We have shown that respiratory mucosal immunization with AdHu5Ag85A induces robust, long-lasting vaccine-specific CD8 T cell responses within the lungs, providing significant protection from bacterial dissemination and from pulmonary TB in multiple animal models. Results from a recent phase I clinical trial have further shown that AdHu5Ag85A induces robust polyfunctional CD4 and CD8 T cell responses in BCG-immunized individuals, highlighting the potential efficacy of this vaccine in humans[85].

In contrast to AdHu5, there have been extensive studies assessing the pre-clinical as well as clinical efficacy of other human adenoviral serotypes as TB vaccine vectors, such as AdHu35. Although such vectors induce robust, multifunctional T cell immune responses, they have proven ineffective in protection – even when administered via aerosol. Recent work has shown that immunization with recombinant AdHu35-based vaccines induces strong type 1 interferon responses which promote natural killer (NK) cell-mediated killing of virally-infected APCs, drastically reducing transgene expression and vaccine efficacy[88]. These findings are in accordance with those seen by Jeyanathan *et al.* which show that type 1 IFN responses elicited by an alternate TB vaccine, VSV85A, drastically impaired vaccine-mediated anti-TB immunity[89]. It is probable that the disappointing efficacy of this AdHu35-vectored vaccine may therefore be correlated with the

robust type 1 IFN-inducing properties of its backbone[89]. This contrasts with the negligible type 1 IFN responses induced by AdHu5Ag8A for example and may explain the disparity in protection between the two vectors. Such undesired innate responses to the viral vectored vaccines which can impede vaccine efficacy warrants thorough investigation of viral vectors prior to TB vaccine design.

1.5.4 - Therapeutic vaccination strategies

Global TB control requires multifaceted approaches to both prevent infection and bolster current TB antibiotic therapies as to enhance patient compliance. Immunotherapeutic approaches represent a newly emerging facet of TB vaccine designed to fulfill the aforementioned task. Therapeutic TB vaccines are designed to be administered as an adjunct to conventional antibiotic therapy in order to enhance/modulate host immune responses against *M.tb*, thereby accelerating bacterial clearance and resolution of disease. This in turn allows for a reduction in the duration of antibiotic regimens without risk of disease relapse[90,91].

The development and assessment of therapeutic TB vaccines is still in its infancy with only a handful of candidate vaccines entering pre-clinical and clinical testing. Summarized below are advancements in this field from the most relevant candidates, RUTI and *Mycobacterium vaccae*.

RUTI. The RUTI vaccine is composed of detoxified cellular fragments of *M.tb*, which is grown under hypoxic conditions in order to expand the antigenic profile of the bacterium[92]. Pre-clinical murine studies have shown that RUTI induces Th1-skewed polyantigenic, polyfunctional CD4 and CD8 T cell responses within the lungs[93]. When administered prophylactically, RUTI provides similar protection as BCG and also reduces progression to active disease from latent infection. When used therapeutically, RUTI synergises with conventional chemotherapy regimens reducing bacterial burden and preventing the formation of granulomatous lung lesions[94].

Phase I clinical trials have provided equally promising results highlighting the ability of RUTI to drive polyantigenic immune responses following subcutaneous administration in BCG naïve, *M.tb* negative volunteers[95]. Although these studies collectively provide strong rationale to assess the therapeutic efficacy of RUTI, this avenue of questioning is not being directly addressed. Instead, RUTI is suggested only to be utilized in latently infected individuals as a means to prevent disease reactivation[90].

The choice to not use RUTI during active disease is unsurprising as this therapeutic vaccine candidate induces moderate-to-severe adverse side effects at the site of injection. Specifically, in the aforementioned phase I clinical trial, RUTI induced sterile granulomatous panniculitis[95]. This exacerbated immune response, primarily due to *M.tb*-derived endotoxins, would be further exacerbated in individuals with active disease.

***Mycobacterium vaccae (M.vaccae)*:** *Mycobacterium vaccae* is a nontuberculous mycobacterium that is rarely pathogenic in humans. Heat-killed variants have been utilized as adjuncts to TB chemotherapy with varying success. Similar to RUTI, *M.vaccae* induces polyantigenic Th1 and cytotoxic CD8 T cell responses to antigens shared by *M.tb*, allowing it to provide cros-protecton[96].

Clinical trials have proven *M.vaccae* is well tolerated in both LTBI and HIV positive individuals, and does not exacerbate immune responses as seen with RUTI in healthy individuals. Phase I and II trials have also shown that *M.vaccae* seems to synergise with antibiotic therapy in improving bacterial clearance, enhancing radiologic clearance of disease, and improving cure rates even in patients with multi-drug resistant (MDR) disease[97].

Although these studies collectively highlight the immunotherapeutic potential of *M.vaccae*, they also highlight the inadequacies of this vaccine. Firstly, *M.vaccae* is only efficacious following administration of multiple doses. In fact, improved clinical outcome in MDR-TB patients was only registered after 12 successive daily doses. Furthermore, there is limited and conflicting data assessing vaccine efficacy between different immunization routes.

There is a steady progress being made towards development of therapeutic TB vaccines. However, a therapeutic TB vaccine with unquestionable efficacy and safety profile remains to be developed. Learning from what has been accomplished so far, next-generation therapeutic TB vaccines must: (a) be safe and not exacerbate immune responses, (b) promote polyfunctional T cell immune responses within the lungs, (c) require as few administrations as possible to improve compliance, and (d) enhance bacterial control and improve clinical outcomes as a means to reduce antibiotic therapy duration.

1.6 - Improving TB vaccine strategies

The main strategy driving the design and development of TB vaccines remains focused on generating immunological responses with proven protective correlates in relevant animal models. Successful candidates that translate into the clinic are first tested for their safety and immunogenicity (mainly in healthy volunteers from developed countries) prior to their protective assessment (mainly in at-risk groups in TB-burdened countries). Deeper understandings of the immunological correlates of anti-TB immunity are central to vaccine development. However, looking beyond the immunological lens at how other factors may impact vaccine accessibility and efficacy is also important, and often overlooked.

1.6.1 - Antigen selection

One of the main challenges in designing recombinant TB vaccines is identifying immunogenic antigen(s) which are protective against *M.tb* infection. Antigen identification for *M.tb* is a complex process due to its multi-stage life cycle[98–102]. As shown in **Figure 7**, the antigenic profile of this bacterium drastically varies as it goes from active replication (during early infection/active disease), to stressed once subjected to immunological/pharmacological pressures, to dormancy/persistence (during latent disease). Such fluctuations in antigen expression represent a major hurdle in successful TB vaccine design as the bacterium can escape vaccine-mediated immune pressures. Such observations highlight a major limitation of vaccines with limited antigenic breadth and thus must be taken into consideration if we are to improve upon existing methodologies. Vaccines expressing or consisting of antigens that span the entirety of the *M.tb* life cycle represent a strategy in not only broadening anti-TB immunity, but providing the foundation for more efficacious prophylactic, therapeutic, and post-exposure vaccine design. To date, a limited number of vaccine candidates have been designed to express such broadened antigen profiles. These candidates combine expression of early/acute-phase antigens (such as members of the Ag85A family), alongside dormancy antigens, and have shown promise in providing broadened anti-TB immunity[103,104]. Regardless, there are numerous additional antigens which remain to be investigated. These include persistence antigens (such as the dosR genes), or during reactivation (such as the rpf genes).

Secondly, the *M.tb* genome expresses multiple functionally redundant proteins that differ in immune dominant epitopes. This is a mechanism of immune evasion, which provides the bacterium a means to avoid the immunological pressures of host immunity and vaccines[100]. In this regard, whereas monovalent vaccines have limited efficacy as they eventually select for escape

variants of the bacterium, multivalent vaccines provide a means to circumvent such selective pressures. Multivalent vaccines generate a more diverse population of T cells recognizing a broader range of *M.tb* antigens, increasing the likelihood of such adaptive immune responses to recognize and kill infected cells. Finally, a practical concern for emerging vaccines is preservation of vaccine efficacy in a genetically outbred population due to the wide variety of HLA haplotypes.

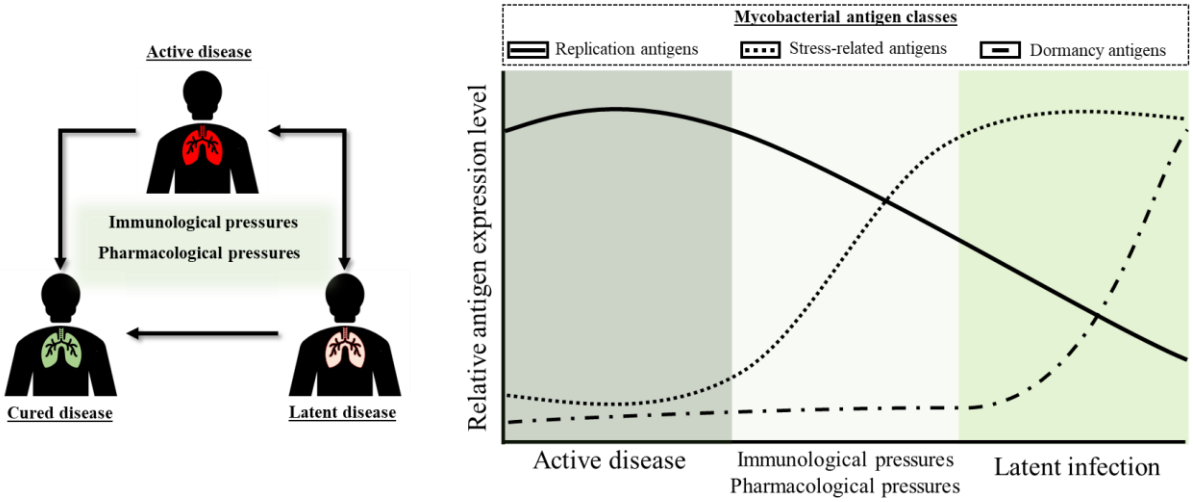


Figure 7. The multi-staged life cycle of *M.tb*. (left panel). Immunological and pharmacological pressures during active disease can either fully cure disease or drive latency. Latently infected hosts can subsequently be cured, or may act as a bacterial reservoir, capable of reactivating into an active disease state. (right panel). Such shifts in the mycobacterial life cycle are associated with significant fluctuations in its antigenic profile. Such shifts are critical considerations in the design of TB vaccines.

A potential issue with monovalent vaccine design is poor immunogenicity in a human sub-population due to restrictions or limitations in HLA expression. Multivalent vaccines aim to induce broad immune responses against multiple antigens as a means to enhance vaccine efficacy in a genetically heterogeneous population.

1.6.2 - Immunization route

Compelling evidence suggests that robust protection against mucosal pathogens such as *M.tb* is heavily reliant on the presence of pathogen-specific immune cells at the primary site of

infection[77,83,105]. As stated above, mycobacterial control is achieved when *M.tb*-specific T cells furnish the lung parenchyma and airways.

As portrayed in **Figure 8**, immunization route largely dictates the anatomical location of antigen-specific T cells[106]. Pre-clinical studies show that parenteral immunization with TB vaccines are capable of driving robust antigen-specific T cell immune responses. However, such cells are primarily restricted to the periphery (restricted in the lumen of the pulmonary vasculature and peripheral lymphoid tissues), unable to enter the lung parenchyma and airway lumen[74,107,108]. Following pulmonary *M.tb* infection, there is a delay in these cells extravasating into the lung parenchyma. This delay is reminiscent of that seen during natural infection, and fails to confer protection against pulmonary TB (**Figure 8, right panel**). For example, parenteral immunization with BCG fails to establish immune responses within the lung interstitium and airways in the early stage of infection, and this may partially contribute with its poor protective capacity against pulmonary TB.

In stark contrast, respiratory mucosal (RM) immunization induces T cell responses that furnish both the lung parenchyma and airways (**Figure 8, left panel**)[106]. Poised at the primary site of infection, these cells rapidly recognize and control mycobacterial infection, thereby thwarting the ability of *M.tb* to establish an infectious foothold. T cells induced following RM immunization can also assume a tissue resident memory (T_{RM}) phenotype, capable of remaining within the respiratory mucosa for long periods of time in an antigen-dependent manner, poised to rapidly respond following infection[107–114]. This speaks to the longevity of the responses induced by RM immunization.

Viral-mediated RM immunization has recently been shown to train the pulmonary innate immune cells[115]. In particular, following respiratory exposure to adenovirus, AMs have been seen to be

take on a memory-like, bactericidal trained phenotype as indicated by upregulation of activation markers like MHC II, defence-ready gene signatures, and greater production of inflammatory cytokines. This study additionally suggests that a properly designed RM vaccine strategy has the added advantage of training the innate immune system to also be better equipped at protecting against both *M.tb*, but also non-specifically against other respiratory pathogens such as *Streptococcus*.[115]

Additionally, RM immunization represents a strategy to circumvent the requirement of needles for administration by replacing it with standard syringe or puffer systems (refer to **Figure 3**).

Despite the overwhelming body of evidence supporting the importance of RM immunization in immunity against TB, it is surprising that a majority of TB vaccine candidates in testing are administered parenterally. A potential explanation for this is regarding the amenability and feasibility – as there are various anatomical restrictions as to whether certain vaccine formulations

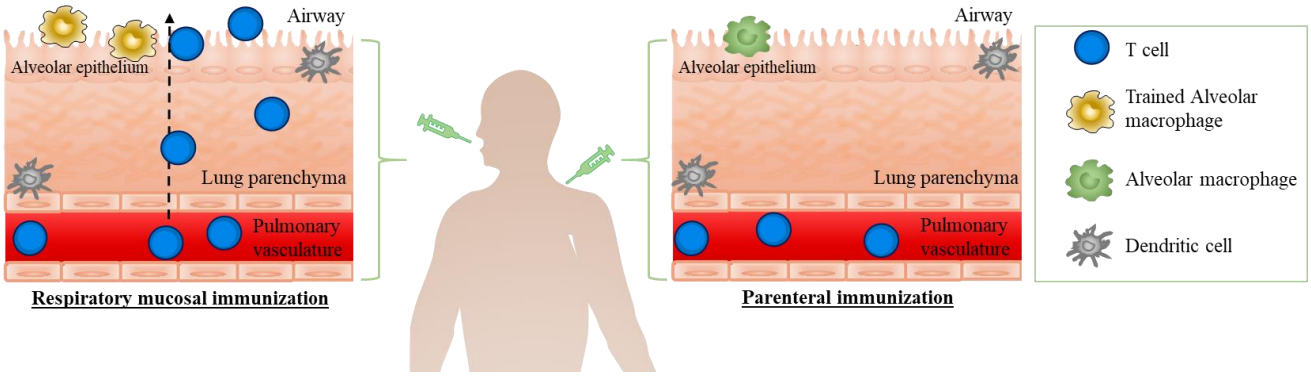


Figure 8. Immunization route and vaccine efficacy. Right panel; Parenteral immunization induces systemic vaccine-specific T cell responses which are not able to access and furnish the lung parenchyma and airways. Left panel; Respiratory mucosal immunization induces vaccine-specific T cell responses which can enter the lung parenchyma and airways taking on a T_{RM} phenotype. Additionally, RM immunization trains the lung AM population, making them poised to more rapidly respond to invading pathogens. Collectively, these cells are poised at the portal of *M.tb* infection and thus can provide immediate protection following infection.

are amenable for RM immunization. This is one of the main reasons as to why viral vectors are promising candidates for TB vaccine design.

1.6.3 - Pre-existing immunity

Unlike the controlled environments used in pre-clinical settings, the human immune system is exposed to an array of infectious, immunomodulatory agents. Depending on the nature of the exposure, this can abrogate a vaccine's intended efficacy, despite its findings in immunologically naïve, pre-clinical animal models.

Exposure to environmental mycobacteria (EM) is a classical example as to how environmental factors significantly impact vaccine efficacy. It is not a coincidence that the efficacy of BCG is lowest in tropical regions where EM exposure is at the highest[116]. This stems from homology between EM, such as *Mycobacterium avium* (*M.avium*), with that of *M.bovis* BCG. Immunologically, EM exposure modulates the immune system such that following BCG immunization there is a drastic abrogation of pro-inflammatory Th1 immunity and a shift to tolerogenic regulatory T cell (Treg) responses as indicated by IL-10 production.

As previously mentioned, AdHu5 has been a promising candidate for TB vaccine design in numerous pre-clinical models[83,86,87]. Clinical application of human adenoviral-based vaccines however has been potentially hindered due to the high prevalence of pre-existing anti-human adenovirus immunity. Up to 100% of individuals in the TB-endemic region of sub-Saharan Africa are pre-immune to AdHu5. Mechanistically, humoral and cellular immune responses intended to be mounted against transgenes expressed by such adenoviral vaccines are also generated against the vector backbone itself. Neutralizing antibody (nAb) responses abolish viral infectivity while cytotoxic CD8 T cell responses kill virally infected cells such as APCs. Collectively this leads to

a significant reduction in vaccine transgene expression, immunogenicity and ultimately protective efficacy[117,118]. The detrimental impact of pre-existing anti-vector immunity on vaccine efficacy is highlighted by the 2007 Merck HIV trial in which the titre of AdHu5 nAb correlated with reduced AdHu5-based vaccine immunogenicity and protective efficacy[119]. Although these conclusions demonstrate that immune status is an important consideration when designing vaccines, it is important to note that pre-existing immunity may not always hinder vaccine efficacy.

For example, a recent clinical phase I clinical trial with AdHu5Ag85A demonstrated not ablation of vaccine immunogenicity despite detectable levels of anti-Adhu5 immunity[85]. Additionally, the magnitude of pre-existing immunity is well known to vary between anatomical sites. For example, respiratory mucosal levels of anti-AdHu5 antibodies is significantly lower than those in circulation. As such, respiratory mucosal immunization route may represent a viable way of circumventing this effect[120].

1.6.4 - Vaccine stability

An effective TB vaccine has little worth if it cannot be feasibly distributed, stored, and implemented in its target populations. Viral-vectored vaccines are mainly stored in aqueous solutions and therefore are dependent on cold chain temperatures at -80°C to retain viral integrity[121]. Cryopreservation is required as molecular movements above these target temperatures drive denaturation of viral genetic and protein components. Such requirements are not feasibly achieved in TB burdened regions due to economic, infrastructural, and geographical restrictions. This emphasizes the need to generate strategies to minimize/eliminate cold chain requirements, and represents one of the major WHO goals.

Lyophilization has long been used as a strategy to stabilize biologics. For example, BCG encapsulated in l-leucine has proven to stabilize the vaccine for upwards of four months with

minimal activity loss[122]. Stabilization strategies however are not homogenous amongst biologics. For example, stabilization of antigen-based influenza vaccines can be done through use of inulin, providing stability for upwards of 3 years at ambient temperatures[123]. These observations highlight how the stabilization strategies must be fine-tuned for the given biologic and for a given requirement.

Rather than suspending viral-vectored vaccines in cryoprotective aqueous media, virions can alternatively be enclosed within amorphous structures composed of organic excipients through a process known as vitrification[124]. This can be achieved through a technique known as spray drying wherein the aqueous viral solution is mixed with carbohydrate excipients, and dispersed/rapidly evaporated into a glassy powder[125]. This process replaces hydrogen bonds initially between the virus and the aqueous media with those between the virus and sugar excipient, thereby stabilizing and preventing molecular movements. Spray drying also allows for fine-tuning physical properties of the generated powders. This allows for generation of particles which are amenable for aerosol/RM mucosal administration. This represents an additional strategy to not only eliminate cold chain, but also generate vaccine formulations which can be delivered needle-free.

1.7 - Chimpanzee adenoviruses as novel platforms for TB vaccine design

Chimpanzee adenoviruses (AdCh) are emerging as promising platforms for vaccine design[126]. This is attributed to their capacity to induce stronger immune responses than their human adenoviral counterparts without being impacted by pre-existing immunity against human adenoviruses[126]. Chimpanzee adenovirus vectors can also be easily amplified in commercially available cell lines used for developing AdHu-vectors, but without the development of replication-competent viruses[127].

To date, four chimpanzee adenovirus serotypes (AdCh3, 6, 63, and 68) have advanced into pre-clinical and clinical testing as vectors for mucosal pathogens including influenza, HIV, and TB. Of these serotypes, AdCh68 is documented as being one of the most immunogenic[113,128–130].

Chimpanzee adenovirus serotype 68 (AdCh68) was first isolated in the 1970's from the mesenteric lymph nodes of chimpanzees. Initial studies have characterized AdCh68's viability as a vaccine platform in direct comparison to AdHu5. One such study has indeed shown that in comparison to AdHu5, AdCh68 shows comparable infectivity, reproducibility, and stably accommodates large transgene inserts. More interestingly, simple immunogenicity studies have shown AdCh68 to induce much greater magnitudes of CD8 T cell responses. Pre-existing immunity studies have also shown that sera from both patients and murine models immunized with multiple human adenoviruses, including AdHu5 do not cross-react with AdCh68. Even though this virus shows sequence homology of upwards of 90% with AdHu5, structural studies have shown that high heterogeneity in the primary nAb-targeted B cell epitopes within the hexon protein exist, explaining why there is no cross reactivity[131].

To date, AdCh68 has been utilized for developing vaccines for rabies and HIV. These studies have provided murine and non-human primate (NHP)-based evidence for potent humoral and cellular immune responses generated by this vector in comparison to AdHu5-based vaccine platforms[132,133]. Murine-based studies utilizing AdCh68 as vectors for intranasal rabies immunization have shown to elicit markedly increased antibody responses in comparison to AdHu5, which translated to more robust protection against lethal rabies challenge at a much lower vaccine dose. Also seen in NHPs, pre-existing AdHu5 immunity completely abolished AdHu5-based vector immunogenicity; AdCh68 vaccine immunogenicity was not affected. Murine studies utilizing AdCh68 as an HIV vaccine vector have shown that this platform elicits much more robust

cytotoxic cellular responses than AdHu5, conferring greater protection, even in the presence of pre-existing human adenovirus immunity.

2.0 - Chapter 2 – Rationale, objectives & overarching hypothesis

2.1 - Rationale

The grand challenges identified by the Bill and Melinda Gates foundation sets the path towards the development and implementation of feasible, accessible, and effective vaccination strategies. Tuberculosis remains one of the focal points of these challenges given its immense global health impact.

Despite much progress in the development of novel TB vaccination strategies, no candidates to-date have proven completely effective. As the major driving force in TB vaccine design comes from our understanding of immune correlates during natural infection, it questions whether this strategy can yield an effective vaccine strategy. As such, there is an urgent need for driving the discovery of newer vaccine strategies to steer away from the status quo which has proven ineffective.

An effective vaccination strategy is most likely to stem from understanding how *M.tb* modulates and adapts to natural immunity during infection. By understanding the antigenic shifts that occur during the life cycle of this bacteria, vaccines can be engineered not only as prophylactics, but also as adjunctive therapeutics to antibiotic therapy.

Most TB vaccines under clinical assessment are parenterally administered through single-to-multiple injections. The inability of this immunization route in inducing robust innate and adaptive immunity within the lung mucosa represents a major factor in the ineffectiveness of these candidates.

In stark contrast, RM immunization can not only be done through needle-free technologies, but is also well documented in generating long-lived or memory innate and adaptive immune responses directly in the lung mucosa[74,108]. Of the vaccine vectors amenable for RM administration, chimpanzee adenoviruses continue to be the most promising.

In addition to their natural tropism for the respiratory mucosal tract, chimpanzee adenoviruses are genetically malleable and less limited in the number of antigens which they can express, do not require the addition of adjuvants to be immunogenic, and pre-existing anti-vector immunity is globally negligible. The developmental landscape of chimpanzee adenoviral-vectored TB vaccines is extremely limited. As such, this dissertation embodies the studies with the following Objectives.

2.2 - Objectives

1) Develop a monovalent chimpanzee adenovirus-vectored TB vaccine for respiratory mucosal application and assess its prophylactic efficacy in murine models pre-exposed to human adenovirus (**chapter 3**).

2) Determine the therapeutic potential of a monovalent chimpanzee adenovirus-vectored TB vaccine in accelerating disease control as adjunct to conventional antibiotic therapy (**chapter 4**).

3) Develop a multivalent chimpanzee adenovirus-vectored TB vaccine that provides broadened immunity against the entire *M.tb* life cycle (**chapter 5**).

4) Determine whether spray drying adenovirus-vectored vaccines with sugar excipients stabilizes the vaccine and eliminates cold chain requirements (**chapter 6**).

2.3 – Overarching hypothesis

We hypothesize that compared to its human adenoviral counterpart, the chimpanzee adenoviral-vectored TB vaccine is superior in its protective immunity for both prophylactic and therapeutic respiratory mucosal applications. A multivalent chimpanzee adenovirus-based TB vaccine targeting various stages of *M.tb* life cycle provides further enhanced protective immunity. Spray drying with a properly chosen excipient formulation can be used to increase the thermostability and feasibility of real-world applications of these vaccines.

3.0 - Chapter 3 - Developing a monovalent chimpanzee adenovirus-vectored TB vaccine for respiratory mucosal application and assessing its prophylactic efficacy in murine models pre-exposed to human adenovirus

Manuscript title: Novel chimpanzee adenovirus-vectored respiratory mucosal tuberculosis vaccine: overcoming local anti-human adenovirus immunity for potent protection.

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Novel chimpanzee adenovirus-vectored respiratory mucosal tuberculosis vaccine: overcoming local anti-human adenovirus immunity for potent TB protection

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Pulmonary tuberculosis (TB) remains to be a major global health problem despite many decades of parenteral use of Bacillus Calmette–Guérin (BCG) vaccine. Developing safe and effective respiratory mucosal TB vaccines represents a unique challenge. Over the past decade or so, the human serotype 5 adenovirus (AdHu5)-based TB vaccine has emerged as one of the most promising candidates based on a plethora of preclinical and early clinical studies. However, anti-AdHu5 immunity widely present in the lung of humans poses a serious gap and limitation to its real-world applications. In this study we have developed a novel chimpanzee adenovirus 68 (AdCh68)-vectored TB vaccine amenable to the respiratory route of vaccination. We have evaluated AdCh68-based TB vaccine for its safety, T-cell immunogenicity, and protective efficacy in relevant animal models of human pulmonary TB with or without parenteral BCG priming. We have also compared AdCh68-based TB vaccine with its AdHu5 counterpart in both naive animals and those with preexisting anti-AdHu5 immunity in the lung. We provide compelling evidence that AdCh68-based TB vaccine is not only safe when delivered to the respiratory tract but, importantly, is also superior to its AdHu5 counterpart in induction of T-cell responses and immune protection, and limiting lung immunopathology in the presence of preexisting anti-AdHu5 immunity in the lung. Our findings thus suggest AdCh68-based TB vaccine to be an ideal candidate for respiratory mucosal immunization, endorsing its further clinical development in humans.

INTRODUCTION

Pulmonary tuberculosis (TB) caused by *Mycobacterium tuberculosis* (*M.tb*) has haunted mankind for thousands of years and still remains a top infectious killer.[1–4] TB causes ~1.4 million deaths and 9 million new cases each year. An estimated one-third of the world population is latently infected by *M.tb* and 5–10% of these people develop active TB some time in their lives. Despite availability of antibiotics, increased multidrug-resistant or extensively drug-resistant TB cases continue to pose a global threat. Bacillus Calmette–Guérin (BCG), which has been used in humans for many decades, remains the only licensed anti-TB vaccine. BCG is given once via the skin shortly after birth in most countries. Although BCG effectively protects against disseminated childhood TB, it has failed to effectively control adolescent and adult pulmonary TB.[3,5] Thus, there is an urgent need to develop novel TB vaccines that can be used for effective boost vaccination following parenteral BCG priming in humans.[6]

One of the immune evasion strategies that *M.tb* utilizes is to significantly slow down the appearance of T-cell immunity in the lung.[7] Such delay or "immunological gap" allows *M.tb* a foothold in the lung before immunity is established,[7–10] and this is likely one of the reasons for the ineffectiveness of parenteral BCG vaccination in humans.[6,7] Therefore, it is believed that

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effective boost vaccination strategies ought to fill such a gap in T-cell immunity in the lung.[6,7,11,12] Recent preclinical research from us and others has identified the respiratory mucosal route of vaccination to be the most effective way to equip the lung, particularly the surface of the respiratory mucosa (the airway luminal compartment), with protective T-cell immunity.[6,11–14] Mucosal vaccination does so via activating mucosal-specialized dendritic cells and upregulating mucosa-homing molecules on T cells.[10,15,16] Nevertheless, almost all of the TB vaccine candidates currently in the TB vaccine pipeline are still being developed as parenteral vaccines for human application.[4] In this regard, the immune adjuvant used for parenteral delivery of protein-based TB vaccines is unsafe for respiratory mucosal applications.[17,18] Replicating mycobacterial-based TB vaccines are known to cause granulomatous inflammation and cannot be easily cleared from the lung, thus deemed unsuitable for respiratory mucosal vaccination. Recent report on the inability of intradermally delivered MVA (modified vaccinia Ankara)-based TB vaccine to enhance protective immunity in a major milestone phase 2b trial[19] speaks further to the urgency and the importance of the effort in developing different vaccination strategies such as mucosal vaccination against pulmonary TB.

However, developing both safe and effective respiratory mucosal-deliverable vaccines represents a unique challenge and has recently been identified, at the National Institutes of Health (NIH)/Aeras Aerosol TB Vaccine Workshop, to be a new priority.[6,13–15,20] Recombinant, replication-deficient virus-vectored TB vaccine platforms represent the most attractive approach for respiratory mucosal vaccination.[12,21–23] Based on its unrivaled potency, the group C human serotype 5 adenoviral vector system (AdHu5) has been widely used for developing vaccines against infectious diseases and cancer.[21,22,24–26] Indeed, an AdHu5-based TB vaccine developed by us was robustly protective, particularly when given via the respiratory tract, in a number of animal models.[6,12,21] This vaccine has also recently been evaluated following intramuscular administration in a phase 1 clinical study.[27] However, a major gap and limitation to the application of AdHu5-based vaccine to humans is the pervasive preexisting anti-AdHu5 immunity resulting from respiratory exposure to AdHu5 virus that not only compromises the potency of the vaccine but may also pose a safety concern when given to HIV high-risk populations.[22,24,25,28–32] Although human adenoviruses of rare serotypes such as AdHu35 may circumvent this issue, such vector is poorly immunogenic because of its strong type 1 interferon (IFN)-inducing ability.[33–35]

Recently, replication-defective chimpanzee-derived adenoviruses (AdCh) have become an attractive alternative to human adenoviral counterparts.[33,35] Neutralizing antibodies against AdCh vectors are rarely found in humans.[29,33–35] Furthermore, as most AdCh viruses utilize the same cell entry receptors as AdHu5, its immunogenicity is comparable to that by AdHu5. The large-scale production is also readily achievable as the same viral packaging cell line for AdHu5 can be used for amplifying AdCh vectors. Thus, AdCh technology has recently been exploited to develop vaccines for malaria, HIV, Ebola, and hepatitis C virus.[33,35–39] However, up to date, AdCh vector technology has not been translated to developing TB vaccines. Furthermore, its potential for respiratory mucosal application and the effect of pulmonary anti-AdHu5 immunity on its potency are still poorly understood.

In this study, for the first time we have applied the new technology for developing a novel replication-defective AdCh-based TB vaccine for respiratory mucosal application. By using preclinical models of human pulmonary TB with or without preexisting anti-AdHu5 immunity, we provide compelling evidence that respiratory-mucosally delivered AdCh-based TB vaccine activates high levels of T-cell responses, provides robust protection, and limits lung pathology at a capacity comparable to or even better than its AdHu5 counterpart. Importantly, contrary to AdHu5Ag85A, respiratory mucosal AdCh68Ag85A-induced protective T-cell immunity is minimally affected by anti-AdHu5 immunity preexisting locally in the lung. Our study supports the AdCh-based TB vaccine to be an ideal candidate for respiratory mucosal immunization in humans and thus warrants its further clinical development.

RESULTS

Molecular construction and characterization of a chimpanzee adenovirus vector AdCh68-based TB vaccine

A replication-deficient chimpanzee type 68 adenovirus was constructed to encode a secreted form of Ag85A, a highly immunogenic secreted antigen of *M.tb* (AdCh68Ag85A), by using a recently described direct cloning technology[40] (**Figure 1a**). Expression of Ag85A is under control of a murine cytomegalovirus promoter and its secretion by mammalian cells is promoted by a human tissue plasminogen signal peptide sequence (tissue plasminogen activator). All Ag85A genetic expression elements in AdCh68Ag85A are identical to those in its human type 5 adenovirus-based TB vaccine AdHu5Ag85A.[41] AdCh68Ag85A was packaged and propagated in 293 cells and purified under the Good Laboratory Practice conditions. Using a monoclonal antibody specific for Ag85A, the production and secretion of Ag85A protein were characterized in lung epithelial A549 cells by western immunoblotting. The expected size of ~30 kDa Ag85A protein was detected in both the culture supernatant and cellular lysate from A549 cells infected with AdCh68Ag85A but not from those infected with empty vector AdCh68 (**Figure 1b**). The levels of Ag85A antigen production by AdCh68Ag85A were found to be comparable to those from the cells infected with the same dose of AdHu5Ag85A (**Figure 1b**). These observations form the basis to compare AdCh68Ag85A with AdHu5Ag85A for their safety, immunogenicity, and protective efficacy *in vivo*.

Respiratory mucosal inoculation with AdCh68Ag85A elicits desired innate immune activation and minimal tissue inflammation in the lung

To begin evaluating the suitability of AdCh68Ag85A for respiratory mucosal vaccination, we first assessed to which degree the vector induced an inflammatory reaction in the lung. To this end, mice were inoculated intranasally (i.n.) with

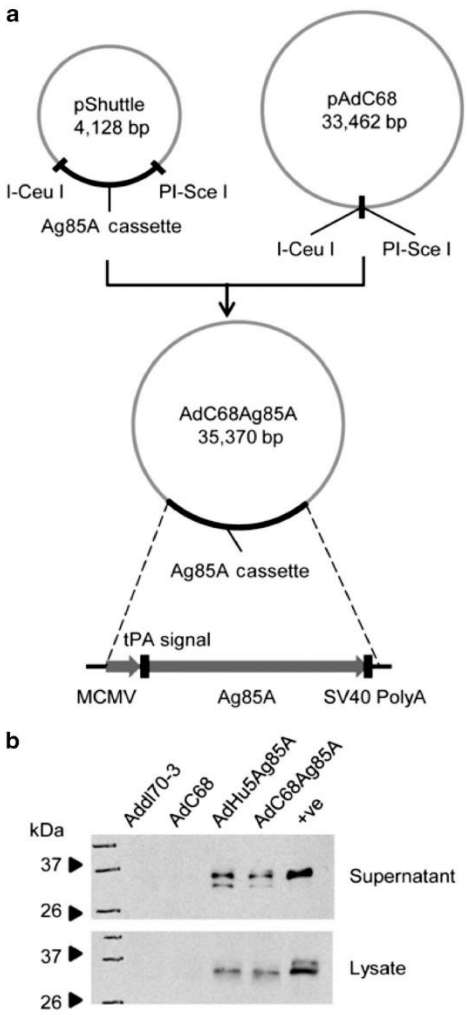

Figure 1 Molecular construction and characterization of AdCh68Ag85A. (**a**) An Ag85A genetic cassette was cloned into the pShuttle plasmid and was then excised with the indicated enzymes. The I-Ceu1 and PI-Sce1-excised insert was subcloned into the I-Ceu1 and PI-Sce1 site of pAdC68 genomic clone to generate recombinant AdCh68Ag85A virus. (**b**) Characterization of Ag85A protein expression levels by AdCh68Ag85A-infected A549 cells was performed in culture supernatant and cellular lysate by using western blots and compared with the cells infected with AdHu5Ag85A. The empty viral vectors Add70-3 and AdC68 were used as negative controls for AdHu5Ag85A and AdCh68Ag85A, respectively. Recombinant Ag85A protein was used as a positive control (+ve). MCMV, murine cytomegalovirus promoter, tPA, tissue plasminogen activator.

AdCh68Ag85A. As a comparison, a group of mice were inoculated with an equal dose of AdHu5Ag85A. By histopathological examination at days 1 and 3 after AdCh68Ag85A, only mild inflammatory infiltrates were seen in the peripheral bronchial and vascular areas compared with naive mice (**Figure 2a**). To further examine the inflammatory and innate immune responses in the lung, we analyzed the major cellular components in bronchoalveolar lavage fluid (BAL). The majority of innate immune cells in the BAL of AdCh68Ag85A lungs were alveolar macrophages at days 1 and 3 (**Figure 2b**). A degree of transient neutrophilic responses was observed only at day 1 but hardly at day 3 (**Figure 2b**). We also examined the responses of innate immune cytokines tumor necrosis factor-α and IFNβ in the BAL. Tumor necrosis factor-α is a type 1 immune cytokine,[7] whereas IFNβ is a type I interferon usually induced during viral infection and has been shown to suppress anti-TB T helper type 1 cell immunity.[42–44] Although intranasal inoculation of AdCh68Ag85A triggered a level of tumor necrosis factor-α production, the level of IFNβ was very low at the both time points (**Figure 2c,d**), in agreement with our previous finding.[42]

Upon comparison we found that the proinflammatory responses induced by AdCh68Ag85 were largely comparable to its human AdHu5Ag85A counterpart in histopathology (**Figure 2a**), inflammatory cellular responses (**Figure 2b**), and innate immune cytokine responses (**Figure 2c,d**). The above data together suggest that although AdCh68Ag85A possesses desired immune adjuvant properties, it is safe for respiratory mucosal administration. Furthermore, like its AdHu5 counterpart, AdCh68Ag85A triggers a negligible level of undesired type 1 IFN responses.

Respiratory mucosal AdCh68Ag85A immunization induces robust T-cell responses in the lung

We next examined the T-cell immunogenicity of AdCh68Ag85A following respiratory mucosal vaccination. The mice were vaccinated i.n. once with AdCh68Ag85A as described above. For comparison, some mice were immunized i.n. with an equal dose of AdHu5Ag85A. T-cell responses were analyzed in the BAL and lung tissue at 2 and 4 weeks after vaccination (**Figure 3a**) by Ag85A tetramer (tet+) and intracellular IFNγ (IFNγ+) immunostaining. AdCh68Ag85A vaccination induced high levels of Ag85A tetramer-positive CD8 T cells (tet+CD8+) in the airway lumen (BAL) and lung interstitium (Lung) at 2 weeks (**Figure 3b**). By 4 weeks, the magnitude of tet+CD8 T cells contracted significantly. AdCh68Ag85A vaccination induced similarly increased responses of IFNγ-producing CD8 T cells in both the BAL and lung upon stimulation with an immunodominant Ag85A peptide (**Figure 3c**). AdCh68Ag85A vaccination also increased CD4 T-cell responses but at much lower levels than CD8 T-cell responses. In comparison, although AdHu5Ag85A vaccination also induced potent CD8 T-cell responses in the BAL and lung, the overall levels of T-cell responses at 2 weeks in the BAL were significantly lower than AdCh68Ag85A. Similar to AdCh68Ag85A, T-cell responses contracted significantly at 4 weeks after AdHu5Ag85A vaccination (**Figure 3b,c**).

To further evaluate the functionality of respiratory mucosal CD8 T cells activated by intranasal AdCh68Ag85A vaccination, we examined the cytolytic capability of these cells by using an *in vivo* intratracheal cytotoxic T cell (CTL) killing assay previously developed by us.[45] Consistent with robust CD8

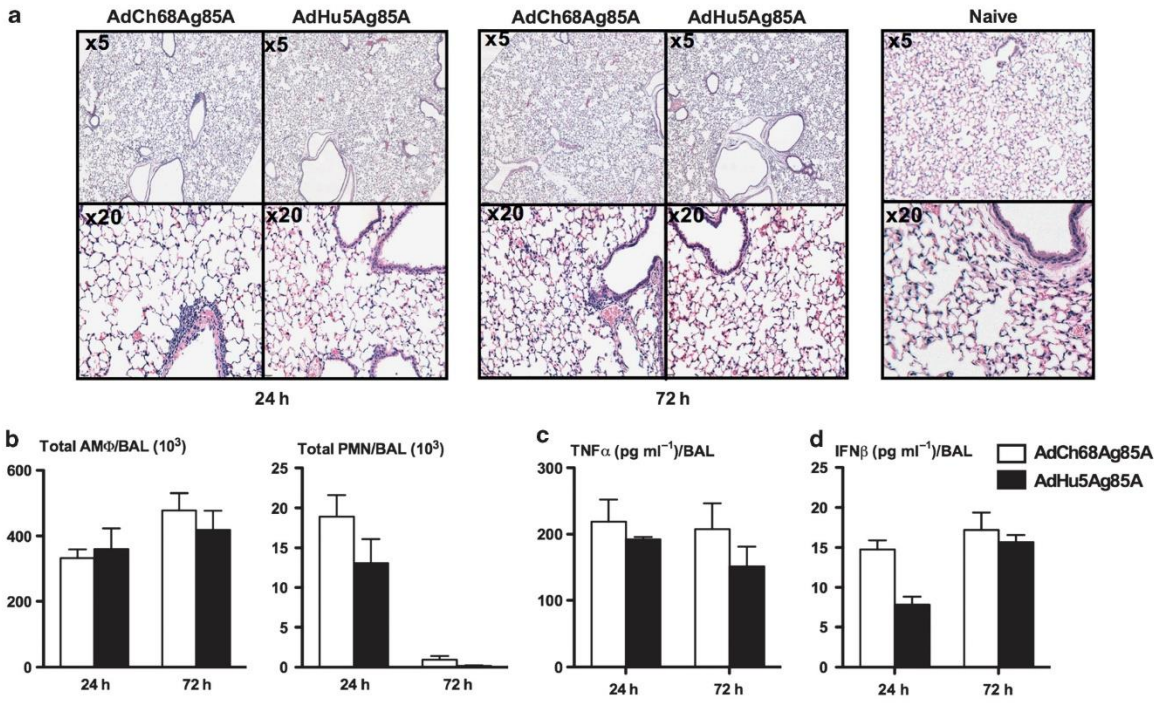

Figure 2 Innate immune activation and minimal lung inflammation by intranasal inoculation of AdCh68Ag85A. Mice were inoculated via intranasal route with 1×10^7 plaque-forming units (PFUs) AdCh68Ag85A or AdHu5Ag85A or left unimmunized (naive). Lungs were harvested at 24 or 72 h after inoculation and processed for histopathologic examination. (**a**) Light hematoxylin and eosin (H&E) micrographs of original magnification $\times 5$ and $\times 20$ of lung sections are shown, representative of three mice per group per time point. (**b**) In separate experiments, the lungs were subject to bronchoalveolar lavage (BAL) and absolute numbers of alveolar macrophages (AMΦs) and polymorphonuclear neutrophils (PMNs) in BAL were analyzed. The concentrations of (**c**) tumor necrosis factor-α (TNFα) and (**d**) interferon-β (IFNβ) in BAL were determined by enzyme linked immunosorbent assay (ELISA). Data in **b**, **c**, **d** are expressed as the mean ± s.e.m. of three to four mice per group per time point.

T-cell responses within the airway lumen by AdCh68Ag85A vaccination (**Figure 3b,c**), >40% of CTL target killing was observed in the BAL at 2 weeks and the level of CTL declined by 4 weeks following vaccination (**Figure 3d**). In comparison, the level of CTL activity by intranasal AdHu5Ag85A was significantly lower at 2 weeks, but not at 4 weeks, than that by AdCh68Ag85A (**Figure 3d**). These data suggest that respiratory mucosal vaccination with AdCh68Ag85A is capable of inducing robust T-cell responses on the respiratory mucosal surface and in the lung that are overall stronger than AdHu5Ag85A vaccination.

Respiratory mucosal AdCh68Ag85A immunization activates CD8 T cells of broadened epitope specificity

As the chimpanzee adenovirus represents a novel viral vector utilized as a TB vaccine platform, we investigated whether AdCh68Ag85A vaccination would activate the T cells reactive to the same immunodominant and subdominant epitopes as AdHu5Ag85A vaccination. To this end, naive mice were vaccinated i.n. with AdCh68Ag85A or AdHu5Ag85A. At 2 weeks, lung mononuclear cells were analyzed by intracellular cytokine staining for IFNγ-producing CD8 T cells in response to a single peptide pool (p1-57) or 6 individual peptide pools (p1-10, p11-20, p21-30, p31-40, p41-50, and p51-57) of Ag85A (**Figure 4a**). Consistent with the data in **Figure 3**, although both AdCh68Ag85A and AdHu5Ag85A triggered high frequencies of antigen-specific CD8 T cells in response to single p1-57 peptide pool, the levels of such responses were higher by AdCh68Ag85A (**Figure 4a**). Significant T-cell responses were also seen with stimulation by individual peptide pools of p11-20, p21-30, and p41-50 following AdCh68Ag85A and AdHu5Ag85A vaccination (the p11-20 pool contains the well-characterized immunodominant H-2^d Ag85A CD8 T-cell epitope MPVGGQSSF or peptide 14/Ep-1). However, AdCh68Ag85A triggered noticeably much greater T-cell responses to p21-30 stimulation than AdHu5Ag85A (**Figure 4a**). These data suggest the emergence of an additional immunodominant Ag85A CD8 T-cell epitope, resulting from respiratory mucosal AdCh68Ag85A vaccination. We have also examined the effects of these two vaccines in H-2^b C57BL/6 mice. At 2 weeks following intranasal vaccination, lung mononuclear cells were analyzed by intracellular cytokine staining for IFNγ-producing CD8 and CD4 T cells in response to 6 individual peptide pools of Ag85A described as above.

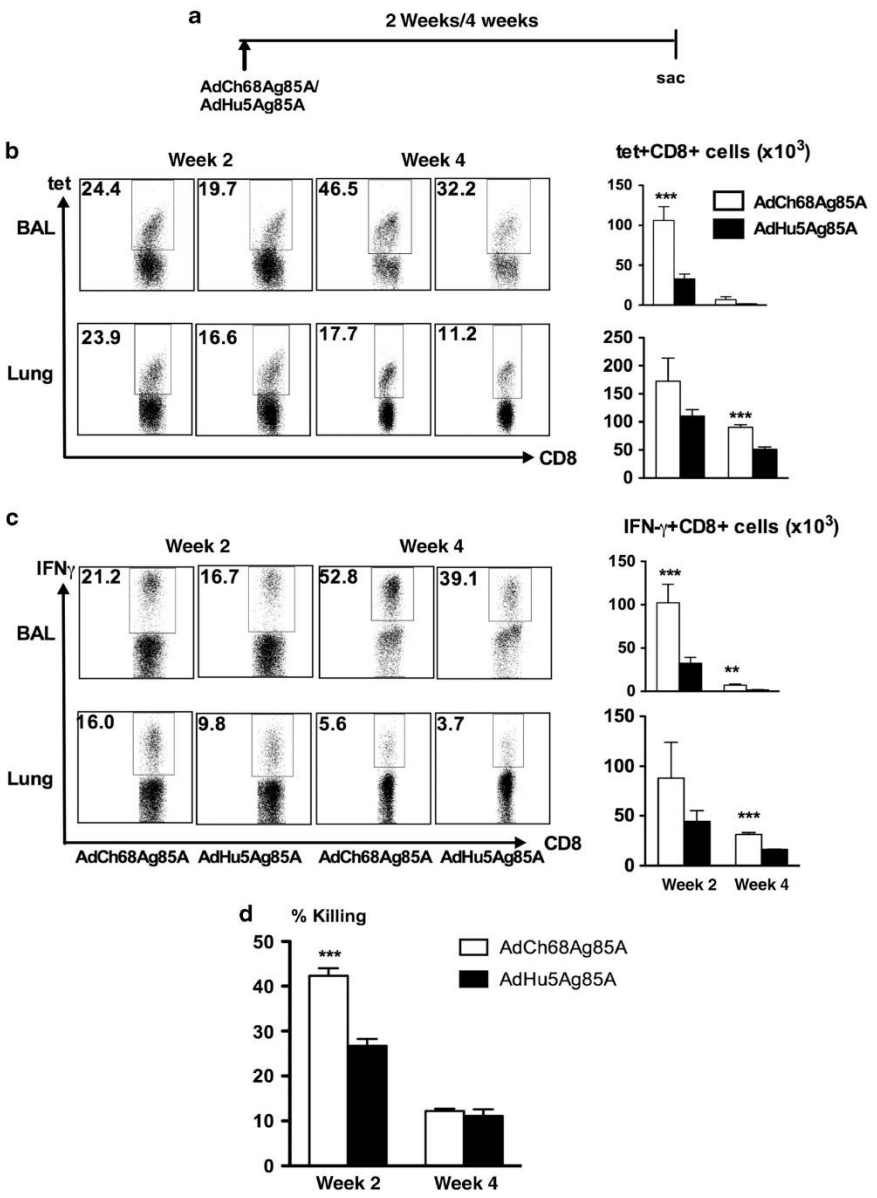

Figure 3 Robust T-cell responses in the lung by respiratory mucosal AdCh68Ag85A immunization. (**a**) Experimental schema. Intranasal immunization with 1×10^7 plaque-forming units (PFUs) AdCh68Ag85A or AdHu5Ag85A was carried out and animals were killed at weeks 2 and 4 after immunization. (**b**) Ag85A tetramer-specific CD8 T-cell responses in the airway lumen (bronchoalveolar lavage (BAL)) and the lung interstitium (Lung). Representative dotplots depicting frequencies of tetramer+ CD8 T cells in the BAL and lung at week 2 and week 4 are shown. Bar graphs show absolute numbers of tetramer+ CD8 T cells per BAL and per lung at week 2 and week 4 after immunization. (**c**) Ag85A-specific interferon-γ (IFNγ)-producing CD8 T-cell responses in the BAL and lung. Representative dotplots depicting frequencies of IFNγ+ CD8 T cells in the BAL and lung at week 2 and week 4 are shown. Bar graphs show absolute numbers of IFNγ+ CD8 T cells per BAL and per lung at week 2 and week 4 after immunization. (**d**) Cytotoxic T-cell (CTL) killing activity in the airway lumen. *In vivo* intratracheal CTL assay was performed at week 2 and week 4 after immunization. Bar graphs show percent *in vivo* killing of carboxyfluorescein succinimidyl ester (CFSE)-labeled CTL target cells. Data in **b**, **c**, **d** are expressed as the mean ± s.e.m. of three to four mice per group per time point. Data in **b**, **c** are representative of three independent experiments. $^{**}P<0.01$; $^{***}P<0.001$ compared with AdHu5Ag85A.

Consistent with previous findings,[46] we found no major histocompatibility complex class I (MHC I)-restricted Ag85A-specific CD8 T-cell responses in B6 hosts following either AdHu5 or AdCh68 vaccination (data not shown). However, there were very small frequencies of MHC II-restricted CD4 T-cell responses detected that were comparable between

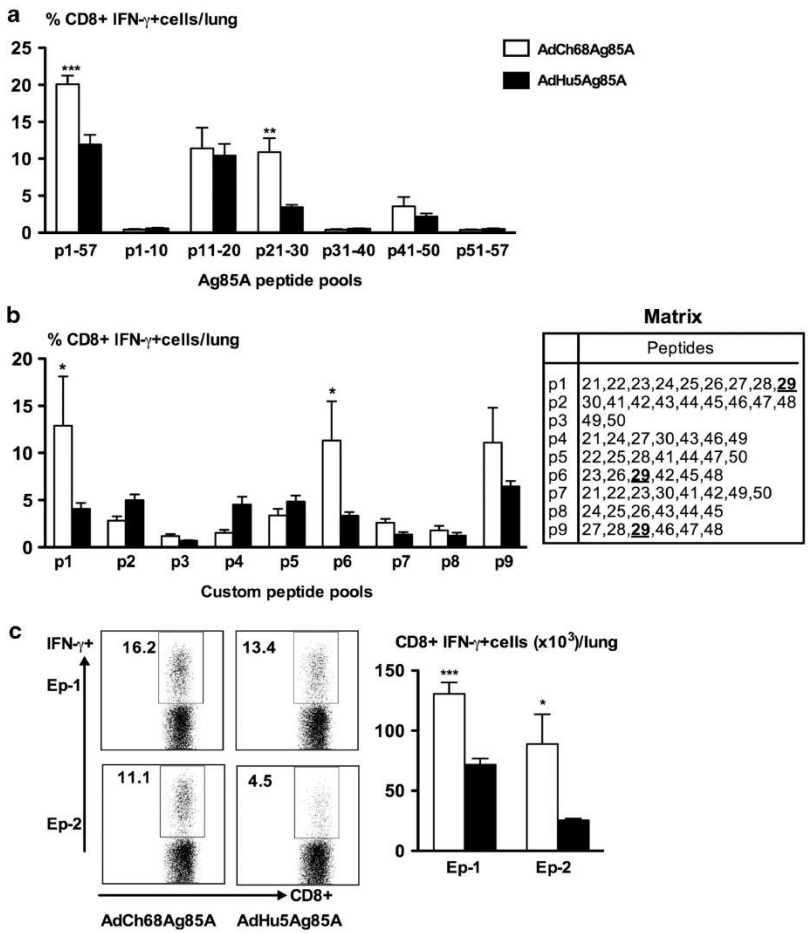

Figure 4 Activation of CD8 T cells of broadened epitope specificity by respiratory mucosal AdCh68Ag85A immunization. Mice were immunized via intranasal route with $1x10^7$ plaque-forming units (PFUs) AdCh68Ag85A or AdHu5Ag85A. Lungs were harvested at week 2 after immunization and CD8 T-cell responses to Ag85A peptide pools or peptide were determined by interferon-γ (IFNγ) intracellular cytokine staining (ICS). (**a**) Bar graph shows frequencies of CD8 + IFNγ + cells in the lung responding to seven peptide pools of Ag85A. (**b**) Bar graph shows frequencies of CD8 + IFNγ + cells in the lung responding to newly designed nine peptide pools. These new peptide pools were designed in such a way that each peptide is present only in three unique pools, according to the Matrix shown in the table. The newly emerged immunodominant peptide 29 is highlighted. (**c**) Representative dotplots show frequencies of CD8 + IFNγ + cells in the lung responding to a conventional immunodominant Ag85A peptide Ep-1 and to a newly emerged immunodominant Ag85A peptide Ep-2. Bar graph shows absolute numbers of CD8 + IFNγ + cells in the lung responding to Ep-1 and Ep-2 peptides. Data in **a**, **b**, **c** are expressed as the mean ± s.e.m. of three to four mice per group, representative of two independent experiments. $^*P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$ compared with AdHu5Ag85A.

AdHu5 and AdCh68 vaccinations (**Supplementary Table S1** online). The level of such MHC II-restricted responses in B6 mice was comparable to that detected in BALB/c mice (data not shown). These data together suggest that AdHu5 and AdCh68 vaccinations predominantly generate high levels of MHC I-restricted CD8 T-cell responses in BALB/c, but not in C57BL/6, hosts.

To further identify the potential additional CD8 T-cell epitopes present in the p21-30 and p41-p50 pools in BALB/c mice, a new set of 9 peptide pools with the peptides p21-p30 and p41-p50 were developed with each pool containing 2–9 peptides according to a previously described peptide-mapping matrix[47] (**Figure 4b**, Matrix). In so doing, each peptide is present only in three unique pools. Significantly raised T-cell responses against pools p1, p6, and p9 were identified only in AdCh68Ag85A-vaccinated, but not AdHu5Ag85A-vaccinated, animals (**Figure 4b**). Thus, the likely new candidate epitope was peptide 29, a 15-mer peptide highlighted in **Figure 4b**, Matrix table. To further verify it, by using an Immune Epitope Database (http://tools.immuneepitope.org/) we identified the highest probable CD8 T-cell epitope sequence in peptide 29 to be VYAGAMSGL (Ep-2). Using its synthesized 9-mer peptide, we confirmed its immunodominance by demonstrating a robust CD8 T-cell response induced only by intranasal

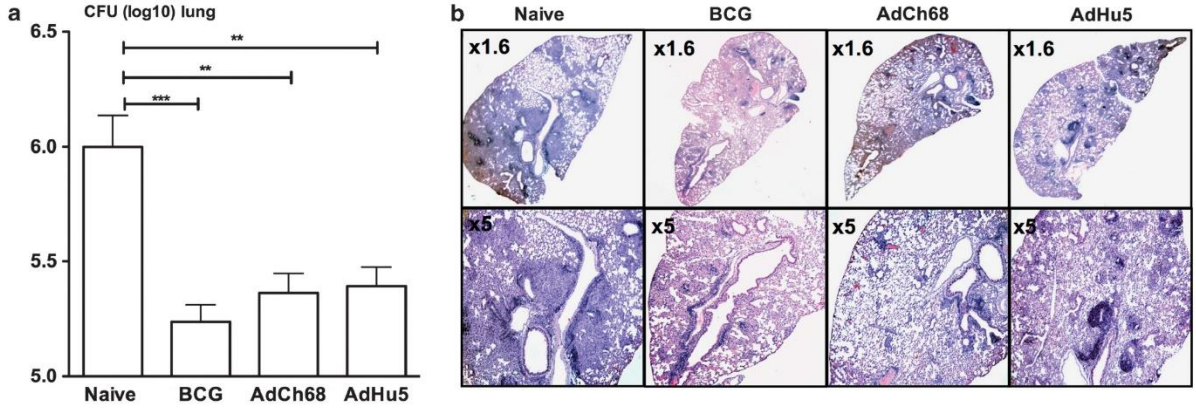

Figure 5 Potent protection against pulmonary tuberculosis (TB) by respiratory mucosal AdCh68Ag85A immunization in naive hosts. (**a**) Naive mice were left unimmunized (naive) or immunized subcutaneously with Bacillus Calmette–Guérin; BCG), intranasally with $1x10^7$ plaque-forming units (PFUs) AdCh68Ag85A (AdCh68) or with AdHu5Ag85A (AdHu5). At 4 weeks after immunization, mice were challenged with *Mycobacterium tuberculosis* (*M.tb*) and the lungs were harvested for analysis 4 weeks after infection. (**a**) Bar graph shows the colony-forming units (CFUs) of *M.tb* in the lung. (**b**) Representative histopathologic images from hematoxylin and eosin (H&E)-stained lung sections of three mice per group are shown at original magnification × 1.6 and × 5. Data in **a** are expressed as the mean ± s.e.m. of four to five mice per group, representative of two independent experiments. $^{**}P<0.01$; $^{***}P<0.001$.

AdCh68Ag85A, but not AdHu5Ag85A, vaccination (**Figure 4c**). In comparison, the CD8 T cells activated by both AdCh68Ag85A and AdHu5Ag85A vaccination well responded to stimulation by the common immunodominant peptide MPVGGQSSF (Ep-1) (**Figure 4c**), although as shown in **Figure 3c**, AdCh68Ag85A triggered higher levels of responses. These data indicate that compared with AdHu5Ag85A, mucosal AdCh68Ag85A vaccination is able to activate CD8 T cells of broader epitope specificity in H-2^d Balb/c hosts.

Respiratory mucosal AdCh68Ag85A immunization provides robust protection against pulmonary tuberculosis in naive hosts

Having demonstrated the potent T-cell immunogenicity of AdCh68Ag85A, we next assessed its protective efficacy against pulmonary TB in naive animals. To this end, naive mice were vaccinated as above with AdCh68Ag85A (AdCh68) or AdHu5Ag85A (AdHu5). As a comparison, some mice were subcutaneously (s.c.) vaccinated with BCG (BCG) or were not vaccinated at all (naive). At 4 weeks after immunization, mice were challenged via the airway with virulent *M.tb*H37Rv. Protective efficacy in the lung was evaluated at 4 weeks after challenge. Indeed, respiratory mucosal AdCh68 vaccination markedly reduced the levels of *M.tb* infection in the lung compared with unvaccinated animals (**Figure 5a**). The magnitude of enhanced protection by AdCh68 was comparable to that by AdHu5 counterpart or by parenteral BCG vaccination (**Figure 5a**).

As the relative amount of *M.tb*-associated lung immunopathology is an even more sensitive and reliable indicator of protection in some other animal models than in murine models of TB, we examined and compared lung immunopathology. In keeping with significantly improved protection by respiratory mucosal AdCh68 vaccination, there was markedly reduced overall granulomatous inflammation and tissue injury in the lung of AdCh68-vaccinated animals, contrasting widespread pronounced granulomatous lesions in the lung of unvaccinated animals (**Figure 5b**). Of note, the lung of AdCh68 animals had less immunopathology than the lung of AdHu5-vaccinated animals (**Figure 5b**). Thus, compared with AdHu5 lungs, the sizes of granulomatous lesion were smaller and there was less bronchial injury in the lung of AdCh68-vaccinated animals. These data together suggest that compared with its AdHu5 counterpart, respiratory mucosal AdCh68Ag85A immunization provides overall further improved protective immunity against pulmonary TB in the lung of naive animals.

Different from its AdHu5 counterpart, respiratory mucosal AdCh68Ag85A immunization is able to provide potent antituberculosis immunity in hosts with preexisting anti-AdHu5 immunity in the lung

AdCh vectors have recently emerged to be an attractive alternative to AdHu5 counterparts for the development of human vaccines because of prevalence of anti-AdHu5 immunity in humans.[22,24,33,35] Indeed, it has been widely accepted that anti-AdHu5 antibodies do not crossreact with AdCh.[22,24] However, it remains largely unclear whether anti-AdHu5 T cells may negatively affect the potency of AdCh-based vaccine.[30] Of importance, little is understood about whether AdCh vector-based vaccine would remain effective when delivered via the respiratory tract—the natural route of human AdHu5 infection—in the context of local anti-AdHu5 immunity. This is an important issue to address as Ad-based TB vaccines were developed primarily as a novel platform for respiratory mucosal delivery in humans. We began to address this issue first by examining to which extent the preexisting

anti-AdHu5 immunity would negatively affect the T cell-activating potency of parenteral (intramuscular) vaccination with AdC68Ag85A or AdHu5Ag85A. Thus, naive mice were first exposed to wild-type AdHu5 virus (WtAd) via the intranasal route (exposed). Such respiratory WtAd exposure led to significant levels of anti-AdHu5 immunity as exemplified by markedly elevated total anti-AdHu5 IgG and IgA antibodies and raised levels of AdHu5-neutralizing antibody titers both locally in the lung (BAL) and systemically in the peripheral blood (serum) (**Supplementary Figure S1**). At 4 weeks after WtAd infection, mice were vaccinated intramuscularly with AdCh68Ag85A (AdCh68) or AdHu5Ag85A (AdHu5) (**Figure 6a**). The control animals were vaccinated similarly without WtAd exposure (unexposed). Ag85A-specific T-cell responses were examined in the BAL, lung, and spleen. As expected,[6,11,12] intramuscular AdCh68 or AdHu5 vaccination of unexposed animals failed to elicit any airway luminal T cells (BAL), whereas WtAd-exposed animals had similarly increased BAL T cells following intramuscular AdCh68 or AdHu5 vaccination (**Figure 6b**). On the other hand, compared with unexposed animals, significantly increased numbers of CD8 T cells were observed in the lung of WtAd-exposed intramuscular AdC68 animals (**Figure 6b**). On the contrary, significantly reduced numbers of CD8 T cells were observed in the lung of WtAd-exposed intramuscular AdHu animals (**Figure 6b**). Similarly, reduced CD8 T cells were seen in the spleen of only WtAd-exposed AdHu5-vaccinated animals, but not WtAd-exposed AdCh68 vaccinated animals (**Figure 6b**). These data together support and extend the previous findings that preexisting circulating anti-AdHu5 immunity dampens the potency of parenterally administered AdHu5 gene transfer vectors but not those based on chimpanzee adenovirus.[24,48]

After demonstrating that different from AdHu5Ag85A vaccination, intramuscular AdCh68Ag85A vaccination can indeed bypass the preexisting anti-AdHu5 immunity to induce potent T-cell responses, we next investigated to which extent this could be reproduced upon respiratory mucosal AdCh68 vaccination in WtAd-exposed animals. To this end, mice were exposed to WtAd via the intranasal route and at 4 weeks after WtAd infection, they were vaccinated i.n. with AdCh68 or AdHu5 vaccine (**Figure 6c**). The (WtAd) unexposed naive mice were vaccinated i.n. as controls. T-cell responses were subsequently examined in the BAL and lung. Of interest, compared with unexposed animals, preexisting anti-AdHu5 immunity in the respiratory mucosa appeared to result in reduced airway luminal (BAL) T-cell responses following either respiratory mucosal AdCh68 or AdHu5 vaccination (**Figure 6d**). However, preexisting anti-AdHu5 immunity had little negative effects on T cells in the lung interstitium of AdCh68-vaccinated animals, whereas it significantly reduced T-cell responses in the lung of AdHu5-vaccinated animals (**Figure 6d**). Overall, intranasal AdCh68Ag85A vaccination triggered much greater antigen-specific T-cell responses in the entire lung (both airway luminal and lung interstitial compartments) than intranasal AdHu5Ag85A vaccination in respiratory WtAd-exposed animals.

To investigate whether the greater T-cell responses seen in the lung of WtAd-exposed, AdCh68-vaccinated animals would lead to better immune protection from pulmonary TB, groups of mice were exposed i.n. to WtAd and subsequently vaccinated via the respiratory mucosal route with AdCh68 or AdHu5 vaccine as above and then challenged via the airway with virulent *M.tb*H37Rv (**Figure 6e**). Relative levels of protective efficacy in the lung were assessed at 4 weeks after *M.tb* infection. Consistent with the protection data shown in **Figure 5a**, (WtAd) unexposed animals were similarly protected in the lung by intranasal AdCh68 or AdHu5 vaccination (**Figure 6f**). Also, consistent with the immunogenicity data (**Figure 6d**), preexisting anti-AdHu5 immunity had little negative effects on immune protection of AdCh68-vaccinated animals, whereas it significantly reduced protection, leading to markedly increased *M.tb* infection in the lung of AdHu5-vaccinated animals (**Figure 6f**). As the above set-up did not directly address whether preexposure to WtAd on its own had an effect on immune protection, in a separate experiment mice were exposed to WtAd and at 4 weeks after exposure mice were left either unvaccinated or vaccinated with AdHu5Ag85A or AdCh68Ag85A. At 4 weeks after vaccination, mice were challenged with *M.tb* and bacterial burden in the lung was assayed. As expected, compared with WtAd-unexposed and unvaccinated mice (naive/unexposed), WtAd exposure alone without vaccination (naive/exposed) had little significant enhancing effects on protection against *M.tb* infection (**Figure 6g**). Importantly, compared with naive/exposed hosts, AdHu5Ag85A vaccination in the presence of anti-AdHu5 immunity (AdHu5/exposed) failed to provide significantly enhanced protection, whereas AdCh68Ag85A vaccination in the presence of anti-Ad5 immunity (AdCh68/exposed) remained robustly protective (**Figure 6g**). The above data together suggest that respiratory mucosal AdCh68Ag85A immunization remains capable of triggering potent protective T-cell immunity against pulmonary TB in the animals with preexisting anti-AdHu5 immunity in the lung. In contrast, the potency of respiratory mucosal immunization with its human counterpart AdHu5Ag85A vaccine is markedly reduced by such local anti-AdHu5 immunity.

Respiratory mucosal AdCh68Ag85A immunization boosts protective T-cell immunity against pulmonary tuberculosis in parenteral BCG-primed hosts

Like a number of other candidate TB vaccines that are currently in the clinical development, AdCh68Ag85A was developed for boost vaccination of parenteral BCG-primed humans. Having systematically investigated the immunogenicity and protective efficacy of respiratory mucosal AdCh68Ag85A vaccination described above, we next set out to examine its effectiveness in boosting protective immunity following parenteral BCG priming. We first examined its effect on T-cell responses. To this end, naive mice were primed s.c. with BCG. At 4 weeks after BCG priming, mice were boosted i.n. with AdCh68Ag85A (BCG/AdCh68) (**Figure 7a**). Control mice received only BCG (BCG) or AdCh (AdCh) vaccination. At 4 weeks after boosting,

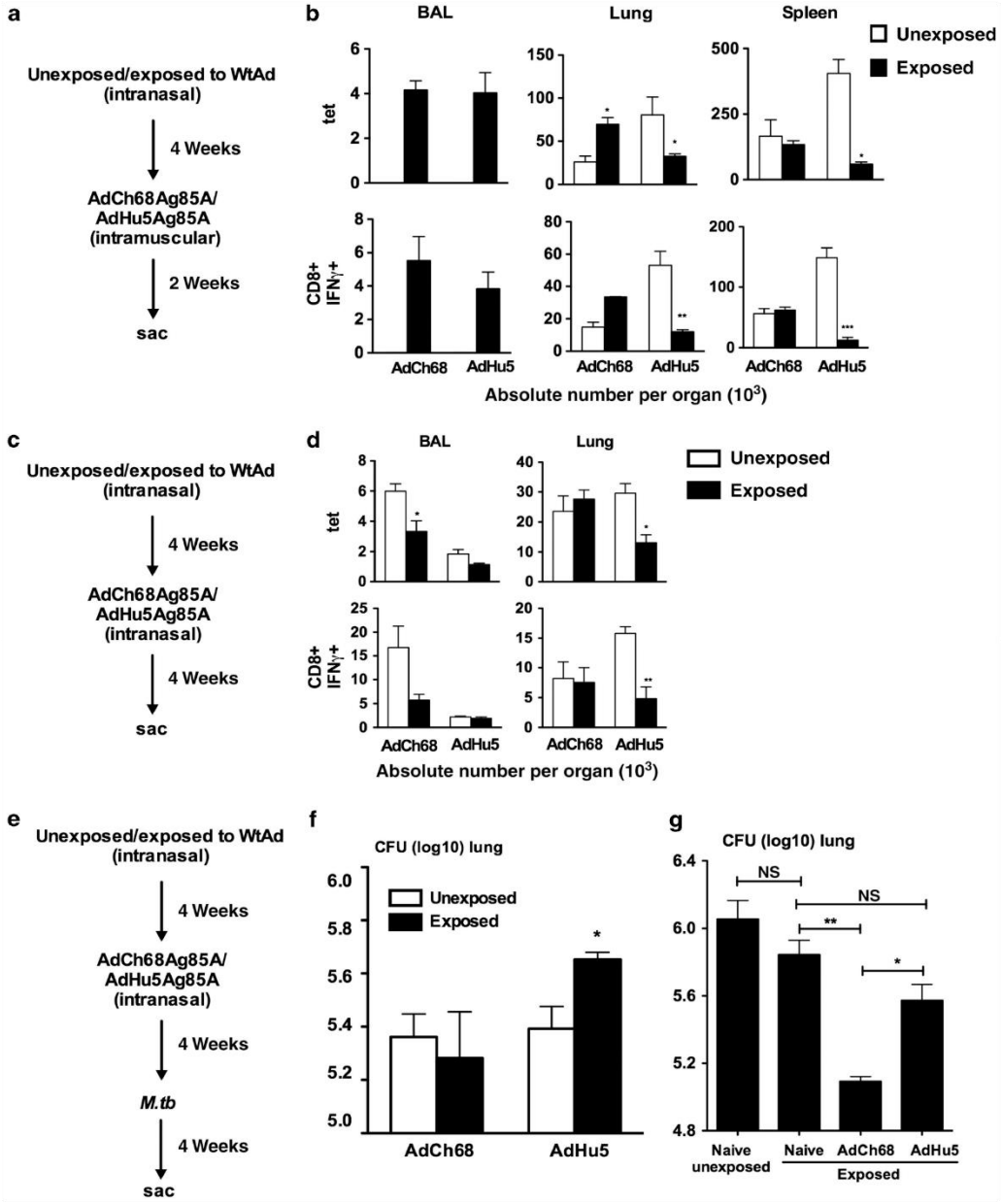

Figure 6 Potent protective immunity against pulmonary tuberculosis (TB) by respiratory mucosal AdCh68Ag85A immunization in hosts with preexisting anti-AdHu5 immunity in the lung. (**a**) Experimental schema for (**b**). Mice were exposed intranasally to wild-type AdHu5 virus (WtAd). The control mice were not exposed to WtAd (unexposed). At week 4 after WtAd exposure, mice were immunized intramuscularly with 1×10^7 plaque-forming units (PFUs) AdCh68Ag85A (AdCh68) or AdHu5Ag85A (AdHu5) vaccine. Bronchoalveolar lavage (BAL), lungs, and spleens were collected for analysis at week 2 after parenteral immunization. (**b**) Bar graphs show absolute numbers of Ag85A tetramer+ and CD8+IFN-γ+ T cells in the airway lumen (BAL), lung interstitium (Lung), and spleen. IFNγ, interferon-γ. (**c**) Experimental schema for (**d**). Mice were exposed intranasally to WtAd. The control mice were not exposed to WtAd (unexposed). At week 4 after WtAd exposure, mice were immunized intranasally with AdCh68 or AdHu5 TB vaccine. BAL and lungs were collected for analysis at week 4 after respiratory mucosal immunization. (**d**) Bar graphs show absolute numbers of Ag85A tetramer+ and CD8+IFN-γ+ T cells in BAL and lung. (**e**) Experimental schema for (**f**,**g**). Mice were set up as described in **c** except that at week 4 after respiratory mucosal immunization, animals were challenged with *Mycobacterium tuberculosis* (*M.tb*) and the levels of infection in the lung were assessed 4 weeks after *M.tb* challenge. (**f**) Bar graph shows the colony-forming units (CFUs) of *M.tb* in the lung. (**g**) Mice were set up as described in **e** except that a group of mice exposed to WtAd but not immunized was included. Bar graph shows the CFUs of *M.tb* in the lung. Data in **b**, **d** are expressed as the mean ± s.e.m. from three to four mice per group, representative of three independent experiments. Data in **f** are the mean ± s.e.m. from four to five mice per group. $^*P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$; NS, not significant $P>0.05$ compared with the unexposed control.

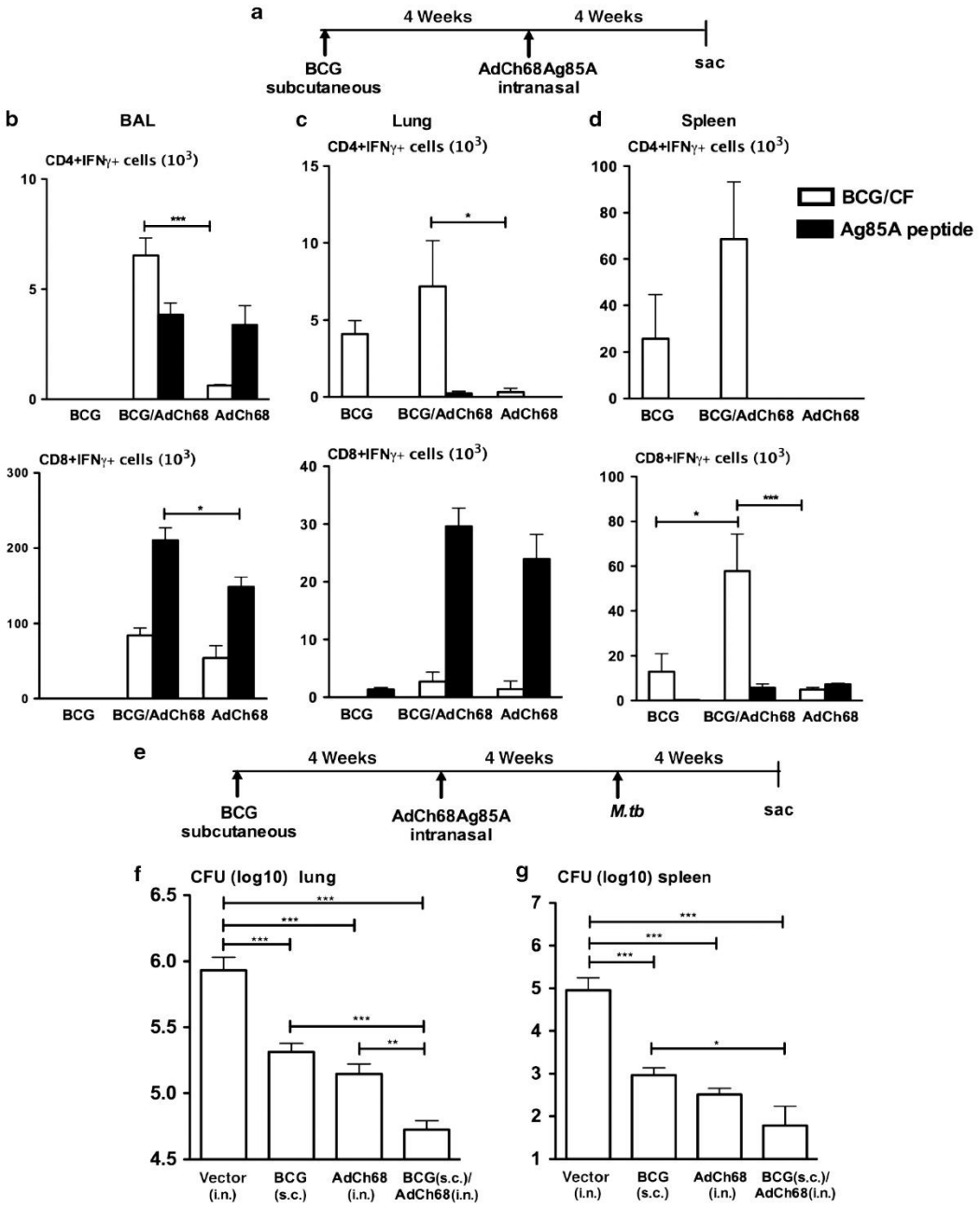

Figure 7 Further enhanced protective immunity against pulmonary tuberculosis (TB) by respiratory mucosal AdCh68Ag85A boost immunization in parenteral Bacillus Calmette–Guérin (BCG)-primed hosts. (**a**) Experimental schema for (**b**–**d**). Mice were prime-immunized subcutaneously (s.c.) with BCG. At week 4, animals were boost-immunized via intranasal (i.n.) route with 5×10^7 plaque-forming units (PFUs) AdCh68Ag85A (BCG/AdCh68). Control groups were immunized only with BCG (BCG) or AdCh68Ag85A (AdCh68). Bronchoalveolar lavage (BAL), lungs, and spleens were collected at week 4 after boost immunization. (**b**–**d**) Bar graphs show absolute numbers of CD4 + IFN-γ + and CD8 + IFN-γ + T cells in (**b**) BAL, (**c**) lung, and (**d**) spleen. The cells were stimulated either with crude mycobacterial antigens (BCG/culture filtrate (CF)) or an Ag85A CD4 or CD8 peptide (Ag85A peptide). (**e**) Experimental schema for (**f**, **g**). Mice were set up as in **a** including BCG s.c., AdCh68 i.n., and BCG (s.c.)/AdCh68 (i.n.) groups except that at week 4 after boost immunization, mice were challenged with *Mycobacterium tuberculosis* (*M.tb*) and the lungs and spleens were collected for analysis. Furthermore, an additional control group was set up, receiving i.n. an empty control AdCh68 vector (vector). (**f**, **g**) Bar graphs show the colony-forming units (CFUs) of *M.tb* in the (**f**) lung and (**g**) spleen. Data in **b**, **c**, **d** are expressed as the mean ± s.e.m. from three to four mice per group, representative of two independent experiments. Data in **f**, **g** are the mean ± s.e.m. from five mice per group, representative of two independent experiments. $^*P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$.

cells isolated from the airway lumen (BAL), lung interstitium (lung), and spleen were stimulated with crude mycobacterial antigens (BCG/culture filtrate) or an Ag85A peptide and analyzed for antigen-specific, IFNγ-producing CD4 and CD8 T-cell responses. Consistent with our previous findings,[8] parenteral BCG priming alone did not elicit airway luminal CD4 or CD8 T-cell responses (**Figure 7b**), whereas it induced a level of crude mycobacterial antigen (BCG/culture filtrate)-reactive CD4 T-cell, but not CD8 T-cell, responses in the lung (**Figure 7c**) and spleen (**Figure 7d**). On the other hand, as expected, intranasal AdCh68 vaccination alone primarily activated Ag85A-specific CD8 T cells in the BAL and lung (**Figure 7b,c**). In comparison, intranasal AdCh68 boost vaccination of parenteral BCG-primed animals (BCG/AdCh68) markedly increased multi-mycobacterial antigen-specific CD4 T cells in the airway lumen (BAL), lung, and spleen (**Figure 7b–d**). It also increased both multi-mycobacterial antigen- and Ag85A-specific CD8 T cells in the BAL and spleen (**Figure 7b–d**). Together, these results indicate that respiratory mucosal AdCh68Ag85A boost immunization can potently enhance CD4 and CD8 T-cell responses in the lung in parenteral BCG-primed animals.

To investigate whether intranasal AdCh68Ag85A-boosted T cell immunity in the lung of BCG-primed hosts could lead to improved immune protection against pulmonary TB, mice were subcutaneous BCG primed and received an intranasal boost with AdCh68 (BCG s.c./AdC68 i.n.) as above and subsequently challenged via the airway with virulent *M.tb*H37Rv (**Figure 7e**). Control groups were treated either with BCG alone (BCG s.c.) or AdCh68Ag85A alone (AdCh68 i.n.) or an empty AdCh68 virus alone (vector i.n.). Relative levels of protective efficacy in the lung and spleen were assessed at 4 weeks after *M.tb* infection. As expected, the vector control animals that were inoculated with the empty AdCh68 virus were not protected, having high levels of *M.tb* infection in the lung (**Figure 7f**), similar to the levels seen in infected naive animals (**Figure 5a**). Again, as shown in **Figure 5a**, subcutaneous BCG priming alone or intranasal AdCh68 vaccination alone each significantly enhanced protection, causing 0.7–0.8log *M.tb* colony-forming unit (CFU) reduction (**Figure 7f**). However, correlating with its improved T-cell immunity (**Figure 7b–d**), AdCh68 boost vaccination of BCG-primed animals further improved protection, leading to an ~1.4log *M.tb* CFU reduction in the lung (**Figure 7f**). Similarly, intranasal AdCh68 boosting also resulted in the best levels of improved protection in the spleen (3log *M.tb* CFU reduction) compared with vector, BCG, or AdCh68 alone (**Figure 7g**). The above data together establish that respiratory mucosal AdCh68Ag85A boost immunization markedly enhances anti-TB T-cell immunity in parenteral BCG-primed hosts.

DISCUSSION

Mounting evidence suggests that any effective TB vaccination strategies ought to be able to close the "immunological gap" instigated by *M.tb* infection whereby the appearance of T-cell immunity is much delayed in the lung.[6,7,11,12] Respiratory mucosal vaccination represents a powerful way to install the respiratory mucosa with T-cell immunity before *M.tb* exposure for enhanced lung protection.[6,11–14] The potency of respiratory mucosal vaccination is not only limited to intracellular infections as its advantage has also recently been recognized for immunity against lung cancer.[49] Indeed, TB vaccine research community has recently identified the development of safe and effective respiratory mucosal TB vaccination strategies to be a new priority.[20] Such renewed effort is timely as parenteral boosting with a vaccinia virus (MVA)-based TB vaccine was unable to improve lung protection in BCG vaccines as recently shown in a phase 2b efficacy trial, the first of its kind to test a new TB vaccine in the world.[19]

Given its natural tropism to the respiratory mucosa and potency, recombinant AdHu5-based vaccine platforms have proven to be most effective and promising for respiratory mucosal vaccination against pulmonary TB based on abundant preclinical data.[11,41,42,45,50–52] However, the high prevalence of preexisting anti-AdHu5 immunity in human populations has become an increasingly recognized limitation and challenge to application of AdHu5-based vaccines in humans.[22,24,25,28–30,32] We have recently reported that preexisting circulating anti-AdHu5 antibodies had no major effect on T-cell activation by intramuscularly administered AdHu5Ag85A vaccine in a human study.[27] Nevertheless, as all trial volunteers had varying degrees of preexisting anti-AdHu5 antibodies,[27] we cannot firmly conclude that responses to the vaccine were not dampened. Furthermore, as wild-type AdHu5 virus is primarily a human respiratory pathogen, the anti-AdHu5 immunity concentrated in lung mucosa could have greater negative effects on respiratory-mucosally administered AdHu5-based vaccine. On the other hand, the results from Step HIV vaccine trials have implied a safety concern in using AdHu5-based vaccine in those HIV high-risk populations who have high levels of preexisting anti-AdHu5 immunity.[32,53,54] Thus, despite the big strides made in basic and preclinical development of new TB vaccines, there exists an important gap in meeting with the conditions and effective applications in humans. These considerations have prompted recently accelerated pace in the development of heterologous chimpanzee adenovirus-based vaccine technology.[22,24,28,33,35]

In this study, by using a new technology[40] we have developed the first chimpanzee adenovirus-vectored TB vaccine as a novel candidate for respiratory mucosal immunization in humans. We find that the ability of a single respiratory mucosal AdCh68Ag85A immunization to activate T cells, provide mucosal protection, and reduce *M.tb*-associated immunopathology in naive animals with or without parenteral BCG priming is comparable to or even better than AdHu5Ag85A counterpart. Of importance, we provide compelling evidence that contrary to AdHu5Ag85A, respiratory mucosal AdCh68Ag85A-induced protective T-cell immunity is minimally affected by anti-AdHu5 immunity preexisting locally in the lung. Given the scarcity of candidate TB vaccines currently amenable to safe and effective respiratory mucosal vaccination in humans,[20] our study holds important implications. The

findings from our study suggest that AdCh68-based TB vaccine is superior to the AdHu5-based counterpart and should be further developed clinically as an ideal TB vaccine candidate for respiratory mucosal immunization in humans. Given the ample experience and infrastructure well established by us and others, its expeditious translation to human studies and applications is expected.

Another noticeable advantage associated with AdCh68Ag85A vaccine is its broadened CD8 T-cell epitope specificity noted in our study. Thus, respiratory mucosal AdCh68Ag85A immunization results in the emergence of a dominant CD8 T-cell epitope corresponding to Ag85A amino acids 146–154 (Ep-2) in addition to its strong inducibility of CD8 T-cell responses to the epitope spanning amino acids 70–80 (Ep-1). In comparison, its AdHu5 counterpart induces a strong response only to Ep-1, with Ep-2 being a subdominant CD8 T-cell epitope. A broadened spectrum of dominant T-cell epitopes including Ep-2 by AdCh68-based immunization is considered an additional benefit to human applications as the epitopes within Ag85A amino acids 141–160 were previously found to be the most immunodominant and MHC promiscuous in *M.tb*-infected humans.[55] We believe that such broadened T-cell epitope specificity by AdCh68-based immunization may have contributed to improved protection over that by AdHu5Ag85A immunization, particularly with regard to reduced lung immunopathology. At the present time, the mechanisms for such difference between AdCh and AdHu5 vectors still remain to be fully understood. Although both AdCh and AdHu5 utilize the same coxsackievirus and adenovirus receptor for infection,[24,56] and we have observed their proinflammatory properties to be comparable, it is possible that other differentially regulated intracellular signaling pathways may play a role. Indeed, previous studies suggest that the nature of formulation or vaccine vector has a profound effect on the immunodominance of T-cell epitopes and the immune protective potential of activated T cells.[57,58] This could be attributed to antigen processing and presentation by antigen-presenting cells to CD8 T cells regulated by genes expressed by the vector.[59,60] Thus, the broaden epitope recognition following AdCh vaccination could be explained in part by the differential inherent prosperities of the viral vectors having a role in antigen processing and canonical epitope presentation. However, whether the broadened epitope specificity of AdCh68Ag85A vaccine may also hold true in humans requires further investigation. Of note, despite enhanced T-cell responses by AdCh immunization relative to AdHu5 immunization, the enhanced bacterial control by the two is comparable in WtAd-unexposed hosts. This suggests that further improved T-cell responses may not always translate into further improved mycobacterial control. Nonetheless, such further improved T-cell responses by AdCh immunization did lead to improved protection with respect to *M.tb*-associated histopathology.

In keeping with previously published studies,[24,38] we find that in contrast to AdHu5 vaccine the potency of parenterally (intramuscularly) administered AdCh vaccine is not negatively affected at all by preexisting systemic anti-AdHu5 immunity. This finding supports that anti-AdHu5 antibodies do not crossreact with AdCh viral vectors. However, it is noteworthy that in the majority of previous studies, preexisting anti-AdHu5 immunity was generated by systemic AdHu5 infection. As AdHu5 virus is a respiratory pathogen in humans and recent evidence suggests a differential immune-regulatory effect by the mucosal route of induction of anti-AdHu5 immunity,[61,62] in this study we generated preexisting anti-AdHu5 immunity by respiratory route of wild-type AdHu5 infection that generated anti-Ad5-neutralizing antibody titers in both the lung and circulation, similar to the levels seen in humans.[62] Thus, this method is not only clinically relevant but is also relevant to the investigation of AdCh-based respiratory mucosal TB vaccination strategies. Recently emerging clinical evidence has also raised the question of whether anti-AdHu5 T cells may crossreact with the antigens from AdCh infection,[30,32,35,63] thus potentially negatively affecting the protective immunity by AdCh-based immunization. Indeed, we find that preexisting anti-AdHu5 immunity present in the lung does have a dampening effect on AdCh68-induced T-cell responses within the airway lumen. However, this dampening effect was found to be limited only to the airway luminal space as the antigen-specific T-cell responses within the lung interstitium were not affected. In contrast, preexisting local anti-AdHu5 immunity significantly reduced the T cells in the lung interstitium of AdHu5-vaccinated hosts. Thus, despite somewhat dampened T-cell responses in the airway lumen by local anti-AdHu5 immunity, the lungs of AdCh68Ag85A-vaccinated hosts retained much greater numbers of *M.tb* antigen-specific T cells than AdHu5Ag85A-vaccinated counterparts. This underpins one of the most important findings in our study that preexisting anti-AdHu5 immunity in the lung does not negatively affect AdCh68Ag85A-induced immune protection against pulmonary TB, whereas it significantly reduces AdHu5Ag85A-mediated protection. In this study we have also demonstrated a dramatic boosting effect by AdCh68Ag85A immunization in BCG-primed animals. Respiratory mucosal AdCh68 immunization boosted both multi-mycobacterial antigen-specific CD4 T cells and Ag85A-specific CD8 T cells that led to markedly further enhanced protection.

In summary, we have developed a novel chimpanzee adenovirus-based TB vaccine for respiratory mucosal immunization and evaluated its potency in animal models of human pulmonary TB with or without preexisting anti-AdHu5 immunity in the lung. We provide compelling evidence that chimpanzee adenovirus-based TB vaccine is superior to AdHu5-based counterpart and thus may represent an ideal candidate for respiratory mucosal immunization in humans. Our findings also hold important implications in developing effective virus-based vaccines against other respiratory infection diseases and cancers for human applications.

METHODS

Animals for *in vivo* models of immunization and pulmonary tuberculosis. Female BALB/c mice, 6 to 8 weeks old, were purchased from Charles River Laboratories (Charles River, St Constant, Quebec, Canada) and housed in a specific pathogen-free level B facility at

McMaster University. All experiments were carried out in accordance with the guidelines from the Animal Research and Ethics Board at McMaster University.

Bioengineering of human and chimpanzee adenovirus-based tuberculosis vaccines. The construction of a recombinant replication-deficient human type 5 adenovirus expressing an immunodominant *M.tb* antigen 85A (AdHu5Ag85A) has been previously described.[41,64] This vaccine has been extensively evaluated as a parenteral or respiratory mucosal TB vaccine in a wide range of preclinical models.[41,42,45,50–52] It has also recently been evaluated in a phase 1 human study.[27]

A replication-deficient chimpanzee type 68 adenovirus was constructed to express an immunodominant *M.tb* antigen 85A (AdCh68Ag85A) by using a direct cloning technology.[40] The genetic Ag85A cassette containing the murine cytomegalovirus promoter, tissue plasminogen activator peptide signal, and polyA sequences used for constructing AdCh68Ag85A (**Figure 1a**) is identical to the one for AdHu5Ag85A. Briefly, a pShuttle plasmid DNA containing the Ag85A cassette was digested with I-Ceu1 and PI-Sce1. The released insert was subcloned by an in-gel ligation technique into the I-Ceu1 and PI-Sce1 site of the genomic DNA clone pAdC68 containing the entire AdCh68 genomic sequences except the E1 and E3 genes. AdCh68Ag85A was packaged and propagated in 293 cells and purified by cesium chloride gradient centrifugation in the same way as for AdHu5Ag85A. The Ag85A protein production and secretion by AdCh68Ag85A-infected mammalian cells was verified and compared with that in AdHu5Ag85A-infected cells by western immunoblotting using a monoclonal antibody specific for Ag85A (**Figure 1b**).[41]

Generation of preexisting anti-AdHu5 immunity in animals by respiratory infection with wild-type human serotype 5 adenovirus. In some experiments, naive mice were infected i.n. with WtAd (1×10^8 plaque-forming units (PFUs)) to generate preexisting anti-AdHu5 immunity before TB immunization.

Respiratory mucosal or parenteral immunization in animals with AdCh68Ag85A, AdHu5Ag85A, or BCG vaccine. Respiratory mucosal route of immunization was carried out by i.n. delivery of AdHu5Ag85A or AdCh68Ag85A (1×10^7 PFUs per mouse).[42,45,50] In selected experiments, mice were immunized parenterally (intramuscular) with the same dose of vaccine. In some experiments a larger dose (5×10^7 PFUs) of AdCh68Ag85A was used. For prime-boost immunization regimen, BCG (Pasteur) (1×10^5 CFUs per mouse) was inoculated s.c.[50] for priming and the animals were then boosted i.n. with Ad-based vaccine.

***M. tuberculosis* preparation and animal models of pulmonary tuberculosis**. *M.tb* bacilli were grown in Middlebrook 7H9 broth 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.[41,42,45,50] Before each *in vivo* use, *M.tb* bacilli were washed with phosphate-buffered saline containing 0.05% Tween-80 twice and passed through a 27-gauge needle 10 times to disperse clumps. Pulmonary infection with *M.tb*H37Rv strain was carried out as previously described.[41,42,45,50] The levels of mycobacterial infection in the lung and spleen were determined by plating serial dilutions of tissue homogenates in triplicates onto Middlebrook 7H10 agar plates. Plates were incubated at 37 °C for 15–17 days before colony enumeration.

Bronchoalveolar lavage and lung and spleen mononuclear cell isolation. Mice were killed by exsanguination. Airway luminal cells were collected through exhaustive BAL as previously described.[42,45] Subsequently, lungs were perfused by injecting Hank's buffer through the right ventricle in order to remove intravascular mononuclear cells. Lungs were digested with collagenase type 1 (Sigma-Aldrich, St Louis, MO) at 37 °C in an agitating incubator. A single-cell suspension was obtained by crushing the digested tissue through 40 μm basket filter. Splenocytes were isolated as described previously.[41,50] All isolated cells were resuspended in complete RPMI-1640 medium (RPMI 1640 supplemented with 10% fetal bovine serum, 1% penicillin–streptomycin, and 1% L-glutamine).

Cell stimulation, tetramer staining, intracellular cytokine staining, and flow cytometry. Mononuclear cells from BAL, lungs, or spleens were cultured in U-bottom 96-well plates at a concentration of 20 million cells per ml for lungs and spleens and 0.5 million cells per ml for BAL. For intracellular cytokine staining,[42,45,50] cells were cultured in the presence of Golgi plug (5 mg ml^{-1} brefeldin A; BD Pharmingen, San Jose, CA) with or without stimulation for 5–6 h with an Ag85A-specific CD8 T cell-specific peptide (MPVGGQSSF)-Ep-1, a newly identified Ag85A-specific CD8 T-cell peptide (VYAGAMSGL)-Ep-2, or Ag85A-specific CD4 peptide (LTSELPGWLQANRHVKPTGS) at a concentration of 1 μg per well. In selected experiments, cells were stimulated with crude BCG and *M.tb* CF at a concentration of 1 μg per well for 24 h with last 6 h in the presence of Golgi plug. After incubation, cells were washed and blocked with CD16/CD32 in 0.5% bovine serum albumin/phosphate-buffered saline for 15 min on ice and then stained with the appropriate fluorochrome-labeled monoclonal antibodies. Cells were then processed according to the manufacturer's instructions (BD Pharmingen). The monoclonal antibodies used included CD8a-phycoerythrin-Cy7, CD4-allophycocyanin-Cy7, IFN-γ-allophycocyanin, and CD3-CyChrome. For tetramer immunostaining, a tetramer for the immunodominant CD8 T-cell peptide (MPVGGQSSF) of Ag85A bound to the BALB/c major histocompatibility complex class I allele H-2L^d (NIH Tetramer Core, Atlanta, GA) was used. Cells were washed and blocked with CD16/CD32 in 0.5% bovine serum albumin/phosphate-buffered saline for 15 min on ice and stained with the tetramer for 1 h in the dark at room temperature. Cells were then washed and stained with surface antibodies. Immunostained cells were run on an LSR II flow cytometer (BD Biosciences, San Jose, CA) and 250,000 events per sample were collected and analyzed on FlowJo software (version 9; Tree Star, Ashland, OR).

T-cell epitope mapping for identification of a newly emerged T-cell epitope following AdCh68Ag85A immunization. To investigate the repertoire of antigen-specific CD8 T cells generated by AdC68Ag85A and AdHu5Ag85A immunization, lung mononuclear cells from immunized mice were *ex vivo* stimulated with pools of 57 peptides of Ag85A[27] by using a previously described method.[47] In the initial screening, lung cells were stimulated separately with six peptide pools (p1-p10, p11-p20, p21-p30, p31-p40, p41-p50, and p51-p57; each peptide is 15 amino acids in length with 10 amino acids overlapping) or single peptide pool containing 1-57 peptides. Each peptide in every pool was kept at a concentration of 1 μg per well. Based on initial screening, two peptide pools (p21-p30 and p41-p50) containing potential CD8 T-cell epitopes were selected to design 9 sets of new peptide pools containing 2–9 peptides/pool in such a way that each peptide is present only in 3 unique pools. These peptide pools were then used for *ex vivo* stimulation of lung mononuclear cells from immunized mice. Based on the magnitude of T-cell reactivity, a 15-amino-acid peptide potentially containing an immunodominant CD8 T-cell epitope was identified and the corresponding amino acid sequence was analyzed using the Immune Epitope Database (http://tools.immuneepitope.org/) for determining the highest probable CD8 T-cell epitope sequence. A novel Ag85A-specific CD8 T cell-specific epitope sequence (VYAGAMSGL) was identified and the corresponding peptide synthesized and used for *ex vivo* antigen stimulation as described above.

***In vivo* intratracheal cytotoxic T-cell assay**. The intratracheal *in vivo* CD8 CTL assay was conducted as previously described.[45] Briefly, 2.5×10^6 antigen-pulsed and 2.5×10^6 unpulsed, CFSE (carboxyfluorescein succinimidyl ester)-labeled splenic CTL target cells were combined to a final 40 μl of phosphate-buffered saline and transferred

intratracheally to the airway of mice vaccinated with Ad-based vaccine for 2 or 4 weeks. The animals were killed at 5 h after target cell transfer. Total cells isolated by bronchoalveolar lavage were run on the FACScan (BD Pharmingen) for assessment of the percentage of CFSE-labeled target cells. The percent *in vivo* killing of CFSE-labeled target cells was defined as the relative loss of such cells after *in vivo* incubation and thus was taken as the measure of CTL.

Assessment of lung inflammation by histology, BAL cytology, and innate cytokine responses. Lung inflammation following respiratory mucosal immunization with AdCh68Ag85A or AdHu5Ag85A was assessed by blinded lung histopathologic examination, BAL differential inflammatory cell counting, and BAL cytokine measurement as previously described.[41,42,45] The levels of BAL cytokine proteins including tumor necrosis factor-α and IFNβ were measured by enzyme linked immunosorbent assay (ELISA; R&D Systems, Burlington, ON, Canada).

ELISA for measuring anti-AdHu5 IgG1, IgG2A, and IgA antibodies. Levels of anti-AdHu5-specific antibodies in BAL fluids and sera were determined by ELISA as previously described.[65] Briefly, Nunc immunoplates were coated with 50 µl per well of wild-type AdHu5 virus-infected HeLa cell lysate (100 µg ml^{-1}) overnight at 4 °C. Serial dilutions of sera or BAL fluids were set up in duplicates and incubated at 37 °C for 1 h. Goat anti-mouse IgG1-biotin or IgG2a-biotin (Southern Biotechnology Associates, Birmingham, AL) or IgA-biotin conjugates (Sigma-Aldrich) were added and incubated for 1 h at 37 °C, followed by streptavidin-conjugated alkaline phosphatase (R&D Systems, Minneapolis, MN) for 1 h at 37 °C. The substrate was added to each well and incubated at room temperature for 15 to 30 min in the dark. After stoppage of reaction, the colorimetric change was evaluated with a microplate reader at 405 nm.

Anti-WtAdHu5-neutralizing antibody measurement. The amount of neutralizing antibody titers present in serum or BAL was determined according to established methods.[27] Samples were serially diluted in culture media starting from neat. Appropriate controls were set up with serum or BAL samples from naive mice (negative) or with just media alone (positive). Each dilution was mixed with AdHu5-expressing *Escherichia coli* β-galactosidase (multiplicity of infection of 5), incubated for 1 h at 37 °C, and applied to confluent A549 epithelial cells in 96-well plates overnight at 37 °C. Neutralizing antibody titers were enumerated with AxioVision4 software (Carl Zeiss, Berlin, Germany). Wt-AdHu5-neutralizing antibody titers were calculated as 1/((number of stained cells × total number of quadrants)/PFU) × sera/BAL dilution.

Data analysis. The differences deemed statistically significant were indicated as $^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$. A two-tailed Student's *t*-test was performed for pair-wise comparisons. One-way analysis of variance and subsequent Tukey's *post hoc* test were carried out for the comparison of multiple groups. All analyses were performed by using GraphPad Prism software (GraphPad Software, San Diego, CA).

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at http://www.nature.com/mi

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DISCLOSURE

The authors declared no conflict of interest.

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4.0 - Chapter 4 - Determining the therapeutic potential of a monovalent chimpanzee adenovirus-vectored TB vaccine in accelerating disease control as adjunct to conventional antibiotic therapy

Manuscript title: Single-dose mucosal immunotherapy with chimpanzee adenovirus-based vaccine accelerates TB disease control and limits its rebound following antibiotic cessation.

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MAJOR ARTICLE

Single-Dose Mucosal Immunotherapy With Chimpanzee Adenovirus-Based Vaccine Accelerates Tuberculosis Disease Control and Limits Its Rebound After Antibiotic Cessation

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Background. The development of strategies to accelerate disease resolution and shorten antibiotic therapy is imperative in curbing the global tuberculosis epidemic. Therapeutic application of novel vaccines adjunct to antibiotics represents such a strategy.

Methods. By using a murine model of pulmonary tuberculosis (TB), we have investigated whether a single respiratory mucosal therapeutic delivery of a novel chimpanzee adenovirus-vectored vaccine expressing Ag85A (AdCh68Ag85A) accelerates TB disease control in conjunction with antibiotics and restricts pulmonary disease rebound after premature (nonsterilizing) antibiotic cessation.

Results. We find that immunotherapy via the respiratory mucosal, but not parenteral, route significantly accelerates pulmonary mycobacterial clearance, limits lung pathology, and restricts disease rebound after premature antibiotic cessation. We further show that vaccine-activated antigen-specific T cells, particularly CD8 T cells, in the lung play an important role in immunotherapeutic effects.

Conclusions. Our results indicate that a single-dose respiratory mucosal immunotherapy with AdCh68Ag85A adjunct to antibiotic therapy has the potential to significantly accelerate disease control and shorten the duration of conventional treatment. Our study provides the proof of principle to support therapeutic applications of viral-vectored vaccines via the respiratory route.

Keywords. antibiotics; immunotherapy; respiratory mucosal immunization; tuberculosis; viral-vectored vaccines.

Pulmonary tuberculosis (TB) remains the leading global infectious cause of death, claiming 1.6 million lives yearly [1]. Although antibiotics are available for treating TB, their success relies on patient adherence to treatment guidelines. Given that effective therapy is complex, requiring the use of multiple antibiotics for a minimum 6 months, many patients fail to properly follow and complete antibiotic therapy [2, 3]. This leads to the relapse of active disease and is a major reason for the emergence of multidrug-resistant and extensively drug-resistant TB cases that are increasingly more difficult to treat and account for significantly higher mortality rates [4, 5].

The development and implementation of effective vaccination strategies is central in combatting the TB epidemic. These vaccines are classified as prophylactic, postexposure (latent infection), and therapeutic vaccines [6–8]. Over the last 2 decades, significant progress has been made mostly in the development of prophylactic vaccines and, to a lesser extent, postexposure vaccine strategies [9]. However, the development of effective TB vaccines for therapeutic applications has lagged far behind.

Therapeutic vaccines are designed to be administered in conjunction with conventional TB antibiotic therapy to hosts with active disease. This strategy aims to boost or redirect the host anti-TB immune response to better control TB disease and/or shorten antibiotic therapy [8, 10–12]. In this regard, a limited number of subunit protein- and viral-based approaches have been explored for their therapeutic application [10, 13–16]. Although these studies have provided insights into the applicability of therapeutic vaccine strategies, they led to either no efficacy [15] or variable efficacies in limiting infection and lung pathology [10, 13, 14, 16]. Of note, all of these therapeutic vaccines had to be given repeatedly via the parenteral route.

We and others have developed viral-vectored TB vaccines for prophylactic applications after parenteral and respiratory mucosal (RM) routes of immunization [17–25]. Via these studies, it has been well established that RM immunization is superior to the parenteral route via positioning protective T cell immunity in the respiratory mucosa before *Mycobacterium tuberculosis*

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(*M.tb*) exposure. In a recent study, we showed that prophylactic vaccination with a chimpanzee adenovirus serotype-68 vectored vaccine expressing *M.tb* antigen Ag85A (AdCh68Ag85A) via the RM route provides robust immunity against *M.tb* challenge by reducing bacterial burden and lung pathology [26]. However, it has remained unclear whether such vaccine strategies can be applied therapeutically via the RM route, in conjunction with antibiotic therapy, to treat established pulmonary TB disease.

In the current study, we have investigated the therapeutic potential of AdCh68Ag85A. For the first time, we demonstrate that a single delivery of RM TB vaccine in conjunction with conventional antibiotic therapy can significantly improve disease treatment and restrict disease relapse after antibiotic cessation. Our findings thus suggest that viral-vectored TB vaccines designed for prophylactic respiratory immunization can also be used for effective immunotherapeutic application.

METHODS

Detailed methods are provided in the online Supplement.

Animal Models

Female BALB/c mice (6–8 weeks old) were housed and used within the biosafety level 3 facility in accordance with guidelines of institutional Animal Research Ethics Board.

Mycobacterium tuberculosis Infection and Antibiotic Therapy

Mycobacterium tuberculosis H_{37}Rv was prepared for infection via the RM route as described [21, 26]. Animals were treated with a triple antibiotic cocktail of rifampicin, isoniazid, and pyrazinamide via drinking water. The nonsterilizing antibiotic regimens were chosen in this immunotherapeutic investigation.

AdCh68Ag85A Immunotherapy

AdCh68Ag85A was administered intranasally (I.N.) or intramuscularly (I.M.) as described [18, 23, 26] to infected animals. Some animals received an empty AdCh68 control vector.

In Vivo T Cell Depletion

CD4 and CD8 T cells were depleted by intraperitoneal (I.P.) injection of anti-CD4 (clone GK1.5) and anti-CD8 (clone 2.43) monoclonal antibodies (mAbs) [27].

Tumor Necrosis Factor α Protein Levels

Tumor necrosis factor α (TNFα) was measured in bronchoalveolar lavage (BAL) fluids and sera using enzyme-linked immunosorbent assay (ELISA).

Mononuclear Cell Isolation and Intracellular Cytokine Staining

Lung mononuclear cells were isolated using our previously published protocol [20, 21, 26]. Intracellular cytokine staining was performed after stimulation with antigens (Ags). Stained cells were acquired on a BD LSRFortessa cytometer, and data were analyzed using FlowJo software, version 10 (TreeStar, Ashland, OR).

Tuberculosis Disease Indices

Lung bacterial burden was evaluated by colony-forming unit (CFU) assay [26]. Lung sections were hematoxylin and eosin-stained for histopathological analysis and quantification or Ziehl Neelsen-stained for visualization of acid-fast bacilli (AFB) [20].

T Cell and Macrophage Distribution by Immunohistochemistry

Immunohistochemical staining of CD4, CD8, and F4/80 was performed on deparaffinized sections using anti-mouse CD4, CD8, and F4/80 mAbs.

Statistical Analysis

Two-tailed Student *t* tests were used for comparison between 2 groups. One-way analysis of variance was used followed by posttest Tukey analysis for multiple-group comparison using GraphPad Prism 8 software. Results were considered significant for P values ≤.05. Area-under-the-curve (AUC) analysis was done to summate changes in bacterial burden over time. Unpaired *t* tests were performed in AUC analysis.

RESULTS

AdCh68Ag85A Respiratory Mucosal Immunotherapy Improves Pulmonary Tuberculosis Disease Control During Antibiotic Therapy

With its demonstrated prophylactic efficacy [26], we investigated whether AdCh68Ag85A could be used as a therapeutic vaccine adjunctive to a triple antibiotic therapy in treating pulmonary TB. To this end, mice infected with *M.tb* for 4 weeks were treated with antibiotics alone (ABx) or in conjunction with intramuscular (ABx I.M. Vac.) or RM (ABx I.N. Vac.) immunotherapy with a single dose of AdCh68Ag85A at 4 weeks postinitiation of antibiotic treatment (Figure 1A). A set of mice was left untreated. All groups of mice were sacrificed at 12 weeks postinfection and were assessed for TB disease indices. As expected, antibiotic treatment significantly reduced pulmonary bacterial burden compared with untreated animals (Figure 1B). Parenteral immunotherapy (ABx I.M.Vac.) failed to significantly further enhance bacterial control. However, RM administration of AdCh68Ag85A (ABx I.N.Vac.) provided further significant reduction in bacterial burden by 1.25 log, reducing pulmonary mycobacterial burden to 3.5 log. Histological analysis revealed markedly reduced lung pathology in ABx I.N.Vac. animals compared with those treated with ABx or in conjunction with parenteral immunotherapy (ABx I.M.Vac.) (Figure 1C).

Given that adjunct RM immunotherapy can significantly improve pulmonary TB disease, we assessed whether such improvement might have been rendered at the expense of overzealous acute tissue inflammation, which could pose a potential safety concern. For this, mice infected with *M.tb* for 4 weeks were treated with ABx or in conjunction with RM AdCh68Ag85A (ABx/I.N.Vac.) and sacrificed 72 hours postimmunotherapy. Bronchoalveolar lavage, lung tissue, and sera were examined

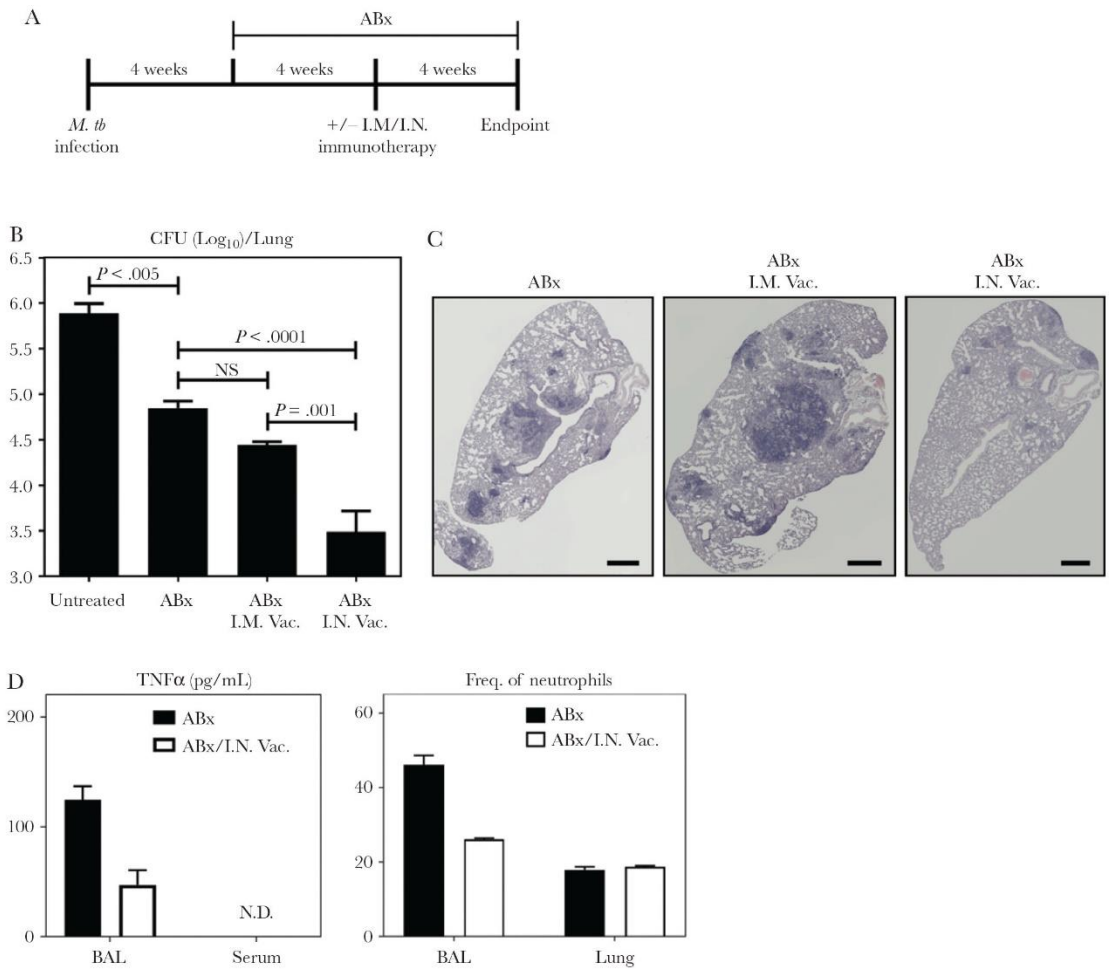

Figure 1. AdCh68Ag85A respiratory mucosal immunotherapy improves tuberculosis (TB) disease control during antibiotic therapy. (A) Experimental schema. At 4 weeks post-*Mycobacterium tuberculosis* (*M.tb*) infection, mice were started on an oral antibiotic (antibiotics alone [ABx]) therapy of rifampicin, isoniazid, and pyrazinamide. A group of these mice was treated either intramuscularly (ABx I.M Vac.) or intranasally (ABx I.N. Vac.) with AdCh68Ag85A at 4 weeks after the initiation of antibiotic therapy. All mice were sacrificed 12 weeks postinfection for assessment of TB disease indices. A set of *M.tb*-infected animals were left untreated as controls (untreated). (B) Bar graph comparing bacterial burden assessed by colony-forming unit (CFU) assay in the lungs of 4 groups of mice. (C) Representative micrographs of lung sections stained with hematoxylin and eosin, comparing the extent of lung inflammation and granulomatous lesions. Scale bar indicates 500 µm. (D) Bar graphs showing levels of tumor necrosis factor α (TNFα) protein in the bronchoalveolar lavage (BAL) fluid and in sera, and frequencies of neutrophils ($CD11b^+Ly6G^+$) in the BAL and lung at 72 hours post-I.N. immunotherapy. Data are expressed as the mean ± standard error of the mean of 6–10 mice/group, representative of 3 independent experiments.

for proinflammatory TNFα levels and neutrophil infiltration. Collectively, in ABx/I.N.Vac. animals, BAL and serological concentrations of TNFα and neutrophil recruitment to the airway and lung did not increase over those treated with ABx (Figure 1D). On the contrary, they appeared even lower in the BAL of ABx/I.N.Vac. animals.

The above data suggest that compared with antibiotic therapy alone, a single dose of AdCh68Ag85A delivered via the RM route, but not via parenteral route, in conjunction with antibiotic therapy significantly accelerates bacterial clearance and reduces lung pathology.

AdCh68Ag85A Respiratory Mucosal Immunotherapy Controls Bacterial Infection in the Lung After Premature Antibiotic Therapy Cessation

Having established the potency of RM immunotherapy adjunctive to continuing antibiotic treatment (Figure 1), we next set out to address whether RM immunotherapy can shorten antibiotic therapy and control antibiotic cessation-associated infection

rebound. To this end, animals were infected with *M.tb* and at week 4 postinfection, all animals were treated with triple antibiotics. In a set of animals, antibiotic therapy continued for a total of 8 weeks (ABx). In another set of animals, antibiotic therapy was prematurely stopped at 4 weeks (ABx cessation). A group of animals received a single dose of RM immunotherapy at the time when antibiotic therapy was stopped (ABx cessation+I.N.Vac) (Figure 2A). All animals were sacrificed 12 weeks postinfection and lungs were assessed for TB disease indices. Premature cessation of antibiotic therapy (ABx cessation) led to a significant rebound of bacterial burden in the lung as indicated by a 1 log increase in CFU compared with animals receiving continuing antibiotic therapy (ABx) (Figure 2B). In contrast, lung bacterial burden in animals treated with RM immunotherapy at the time of antibiotic cessation (ABx cessation+I.N.Vac) did not undergo rebound with CFU kept similar to that in ABx animals with continuing antibiotic therapy. In support, the lungs of ABx cessation+I.N.Vac animals had reduced granulomatous lesions both in size and number compared with ABx cessation animals (Figure 2C). These data suggest that RM immunotherapy helps control TB disease in the lung even after premature antibiotic cessation.

Adjunct Respiratory Mucosal Immunotherapy Accelerates Bacterial Clearance and Curbs Bacterial Rebound During Chronic Pulmonary Tuberculosis

Having established that adjunct AdCh68Ag85A RM immunotherapy is able to help control TB disease in a 12-week infection model (Figures 1 and 2), we next investigated the utility of this immunotherapeutic strategy in a protracted model of chronic TB disease. To this end, 4-week *M.tb*-infected mice were treated with ABx or ABx plus a single RM immunotherapy performed at week 8 (ABx/Vac.) and were sacrificed at 4, 12, 16, and 20 weeks postinfection (ABx continuation phase) (Figure 3A). We observed that both ABx and ABx/Vac. led the bacterial burden in the lungs to continue to decrease (Figure

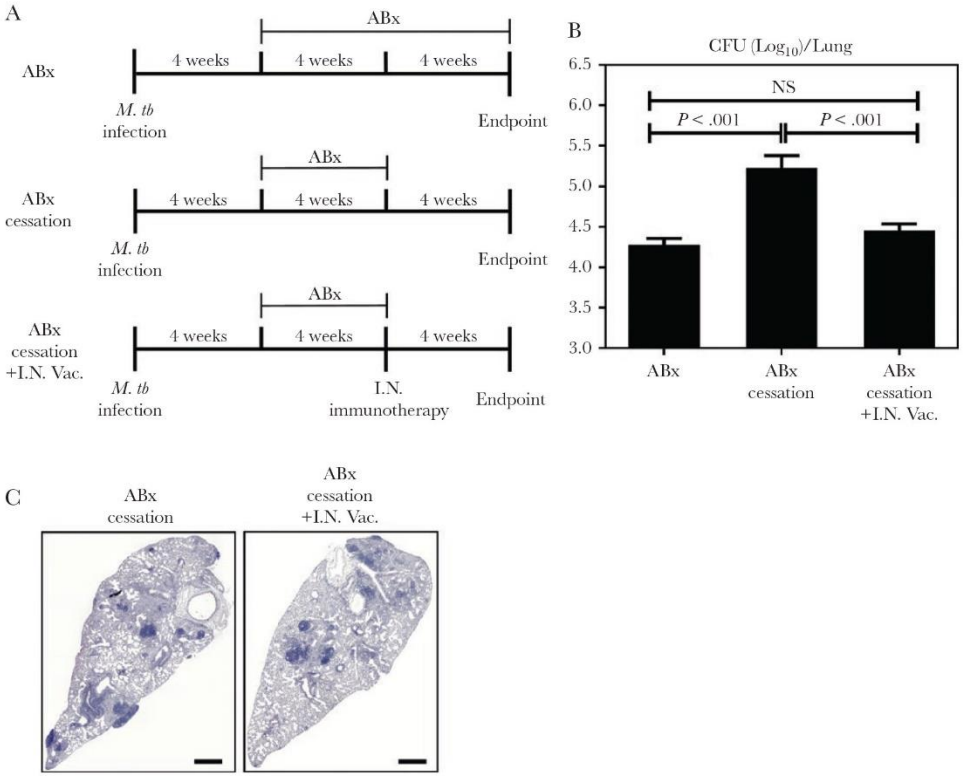

Figure 2. Respiratory mucosal immunotherapy controls bacterial infection in the lung after antibiotic cessation. (A) Experimental schema. At 4 weeks post-*Mycobacterium tuberculosis* (*M.tb*) infection, mice were started on oral antibiotic therapy. Groups of mice were treated for 4 (antibiotics alone [ABx] cessation) or 8 (ABx) weeks. In a set of animals, antibiotic therapy was ceased after 4 weeks and a single dose of AdCh68Ag85A was administered intranasally (ABx cessation+I.N.Vac.). All mice were sacrificed 12 weeks postinfection for assessment of tuberculosis disease indices. (B) Bar graph comparing bacterial burden in the lungs of various groups of mice. (C) Representative micrographs of lung sections stained with hematoxylin and eosin, comparing the extent of lung inflammation and granulomatous lesions. Scale bar indicates 500 μm. Data are expressed as the mean ± standard error of the mean of 6–10 mice/group. CFU, colony-forming units.

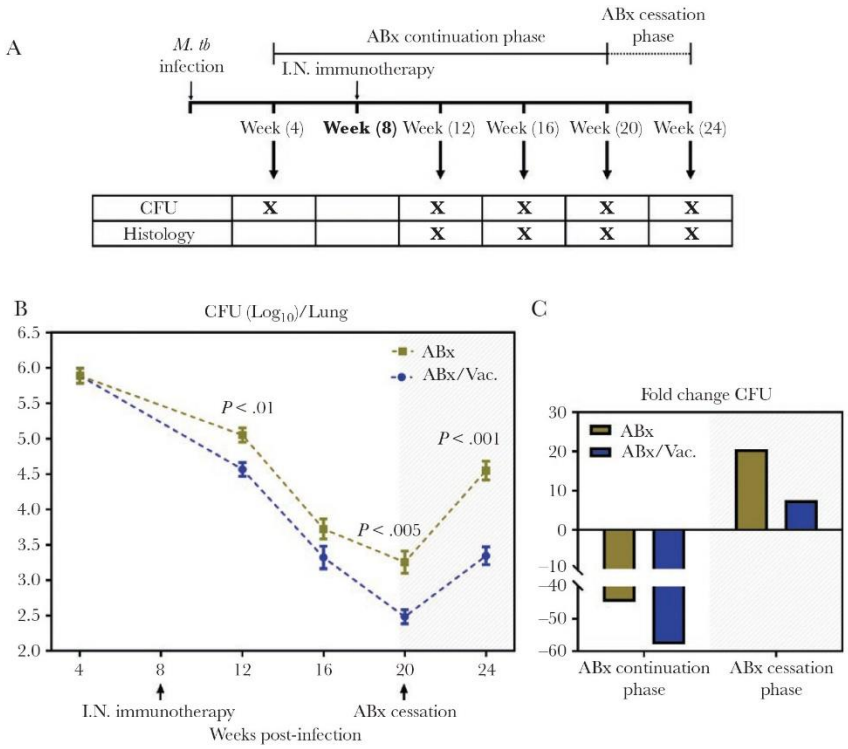

Figure 3. Adjunct respiratory mucosal immunotherapy accelerates bacterial clearance and curbs bacterial rebound during chronic pulmonary tuberculosis. (A) Experimental schema. At 4 weeks postinfection, mice were started on an oral antibiotic therapy. Groups of mice were treated for 16 weeks. In a set of animals, a single dose of AdCh68Ag85A was administered intranasally (I.N.) at 4 weeks after the initiation of antibiotic therapy. Animals were sacrificed for analysis at specified time points. In some animals, antibiotic therapy was ceased at 20 weeks postinfection. Tuberculosis disease indices were assessed in these animals 4 weeks after the cessation of antibiotic therapy (week 24). (B) Line graph showing kinetic changes in bacterial burden in the lung. Unshaded area indicates antibiotic continuation phase and shaded area indicates antibiotic cessation phase. (C) Bar graph showing the mean fold changes in lung bacterial burden during the antibiotic continuation phase and subsequently during the antibiotic cessation phase. Data are expressed as the mean ± standard error of the mean of 10–12 mice/group, representative of 1 to 3 independent experiments (depending on the time point). ABx, antibiotics alone; CFU, colony-forming units; *M.tb*, *Mycobacterium tuberculosis*.

3B). However, the bacterial clearance in animals with adjunct RM immunotherapy (ABx/Vac.) was significantly accelerated (AUC = 9.16 vs 9.635, $P = .001$), resulting in a significantly lower bacterial burden at 12 and 20 weeks postinfection compared with ABx animals (Figure 3B). Thus, whereas ABx alone reduced the bacterial burden by 40-fold over the antibiotic continuation phase, conjunctive ABx/Vac. therapy brought it down by 60-fold (Figure 3C).

We next determined whether adjunct immunotherapy could control disease rebound after premature antibiotic cessation in this protracted TB model. Thus, by the experimental design described above, antibiotic therapy was stopped in a group of animals at 20 weeks postinfection, and animals were sacrificed and examined for the extent of disease rebound 4 weeks later (antibiotic cessation phase) (Figure 3A). The ABx animals showed a rapid bacterial rebound (1.5 log increase from the time of antibiotic cessation) equating to a 20-fold increase in bacterial burden (Figure 3B and C). In contrast, ABx/Vac. animals showed a significantly restricted bacterial rebound (0.75 log, AUC = 3.324 vs 4.137, $P < .0001$), equating to only a 10-fold increase (Figure 3B and C).

Because it is well documented that natural *M.tb* infection occurs after exposure to very few bacilli [28, 29], we further determined the efficacy of AdCh68Ag85A RM immunotherapy in an infection model set up by a much reduced dose of *M.tb* (100 CFU). Infected animals were subsequently treated with ABx or in conjunction with RM immunotherapy (ABx /I.N. Vac.) similarly as described above except that they were sacrificed at 12 and 16 weeks postinfection (ABx continuation phase) (Figure 4A). In agreement with the earlier findings (Figure 3), both ABx and ABx /I.N.Vac. led to a sharp decline in lung bacterial burden over time (Figure 4B). However, ABx/I.N.Vac. animals had the most significant CFU reduction in comparison to ABx-treated animals (AUC = 2704 vs 2995, $P = .01$), even with some animals with below-limit of CFU detection at 16 weeks postinfection (Figure 4B, dotted black line). Furthermore, when

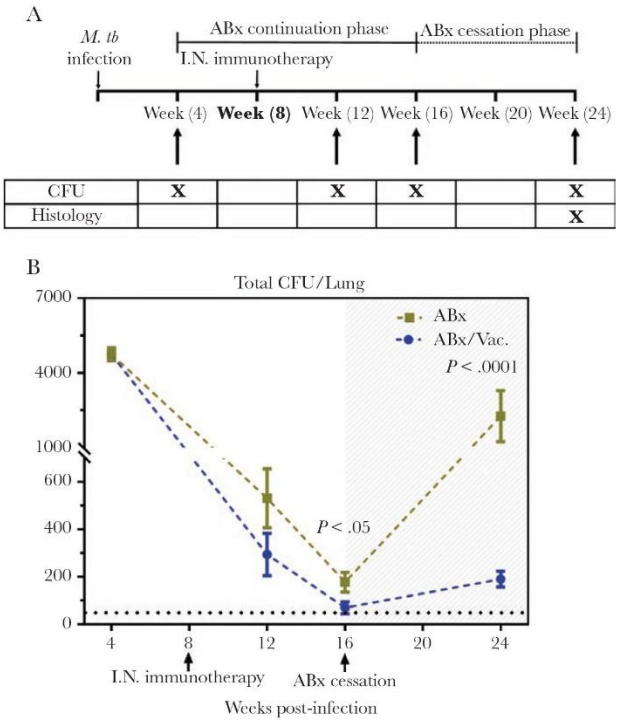

Figure 4. Efficacy of AdCh68Ag85A respiratory mucosal immunotherapy in a low infection dose model. (A) Experimental schema. Mice were infected and treated as described in Figure 3A (schema) but with a low dose of *Mycobacterium tuberculosis* (*M.tb*) infection (100 colony-forming units [CFU]), and antibiotic therapy ceased at week 16 postinfection. At specified time points, tuberculosis disease indices were assessed. (B) Line graph showing kinetic changes in bacterial burden in the lung. Unshaded area indicates antibiotic continuation phase and shaded area indicates antibiotic cessation phase. Dotted horizontal line represents the limit of detection (50 CFU). Data are expressed as the mean ± standard error of the mean of 6–12 mice/group, representative of 1 to 3 independent experiments (depending on the time point). ABx, antibiotics alone; I.N., intranasal.

assessed at 8 weeks (24 weeks postinfection) after antibiotic cessation (Figure 4A), ABx/I.N.Vac. animals showed a remarkably limited rebound as indicated by minimal detectable CFU (AUC = 129.6 vs 1220, $P < .005$) (Figure 4B) and markedly reduced lung pathology (Supplementary Figure 1) in comparison to ABx animals.

The above data together indicate an even greater protective role by adjunct RM immunotherapy in a more clinically relevant low-dose infection model.

Adjunct Respiratory Mucosal Immunotherapy Reduces Tuberculosis-Associated Tissue Pathology and Enhances CD8 T Cell Infiltration in the Lung

The extent of lung immunopathology is a critical index in assessing TB disease and vaccine-induced protection and even more so in the setting of TB immunotherapy [16, 18, 30]. Thus, besides quantifying bacterial burden, we performed an in-depth histopathological examination of the lungs from our protracted TB disease model illustrated in Figure 3A. Antibiotic therapy alone reduced TB-associated lung pathology shown by decreasing granulomatous regions and inflammation over time (Figure 5A, B). In comparison, adjunct RM immunotherapy (ABx/Vac.) significantly accelerated the resolution of lung pathology as indicated by major reductions in the severity and number of granulomatous lesions and overall reduced pulmonary inflammation at 12, 16 and 20, weeks postinfection (Figure 5A and B). Using 2-color immunohistochemistry, we examined the distribution of macrophages ($F4/80^+$) and $CD4^+/CD8^+$ T cell subsets in consecutive lung sections obtained at 12 weeks postinfection. We found abundant presence of macrophages in the regions outside granulomatous lesions of ABx animals (Figure 5C, red arrows). In comparison, fewer macrophages were detected and were mostly localized within granulomatous regions in ABx/Vac. animals (Figure 5D, red arrows). On the other hand, considerably more CD8 T cells were found around and within granulomatous lesions in the lung of ABx/Vac. animals (Figure 5D, yellow arrows). This contrasted greatly with a high CD4 versus CD8 T cell ratio and much less intensity of CD8 T cells in lung lesions of ABx animals (Figure 5C, yellow arrows).

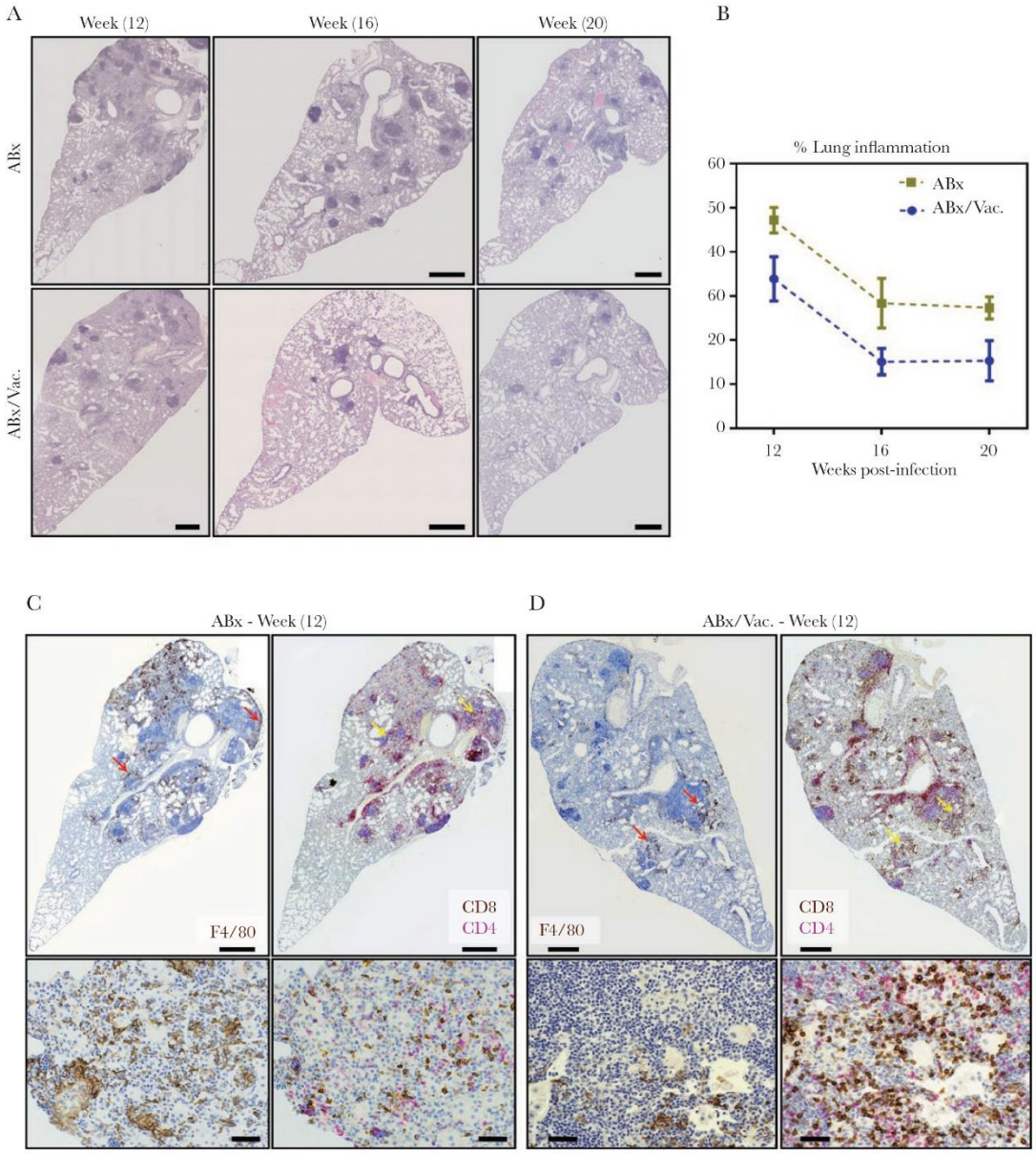

Figure 5. Adjunct respiratory mucosal immunotherapy reduces tuberculosis-associated tissue pathology and enhances CD8 T cell infiltration in the lung during antibiotic continuation phase. Experiments were set up as depicted in experimental schema Figure 3A. (A) Representative micrographs of lung sections stained with hematoxylin and eosin, comparing the extent of lung inflammation and granulomatous lesions at weeks 12, 16, and 20 weeks postinfection. Scale bars indicate 500 µm. (B) Line graph showing semiquantified area of lung inflammation. Displayed values are averages from 3 micrographs per mouse. (C and D) Representative micrographs of immunohistochemically stained lung sections at 12 weeks postinfection visualizing the spatial distribution of F4/80 macrophages (brown stain) and of CD4 (red stain) and CD8 (brown stain) T cells costained in consecutive sections. Red arrows highlight macrophage-rich areas. Yellow arrows highlight T cell-rich areas. Top panel scale bars indicate 500 µm; bottom panel scale bars indicate 50 µm. ABx, antibiotics alone.

We also assessed lung histopathology upon disease rebound at 4 weeks postantibiotic cessation (24 weeks postinfection) (Figure 3A). In ABx animals, antibiotic cessation led to much worsened immunopathology as shown by large consolidated granulomatous lesions in the lung (Figure 6A). At higher magnification, these lesions were characterized by islands of lipid-laden foamy

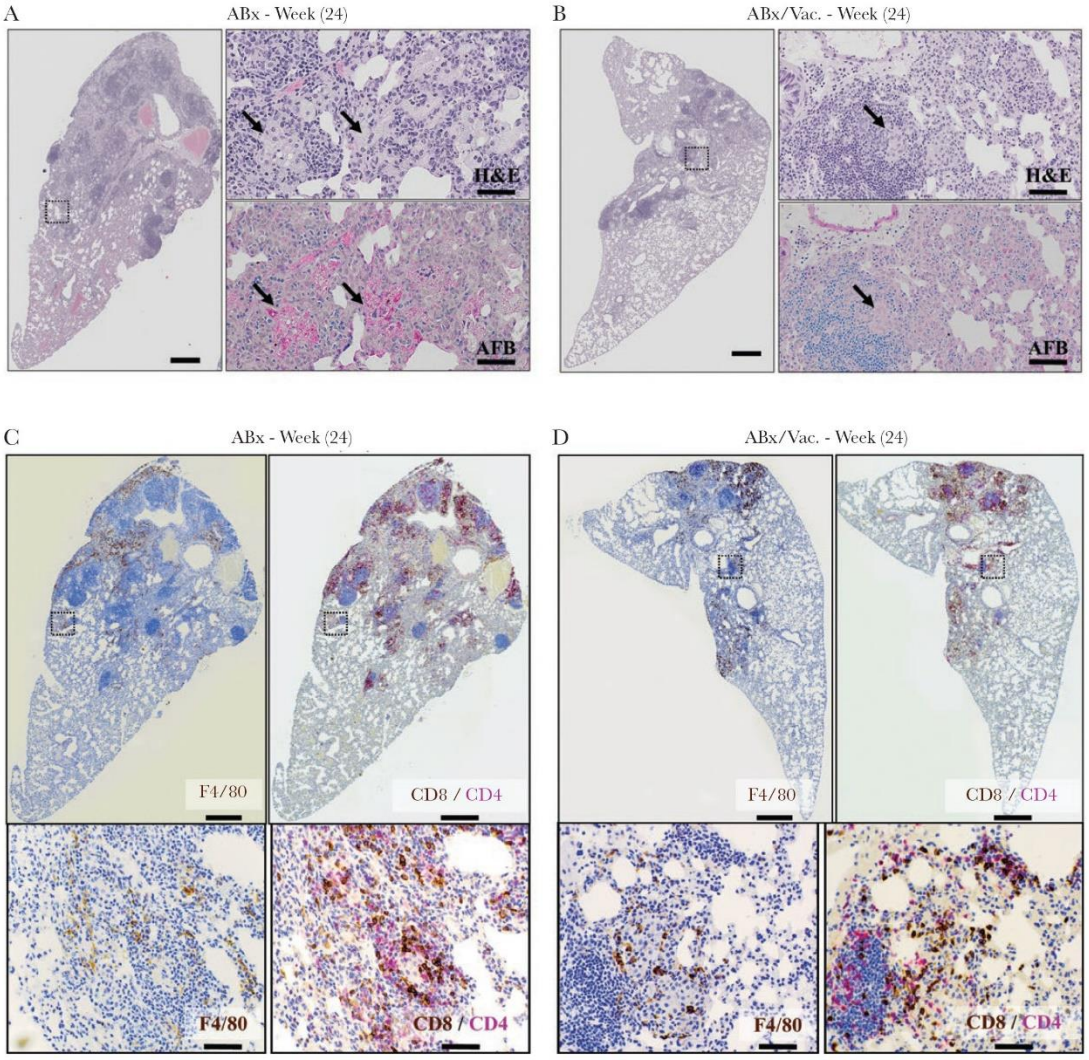

Figure 6. Adjunct respiratory mucosal immunotherapy reduces tuberculosis-associated tissue pathology in the lung during antibiotic cessation phase. Experiments were set up as depicted in experimental schema Figure 3A. (A and B) Representative micrographs of lung sections stained with hematoxylin and eosin (H&E), comparing the extent of lung inflammation and granulomatous lesions at 4 weeks postantibiotic cessation (24 weeks). Scale bars indicate 500 μm. Higher magnifications of H&E micrographs depict abundant foamy macrophages (H&E) colocalized with acid-fast bacilli (AFB) (black arrows). Scale bars indicate 50 μm. (C and D) Representative micrographs of immunohistochemically stained lung sections at 4 weeks postantibiotic cessation (24 weeks) visualizing the spatial distribution of F4/80 macrophages (brown stain) and CD4 (red stain) and CD8 (brown stain) T cells costained in consecutive sections. Scale bars indicate 500 μm. ABx, antibiotics alone.

macrophages surrounded by mononuclear cells (Figure 6A, black arrows). Upon Ziehl-Neelsen staining for identifying AFB, these macrophages were densely packed with mycobacterial AFB and their products (Figure 6A, black arrows and red bacilli). In contrast, the lungs of ABx/Vac. animals showed very limited pathological rebound with much less granulomatous lesion, few foamy macrophages, and minimally detectable AFB (Figure 6B, black arrows), consistent with at least 10-fold fewer CFU in the lungs of ABx/Vac. animals in the ABx cessation phase (Figure 3C). Immunohistochemistry provided similar findings as in Figure 5C with macrophages encasing granulomatous lesions largely void of CD4 and CD8 T cells in ABx animals (Figure 6C, dotted black box). In contrast, the lungs of ABx/Vac. animals had fewer macrophages and a CD8 T cell-dominant response within the lesions (Figure 6D, dotted black box).

Taken together, the above data suggest that adjunct RM immunotherapy effectively controls TB disease even after premature antibiotic cessation, not only via reducing

mycobacterial bacillary burden but, importantly, via mitigating immunopathology in the lung. This vaccine-induced immunotherapeutic effect is closely associated with CD8 T cell infiltration in the lung.

Adjunct Respiratory Mucosal Immunotherapy Enhances Antigen-Specific CD8 T Cell Responses in the Lung and Confers Protection in a T Cell-Dependent Manner

Our results thus far have suggested a role of vaccine-induced CD8 T cells in accelerated resolution of TB disease and limiting disease rebound by adjunct AdCh68Ag85A immunotherapy. To investigate the mechanisms underlying the therapeutic efficacy of AdCh68Ag85A, we first examined T cell responses in the lung of animals treated with ABx or in conjunction with RM immunotherapy (ABx/Vac.) by flow cytometry (Figure 7A). Immunotherapy caused a shift from an overall CD4 T cell-dominated response in the lung of ABx animals to a CD8 T cell-dominated response in ABx/Vac. animals (Supplementary Figure 2). By Ag-specific Ag85A CD8 tetramer (Tet) immunostaining or by specific Ag-stimulated intracellular cytokine straining of total lung mononuclear cells, we found that although there was a very small frequency of CD8 T cells specific for Ag85A in ABx lungs, more than 50% of the CD8 T cells in the lung of ABx/Vac. animals were Ag85A-specific, resulting in a much greater number of Tet^+ CD8 T cells (Figure 7B). These Ag-specific cells were functionally activated as indicated by their ability to produce interferon-γ upon stimulation (Figure 7C, dot plots). In comparison, Ag85A-specific CD4 T cell responses were negligible (Figure 7C, bar graph), thus supporting the immunohistochemical findings (Figure 5D). Of interest, although CD4 T cells dominated the lung in ABx animals, only a very small number of them were *M.tb*-specific, comparable to those in ABx/Vac. animals (Supplementary Figure 3). On the other hand, *M.tb*-specific CD8 T cell responses were markedly increased in ABx/Vac. animals compared with ABx animals.

We next further investigated the role of T cells in protective immunotherapeutic effects of AdCh68Ag85A. To this end, mice infected with *M.tb* for 4 weeks started on antibiotics in conjunction with RM immunotherapy with AdCh68Ag85A (ABx/Vac.). A subset of ABx/Vac. animals received weekly I.P. injections of anti-CD4/CD8 antibodies until experimental endpoint (ABx/Vac. T cell depleted), and an additional subset of animals received RM empty viral vector lacking the Ag85A gene (ABx/Empty vector) instead of AdCh68Ag85A. All animals were sacrificed 12 weeks postinfection and assessed for TB disease indices (Figure 7D). We found that T cell ablation in ABx/Vac. animals was associated with a significant increase in pulmonary bacterial burden in comparison to their undepleted counterparts. On the other hand, the animals receiving only a control viral vector (ABx empty vector) also had heightened bacterial burden in the lung (Figure 7D). Taken together, the above data suggest that RM immunotherapy-induced Ag85A-specific T cells contribute directly to the protective therapeutic efficacy of AdCh68Ag85A.

DISCUSSION

The emergence of multidrug-resistant to total drug-resistant strains of *M.tb* and the paucity of new drugs call for developing new therapeutic strategies [1, 3]. In this study, we show that a single dose of adjunct AdCh68Ag85A RM immunotherapy accelerates the resolution of pulmonary TB disease and limits disease rebound after premature antibiotic cessation in a model of chronic TB. Respiratory mucosal immunotherapy-induced Ag-specific T cells contribute to its protective therapeutic efficacy. These data indicate that virus-based TB vaccines designed for prophylactic RM immunization have the potential for therapeutic applications.

Our study represents the first to show the potency of respiratory route of immunotherapy and its superiority over the parenteral route. Failure of parenteral immunotherapy to enhance TB disease control by antibiotics supports the importance of quantity and quality of Ag-specific T cells in the lung [7, 19, 31]. A previous study has also shown the inability of repeated parenteral immunotherapies with adenoviral (Ad26/Ad35)-vectored TB vaccines to enhance protection [15]. High Ag exposure associated with repeated parenteral immunotherapies and ongoing TB infection may lead to impaired T cell functions [32] and severe local adverse effects [9]. Such situations may likely be worsened during antibiotic therapy. By comparison, besides its potency in directly furnishing infected lungs with T cells, adenoviral-based RM immunotherapy may train/activate lung macrophages such that they not only have enhanced mycobactericidality but also become better responders to Ag-specific T cells [7, 27]. Indeed, we show here that a single dose of adjunct mucosal immunotherapy markedly reduces both bacterial burden and pathology in the lung. This approach appears advantageous over a repeated parenteral immunotherapeutic strategy that significantly reduced lung pathology but had a much less effect on bacterial control [16].

Consideration of the timing of immunotherapy during antibiotic therapy is also of importance, in particular to the safety and efficacy of RM immunotherapy [33]. In our study, RM immunotherapy was carried out until after 4-week antibiotic therapy. This was to ensure that antibiotic therapy had markedly reduced bacterial infection and antigenic load and associated inflammation in the lung. This helps avoid not only the morbidity resulting from vaccine-enhanced inflammation [16, 30] but also T cell exhaustion [32].

The protective correlates of anti-TB immunity have remained to be established. Our current study provides the evidence that RM immunotherapeutic vaccine-induced infiltration of T cells, particularly CD8 T cells, in TB lesions plays an important protective role. A recent study showed that *M.tb*-specific CD8 T cells recognize and inhibit *M.tb*

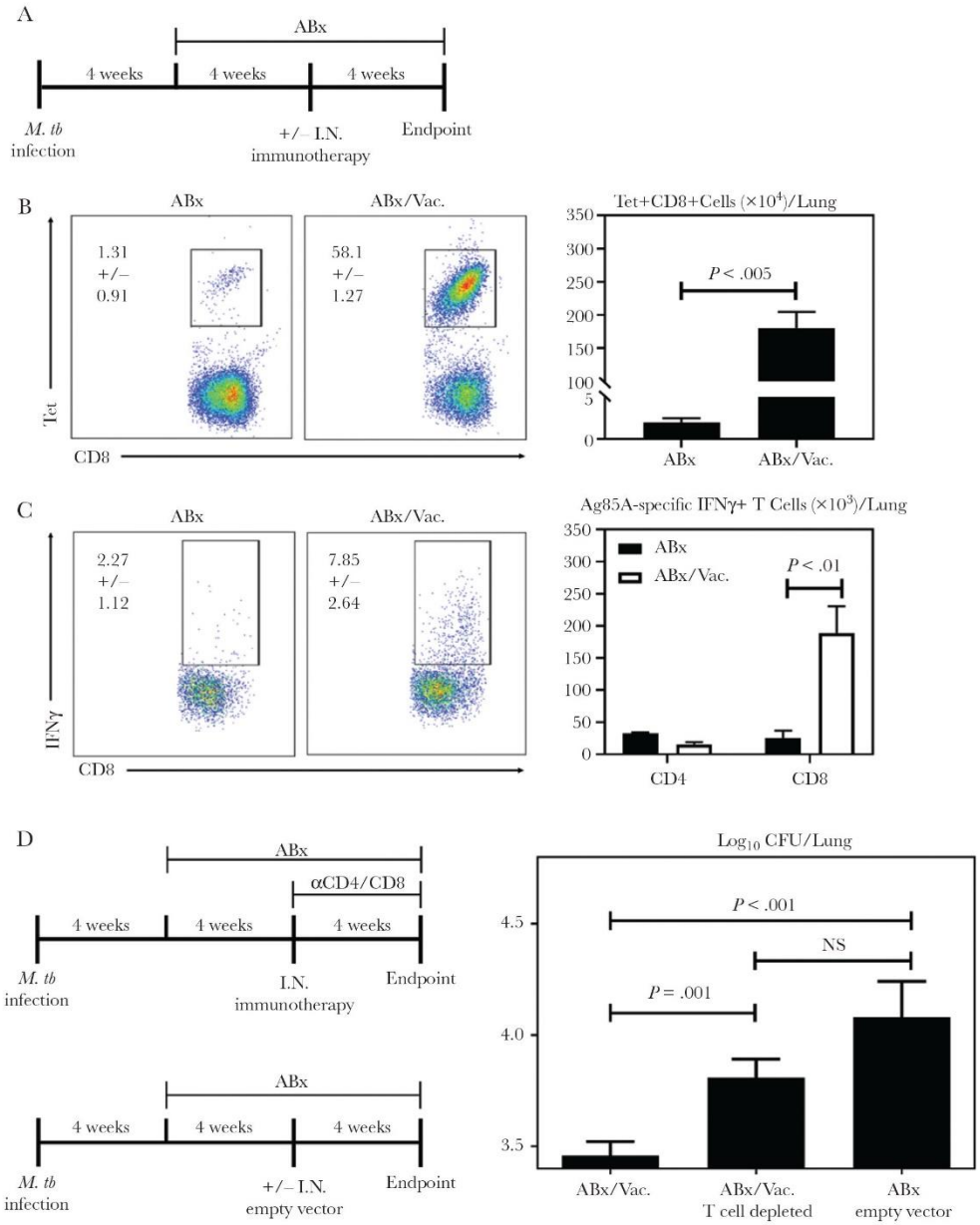

Figure 7. Adjunct respiratory mucosal (RM) immunotherapy enhances antigen (Ag)-specific CD8 T cell responses in the lung and confers protection in a T cell-dependent manner. (A) Experimental schema. Mice were set up as depicted in experimental schema Figure 3A. (B) Representative dot plots depicting the frequencies and bar graph depicting absolute numbers of Ag85A-specific CD8 T cells as assessed by tetramer immunostaining. (C) Representative dot plots depicting the frequencies and bar graph depicting absolute numbers of Ag85A-specific IFNγ^+ T cells as assessed by intracellular cytokine staining after recombinant Ag85A stimulation of lung mononuclear cells. (D) Experimental schema. Mice were infected and treated with antibiotics as per Figure 1A. One set of animals received RM immunotherapy 4 weeks after the initiation of antibiotics (ABx/Vac.). T cells were subsequently depleted weekly after immunotherapy (ABx/Vac. T cell depleted). A set of animals received a single dose of AdCh68 empty control vector in place of AdCh68Ag85A (ABx/empty vector). Data are expressed as the mean ± standard error of the mean of 3 mice/group for B or C and 6–10 mice/group for D. ABx, antibiotics alone; CFU, colony-forming units.

growth in infected macrophages [34]. Our data suggest that inducing Ag85A-specific CD8 T cells is a useful criterion for immunotherapeutic development. In addition to T cells, it is likely that vaccine-trained innate immune cells may also play a role because we have recently shown that adenoviral infection in the lung induces memory macrophages with enhanced antimicrobial activities [27].

The success of current TB antibiotic regimens is challenged by the length of therapy required to cure disease without rebound. Even with the implementation of Direct Observed Treatment, short course, treatment failures spike as high as 40% for individuals with drug-resistant TB [2, 35, 36]. Treatment-shortening regimens are therefore highly sought after to improve antibiotic treatment success. Different from other immunotherapeutic studies published to-date, we show here that a single-dose immunotherapy with AdCh68Ag85A not only enhances infection control by antibiotics, but it also restrains disease rebound after antibiotic stoppage in a protracted TB disease model.

Our study establishes the therapeutic efficacy of AdCh68Ag85A in the context of pulmonary TB. Although this is the most prevalent form of the disease, TB in humans can manifest in extrapulmonary sites, and it would be relevant to examine the relationship of its local therapeutic effects to extrapulmonary infection in future studies [37]. Furthermore, future work should also investigate whether our therapeutic vaccine strategy differentially affects replicating and dormant/persistent *M.tb* bacilli. This is of particular relevance because the latter underinvestigated mycobacterial population is thought to be a major contributor to the disease relapse post-antibiotic therapy [33, 38–40].

CONCLUSIONS

In conclusion, we have provided strong evidence to support the safe and effective immunotherapeutic application, via the respiratory route, of a recombinant chimpanzee adenovirus-based prophylactic TB vaccine. Further clinical translation shall help develop strategies to shorten antibiotic therapy and curb the emergence of drug-resistant disease.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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5.0 - Chapter 5 - Developing a multivalent chimpanzee adenovirus-vectored TB vaccine that provides broadened immunity against the entire *M.tb* life cycle

Manuscript title: Markedly improved protection against established pulmonary tuberculosis by a multi-antigenic chimpanzee adenovirus-vectored vaccine targeting both acute and resuscitation antigens.

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Markedly improved protection against established pulmonary tuberculosis by a multi-antigenic chimpanzee adenovirus-vectored vaccine targeting both acute and resuscitation antigens

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Abstract

Although the multi-staged life cycle of *Mycobacterium tuberculosis* makes it an exceedingly difficult bacterium to treat, it provides avenues for identifying antigenic targets for vaccine design. Mycobacterial persistence represents a stage of phenotypic resistance against host and pharmacological pressures. Evasion of such stresses allows *M.tb* to eventually resuscitate, thereby leading to active disease. Resuscitation is mediated by a series of enzymes known as resuscitation promoting factors, which makes them attractive vaccine antigens. In this study, we have developed a multi-antigenic chimpanzee adenovirus-vectored TB vaccine that expresses two early infection antigens (Ag85A and TB10.4) and one resuscitation antigen (rpfB) in order to broaden protective immunity against *M.tb*. We experimentally prove that such a formulation not only provides robust immunity against actively replicating bacilli, but also markedly reduces the population of persistent *M.tb*. Our study provides novel evidence supporting the importance of antigen selection in TB vaccine design.

Introduction

Etched throughout human history, *Mycobacterium tuberculosis* (*M.tb*) continues to impact millions of lives annually. In 2018 alone, over 10 million individuals became newly infected, and over 1.5 million succumbed to pulmonary tuberculosis (TB)[1]. The global TB burden has continued to expand, with increased prevalence of drug-resistant disease due to treatment default/failure. Such statistics accentuate inadequacies within current preventative and therapeutic anti-tuberculosis regimens.

Bacillus Calmette-Guerin (BCG) remains the only clinically approved TB vaccine. Although this vaccine is indispensable in curbing childhood disseminated forms of disease, it largely fails to protect against adult pulmonary TB[1]. As such, major strides in developing novel vaccines to address this gap in protection are highly sought after. Recently, two vaccine platforms have been evaluated for their protective efficacy in phase 2B trials. Unfortunately, these trials either showed no enhancement in protection (MVA85A), or showed promising efficacy, but confined to a narrow cohort of participants ($M72/ASO1_E$)[2,3]. Currently there are over 10 other vaccine candidates at various stages of clinical evaluation[1]. Some of these platforms are nearly identical to those already proven to have limited efficacy. Thus, questions arise whether such formulations are capable of eliciting broad protective immunity.

Designing novel TB vaccines must be done with an intimate understanding of the mycobacterial life cycle. Throughout the course of infection, *M.tb* is faced with a plethora of host and environmental stresses such as nutrient deprivation and oxygen depletion[4–6]. To survive, *M.tb* bacilli must metabolically and physiologically shift to a quiescent state of persistence. Mycobacterial adaptation from active replication to persistence is associated with major shifts in antigen expression. Therefore, we must broaden the spectrum of antigens utilized in vaccine design

to encompass antigens from persistence, eliminating a mechanism of pathogen evasion from the host immune reponse.

A majority of vaccines in the developmental pipeline utilize antigens secreted by *M.tb* during active growth, mainly members of the Ag85 complex and the RD1 locus. They are strongly immunogenic in humans and are focal to nearly all vaccine development strategies[7]. Drastic antigenic shifts however (as seen during mycobacterial persistence), lower the ability of the adaptive response primed against these active growth antigens to recognize infected target cells, thereby circumventing the host immune response. Persistent mycobacteria are also phenotypically resistant to front-line chemotherapies, thereby making them exceedingly difficult to eliminate. As such, development of vaccination strategies to eliminate persistent mycobacteria would have significant clinical implications.

Persistence phase antigens can be effectively exploited as targets for vaccine design, as seen with a small percentage of candidates currently under assessment[8–10]. By directing immune responses against persistence antigens, these vaccines aim to eliminate quiescent mycobacteria, preventing disease reactivation and expanding breadth of protection. The development of vaccines to target non-replicating populations of bacteria is foundational to broadening the scope of vaccine elicited antigens, drastically changing treatment regimens if translated effectively. However, these studies have thus far failed to directly assess vaccine efficacy against persistent mycobacteria.

Traditional methods used to assess the protective efficacy of TB vaccines utilize conventional colony forming unit (CFU) assays on solid agar. However, a growing body of evidence strongly suggests that this assay does not capture the heterogeneous mycobacterial population as persistent mycobacteria fail to grow on solid agar media[11–13]. Therefore, further steps must be taken to directly measure changes in persistent populations through utilization of liquid cultures rich in

mycobacterial resuscitation factors. No tuberculosis vaccine studies have shown direct efficacy against persister populations to-date. This would ultimately mask a potential lack of efficacy, as it fails to discern whether vaccination truly lowers mycobacterial burden, or merely pushes bacilli to a persistent state undetectable with traditional methodologies.

Resuscitation to an actively replicating state is further facilitated by the degradation of host immunity and/or removal of pharmacological pressures, dictated in part by the function of a family of enzymes known as resuscitation promoting factors (rpf). Of the five known members of the rpf family (rpfA-E), rpfB is pivotal to the early resuscitation events, highlighting an attractive target in vaccine design[6,12,14–16].

Our previous work following respiratory mucosal (RM) immunization with a monovalent chimpanzee adenovirus-vectored TB vaccine expressing Ag85A (AdCh68Ag85A) demonstrated both prophylactic immunity against TB, and accelerated anti-TB antibiotic therapy when used as an immunotherapeutic[17,18]. To further enhance immunity against TB and address existing gaps in vaccination platforms, we prioritized targeting all phases of the *M.tb* life cycle. In this study we have redesigned this AdCh68 vector to express both an additional immunogenic antigen expressed during early infection (TB10.4), and a unique antigen expressed during resuscitation (rpfB). We show that RM immunization with this trivalent vector, AdCh68Ag85A:rpfB:TB10.4, establishes polyfunctional, long-lasting residential memory T cell immunity at the respiratory mucosa. Not only does the addition of additional mycobacterial antigens enhance the protective efficacy of this vaccine, but for the first time shows that such a strategy can be designed in a way to specifically eliminate persistent mycobacteria.

Results

Molecular construction and characterization of a trivalent chimpanzee adenovirus serotype 68-based TB vaccine

Our previous studies have shown that a monovalent chimpanzee adenovirus 68-vectored TB vaccine expressing the immunogenic *M.tb* Antigen 85A (AdCh68Ag85A) provides both prophylactic and therapeutic efficacy against pulmonary TB infection[17]. To further expand the antigenic breadth of this vaccine as to cover the multi-staged life of *M.tb*, we went on to re-engineer this monovalent platform to express two additional *M.tb* antigens: EsxH (TB10.4), a virulence factor, and Rv1009 (rpfB), a protein involved mycobacterial resuscitation from dormancy/persistence[12,15,19].

This trivalent vector, AdCh68Ag85A:rpfB:TB10.4 (herein referred to as Tri. AdCh68), was constructed utilizing a previously-documented cloning technique[20]. Genes encoding all three antigens were engineered to be translated as a single polypeptide with each antigen separated by a 4X-glycine linker (**Figure 1A**). An N-terminus tissue plasminogen signal peptide sequence (tPA) was also added to target the entire protein for secretion. All regulatory elements, including the murine cytomegalovirus (MCMV) promoter and termination sequences were identical to that of monovalent AdCh68Ag85A, to allow a direct basis for comparison.

Tri. AdCh68 was packaged and propagated in HEK 293 cells prior to cesium chloride gradient purification. Transgene integrity was assessed by comparing transgene expression in the cellular lysate of A549 cells infected for 48 hours with Tri. AdCh68 (**Tri.**) or with Mono. AdCh68 (**Mono.**), as a control (**Figure 1B, top schema**). As expected, the trivalent transgene cassette was approximately 2kbp larger than that of its monovalent counterpart, accounting for the addition of

rpfB and TB10.4 (**Figure 1B, bottom gel pattern**). Expression at the protein level was additionally verified by western blot analysis (**data not shown**). Given our validation of the molecular integrity of Tri. AdCh68, we next sought to characterize the safety and immunogenicity of this vaccine relative to its monovalent counterpart.

Mucosal immunization with Tri. AdCh68 is safe, eliciting minimal pulmonary inflammation

Vaccine-mediated immunity against pulmonary TB is in part dictated by immunization route[21]. We previously have shown that respiratory mucosal (RM), but not parenteral prophylactic, and therapeutic immunization with Mono. AdCh68 is safe and well-tolerated[17,18]. To further such findings to our trivalent construct, we assessed various indices of lung inflammation 24 hours following RM immunization with Tri. AdCh68 (**Tri.**) in the lung and airways (bronchoalveolar lavage, BAL) of mice. As a control, a group of animals was either left untreated (**Naïve**) or received an equivalent dose of Mono. AdCh68 (**Mono.**). Histopathological analysis agreed with previously published work, indicating minimal adverse cellular infiltration around vascular and bronchial areas of immunized mice comparable to unvaccinated controls (**Figure 1D, black arrows**). Flow cytometric analysis of airway and lung tissue cells further agreed with these qualitative findings, indicating minimal, neutrophilic and monocytic infiltration in the lung and airway of immunized animals similar to unvaccinated controls (**Figure 1E**). We additionally measured soluble levels of TNFα, a pro-inflammatory cytokine, and IFNβ, an innate cytokine that can impair anti-TB Th1 immune responses[22] in the BAL fluid. Once again, negligible levels of both cytokines were detected following immunization with Tri AdCh68. (**Figure 1F)**.

Collectively, Tri. AdCh68 immunization elicited histopathology, inflammatory cellular, and innate cytokine responses in the lung and airway similar to both its Mono. AdCh68 counterpart, and

unvaccinated mice. Our findings support that Tri. AdCh68 is safe and amenable for administration via the RM route.

RM immunization with Tri. AdCh68 induces potent antigen-specific T cell responses with no observable antigenic competition

We have previously shown that the anti-TB efficacy of Mono. AdCh68 is reliant on Ag85A-specific CD8 T cell responses. When engineering multivalent vectors, assessing for antigenic competition must be done to determine whether adaptive immune responses are skewed/impaired for previously characterized immunogenic epitopes. We therefore sought to characterize antigen-specific T cell responses elicited by Tri. AdCh68 in comparison to its monovalent counterpart.

Mice were RM immunized with Tri. AdCh68 (**Tri.**), and as a control, a second group of animals received an identical RM dose of Mono. AdCh68 (**Mono.**). All animals were sacrificed 2 weeks post-immunization and antigen-specific T cell responses were assessed in the airways (**BAL**) and lung tissue (**Lung**) by flow cytometry following *ex vivo* stimulation with vaccine antigens (**Figure 2A**). In agreement with our previously published study[17,18], Mono. AdCh68 induced a CD8-dominant Ag85A response in both the airways and lungs (**Figure 2B** and **2C**, respectively). The frequency and total number of Ag85A-specific CD8 T cell responses were comparable to that induced by Tri AdCh68. This indicates that the expression of additional antigens (TB10.4 and rpfB) did not induce any observable antigenic competition, and therefore Ag85A-specific T cell responses remained intact.

Furthermore, RM immunization with Tri. AdCh68 generated predominantly CD8 T cell responses against both TB10.4 and rpfB in congruence with the responses against Ag85A (**Figure 2B/C**).

The frequency and absolute number of CD8 T cell responses specific for TB10.4 were similar to those against Ag85A, with rpfB responses being smaller.

When assessing the total magnitude of vaccine-specific immune responses (**Figure 2B/C**, bar graphs), RM Tri. AdCh68 immunization generated 4-fold greater responses in the airway, and 2-fold greater responses in the lungs than those induced by its monovalent counterpart. Overall our initial immunogenicity studies indicate that RM immunization with Tri. AdCh68 induces markedly larger, and antigenically-broader T cell response than its monovalent counterpart.

RM immunization with Tri. AdCh68 establishes lasting, multifunctional tissue resident memory T cell responses within the respiratory tract

Our findings thus far indicate that RM immunization with Tri. AdCh68 generates potent T cell immunity against Ag85A, TB10.4, and rpfB. To further profile the immunogenicity of this vaccine, we next determined the longevity and functionality of such responses. A growing body of evidence suggests that tissue resident memory T cells (T_{RM}) established at the respiratory mucosa following RM immunization is capable of rapidly providing efficacious immunity against subsequent infection[23,24]. Given the robust mucosal T cell immune responses following RM Tri. AdCh68 immunization, we next characterized the T_{RM} responses in respiratory mucosal following Tri. AdCh68 immunization.

Tissue resident memory T cells are defined in part by the expression of surface integrins that facilitate cell trafficking and retention in the mucosal tissues. In this study T_{RM} are defined by their expression of CD69, and CD49a, while CD8 and CD4 T_{RM} are further defined by expression of CD103 and CD11a, respectively[23–28]. To further address the spatial distribution of bona fide antigen-specific T_{RM}, we utilized an intravascular (I.V.) fluorescent α-CD45 staining technique

that differentiates leukocytes within the pulmonary vasculature from those in the lung parenchymal tissue (LPT)[26,29,30].

Animals were RM immunized with either Tri. AdCh68 (**Tri.**) or Mono. AdCh68 (**Mono.**) and were sacrificed 6 weeks later (**Figure 3A**). Immediately prior to sacrifice, I.V. staining was performed, and lung and BAL were harvested. Isolated mononuclear cells were assessed by flow cytometry following *ex vivo* vaccine antigen stimulation for expression of T_{RM} markers (**Figure 3B**). Given our extensive understanding of how RM immunization drives robust T_{RM} establishment, we observed the vast majority of antigen-specific CD4 and CD8 T cells expressing some combination of T_{RM} markers (**Figure 3C**).

Coinciding with the memory phase, we documented a marked contraction in antigen-specific T cells in animals immunized with either vaccine. In terms of functionality, antigen-specific CD4 T cells induced by Tri. AdCh68 were predominately monofunctional, producing IFNγ. In contrast, CD8 T cells were mainly polyfunctional, producing two or more of - IFNγ, TNFα, and/or IL-2 (**Figure 3D**).

RM immunization with trivalent AdCh68 markedly boosts antigen-specific immune responses in parenterally BCG-primed hosts

Given the widespread utilization and importance of BCG in the global immunization program, we next sought to characterize immune responses boosted by RM Tri. AdCh68 immunization in parenterally BCG-primed hosts. To this end, animals were either subcutaneously (s.c.) primed with BCG alone (**BCG**), or were subsequently RM-boosted with either monovalent (**BCG/Mono**.), or trivalent (**BCG/Tri**.) AdCh68 four weeks post-BCG. Two weeks post-boost, all animals were sacrificed and cells were isolated from the airway (**BAL**), and lung (**Lung**). Pulmonary myeloid

cells were stimulated *ex vivo* with crude mycobacterial antigens (BCG/culture filtrate) or recombinant vaccine antigens, and were analyzed for antigen-specific T cell responses by flow cytometry (**Figure 4A**).

As previously documented[31], parenteral BCG immunization alone failed to establish mycobacterial antigen-specific T cell responses within the airways, whereas it induced equivalent levels of mycobacterial CD4 and CD8 responses in the lung tissue (**Figure 4B/C, bar graphs**). In agreement with our previously published work[17], RM boosting with Mono. AdCh68 increased mycobacterium-specific CD4 and CD8 responses in the airway and in the lung tissue. In stark contrast, RM-boosting with Tri. AdCh68 induced 5-fold greater mycobacterial-specific CD4 and CD8 T cell responses than those induced by monovalent AdCh68 in the airways, and nearly 20-fold greater in the lung tissue.

We further quantified vaccine antigen-specific responses in BCG-primed animals (**Figure 4B/C, flow plots**). As expected, RM boosting with Mono. AdCh68 enhanced Ag85A-specific T cell responses in both the airways and lungs in comparison to BCG alone. Minimal TB10.4 and rpfB-specific responses were also registered, accounting for the responses elicited by BCG. Interestingly, boosting with Tri. AdCh68 did not boost Ag85A-specific responses, but TB10.4 and rpfB responses were markedly boosted in comparison to BCG naïve hosts.

Short-course immunotherapy with trivalent AdCh68 in a low-dose infection model selectively eliminates persistent *M.tb* bacilli

Given that our trivalent vaccine generates robust, multifunctional immune responses against the resuscitation promoting factor rpfB, we next sought to assess whether this vaccine was capable of eliminating persister mycobacteria in a model of vaccine immunotherapy. By utilizing our

established immunotherapy model[18], animals were infected with a low dose *M.tb* (100 CFU), and subsequently treated with antibiotics alone (ABx) or in conjunction with RM immunotherapy with either mono. AdCh68 (**ABx / Mono.**) or tri. AdCh68 (**ABx / Tri.**). Animals were sacrificed 12 weeks post-infection, and lungs were isolated for assessment of mycobacterial disease indices (**Figure 5A**).

To distinguish different mycobacterial subpopulations, we utilized a conventional agar CFU assay and compared it to a limiting dilution Most Probable Number (MPN) assay[32] utilizing liquid cultures supplemented with or without culture supernatants enriched in mycobacterial rpfs to resuscitate and quantify persister bacilli (**Figure 5B)**.

Respiratory mucosal immunotherapy with both Mono. or Tri. AdCh68 provided a significant reduction in pulmonary mycobacterial burden in comparison to antibiotics alone by conventional CFU assay (**Figure 5B, conventional CFU**). This trend was similarly seen in liquid cultures lacking rpfs by MPN (**Figure 5C, MPN, Control Media**). In stark contrast, by utilizing liquid cultures enriched with these resuscitation factors, we documented no significant differences in bacterial burden between animals treated with and without Mono. AdCh68. However, a major difference was observed in Tri. AdCh68 boosted mice, indicating a significant reduction of the total mycobacterial population following Tri. AdCh68 immunotherapy (**Figure 5B, MPN, Resuscitation Media**).

Discussion

In this study, we have presented a novel vaccine platform designed to confer longitudinal immunity against pulmonary TB by directing immune responses against an occult population of persistent mycobacteria. We have engineered a chimpanzee adenoviral-vectored vaccine expressing antigens from different stages of the *M.tb* life cycle. Through this, we present an approach that not only protects against the early stages of infection (Ag85A and TB10.4), but also against mycobacterial resuscitation (rpfB) from dormancy.

Mycobacterium tuberculosis expresses five unique resuscitation promoting factors, rpfA - E[33]. Through altering the cell wall, these proteins work in a concerted manner to promote mycobacterial resuscitation. Their expression profile and activity were originally discovered *in vitro*, with recent studies translating these findings to human pulmonary and lymphoid tissues[4,12,13]. Immunogenically, rpf-specific immunity has been documented in humans, with higher responses in latently infected individuals, suggesting a role in maintaining dormancy[34]. We chose rpfB as the representative persistence antigen in this study due to (1) its robust expression and functionality in mycobacterial resuscitation, and (2) documented immunogenicity in latently infected humans.

We show for the first time that inclusion of persistence phase antigens in a viral vectored vaccine provides robust immunity against persistent mycobacteria. rpfB however represents one of many persistence antigens which may prove more protective, such as the genes of the DosR regulon. In addition to the selected antigens, the construction strategy utilized during vector design (including the vector itself) is a major consideration in vaccine efficacy. Certain antigens may not provide protection unless designed in-tandem with additional antigens. This is highlighted by the study from Aagaard *et al*. where the persistence antigen Rv2660c was only protective when expressed as a polypeptide with two additional antigens. In our study, we express Ag85A, rpfB, and TB10.4

as a single polypeptide, separated by glycine linkers. However, it remains to be addressed whether this design is the most optimal and as such, remains an active area of investigation.

Persistence is both a stochastic and inducible state by which mycobacteria endure immunological and pharmacological insults. The inability of BCG to protect against pulmonary TB therefore may stem from its ability to weakly drive immunity against persistence antigens. This is supported by our findings, where we document weak responses to rpfB in BCG-immunized hosts. Remarkably, when BCG primed animals were boosted with Tri. AdCh68, we were able to register rpfB-specific responses which were many magnitudes higher relative to BCG naïve animals. With the near universal administration of BCG, our findings provide strong merit in utilizing antigens such as rpfB when designing booster vaccines as to enhance immunity against persistence/resuscitation.

To our knowledge, all TB vaccine studies to-date have only quantified protection by assessing mycobacterial burden through conventional solid agar CFU assays, failing to address whether vaccination has any impact on persistent mycobacterial populations. As immunological pressures are known to drive persistence, it remains unclear whether these vaccines truly reduce mycobacterial burden or instead rather contribute to persistence.

Our study represents the first TB vaccine to directly quantify reductions in persistent mycobacteria in addition to conventional assays, thereby providing a more comprehensive measure of prophylactic vaccine efficacy. Through these assays, we show that both our monovalent and trivalent vaccines provided marked reductions in pulmonary mycobacterial burden as measured by standard CFU assay. However, by quantifying persistent mycobacteria, we showed that our trivalent, but not monovalent vaccine was able to directly reduced persistent mycobacteria.

There are limitations to any animal model used for assessing the efficacy of a TB vaccine. Although the murine model is practical with a plethora of available immunological reagents, it does have its shortcomings. Numerous key pathological TB hallmarks are not recapitulated in the standard BALB/c or B6 model. The impact of this is particularly evident when trying to understand how mycobacterial heterogeneity may impact vaccine efficacy. As such, future studies may extend towards the use of the C3HeB/FeJ model which shows numerous pathological indicators of disease, such as necrosis, caseation and liquefaction[35–39]. This would expand the assessment of the vaccine to survival, and towards a more realistic and translatable understanding of vaccine efficacy.

Through this study, our goals were to not only describe a novel TB vaccine with broad applicability, but to emphasize the importance as to how TB vaccines are constructed and assessed. We believe that "mycobacteria-centric" approach wherein you understand how the bacteria adjusts and adapts to pressures (and how you measure such changes) provide exciting and more realistic avenues for vaccine development.

Methods

Animal models for *in vivo* studies

Female BALB/c mice (6-8 weeks old) were purchased from Charles River (Wilmington, MA, USA). Female C3HeB/FeJ mice (6-8 weeks old) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). All experimental mice were housed within either the level 2 or level 3 containment facility at McMaster University, with all experiments being conducted in accordance with the McMaster University Animal Research Ethics Board.

Molecular construction and validation of trivalent chimpanzee adenovirus vaccine

A replication-deficient chimpanzee serotype 68 adenovirus was constructed to express three *M.tb* antigens - Antigen 85A, resuscitation promoting factor B, and TB10.4; each antigen is separated by linkers composed of 4 glycine residues (trivalent AdCh68Ag85A:rpfB:TB10.4), using previously described technology[20,40]. The transgene cassette was cloned to express the murine cytomegalovirus promoter (MCMV), and a tissue plasminogen activator peptide signal. Apart from the expressed antigens, this trivalent vector is molecularly identical to monovalent AdCh68 expressing Ag85A alone. Briefly, a pShuttle plasmid was engineered to express the transgene cassette and amplified in DH5α *E.coli* (ThermoFisher, Waltham, MA, USA). The entire transgene cassette was excised and subcloned into the DNA clone pAdCh68 (ΔE1/E3) by I-Ceu1/PI-Sce1 digest and subsequent in-gel ligation. Trivalent AdCh68 was subsequently packaged and propagated in HEK 293 cells and purified by cesium chloride centrifugation. Transgene expression was validated by PCR on the supernatants and lysates of infected A549 mammalian cells.

Respiratory mucosal immunization with AdCh68 vaccines

Respiratory mucosal immunization was done by intranasal instillation of $1x10^7$ PFU of either AdCh68Ag85A or AdCh68Ag85A:rpfB:TB10.4 in a total volume of 25uL of sterile phosphate buffered saline (PBS). In select experiments, BCG (Pasteur) immunization was performed subcutaneously at a dose of $1x10^5$ CFU in 100uL of sterile PBS.

Measure of TNFα type 1 IFN protein levels

BAL fluid was collected from centrifugation of bronchoalveolar lavage (BAL). Soluble TNFα and IFNβ protein levels were measured in BAL fluids using ELISA as per manufacturer's instruction (R&D Systems, Minneapolis, MN, USA).

Pulmonary *M. tuberculosis* infection and antibiotic therapy

Mice were infected via the respiratory mucosal route with $1x10^4$ colony-forming units of *M. tuberculosis* H37Rv (ATCC27294) in a total volume of 25uL of sterile PBS. Mice which received antibiotics for immunotherapy studies did so in an oral Medidrop (Clear H2O, Westbrook, ME, USA) solution of rifampicin (10mg/kg), isoniazid (25mg/kg), and pyrazinamide (150mg/kg). Unless stated otherwise, pyrazinamide was stopped 4 weeks after antibiotic initiation.

Mononuclear cell isolation

Lung and BAL mononuclear cells were isolated as previously described. Briefly, lungs were cut into small pieces and digested with 150 units of collagenase type 1 (Life Technologies, Grand Island, NY, USA) in RPMI medium at 37ºC with agitation for an hour. Digested lung pieces were then crushed through a 100µm filter and red blood cells were removed by treatment with an ACK lysis buffer. BAL cells were isolated by centrifugation. Splenic mononuclear cells were isolated by crushing the organ through a 100µm filter, with red blood cells being removed by treatment with an ACK lysis buffer. Cells were resuspended in RPMI supplemented with 10% FBS, 1% pen-strep, 1% L-glutamine.

Cell stimulation, intracellular cytokine staining, and flow cytometry

Mononuclear cells were cultured in U-bottom plates at a concentration of 20 million cells per mL. For stimulation, 5ug/well of either recombinant Ag85A, rpfB, and/or TB10.4 were used. Cells were stimulated for a total of 6 hours in the presence of brefeldin A (5mg/mL; BD Pharmingen, San Jose, CA, USA). In select experiments, BCG-specific immune responses were assessed by stimulation with crude BCG and *M.tb* culture filtrate at a concentration of 1ug/mL, in the presence of brefeldin A. Following incubation, cells were washed and blocked with CD16/CD32 FcBlock in 0.5% bovine serum albumin/PBS for 15 minutes on ice, prior to being stained with the experimental-specific flourochrome-labelled monoclonal antibodies, according to the manufacturer's instructions (BD Pharmingen). This included: T cell panel - CD3-V450, CD8a–PE–Cy7, CD4– APC–Cy7, IFN-γ–APC, TNFα-FITC, PE-IL2. Neutrophil panel – CD45-APC-Cy7, CD11b-PE-Cy7, and Ly6G-BV605 (all from BD Biosciences, San Jose, CA, USA). All flow cytometry data were collected using a Fortessa Cytometer and FACSDiva software (BD Biosciences, San Jose, CA, USA) and analyzed using FlowJo software version 10 (Tree Star, Ashland, OR, USA).

Measurement of tuberculosis disease outcome

Lung bacillary load was assessed by plating serially-diluted lung homogenates on Middlebrook 7H10 agar plates supplemented with 10% OADC growth supplement (BD Biosciences, San Jose, CA, USA), 5μg/mL ampicillin and 50μg/mL cycloheximide (Sigma-Aldrich, St. Louis, MO, USA). Lung pathology was assessed histologically using lungs embedded in paraffin and assessed following either hematoxylin and eosin (H&E) or Ziehl-Neelson acid-fast staining. Histological samples were visualized by the Zeiss M2 Imager System (Zeiss, Toronto, ON, Canada). Pulmonary inflammation was determined qualitatively by using Image J software (NIH,

http://rsb.info.nih.gov/nih-image/) by measuring the areas of dense inflammatory infiltrates relative to the total lung sample area. For each animal, 3 independent lung slices were measured prior to being averaged.

Mycobacterial resuscitation assay

Lung homogenates from infected animals were cultured in flat bottom plates in either a control media (7H9 media supplemented with 10% (vol/vol OADC (BD Biosciences) and 0.05% Tween80) or a resuscitation media. Resuscitation media was extracted from culture supernatants isolated from *M.tb* cultures grown in supplemented 7H9 media to mid-exponential stage (OD_{600nm} 0.9-1.0). Briefly, bacteria were removed by centrifugation prior to being filtered twice through a 0.2um filter. Resuscitation media was composed of 50% of control media (vol/vol).

Statistical analysis

Two-tailed Student *t* tests for comparison between 2 groups. 1-way analysis of variance followed by post-test Tukey analysis for multiple-group comparison using GraphPad Prism 8 software (Version 8, La Jolla, CA, USA). Results were considered significant for P values ≤ 0.05. Area-under-curve (AUC) analysis was done to summate changes in bacterial burden over time. Unpaired *t* tests were performed using AUC analysis.

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Figure legends

Figure 1. ***Molecular construction and safety assessment of Tri. AdCh68.*** **A.** Molecular schematic of Tri. AdCh68. in comparison to Mono. AdCh68. Transgene cassettes in both vectors are under the control of the MCMV promoter, with a tPA signal to facilitate transgene secretion. Each antigen in Tri. AdCh68 is separated with a 4x glycine linker. **B.** Transgene expression. A549 cells were *in vitro* infected with equivalent doses (MOI 1) of Tri. or Mono. AdCh68 and supernatants and cell lysates were collected 24 hours post-infection. Transgene integrity was assessed by PCR. Mono. AdCh68 transgene cassette is expected at ~2kbp (size of Ag85A), whereas Tri. AdCh68 cassette is expected at ~3.5kbp (Size of Ag85A plus rpfB and TB10.4). **C.** Experimental schema for safety assessment. BALB/c mice were intranasally vaccinated with either Tri. or Mono. AdCh68 and BAL and lungs were collected 24 hours post-vaccination. **D.** Representative micrographs of lung sections stained with hematoxylin-eosin (H&E), comparing the extent of lung inflammation post-immunization. **E.** Bar graphs showing levels of neutrophils and inflammatory monocytes in the BAL and lung 24 hours post-vaccination. **F.** TNFα and IFNβ protein levels in the BAL 24 hours post-vaccinaton. Data are expressed as the mean±S.E.M. of 3-5 mice/group, representative of three independent experiments.

Figure 2. ***Respiratory mucosal immunization with Tri. AdCh68 induces robust, multi-antigenic immune responses.*** **A.** Experimental schema. Mice were RM immunized with either Tri. or Mono. AdCh68 and were sacrificed 2 weeks post-immunization. **B/C.** Representative histograms depicting the frequencies, and bar graph depicting absolute number of Ag85A, rpfB, or TB10.4-specific IFNγ+ T cell responses as assessed post-antigen stimulation by flow cytometry in the BAL (B), and lung (C). Data are expressed as the mean±S.E.M. of 3-5 mice/group, representative of three independent experiments.

Figure 3. ***Respiratory mucosal immunization with Tri. AdCh68 induces multifunctional tissue resident memory T cell responses.***

A. Experimental schema. Mice were set up as depicted in experimental schema Figure 2A, with animals being sacrificed 6 weeks post-immunization. 3 minutes prior to sacrifice, animals received intravascular fluorescent α-CD45 to distinguish vascular-restricted leukocytes from those in the lung tissue **B.** Intravascular staining flow schematic to distinguish lung parenchyma restricted cells from those in the lung vasculature. **C.** Pie graphs representing the relative percentage of antigen-specific resident memory CD4 (left panel) and CD8 (right panel) T cells in the BAL and lung parenchymal tissue (LPT). **D.** Bar graph (left panel) depicting absolute number of Ag85A, rpfB, or TB10.4-specific IFNγ+ T cell responses as assessed post-antigen stimulation by flow cytometry in the lung. Pie graphs (right panel) representing the relative percentage of CD4 and CD8 T cells expressing IFNγ, TNFα, and/or IL-2. Data are expressed as the mean±S.E.M. of 3-5 mice/group, representative of three independent experiments.

Figure 4. ***Respiratory mucosal immunization with Tri. AdCh68 markedly boost BCG-induced immunity.*** **A**. Experimental schema. Mice were subcutaneously primed with BCG prior to being boosted 4 weeks later with either Tri. or Mono. AdCh68. Animals were sacrificed 2 weeks post-boost. **B.** Bar graphs (left) representing the absolute number of BCG-specific CD4 and CD8 T cell responses in the BAL as assessed by culture filtrate/crude BCG stimulation. Representative dot plots (right) of Ag85A, rpfB, or TB10.4-specific IFNγ+ T cell responses as assessed post-antigen stimulation by flow cytometry in the BAL. **C.** Similar to that of B, but in the lung. Data are expressed as the mean±S.E.M. of 3-5 mice/group, representative of three independent experiments.

Figure 5. ***RM immunotherapy with Tri. AdCh68 improves TB disease control during antibiotic therapy by eliminating persistent mycobacteria.*** **A.** Experimental schema. At four weeks post-*M.tb* infection, mice were started on an oral antibiotic (ABx) therapy of rifampicin, isoniazid, and pyrazinamide. A group of these mice was treated via the RM route with either Tri. AdCh68 or Mono. AdCh68. at 4 weeks after the initiation of antibiotic therapy. All mice were sacrificed 12 weeks post-infection for assessment of mycobacterial burden. **B.** Bar graphs representing bacterial burden in the lungs through conventional agar CFU assay or in liquid culture enriched with mycobacterial resuscitation promoting factors. Data are expressed as the mean±S.E.M. of 10 mice/group, representative of one experiment.

Figure 1 – Molecular characterization of trivalent AdCh68

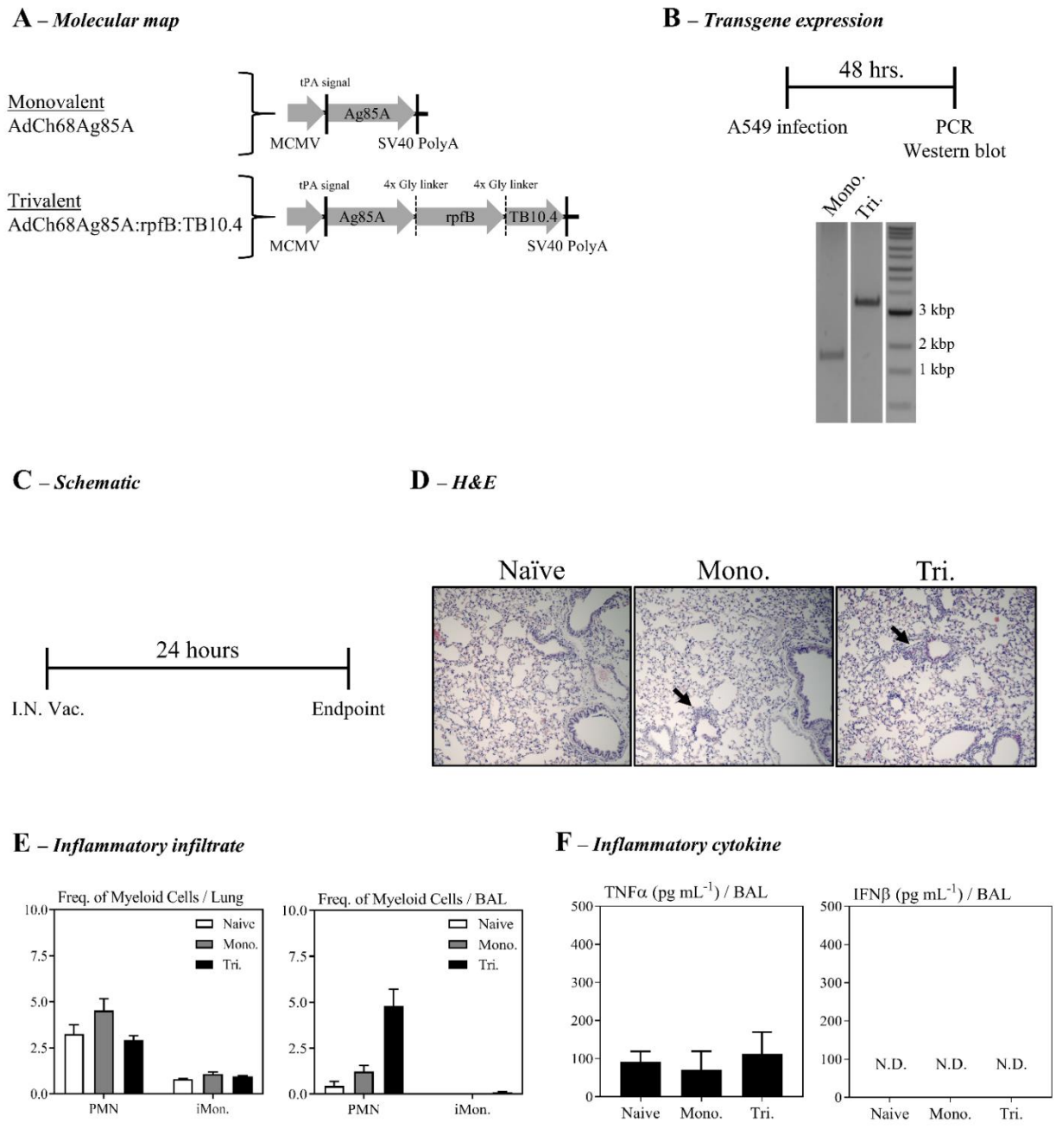


Figure 2 – Immunogenicity of Tri. AdCh68 in the BALB/c model

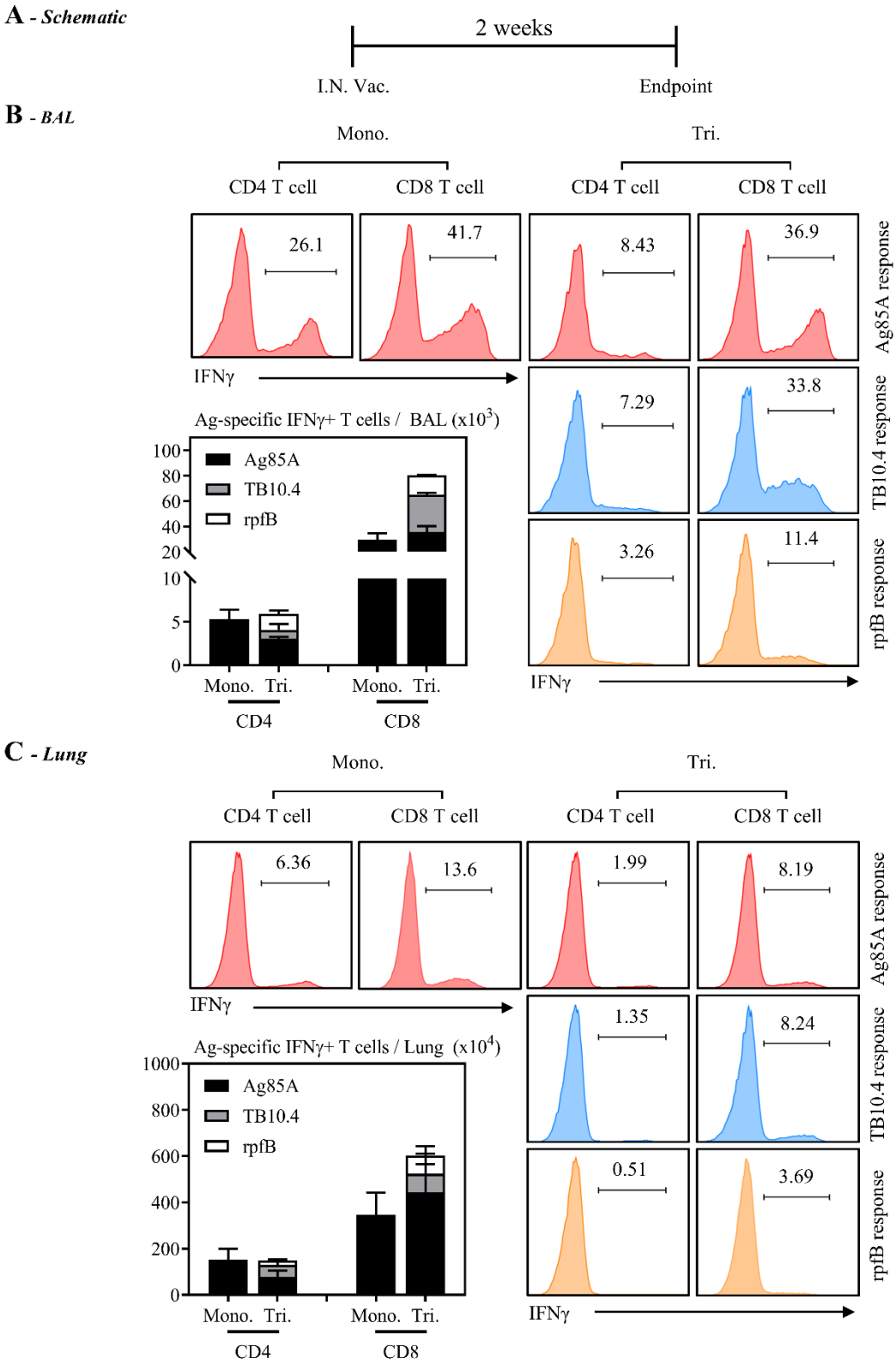


Figure 3 – Memory and multifunctionality

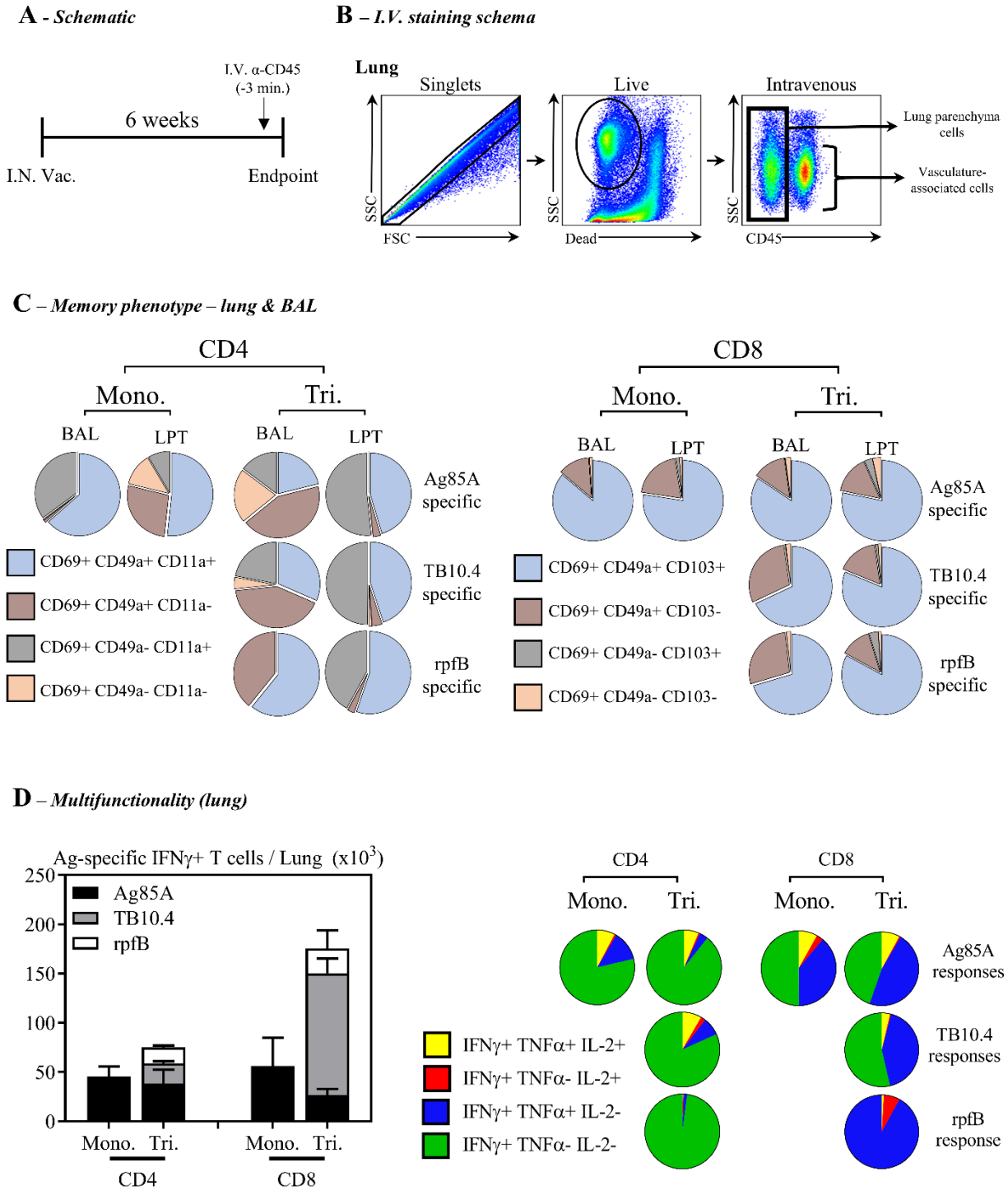


Figure 4 – BCG-boosting efficacy of Trivalent AdCh68

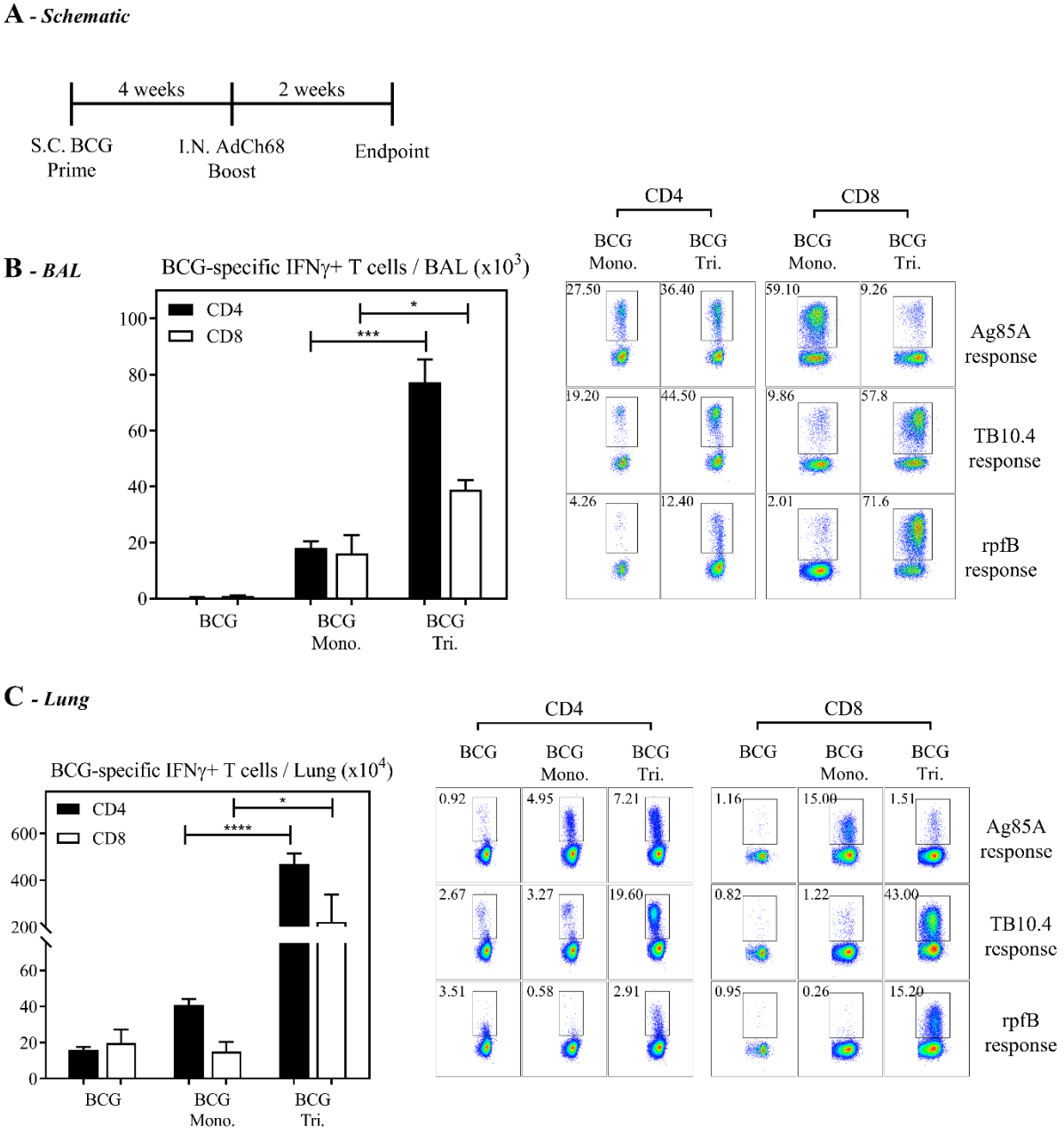
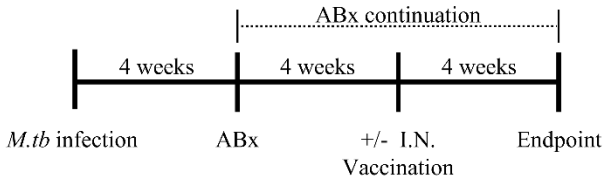
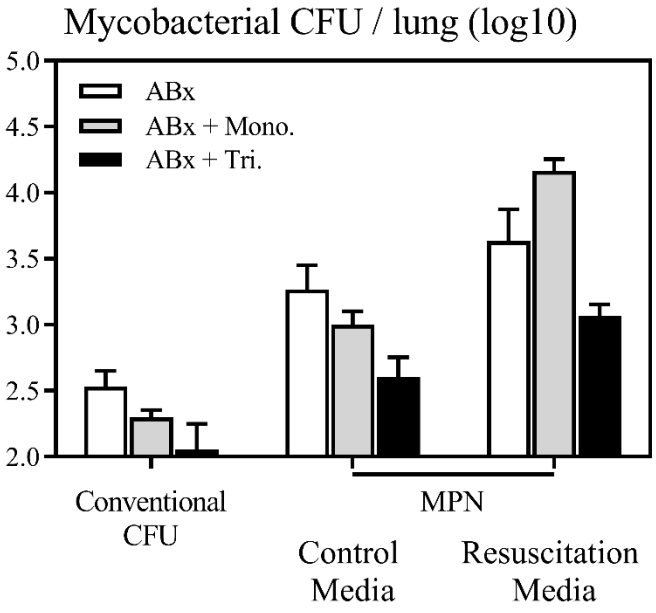


Figure 5 – Trivalent AdCh68 immunotherapy

A - ***Schematic***



B – ***Lung CFU (conventional vs. persistent)***



6.0 – Chapter 6 - Determining whether spray drying adenovirus-vectored vaccines with sugar excipients stabilizes the vaccine and eliminates cold chain requirements

Manuscript title: Spray dried human and chimpanzee adenoviral-vectored vaccines are thermally stable and immunogenic *in vivo*.

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Spray dried human and chimpanzee adenoviral-vectored vaccines are thermally stable and immunogenic *in vivo*

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ABSTRACT

Cold chain-free vaccine technologies are needed to ensure effective vaccine delivery and coverage, particularly in resource-poor countries. However, the immunogenicity and thermostability of spray dried live viral vector-based vaccines such as recombinant adenoviral-vectored vaccines remain to be investigated. To address this issue, we have spray dried human adenoviral (AdHu5)- and chimpanzee adenoviral (AdCh68)-vectored tuberculosis vaccines in a mannitol and dextran matrix. Spray dried powders containing these two vaccines display the morphologic and chemical properties desired for long-term thermostability and vaccination. Upon reconstitution, they effectively transfected the cells *in vitro* with relatively small losses in viral infectivity related to the spray drying process. Following *in vivo* vaccination, AdHu5- and AdCh68-vectored vaccines were as immunogenic as the conventional fresh, cryopreserved liquid vaccine samples. Of importance, even after cold chain-free storage, at ambient temperatures and relatively low humidity for 30 and 90 days, the vaccines retained their *in vivo* immunogenicity, while the liquid vaccine samples stored under the same conditions lost their immune-activating capability almost entirely. Our results support further development of our spray drying technologies for generating thermally stable adenoviral-vectored and other viral-vectored vaccines.

1. Introduction

Adenoviruses (Ad) are widely used as vectors for delivering vaccines against infectious diseases of global importance such as tuberculosis (TB) [1,2]. Such infection diseases remain a daunting global threat, particularly in the resource-poor parts of the world, such as South Africa and Southeast Asia. As such, there remains an urgent need to develop not only novel vaccination strategies but also cold chain-free technologies to ensure effective delivery of Ad-based vaccines to resource-poor areas. Ad vectors possess advantages of high gene plasticity, ease of development, excellent safety profiles, and potency of driving robust T cell immunity [3,4]. As multiple pre-clinical and epidemiological studies have highlighted the importance of T cells in anti-TB immunity, Ad are one of the most widely used viral-vectors for TB vaccine delivery [3,5]. In particular, enhanced protection against TB has been demonstrated by us and others with two Ad TB vaccines: a human Ad serotype 5 encoding the *M. tb* antigen 85A (AdHu5Ag85A) and a chimpanzee Ad serotype 68 encoding Ag85A (AdCh68Ag85A) [5–8].

At the present time, cryopreservation is required for Ad vectors' long-term stability and efficacy [9,10]. This poses a serious issue for its ultimate application in resource-poor countries where TB vaccines are needed the most. Circumvention of these constraints requires increased thermostability of Ad-based vaccines, which can be achieved through controlled drying of the liquid formulation with vitrifying excipients into glassy powders [11–13]. While lyophilisation has been the preferred method of drying biologics, perhaps due to well established processes in industry [14], spray

Abbreviations: AdHu5, human serotype 5 adenoviral vector; AdCh68, chimpanzee serotype 68 adenoviral vector; Ag85A, *Mycobacterium tuberculosis* antigen 85A; CO_2, carbon dioxide; d(0.5), volume median diameter; DSC, differential scanning calorimetry; *M. tb*, *Mycobacterium tuberculosis*; PBS, phosphate buffered saline; pfu, plaque forming unit; RSD, relative standard deviation; SEM, scanning electron microscopy; TB, tuberculosis; T_g, glass transition temperature; UV, ultraviolet.

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drying has increasingly become more attractive due to its advantages over lyophilisation, including cheaper process costs and extended process control for pharmaceutical responses such as particle size, yield, and viral vector activity [15–17].

Spray drying is a continuous process using high temperature and pressurized gas to form powder products of controlled particle size from stock solutions [18]. For thermally stable vaccines, the excipients present in the stock solution, commonly sugars, amino acids and surfactants, precipitate out of solution as the sprayed droplets dry. The thermal stability of the biologic is then increased through entrapment within the precipitated powder [19]. The immobilization of the virus reduces the rate of protein denaturation and biologic aggregation, maintaining vector efficacy over the long term. Effective thermally stable powders are characterized by the mobility of the biologic within the particle matrix [18,20].

Thermostability of various spray dried powders containing bacterial cell-, protein-based or measles virus-based vaccines has been previously demonstrated by *in vitro* and benchtop testing [11,12,21–23]. We have recently shown a markedly increased thermostability of spray-dried model AdHu5 vector *in vitro* following three months of storage at room temperature and even for several days at temperatures as high as 55 °C [12]. There have also been a few studies evaluating *in vivo* performance in small animal models of spray dried protein- or inactivated virus-based vaccines but since these vaccines were tested *in vivo* immediately after spray drying, the issue regarding their thermostability as pertaining to vaccine immunogenicity was not addressed [24–26]. Thus, to date it has remained unclear whether spray dried live viral-vectored vaccines such as Ad-vectored vaccines are thermally stable and retain the full immune-stimulatory capability in animals. This is a relevant question which cannot be answered *in vitro* as spray dried live viral-vectored vaccines require the biologic competency of viral surface protein structure for efficient infection of its target cells and subsequent efficient expression of the encoded antigens to activate the immune system.

Thus, the objective of our current study was to investigate the thermostability and *in vivo* immunogenicity of spray dried live viral-vectored vaccines. By using a mannitol and dextran stabilizing excipient matrix, we spray dried AdHu5Ag85A and AdCh68Ag85A vaccines. We show for the first time that spray dried powders containing these two vaccines display the chemical properties desired for long-term thermostability and vaccination. Upon reconstitution, they effectively transfected the cells *in vitro* with relatively small losses in viral activities. Following *in vivo* vaccination, they were as immunogenic as the conventional cryopreserved liquid vaccine samples. Even after storage at ambient temperature for 30 and 90 days, they retained immunogenicity *in vivo*, while the liquid vaccine samples stored so lost their immunogenicity. Our findings support further development of our spray drying technology to produce thermally stable viral-vectored vaccines for clinical applications.

2. Materials and methods

2.1. Chemicals and adenoviral-vectored vaccines

D-mannitol and dextran (M_r 40,000 kDa) were purchased as USP grades from Sigma-Aldrich (Ontario, Canada). Culture media was prepared from F11 culture medium (in house according to protocol by the supplier, Life Technologies; Ontario, Canada) with 10% fetal bovine serum and 1% streptomycin/penicillin (Invitrogen; Ontario, Canada). Recombinant replication-defective human type 5 and chimpanzee type 68 adenoviruses expressing *M.tb* antigen 85A (AdHu5Ag85A/AdCh68Ag85A) were produced in the vector facility of McMaster Immunology Research Centre, as described previously [6,7]. The ratio of viral particles (vp) to plaque forming units (pfu) was 150/1 and 521/1 for AdHu5Ag85A and AdCh68Ag85A, respectively.

2.2. Spray drying of adenoviral-vectored vaccines

Powder vaccines were produced by spray drying with a Mini Spray Dryer B-290 (Büchi; Switzerland) using our recently reported method [12,16]. The feed solution was composed of mannitol and dextran (at a weight ratio of 67% mannitol and 33% dextran) along with either AdHu5 or AdCh68 vaccine (concentration of 1×10^6 pfu/mg dry powder). The spray dryer was operated at a nozzle inlet temperature of 120 °C, spray gas flow rate of 439.11 L/h and feed solution flow rate of 217.5 mL/h.

2.3. Powder vaccine storage

Spray dried powders were stored for differing durations at an ambient temperature of 20 °C and relative humidity of <10%. Storage humidity was controlled through gel desiccants within a sealed container. Samples were stored individually in closed 2 mL Nalgene General Long-Term Storage Cryogenic Tubes (Nalgene; Ontario, Canada).

2.4. Particle size and morphology

Spray dried particle size and morphology was examined using a TESCAN VP scanning electron microscope (SEM) (TESCAN, Czech Republic) as we recently described [12]. Samples containing adenovirus-vectored vaccine were inactivated through exposure to UV light for 30 min prior to imaging. A Malvern Mastersizer 2000G (Malvern Instruments; United Kingdom) equipped with a He-Ne laser was used to measure particle size and size distribution. Powder formulations were dispersed in anhydrous ethanol at a concentration of 1.0 mg/mL. Powder particle size was determined by the volume median diameter, d(0.5).

2.5. Thermal properties of spray dried powder vaccines

Thermograms for the powders were measured by differential scanning calorimetry (DSC). Samples of 5–10 mg were weighed into hermetically sealed aluminum pans and analyzed with a Q200 Differential Scanning Calorimeter (TA Instruments; New Castle, DE). Samples were tested following a heat-cool-heat ramp procedure from 4 °C to 275 °C using a rate of 10 °C/min. All procedure steps were conducted under a nitrogen purge gas flow rate of 50 mL/min. Sample glass transition temperature (T_g) was determined using TA Universal Analysis software (TA Instruments; New Castle, DE).

2.6. In vitro testing of spray dried vaccines

Infectivity of spray dried vaccines was assessed *in vitro* as previously described [12]. Briefly, HEK 293 cells seeded to confluency in 24 well tissue culture plates were incubated with spray dried powdered containing either AdHu5Ag85A or AdCh68Ag85A which were reconstituted in PBS++ (1X PBS supplemented with 1% $CaCl_2$ and 1% $MgCl_2$). The reconstituted solution was aspirated following viral adsorption and a MEM/F11 agar overlay solution was added to each well. Samples were incubated at 37 °C/5% CO_2 for 14 days, at which viral plaques were counted under the microscope and viral titre was calculated with the Reed-Muench method [27].

2.7. In vivo evaluation of immunogenicity of spray dried vaccines

Female BALB/c mice, 6–8 weeks old, were purchased from Charles River Laboratories (Charles River, St. Constant, Quebec, Canada) and housed in a specific pathogen-free level B facility at McMaster University. All experiments were carried out in accordance with the guidelines from the Animal Research and Ethics Board at McMaster University. Immunization was carried out by the intramuscular route through delivery of the vaccine into the hind legs of the animals. Tested vaccine samples were either fresh liquid control (1×10^7 pfu), stored liquid control (initial concentration of 1×10^7 pfu stored for 30 days at ambient temperature), or reconstituted spray dried powders (adjusted viral vector concentration to account for spray dry process loss, as determined by *in vitro* testing, stored for 0, 30 and 90 days at ambient temperature). Control samples of the vaccines were performed in 100 μL of sterile PBS and the spray dried vaccine was fully reconstituted in 100 μL of sterile PBS solution. The 10^7 dose was chosen based on our recent published study where the same dose of both AdHu5Ag85A and AdCh68Ag85A was used [7]. Testing of intramuscular immunization with increasing doses (1×10^6, 1×10^7 and 1×10^8 pfu) of AdHu5Ag85A also found the dosage of 10^7 pfu to be at an unsaturated point of *in vivo* dose responses (Fig. S1).

2.8. Blood, spleen, and lung mononuclear cell isolation

Mice were sacrificed by cervical dislocation. Blood was harvested from the abdominal artery and mononuclear cells were isolated. Splenocytes were isolated as described previously [6]. Lungs were digested with collagenase type 1 (Sigma-Aldrich, St Louis, MO) at 37 °C in an agitating incubator. A single-cell suspension was obtained by crushing the digested tissue through a 40 μm basket filter. All isolated cells were re-suspended in a complete RPMI-1640 (RPMI 1640 supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, 1% L-glutamine).

2.9. Tetramer immunostaining and flow cytometry

Mononuclear cells from the blood, spleen and lungs were processed, immunostained and analyzed as previously described [6,7]. Briefly, cells were plated into U-bottom 96-well plates at a concentration of 20 million cells per mL. Cells were washed and blocked with CD16/CD32 in 0.5% bovine serum albumin/phosphate-buffered saline for 15 min on ice and then stained with the appropriate fluorochrome-labelled monoclonal antibodies. Cells were then processed according to the manufacturer's instructions (BD Biosciences, San Jose, CA). The monoclonal antibodies used included CD8a-phycoerythrin-Cy7, CD4-allophycocyanin-Cy7, and CD3-CyChrome. For tetramer immunostaining, a tetramer for the immunodominant CD8 T-cell peptide (MPVGGQSSF) of Ag85A bound to the BALB/c major histocompatibility complex class I allele H-2L (NIH Tetramer Core, Atlanta, GA) was used. Immunostained cells were run on an LSR II flow cytometer (BD Biosciences, San Jose, CA) and 250,000 events per sample were collected and analyzed on the FlowJo software (version 10; Tree Star, Ashland, OR).

3. Results

3.1. Spray dried human- and chimpanzee-adenoviral vectored TB vaccines are thermally stable

3.1.1. Powder size and morphology

We have recently reported that a chosen excipient blend of mannitol and dextran markedly increases the thermostability of a human serotype 5 adenoviral (AdHu5) vector expressing the LacZ marker following spray-drying [12]. However, this excipient blend has not been tested for AdHu5-vectored vaccines and other adenoviral species such as a chimpanzee serotype C68 adenoviral (AdCh68) vector. To this end, the two candidate TB vaccines, AdHu5Ag85A and AdCh68Ag85A, were spray dried in our mannitol and dextran excipient, and their thermostability was compared in particle size and surface morphology at day 0 and day 90 post-storage at ambient temperature (20 °C) and <10% relative humidity. By SEM examination, the day 0 particles showed no differences between AdHu5Ag85A (Fig. 1A) and AdCh68Ag85A (Fig. 1B), and all particles were observed to have morphologies and sizes consistent with previous work on spray dried biologics [11,18,24]. The particles exhibited high sphericity, minimal aggregation, and no collapsed particles, which indicates good diffusion control during spray drying. All particles were generally less than 10 μm in diameter.

After storage for 90 days at ambient temperature, both types of viral particles showed unchanged morphology (Fig. 1C/D). By using laser diffraction techniques, we conducted a more detailed size analysis. The size distribution was consistent among all samples, being monomodal with a small 'knee' and showing a high percentage of fines. At day 0 (d0), the spray dried particles were generally less than 10 μm with a median diameter of d(0.5) = 5.2 μm and d(0.5) = 5.0 μm for AdHu5Ag85A (Fig. 2A) and AdCh68Ag85A (Fig. 2B) samples, respectively. After 90 days of storage, little changes were seen in particle size with a median diameter of d(0.5) = 4.9 μm and d(0.5) = 5.3 μm for AdHuAg85A (Fig. 2C) and AdCh68Ag85A (Fig. 2D) samples, respectively. The uncertainty of the particle size measurement was 4.1% RSD.

3.1.2. Thermal properties of spray dried powders

The glass transition temperature (T_g) was measured for the spray dried AdHu5Ag85A and AdCh68Ag85A vaccines at day 0 and 90 days after storage at ambient temperature. At day 0, mannitol/dextran particles encapsulating AdHu5Ag85A and AdCh68Ag85A had comparable T_g values of 135.7 °C and 139.8 °C, respectively (Table 1). The measurement uncertainty was 3.7% RSD. For spray dried mannitol/dextran powders, dextran is largely the glass-forming amorphous component as mannitol readily crystallizes [28,29]. Our T_g values are comparable to previously reported values for powders of dextran mixed with other carbohydrates [30,31]. At 90 days post-storage at ambient temperature, the T_g values were 134.4 °C and 136.5 °C for spray dried AdHu5Ag85A and AdCh68Ag85A, respectively (Table 1). The T_g did not differ significantly from day 0 to day 90 with the two vaccines as the differences between all values were much smaller than 2× RSD. The small deviations within measured T_gs can be largely attributed to the variance of residual moisture within spray dried samples, as water is an effective plasticizer.

The above data suggest that the crystalline morphology of spray dried mannitol/dextran is not altered by the incorporation of the viral vectors and furthermore, there is no significant change in particle morphology over the storage conditions employed.

3.2. Spray dried human- and chimpanzee-adenoviral vectored TB vaccines demonstrate a relatively small loss of in vitro infectivity

The efficacy of viral-vectored vaccines is dependent on their ability to viably infect cells. To determine the effect of the spray drying process on infectivity of the two tested viral-vectored vaccines, a standard *in vitro* HEK 293 plaque forming assay was used with the samples collected right after spray drying (d0). By comparing with the infectivity before spray drying, the relative average losses of infectivity upon spray drying were determined to be comparable at 0.4 and 0.3 log for AdHu5Ag85A and AdCh68Ag85A,

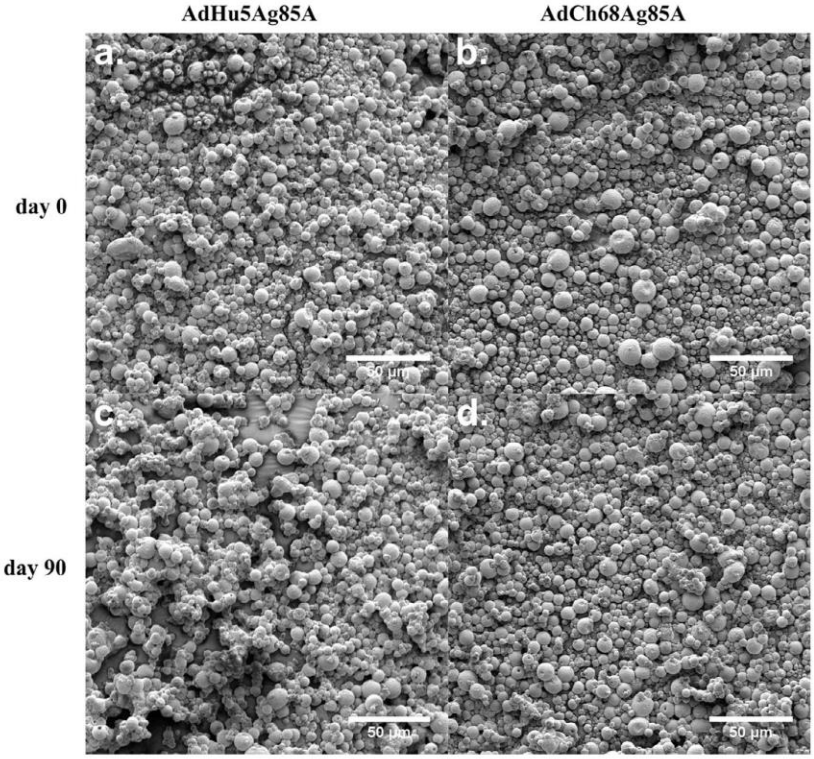

Fig. 1. Scanning electron micrographs taken at 1000× magnification for mannitol/dextran powders encapsulating AdHu5Ag85A (a) or AdCh68Ag85A vaccine (b) immediately after spray drying, and for mannitol/dextran powders encapsulating AdHu5Ag85A (c) and AdCh68Ag85A vaccine (d) after ninety days of storage at ambient temperature and low relative humidity.

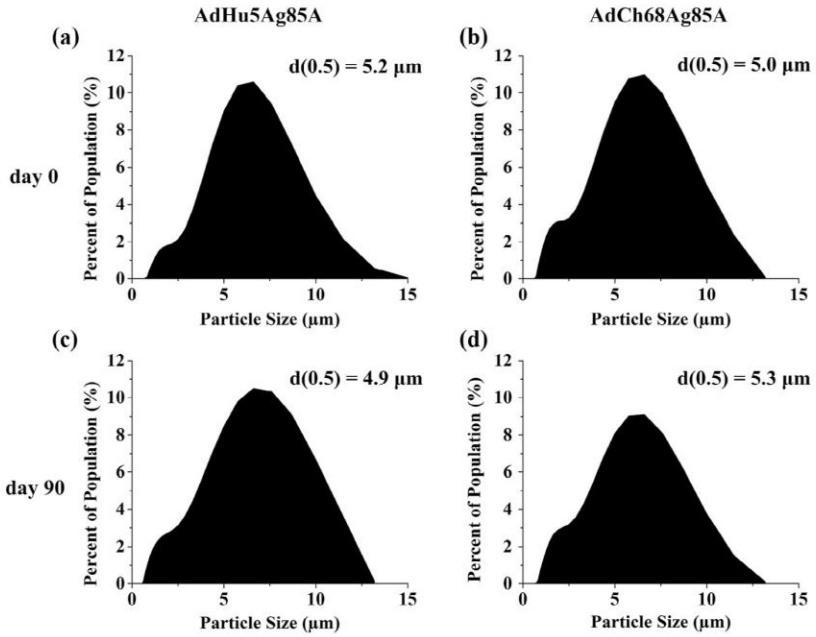

Fig. 2. Distribution of particle size of mannitol/dextran powders encapsulating AdHu5Ag85A (a) or AdCh68Ag85A vaccine (b) determined immediately after spray drying, and the mannitol/dextran powders encapsulating AdHu5Ag85A (c) and AdCh68Ag85A vaccine (d) determined after ninety days of storage at ambient temperature and low relative humidity.

Table 1
Measured particle glass transition temperature (T_g) for mannitol/dextran particles containing either AdHu5Ag85A or AdCh68Ag85A. Particle T_g was measured immediately after spray drying, and after ninety days of storage at ambient temperature and low relative humidity.

Sample	T_g (Day 0, °C)	T_g (Day 90, °C)
Mannitol + Dextran/AdHu5Ag85A	135.7	134.4
Mannitol + Dextran/AdCh68Ag85A	139.8	136.5

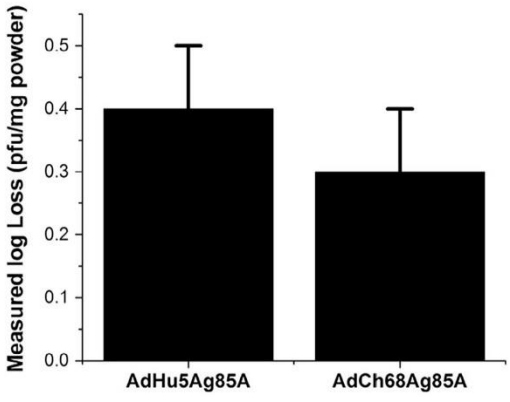

Fig. 3. Measured log losses of infectivity (pfu/mg powder) for spray dried AdHu5Ag85A and AdCh68Ag85A samples (day 0). Samples were measured immediately after spray drying by using cultured 293 cells. Data are presented as mean ± SEM of the mean of three samples.

respectively (Fig. 3). These data are in basic agreement with our previous findings [12] and suggest that the process of spray drying with a mannitol/dextran formulation leads to a relatively small loss of infectious particles of AdHu5Ag85A and AdCh68Ag85A vaccines.

3.3. Spray dried human- and chimpanzee-adenoviral vectored TB vaccines are as immunogenic in vivo as their liquid counterparts

Since the *in vitro* infectivity testing cannot inform of a vaccine's immunogenicity, it is relevant to determine whether the spray drying process may have negatively affected the immunogenicity of spray dried AdHu5Ag85A and AdCh68Ag85A vaccines. To this end, the spray dried powders were reconstituted in buffer immediately after spray drying (day 0) and injected intramuscularly (I.M.) to naïve mice. As a comparison, the same doses of corresponding liquid-formulated vaccines were used. The antigen (Ag)-specific $CD8^+$ T cells were examined in the blood, spleen and lungs by Ag85A tetramer (Tet^+) immunostaining at two weeks following vaccination (Fig. 4A). Levels of Ag-specific $CD8^+Tet+$ T cells remained highly comparable between the spray dried and liquid control samples of the same vaccine across the spleen (Fig. 4B), blood (Fig. 4C), and lung tissue (Fig. 4D). Furthermore, there were no significant differences in T cell immunogenicity between spray dried AdHu5 and AdCh68 vaccines (Fig. 4B/C/D). These results clearly indicate that the spray dried adenoviral vaccines, irrespective of the adenoviral species, retain their *in vivo* immune-activating capabilities well, when assessed immediately following the spray dry process.

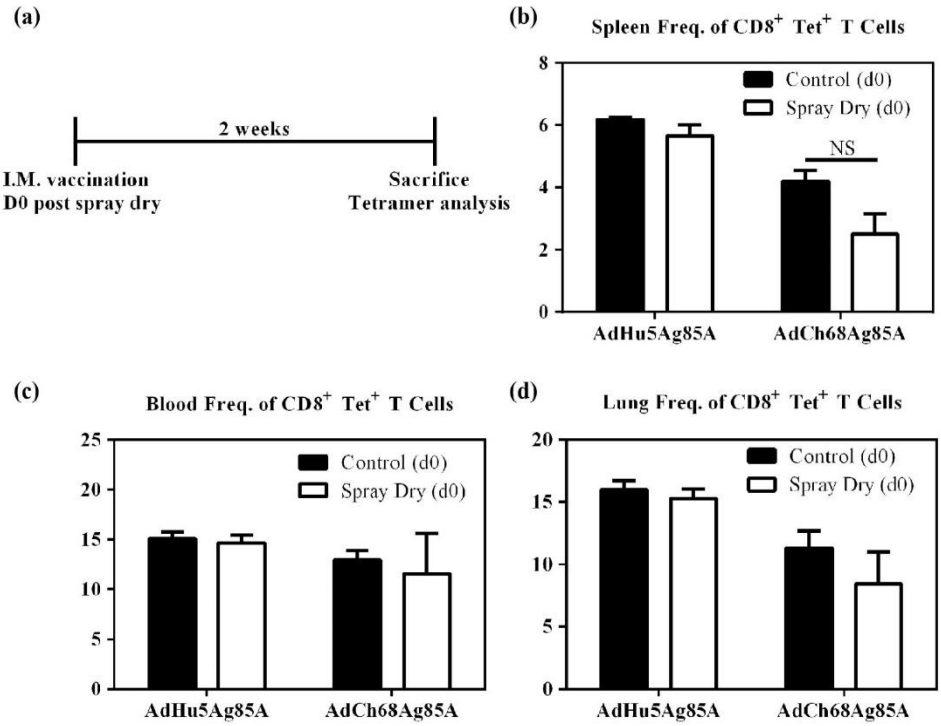

Fig. 4. AdHu5Ag85A and AdCh68Ag85A vaccines immediately after spray-drying induce immune responses *in vivo* comparable to their fresh cryopreserved liquid vaccine controls. (a) Experimental schema. Intramuscular immunization with 1×10^7 pfu of either spray dried AdHu5Ag85A or AdCh68Ag85A or an equivalent amount of fresh cryopreserved liquid vaccine control, was carried out and animals were sacrificed 2 weeks post-immunization. Frequencies (%) of Ag85A tetramer-specific $CD8^+$ ($CD8^+Tet+$) T cells in the spleen (b), blood (c) and lung (d) were measured. Data are presented as mean ± SEM of the mean of three animals/vaccine/condition.

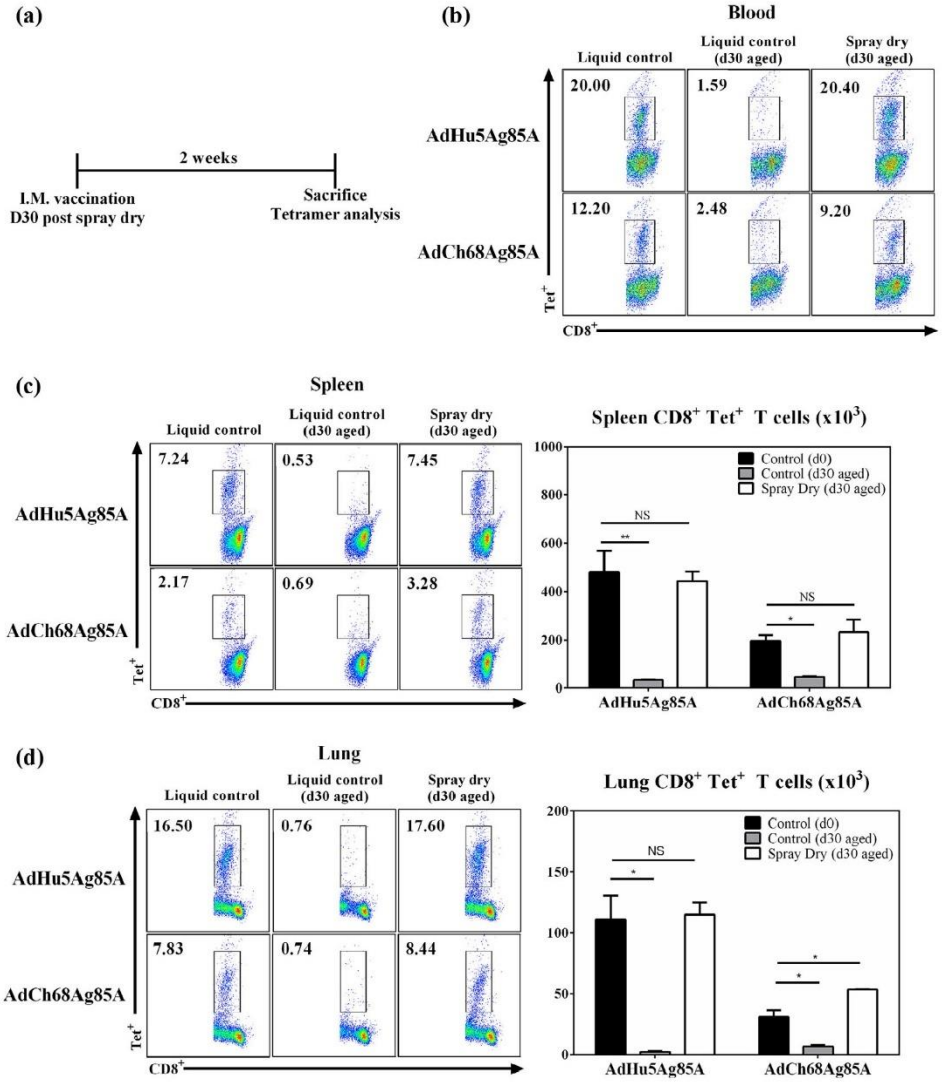

Fig. 5. Contrast to their liquid counterparts, spray dried AdHu5Ag85A and AdCh68Ag85A vaccines post-30-day thermal storage, remain immunogenic *in vivo*. (a) Experimental schema. Intramuscular immunization with 1×10^7 pfu of either spray dried AdHu5Ag85A or AdCh68Ag85A or an equivalent liquid vaccine control that had been stored for 30 days at ambient room temperature and low relative humidity, or with the same amount of fresh cryopreserved liquid vaccine control. Animals were sacrificed 2 weeks post-immunization. Frequencies (%) and absolute numbers of Ag85A tetramer-specific $CD8^+$ ($CD8^+Tet+$) T cells in the blood (b), spleen (c) and lung (d) were determined. Data are expressed as representative dotplots (frequencies) and mean ± SEM of the mean of absolute numbers of T cells from three animals/vaccine/condition.

In contrast to their liquid counterparts, spray dried human- and chimpanzee-adenoviral vectored TB vaccines post-thermal storage, remain immunogenic *in vivo*

To investigate the thermostability, as pertaining to *in vivo* immunogenicity, of spray dried AdHu5Ag85A and AdCh68Ag85A, spray dried vaccines and liquid controls were stored first for 30 days under ambient temperature and low relative humidity conditions before *in vivo* testing. Day 0 liquid vaccine (Control (d0)) and day 30 liquid (Control d30 aged) vaccine stored at ambient temperature were used in parallel as comparison. At two weeks post-immunization, Ag-specific $CD8^+$Tet+ T cell responses were assessed in the blood, spleen and lungs (Fig. 5A). In the case of AdHu5Ag85A (Spray dry d30 aged), the reconstituted spray dried vaccine induced levels of T cell responses, in frequencies and absolute numbers, after 30 days of storage that were comparable to those by control day 0 liquid in blood (Fig. 5B), spleen (Fig. 5C) and lung tissue (Fig. 5D). The same held true for AdCh68Ag85A vaccine (Fig. 5B–D). In sharp contrast, as expected, the control d30-aged liquid AdHu5Ag85A or AdCh68Ag85A vaccine stored under the same conditions as spray dried vaccines lost their respective immunogenicity *in vivo* almost completely (Fig. 5B–D).

Having demonstrated the maintained day-0 *in vitro* infectivity (Fig. 3) and day-30 *in vivo* immunogenicity (Fig. 5) of spray dried AdHu5- and AdCh68-vectored TB vaccines, we set out to further investigate the thermostability, in terms of immunogenicity, of spray dried vaccine products by extending their storage time to 90 days at ambient temperature and low relative humidity (Spray dry d90 aged). Day 0 liquid vaccines (Control (d0)) were used as comparison. Day 90-aged liquid controls were not included since the liquid vaccines stored lost most of their immunogenicity by

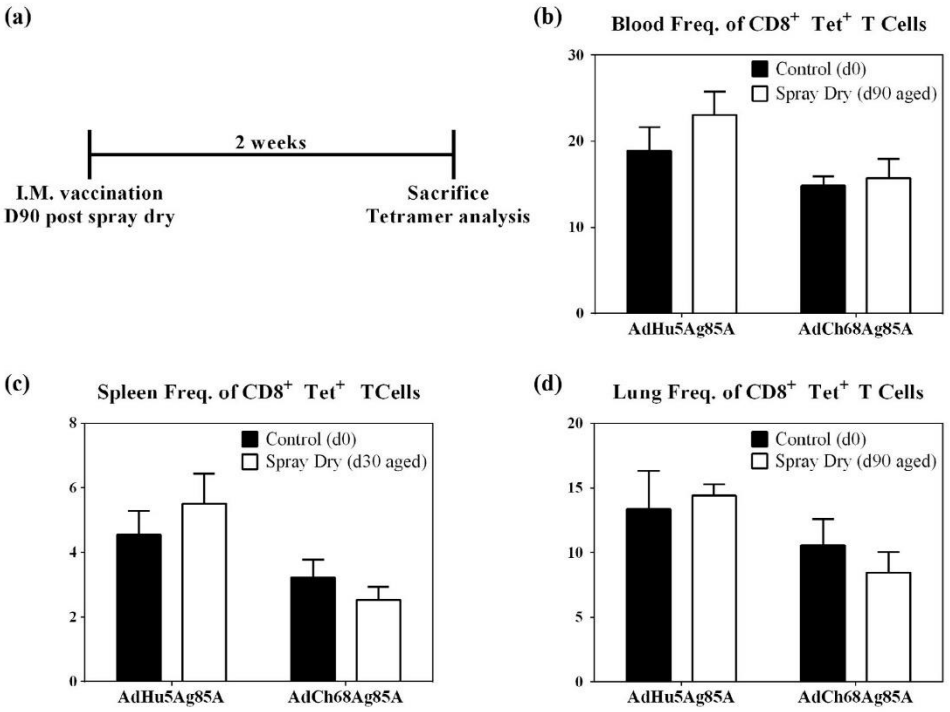

Fig. 6. Spray dried AdHu5Ag85A and AdCh68Ag85A vaccines post-90-day thermal storage remain immunogenic *in vivo*. (a) Experimental schema. Intramuscular immunization with 1×10^7 pfu of either spray dried AdHu5Ag85A or AdCh68Ag85A that had been stored for 90 days at ambient room temperature and low relative humidity, or with the same amount of fresh cryopreserved liquid vaccine control. Animals were sacrificed 2 weeks post-immunization. Frequencies (%) of Ag85A tetramer-specific $CD8^+$ ($CD8^+Tet+$) T cells in the blood (b), spleen (c) and lung (d) were determined. Data are expressed as mean ± SEM of the mean of three animals/vaccine/condition.

30 days (Fig. 5). Upon examination of Ag-specific $CD8^+Tet+$ T cells at two weeks post-immunization (Fig. 6A), we found the levels of T cell responses by 90 day-aged spray dried AdHu5Ag85A or AdCh68Ag85A vaccines were very similar to those by day 0 liquid control vaccines in the blood (Fig. 6B), spleen (Fig. 6C) and lung tissue (Fig. 6D). These data together suggest that spray dried AdHu5- and AdCh68-vectored vaccines when encapsulated in a mannitol/dextran excipient formulation possess markedly increased thermal stability with well maintained immunogenicity.

4. Discussion

Thermostability of vaccines, particularly when stored under cold chain-free conditions, remains a challenge for their effective distribution in the resource-poor regions where they are needed the most [2]. Thus, the purpose of our current study was by using mannitol and dextran as stabilizing excipients for spray drying, to evaluate the thermostability and immunogenicity of AdHu5- and AdChC68-vectored vaccines. Spray dried powders containing these recombinant viruses display properties necessary for application as thermally stable viral vector-based vaccines. Upon reconstitution, they effectively transfected cells *in vitro* with modest losses in viral infectivity related to the spray drying process. Furthermore, following *in vivo* vaccination, they were as immunogenic as the conventional fresh, cryopreserved liquid control samples. Of importance, even after storage at ambient temperatures for 30 and 90 days, they did not suffer reduced immunogenicity *in vivo*, while the liquid vaccine samples stored under the same conditions lost their *in vivo* immune-activating capability.

Effective vaccine administration requires the biologic to maintain its immunogenic activity after the spray drying process. Previous studies have demonstrated retained immunogenicity for selected types of vaccines immediately after processing through various drying methods [24,25,32]. However, few studies have examined the immunogenicity and thermostability of spray dried live viral-vectored vaccines, hence our current study.

Infectivity losses *in vitro* from our spray drying for AdHu5Ag85A- and AdCh68Ag85A-containing particles are considered to be minor (measured at 0.4 ± 0.1 log and 0.3 ± 0.1, respectively) attributable to the excellent stabilizing effects of the mannitol and dextran excipients. Preservation of viral vector activity was maintained through replacing the water-biologic hydrogen bonds with sugar-biologic hydrogen bonds, and by immobilizing the encapsulated biologic within the glassy matrix [12]. Vaccine immunogenicity is dependent on the stabilization of viral surface protein structure necessary for *in vivo* viral interaction with immune cells [33,34] and the denaturing of such viral proteins and viral aggregation interfere with many crucial viral mechanisms [35–37]. Thus, addition of the hydrogen-bonding sugars, mannitol and dextran, acts to maintain protein stabilization through drying. It is known that differing biologics potentially exhibit differing stabilizing profiles within the same excipient matrix for spray drying. The particular proteins comprising adenoviral structures differ between types, though the icosahedral shape with fibre and knob attachment remains consistent [38,39]. Thus, subtle differences separate adenoviral serotypes, while the general structure is definitive. However, in our study we did not see significant differences in spray dried particle properties and thermostability between the human adenoviral (AdHu5)- and chimpanzee adenoviral (AdCh68)-vectored vaccines. This suggests that the sensitivity of the biologic with respect to the stabilizing matrix formulation is similar among structurally related adenoviral viral vectors.

An aqueous medium does little to constrict molecular movements of the stored viral vectors, resulting in denaturing and aggregation of viral vector proteins when stored at ambient temperature [40,41]. Furthermore, the rates of protein unfolding within aqueous media are increased at elevated temperatures and in the presence of synthetic surfaces [41]. Conversely, a glassy matrix can prevent the unstructuring of viral vector proteins by restricting mobility, as shown through the witnessed stability of spray dried vaccines [42–44]. The stability of this immobilizing medium can be measured through its glass transition temperature, T_g, which determines the temperature when the amorphous phase of the matrix – where the adenovirus resides – transitions from a restrictive glassy state to one that allows for greater molecular mobility [45]. It is thus at temperatures well below the T_g where biologic stability is best maintained as a result of restrictions on denaturing and aggregation [46]. The high particle T_g values immediately after spray drying and after ninety days of storage, as shown in Table 1, are indicative of good retention of the matrix glassy properties without plasticization by the viral vectors or ambient moisture. Low relative humidity was necessary for storage because moisture uptake readily occurs within hygroscopic sugars [12].

Particle stability was further implied through morphology and size of the powders throughout storage. Consistent particle morphology (Fig. 1) and size (Fig. 2) were apparent after storage, which is critical for maintaining an immobile matrix. Other studies have shown that matrices can become increasingly tacky over time, leading to particle caking and aggregation [43]. In such cases, there is a corresponding decrease in the stabilizing ability of the matrix based on increased mobility of the encapsulated biologics [12,24,44]. Thus, maintenance of particle size and morphology is considered a practical indicator of a functional vaccine, which agrees with the observations we made. Although our measured viral particle sizes of <10 μm are potentially amenable to respiratory inhalational delivery as dry powder vaccines, this was not the objective of the current study. This is why the thermostability and immunogenicity of spray dried adenoviral vaccines was evaluated only after liquid reconstitution and following intramuscular injection in our current study. It would be of interest to investigate the aerosol properties and vaccine potency as an inhalable dry powder product in a separate future study.

In summary, we have developed a mannitol and dextran excipient matrix for effectively spray drying the two adenoviral vaccine vectors of human and chimpanzee origin. We demonstrate that both spray dried vaccines well retain their infectivity and immunogenicity even after stored, cold-chain free, for 90 days. Our results support further development of spray drying technology for adenoviral-vectored and other viral-vectored vaccines.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2017.04.026.

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7.0 – Chapter 7 – Discussion

7.1 - Advancements towards the design of novel TB vaccines

There was a time when a diagnosis of tuberculosis was considered a death sentence. The discovery and implementation of effective drug regimens, and global introduction of BCG in the 1940s provided hope in controlling and eradicating this disease[1]. In conjunction to social and economic infrastructure improvements, TB rates have declined to very low levels in most countries throughout western Europe and North America.

Despite monumental scientific and socioeconomic advancements, the control, let alone eradication, of TB remains an aspiration for many countries. With the goalposts of the END TB initiative being constantly pushed further away, there is a major need for technological breakthroughs to meet the revised END TB goal in 2035. The goals and challenges identified by the WHO, and the Bill and Melinda Gates Foundation provide clear targets and requirements to reach this goal, as detailed in **Figures 2** and **3**.

Understanding the protective gaps in natural immunity against TB (as previously discussed) provides avenues for developing improved vaccination strategies[74]. As such, it is relevant to highlight that most vaccine candidates currently under evaluation have not taken this into consideration. As such, the studies presented within this dissertation collectively show that effective TB vaccines can be developed through consideration of the *M.tb* life cycle, immunization route, and antigen selection.

7.1.1 - Prophylactic vaccine development

In Chapter 3, we present a strategy in designing a monovalent chimpanzee adenovirus serotype 68 vaccine expressing Ag85A (AdCh68Ag85A) for prophylactic application via the respiratory

mucosal route against pulmonary TB[129]. We demonstrate that this vaccine is safe and amenable for respiratory mucosal administration. When directly compared to its well-assessed AdHu5 counterpart (AdHu5Ag85A), we additionally show that RM AdCh68Ag85A immunization generates significantly greater antigen specific CD8 T cell responses within the lung. Furthermore, we not only show that this vaccine provides superior immunity against pulmonary TB, but demonstrate that antigen specific CD8 T cells are directly responsible for the protection seen. Furthermore, given the pervasive nature of pre-existing human adenovirus immunity in TB burdened countries, we additionally assessed AdCh68Ag85A in a model of pre-existing anti-AdHu5 immunity and showed its ability to protect against TB.

Collectively, our study in Chapter 3 presents strong evidence that as a respiratory mucosal-deliverable vaccine, the chimpanzee adenovirus-vectored TB vaccine, has advantages over its human adenovirus counterpart for human applications. It also further supports the role of CD8 T cells in TB immunity. These conclusions warrant further pre-clinical and clinical development of this vaccine.

Prophylactically administered vaccines are designed to drive protective immune responses which either prevent infection and/or minimize clinical disease. Prophylactic vaccines are imperative in TB control, but as detailed in **Figure 6**, they represent one of multiple strategies required in disease control. Given our findings in Chapter 3 showing the prophylactic potential of AdCh68Ag85A, we next sought to determine its therapeutic potential.

7.1.2 - Therapeutic vaccine development

As detailed in **Figure 5**, the success of TB antibiotic therapy is hindered by many factors, with lengthy treatment duration being one of the major examples. As such, development of strategies which can accelerate disease treatment and therefore shorten antibiotic therapy duration are highly

sought after[59,134]. One such strategy is the application of therapeutic vaccines[71]. As such, in Chapter 4, we determined whether respiratory mucosal immunotherapy with AdCh68Ag85A in conjunction with conventional antibiotic therapy was capable of accelerating TB disease clearance and ultimately shortening the duration of antibiotic therapy required to treat disease[135].

Our work demonstrates that a single RM dose of AdCh68Ag85A accelerates bacterial clearance and improves lung pathology in comparison to antibiotic alone, capable of shortening the length of conventional therapy. For the first time, our study indicates the respiratory mucosal, but not parenteral, therapeutic vaccination to be effective, and supports its application as an adjunct therapy to antibiotic treatment. In accordance with our findings in Chapter 3, we provide the evidence that immunotherapy with AdCh68Ag85A induces robust Ag85A-specific CD8 T cell responses within the respiratory mucosa which are directly correlated with this vaccine's therapeutic efficacy. In addition to their presence within the lungs, our immunohistochemical analysis additionally reveals that CD8 T cell were enriched within the granulomatous lesions.

Our findings strongly support that when delivered via the RM route, AdCh68Ag85A is an efficient prophylactic and therapeutic TB vaccine candidate. It is worthwhile to point out that the findings presented in Chapters 3 and 4 were from murine models. Further investigation in other animal models such as guinea pig and non-human primate models will prove helpful (see below).

7.1.3 - Multivalent vaccine development

Although it represents an important first step to develop and evaluate the monovalent AdCh68 vectored TB vaccine, as detailed in **Figure 7** and discussed in Chapter 5 the complexity of the *M.tb* life cycle and the antigenic shifts associated with it are often overlooked in TB vaccine design. Immune responses elicited by vaccines with narrow antigenic breadth therefore are prone to becoming ineffective as such antigenic shifts occur. Antigen 85A is a well characterized antigen

expressed during the early stages of active disease, with its expression dropping during persistence[13]. As such, given our limited antigen breadth in our monovalent AdCh68Ag85A vaccine, this brings up the question as to whether increasing the antigenic breadth of this vaccine may further improve its protective capacity.

Therefore, in Chapter 5 we redesigned our monovalent AdCh68 construct to express a total of 3 antigens – an early-stage antigen and a chronically-expressed antigen (Ag85A and TB10.4 respectively), and an antigen expressed during resuscitation and in the later stages of infection (rpfB). We demonstrate the ability of this vaccine to furnish the lung with T cells specific to all 3 antigens. In addition to the enhanced antigenic breadth of immune responses by this trivalent AdCh68 vector, we additionally show the broad functionality of these responses, and importantly demonstrate that BCG-specific responses can be boosted to a significantly greater magnitude than by its monovalent AdCh68 counterpart. These have major implications given the widespread utilization of BCG and may fill an immunological gap. As discussed in Chapter 5, the lack of persistence antigen responses elicited by BCG may represent a factor in its limited protective efficacy against pulmonary TB.

In addition to our immunological findings, our protective studies show that through simply designing a vector to express persistence antigens, we can drive immunological responses to eliminate both actively replicating and persistent bacteria. Given the abundant presence, and implication of persistent mycobacteria in TB disease burden, our strategy represents the first of its kind where a vaccine can be designed to target this population. As explained later on, our strategy presented in Chapter 5 opens avenues for more relevant, and translational TB vaccine research.

7.1.4 - Improvements in vaccine stabilization

Chapters 3 through 5 demonstrate that efficacious and broadly applicable vaccines can be developed through addressing the gaps in immunity against TB. In addition to this, however, strategies must be developed which allow such vaccines to reach their intended geographic locations, should one of such vaccine candidates be chosen for real-world applications in the near future. Tuberculosis-ridden regions are usually geographically restricted and lack the infrastructure required to stabilize, store, and distribute temperature-sensitive vaccines. In particular, viral-vectored vaccines (as those focused on in this dissertation) are in an aqueous solution and therefore they heavily rely on cold chain conditions to retain their activity. As such, in Chapter 6 we built upon the work by Leclair *et al.* and characterized the ability of sugar excipients in stabilizing adenoviral vaccines through a method known as spray drying[136,137].

Our results provide ample evidence indicating that both spray dried chimpanzee and human adenoviruses with a mannitol-dextran excipient blend exhibit much enhanced thermal stability. These spray dried vectors were able to withstand ambient temperatures and low relative humidity for up to 3 months, with little loss in viral activity. Our findings have major practical implications for those novel viral-vectored vaccines that may eventually advance to the stage of field efficacy assessment in TB-endemic regions.

7.2 - Towards improving TB vaccine design and assessment

The strategies presented within this dissertation describes approaches in the development, assessment, and application of novel TB vaccines. A number of questions pertaining to these studies however remain to be answered; These will be discussed in this section.

7.2.1 - Beyond conventional T cell immunity

Our studies solely focus on the generation, functionality, and direct contribution of vaccine-induced T cell responses in anti-TB immunity. Although we have provided ample evidence for the role of T cell immunity in vaccine efficacy (prophylactic – Chapter 3, and therapeutic – Chapter 4), this does not rule out the contribution of other cells/factors in the documented protection.

B cell immunity, including antibody responses, are often overlooked given the phagosomal nature of *M.tb* which masks it from direct antibody recogniction[138]. Recent studies however challenge this view and suggest that B cell responses can be protective, capable of even providing immunity against active disease. For example, antibodies generated against mycobacterial cell wall glycolipids like manLAM have been seen to be protective[139,140]. This suggests that vaccines generated to target the right mycobacterial antigen may drive both T and B cell responses that are collectively protective.

Furthermore, recent work has also shown that the individuals who are chronically exposed to *M.tb* but do not develop TB have unique signatures of B cell immunity[141]. These include the high avidity class-switched IgG and IgA with unique Fc glycosylation specific to *M.tb*-specific antigens, and up-regulation of co-stimulatory molecules involved in B cell maturation, CD40L/CD154.

Although we have not dissected vaccine-specific B cell responses in our work, based on our current understanding it is likely that RM immunization also induces robust antigen-specific IgA responses at the respiratory mucosa. While our previously published work with AdHu5Ag85A has suggested a minimal role of B cells in anti-TB immunity, this may heavily depend on the vaccine antigen(s) and remains to be addressed[142].

In addition to the B cell response, the innate immune system plays a paramount role in anti-TB immunity. Alveolar macrophages (AMs) are the first immune cell to be infected and as such, represent a realistic target for mycobacterial control. Classically, this is thought to be mediated in the context of Th1 immunity and cytokines capable of activating the bactericidal functions of these cells.

Recent studies however suggest that the pulmonary innate immune system, and in particular AMs, can be directly augmented through RM immunization. Recent work by us has shown that these cells can be directly "trained" following RM immunization with AdHu5Ag85A, assuming an augmented, memory-like phenotype[115]. Upon secondary exposure to bacterial pathogens, these memory AMs were seen to rapidly respond to and control infection independent of the adaptive immune responses. This and other studies therefore suggest that the AM population may be trained in such a way to have a defense-ready signature.

Trained innate immune responses were not characterized in the work presented in this dissertation and as such, we cannot rule out its roles in the protection documented in Chapters 3 through 5. It would be particularly interesting to assess the protective contribution of trained innate immunity relative to adaptive immunity following therapeutic immunization.

As previously discussed, the innate immune system experiences strong *M.tb*-mediated immune suppression, thereby expanding the niche for mycobacterial growth. Given our overwhelming findings that RM immunization drives a trained innate immune phenotype in AMs, it would be imperative to assess whether such training could counteract/minimize *M.tb*-orchestrated suppression. This would be of particular relevance in the context of therapeutic immunization.

7.2.2 - Improved animal models for TB vaccine research

Our studies thus far have been exclusively carried out in the BALB/c mouse model. This stems from its ease of handling, cost, and availability of immune reagents. Additionally, immunological responses following *M.tb* infection are similar to that in humans, with a T cell-dependent phenomenon in anti-TB immunity. Unfortunately, where this model falls short is the inability to recapitulate human disease pathology[143–145]. This not only includes the inability to form organized granulomas, but also the complete lack of granulomatous necrosis, caseation, and cavitation[4].

The inability to recapitulate the hallmarks of human TB pathology may cloud the translatability of our findings – in particular, our therapeutic vaccination study (Chapter 4). The structure of the human granuloma includes a core composed of a heterogenous population of macrophages that is surrounded by a lymphocytic cuff and encompassed in a fibrous capsule. Granulomas are heterogenous in their size, immune composition, and mycobacterial heterogeneity and burden. Under certain conditions, the central core can become necrotic[146]. Necrosis and caseation significantly alter the biochemical and microbiological microenvironment of the granuloma. This stems from the abundant hypoxia, limited nutrient availability, excessive cellular debris, and a large abundance of extracellular mycobacteria. The consequence of this is two-fold: (1) Heterogeneity in the mycobacterial population, with a shift towards persistence, and (2) Altered antibiotic drug diffusion and metabolism[26,146,147].

The altered biochemical microenvironment of the granuloma, alongside the high concentration of persistent mycobacteria, not only acts as a physical barrier for antibiotic diffusion but also impedes antibiotic metabolism (e.g Pyrazinamide to its active form pyrazinoic acid). As these events do not occur within the BALB/c mouse model, one may argue against the translatability of our therapeutic

study (Chapter 4). However, an alternative conclusion may be that the immunotherapy may control infection, thereby preventing these necrotic events from occurring in the first place.

Other animal models including humanized mice, and NHP that better recapitulate human TB pathology may prove more translatable for assessing the prophylactic and therapeutic efficacy of our vaccine constructs[87,143,148,149]. However, a balance must be struck with its feasibility. As discussed in Chapter 5, the C3HeB/FeJ model forms necrotic, caseous granulomatous lesions and may represent a more realistic model for vaccine assessment[150–152].

7.2.3 - Mycobacterial-centric approach to vaccine design and refinement

The collective approach in the above studies has been to develop vaccine strategies that address the gaps in natural immunity against TB. Given our rationale and focus in Chapter 5, there is a complementary approach that has yet to be investigated – understanding mycobacterial adaptation to vaccine-mediated pressures.

The availability of technologies like RNAseq allows for measuring changes in expression of *M.tb* genes in response to stimuli, infection, and environmental changes[153,154]. To date, studies pertaining to TB have mainly focused on *M.tb*-centric changes in *in vitro* conditions, with only a handful assessing such changes in cell lines[155–157].

Implementing this technology in our model (particularly Chapter 5), it would be of interest to compare gene changes between unimmunized and RM immunized hosts in both mycobacterial genes, and host immune signatures. Such an approach may allow for identification of novel antigenic targets for improving vaccine design / developing novel boosters.

8.0 – References

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9.0 – Appendix

9.1 - Methods and clinical development of adenovirus-vectored vaccines against mucosal pathogens

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REVIEW ARTICLE

Methods and clinical development of adenovirus-vectored vaccines against mucosal pathogens

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Adenoviruses represent the most widely used viral-vectored platform for vaccine design, showing a great potential in the fight against intracellular infectious diseases to which either there is a lack of effective vaccines or the traditional vaccination strategy is suboptimal. The extensive understanding of the molecular biology of adenoviruses has made the new technologies and reagents available to efficient generation of adenoviral-vectored vaccines for both preclinical and clinical evaluation. The novel adenoviral vectors including nonhuman adenoviral vectors have emerged to be the further improved vectors for vaccine design. In this review, we discuss the latest adenoviral technologies and their utilization in vaccine development. We particularly focus on the application of adenoviral-vectored vaccines in mucosal immunization strategies against mucosal pathogens including *Mycobacterium tuberculosis*, flu virus, and human immunodeficiency virus.

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INTRODUCTION

Since their discovery nearly half a century ago, adenoviruses have quickly become attractive vectors for vaccine development. Adenoviruses originally showed tremendous promise as gene replacement therapy vectors due to their ability to be easily genetically modified, their excellent safety profiles, broad tissue tropism, and ability to drive robust, sustained transgene expression.[1] Their efficacy in clinical settings was unfortunately short-lived due to the robust host innate and adaptive immune responses against adenoviral and transgene products shortly after their administration. The robust immunogenicity of adenoviruses, combined with their favorable safety profiles, however, prompted a rapid transition of adenoviruses from being the gene therapeutic vector to the widely used vaccine platform to-date.[2]

Adenoviruses are efficacious vaccine vectors against diseases in which traditional vaccine development strategies have proven ineffective.[3] These include, but not limited to, diseases such as tuberculosis (TB) and the human immunodeficiency virus (HIV).[4] Adenoviral-based vaccines also have the potential to eventually replace existing vaccine platforms which are either financially and/or technically challenging to generate (such as the seasonal influenza vaccine), and less efficacious platforms which have to be administered multiple times in order to engender protective immunity (such as the rabies vaccine).[4]

In this review we discuss the most often used adenoviral-based vaccine technologies and their pre-clinical and clinical development in the fight against mucosal pathogens. We focus on three mucosal pathogens, *Mycobacterium tuberculosis*, influenza, and HIV, given the high global prevalence, unmet vaccine needs, and well-documented effort in preclinical and clinical vaccine development using adenoviral technology for these pathogens.

ADVANTAGES OF ADENOVIRUSES AS PLATFORMS FOR VACCINE DESIGN

Adenoviruses possess the features which make them favorable platforms for vaccine design. Firstly, recombinant adenoviral vectors have excellent safety records which are apparent by their initial use as vectors for gene therapy in humans.[5] Combined with their broad tissue tropism, adenoviral vectors have been widely explored for developing vaccines desired to be delivered via the respiratory mucosal route against mucosal pathogens such as *M. tuberculosis* and influenza.[6,7] Secondly, adenoviruses are highly immunogenic, capable of driving robust, long-lasting immune responses to vector-encoded antigens. Adenoviruses are particularly effective in inducing potent CD8, and to a lesser extent, CD4 T-cell responses.[4,8] This makes them attractive platforms for the vaccines against intracellular pathogens to which cellular immune responses are indispensable for protection.[4] The intense interest in using adenoviral vectors for vaccine design is not restricted to only using the human serotype 5 adenovirus (AdHu5) or other human serotypes. Nonhuman adenoviruses are being increasingly explored for vaccine development. The following sections discuss the tools and procedures available for the generation of such vectors.

MOLECULAR BIOLOGY OF ADENOVIRUSES

Adenoviruses are species-specific with multiple serotypes in each species. Adenoviruses are further classified into different subgroups

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based on genetic homology between serotypes.[9] Currently 57 different human adenovirus serotypes have been identified, being classified into 7 subgroups.[9,10] Of these, AdHu5, a group C adenovirus, is both the most common serotype to infect humans and the most widely used serotype for recombinant vaccine design owing to its superior immunogenicity in comparison to other human adenovirus serotypes.[2,11] However, other human adenovirus serotypes and nonhuman adenoviruses have increasingly been used for vaccine design due to the global prevalence of preexisting anti-AdHu5 immunity in humans which has been clinically seen to negatively impact vaccine efficacy.[12–14]

Adenoviruses are double-stranded DNA viruses with linear genomes ranging between 34 and 43kbp in size. Both linear strands of the viral genome code for viral polypeptides thereby compensating for the viruses' relatively compact genome size. The genome encodes five early gene transcription units (dictated as E1a, E1b, E2, E3, and E4) and a single late transcription unit that is subdivided into L1 through L5 (Figure 1). The early transcription units play critical roles in viral DNA replication and evasion of host immunosurveillance while the late transcription units primarily encode viral structural components.[10] Readers are directed to ref. [10] for a detailed review on the molecular biology of adenoviruses.

GENERATION OF RECOMBINANT ADENOVIRUSES

Recombinant adenoviral vaccines are generated through the insertion of a transgene cassette into the adenoviral backbone. Transgene cassettes are constructed to express one or more foreign antigens-of-interest under the control of a given promoter (usually the cytomegalovirus (CMV) promoter due to its ability in driving robust and sustained transgene expression). Depending on numerous factors such as the size, composition, or complexity and the expression pattern of the chosen promoter, different promoters may be utilized.[15–17] Readers are directed to refs. [15–17] for other commonly used promoters.

Adenoviruses are able to efficiently package 105% of their original genome in viable virion before becoming unstable.[18] This allows for up to a 2,000-bp transgene cassette to be inserted into the adenoviral backbone. As regulatory elements within transgene cassettes (such as the promoter) can encompass over 1,000 bp in size, this can limit the size and number of heterologous antigens. As such, the adenoviral genome must be manipulated to accommodate larger transgene cassettes. The majority of recombinant adenoviral vaccine vectors are known as first-generation vectors as they are deficient in the E1 transcription unit. Deletion of E1, which is critical in viral replication, not only bolsters the safety of adenoviral vectors, but further enhances vector capacity allowing for accommodation of transgene cassettes up to 5,000 bp in size.[18,19] A majority of first generation vectors are also deficient in E3, further increasing the potential size of transgene cassettes to 7,500 bp in size.[18,19] The deletion of multiple transcription units provides several advantages. For instance, it enhances vector capacity for generating multivalent vaccines against complex pathogens. Secondly, it reduces the number of viral-encoded antigens, resulting in a diminished magnitude of antivector immunity.

Adenoviral vectors can be molecularly designed to lack all viral genes and only express essential elements for viral replication and packaging. These gutted vectors are recoverable with the use of helper adenoviruses which provide *in trans* complementation of the viral genes needed to properly package the gutted adenovirus.[1,17] Such vectors are not only highly flexible, capable of expressing transgene cassettes up to 36 kbp in size, but due to the lack of viral genes, induce drastically reduced antivector immunity, thus allowing for longer and more efficient transgene expression than the first-generation vectors.[20] These vectors also have favorable safety profiles following the respiratory mucosal delivery as shown in large animal models.[21] As such, such gutted adenoviral vectors continue to represent promising platforms for gene replacement therapy. However, their application for vaccination is limited due to their reduced immune adjuvant effects,[22] the technical difficulty in their large-scale production, and the potential negative effect on quality memory immune responses resulting from prolonged high levels of transgene-encoded immunogens.

There are the two main strategies currently utilized in the design and development of recombinant adenoviral vectors.[23] The first involves homologous recombination between the adenoviral genome and a transgene cassette-expressing shuttle plasmid within mammalian systems. The second involves directly cloning a transgene cassette into the adenoviral backbone, bypassing the need for homologous recombination.

Method 1: homologous recombination in mammalian systems

The most widely used method in adenoviral vector development involves homologous recombination between a shuttle plasmid

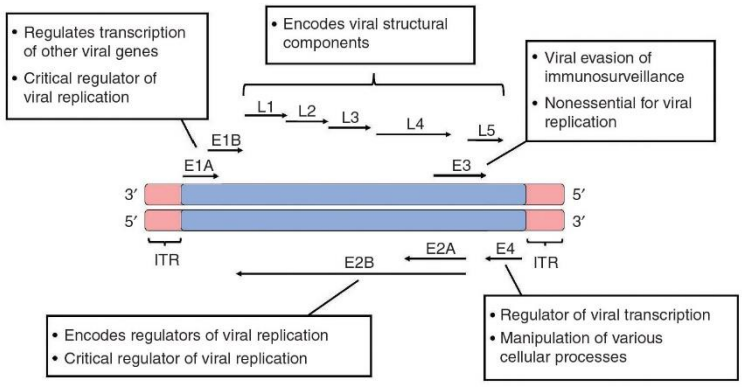

Figure 1 Organization and function(s) of the adenoviral genome. The organization, orientation, and broad function(s) of the adenoviral transcriptional units are depicted. Knowledge of the entire adenoviral genome is critical in the downstream construction of adenoviral-vectored vaccines.

carrying a given transgene cassette and the adenovirus backbone.[18] This strategy is relatively straightforward and can be easily performed with a variety of commercially available protocols, reagents, and kits.

Generation of adenoviral vectors is commonly carried out in mammalian cell lines. A shuttle plasmid is constructed to express the 5′ end of the adenoviral genome in which the E1 transcription unit is replaced with a transgene cassette.[18,19] This shuttle plasmid is transfected into a mammalian system alongside either a rescue plasmid DNA containing an E1-deleted adenoviral genome which is designed to slightly overlap with the 3′ region of the shuttle plasmid or the wild-type adenoviral genome with cleavages in the E1 region. Removal/cleavage of E1 greatly reduces the risk of generating replication-competent viruses.[18] Within the mammalian system, homologous recombination between the shuttle plasmid and the adenoviral vector leads to incorporation of the transgene cassette into the adenoviral backbone (Figure 2). The shuttle plasmid can also be generated in such a way that the E3 transcription unit is replaced with a given transgene cassette.[18]

As the lack of E1 renders recombinant adenoviral vectors replication-deficient, vector amplification and propagation must be done within cell lines which can provide E1 *in trans*. The HEK 293 cell line, a modified human embryonic kidney cell line, constitutively expresses E1 from AdHu5 has been the staple workhorse in the generation of recombinant adenoviral vectors for both preclinical and clinical applications.[24] The 293 cell line, however, is not without its shortcomings. Firstly, the E1 protein expressed by this cell line is from AdHu5. As such, this cell line cannot fully complement the recovery of all non-AdHu5 adenoviruses. This is particularly evident for group B adenoviruses (such as AdHu35) and certain nonhuman adenoviruses shown poorer recovery yields when propagated in 293 cells.[25] This, however, can be overcome by two methods: (i) trans-complementing the E1 protein specific to the adenovirus through transfecting the cell line with an additional vector expressing the adenovirus-unique E1 protein, and (ii) using a different cell line. Readers are directed to ref. [24] for a comprehensive review on the various available adenoviral producer cell lines.

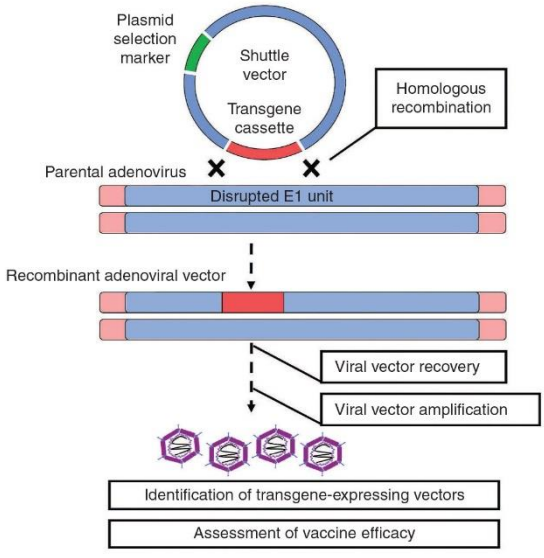

Figure 2 Development of adenoviral-vectored vaccines through homologous recombination. The most commonly used strategy in the generation of E1-deleted human adenoviral vectors (homologous recombination in a viral packaging cell line) is depicted.

Furthermore, propagation of E1-deficient adenoviral vectors on the 293 cell line can lead to a small degree of contamination with replication-competent adenovirus, which may be one of the questions to address when preparing such vaccines for human applications.[24,26] The main cause of replication-competent adenovirus (which is particularity more prevalent for AdHu5 vectors) is due to significant homology between the regions flanking E1 expressed by the cell line and the majority of Ad vectors developed via homologous recombination.[26] The commercially available Per.C6 cell line is an alternative cell line for the clinical development of adenoviral vectors as it reduces the risk of replication-competent adenovirus formation significantly.[24]

Homologous recombination in mammalian systems is not highly efficient which can lead to generation of recombinant viral vectors which lack transgene cassette expression. As such, multiple rounds of viral purification are required to recover and amplify recombinant vectors which properly express the encoded transgene. This can be partly circumvented through implementation of selection strategies (such as the use of β-galactosidase or florescent markers[27,28]), but are not ideal for development of clinical-grade vectors. Issues relating to ineffective recombination have also been addressed through utilization of bacterial systems (such as the pAdEasy system for AdHu5 vector development) but this strategy is technically more challenging and also more restricted in terms of the adenovirus serotypes available.[29–31] Readers are directed to refs. [29–31] for detailed reviews and protocols of homologous recombination in bacterial systems for adenoviral vector development.

Method 2: direct molecular cloning of the adenoviral genome

Despite the widely used homologous recombination method, reconstruction of the entire adenoviral genome into a plasmid represents an alternative strategy which can address some of the issues associated with homologous recombination.

This strategy involves the molecular cloning of the entire adenoviral genome into a plasmid vector.[32] Vectors generated this way can be altered *in vitro*, allowing for direct cloning of transgene cassettes into the adenoviral backbone. Although this strategy is technically more challenging as it requires access to a wider array of molecular biology reagents and techniques, it has multiple advantages. Firstly, transcription units can be easily omitted during vector construction and replaced with cloning (linker) regions. This allows users to develop recombinant vectors which can accommodate multiple transgene cassettes of varying sizes (as per user's discretion).[33] Secondly, it is a one-step straightforward way to rescue the virus without relying on the chances of homologous recombination. Thirdly, this strategy has significant clinical implications as the molecular cloning of the entire adenoviral genome into a plasmid ensures that potential infectious contaminants which may be present in an original adenoviral preparation used for homologous recombination method are completely eliminated.[27] This is particularly useful in the development of non-human adenoviruses which are originally isolated from tissues that may harbor unknown or undetectable pathogens. Detailed protocols outlining this method are available in refs. [27,32,33]. Figure 3 outlines this strategy. Readers are directed to ref. [33] for an up-to-date and in-depth protocol in the generation of both recombinant human and chimpanzee adenoviral vectors.

Molecular cloning of the entire adenoviral genome into a plasmid requires a complete and accurate sequence map of the original viral genome as the location and rarity of restriction sites are critical in

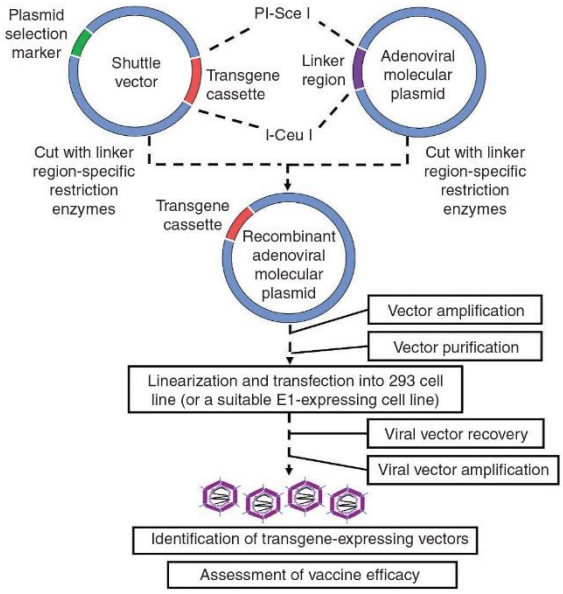

Figure 3 Development of adenoviral-vectored vaccines through a direct-cloning strategy. Generation of adenoviral vectors through direct *in vitro* molecular cloning of the entire adenovirus is depicted. This represents an alternative strategy in the generation of adenoviral vectors, bypassing the need for homologous recombination.

vector construction. This strategy is technically challenging due to the large size of the adenoviral genome and the limited number of genome-wide unique restriction sites to be used for vector reconstruction.[32] Circumvention of this issue involves cloning the entire adenoviral genome in a step-by-step approach through exploitation of restriction sites which are exclusive to given sections of the adenoviral genome. Adenoviral transcription units which are omitted during vector reconstruction may be replaced by linker sequences that are used for the insertion of transgene cassettes. These linker sites are composed of rare restriction cut sites which are not found within the adenoviral genome. Cut sites such as PI-Sce I and I-Ceu I have been traditionally used due to their absence in a variety of human and nonhuman adenoviruses.[33]

Following construction of the adenoviral plasmid, a shuttle vector is engineered to express a given transgene cassette with similar restriction sites as those found within the linker sequences. The shuttle vector can be amplified and the transgene cassette subsequently be excised from the shuttle vector and directly ligated into the adenoviral plasmid using standard molecular cloning techniques.[33] The recombinant adenoviral plasmid can subsequently be transformed into a suitable bacterial cell line and amplified, and then inserted into a viral packaging cell line for recovery.

PRODUCTION OF CLINICAL-GRADE RECOMBINANT ADENOVIRUS-BASED VACCINES

Generation of vectored vaccines that meet the quality and quantity demands for clinical trials can be met with multiple logistical and financial roadblocks. These include the feasibility in vector scaling, availability of proper equipment and facilities, and optimized/standardized Good Manufacturing Practices protocols. Due to the extensive clinical history of adenoviral-vectored therapies (ranging from gene therapy to modern vaccine trials), the ability to scale-up and purify human or nonhuman adenoviral-based vectors from preclinical to clinical studies has become a much more standardized practice compared with other viral vectors.[34] This is made possible by the wide availability of quality-controlled cell lines which generate high titre viral batches and of highly scalable clinical-grade purification strategies (such as ion-exchange or size-exclusion chromatography) which allow for generation of high purity and quality vaccine stocks.[35,36] The strategies in generating such clinical-grade vectors are well described in refs. [34–36].

PRECLINICAL AND CLINICAL DEVELOPMENT OF ADENOVIRUS-BASED VACCINES AGAINST TUBERCULOSIS

The development of vaccines against TB remains a daunting task with the only clinically approved vaccine, Bacillus Calmette Guerin, failing to protect against the pulmonary form of the disease. With over nine million new cases of active disease and over 1½ million deaths every year, there has been a dire need for the development of improved TB vaccine platforms.[37]

It is well characterized that protection against pulmonary TB requires the establishment of long-lasting adaptive cellular immune responses at the respiratory mucosa.[7,38] Preclinical and human epidemiological studies have shown the importance of type-1 helper $CD4^+$ T cell (Th1) and $CD8^+$ T-cell responses in anti-TB immunity through the production of type-1 cytokines such as interferon γ (IFNγ) and tumor necrosis factor alpha (TNFα), and their cytotoxic effector functions.[39] Importantly, such immune responses must be established at the primary site of infection, the lungs, to be protective. As immunization route determines the anatomical location of vaccine-specific T cells, numerous studies have shown that immunization via the respiratory mucosal route is desirable for effective anti-TB immunity.[40–44] Collectively these findings suggest that the success of future TB vaccines relies on their ability to induce long-lasting multifunctional T-cell responses situated at the respiratory mucosa before or shortly after *M. tuberculosis* exposure.

Adenoviruses are one of the most widely used viral-vectored platforms for TB vaccine development. This is not only due to their ability to induce robust T-cell responses, but also due to their natural tropism to the respiratory mucosa, which when combined with their established safety profiles, makes them highly amenable for respiratory mucosal vaccination. A number of human adenovirus serotypes, as well as two recently developed recombinant chimpanzee adenovirus-based TB vaccines are currently under evaluation in both preclinical and clinical settings.

AdHu5-based TB vaccines

AdHu5 is the most immunogenic and utilized serotype for TB vaccines.[11] One of the best-characterized AdHu5-based vaccine candidates is a first-generation (E1/E3-deficient) recombinant AdHu5 vector expressing an immunodominant *M. tuberculosis* antigen 85A under the control of the cytomegalovirus promoter (referred to as AdHu5Ag85A).[40,41,45]

AdHu5Ag85A has been extensively characterized as a respiratory mucosal vaccine in multiple animal models, ranging from murine models for basic immunogenicity and protective efficacy studies to nonhuman primate models which represent the most clinically relevant model for assessing TB vaccines.[41,44,46,47] Multiple studies have collectively shown that respiratory mucosal immunization with AdHu5Ag85A is safe, even in immune-compromised hosts, highlighting the potential use of such first-generation vectors in immunocompromised populations.[48]

Murine studies have shown that respiratory mucosal immunization with AdHu5Ag85A was immunogenic, inducing robust

cytotoxic IFNγ$^+$TNFα$^+$CD8$^+$ T cell, and to a lesser extent CD4$^+$ T-cell responses directly at the respiratory mucosa.[41,43] The effector memory CD8$^+$ T cells induced by this strategy persisted at the respiratory mucosa and in an antigen-dependent manner for many months.[43] Alongside murine protections studies, guinea pig, bovine, and a recently published nonhuman primate studies have shown significantly greater bacterial control and survival of animals immunized via the respiratory mucosal route with AdHu5Ag85A.[41,46,47,49] The potential of AdHu5Ag85A has been further supported by a recently completed phase-1 clinical trial where intramuscular administration of AdHu5Ag85A induced polyfunctional CD4$^+$ and CD8$^+$ T-cell responses particularly in Bacillus Calmette Guerin+ human volunteers.[50] A second clinical trial assessing the safety and immunogenicity of AdHu5Ag85A delivered by inhaled aerosol is to be launched soon in Canada.

Despite their efficacy as vaccine vectors, such (and other) AdHu5-based vaccines are not without their inherent limitations. Preexisting immunity to the adenoviral backbone may dampen the potency of adenoviral-based vaccines.[13] Such preexisting AdHu5 immunity is particularly prevalent in the TB endemic regions, which has been seen to limit the efficacy AdHu5-based vaccines.[14,51,52] Although utilization of higher vaccine doses can overcome this limitation, this situation may limit its respiratory mucosal application in humans where the smallest safe effective doses are desired. Such considerations have prompted the development of adenovirus-vectored vaccines based on rarer, less prevalent human serotypes or nonhuman adenoviral species.

Human serotype 35 adenovirus-based TB vaccines

One approach designed to circumvent preexisting anti-AdHu5 immunity is to use rare human serotypes that humans have low global preexisting immunity against.[53] Human adenovirus serotype 35 (AdHu35), a group B adenovirus, represents such a vector which is currently being assessed as a platform for TB vaccine design.[25]

rAd35-TBS is currently the most advanced recombinant AdHu35-based TB vaccine. Based on an E1/E3-deleted AdHu35 vector, this vaccine takes advantage of the genetic plasticity of adenoviral vectors and expresses three different *M. tuberculosis* antigens under the control of the cytomegalovirus promoter.[25,54] Similar to AdHu5Ag85A, this vaccine has also been evaluated as a respiratory mucosal vaccine.[55] The studies in nonhuman primate models showed its favorable safety profile and its ability to elicit long-lived antigen-specific immune responses within the respiratory mucosa. Both CD4$^+$ and CD8$^+$ T cells elicited by vaccination were polyfunctional, secreting IFN-γ$^+$, TNF-α$^+$, and IL-2$^+$, with CD8$^+$ T cells possessing cytotoxic activity, as measured by granzyme B staining.[55,56] Hokey *et al.* further studied rAd35-TBS following multiple homologous boost immunizations showing repeated aerosol vaccinations to be well tolerated and elicit the persisting polyfunctional antigen-specific T cells in the lung. It is important to note that in the majority of these studies, rAd35-TBS had to be administered repeatedly, indicative of the poor immunogenicity of the AdHu35 vector, relative to the AdHu5 vector. As a result, following intranasal vaccination it induced only a moderate level of protection in a murine model[54] and provided no protection in aerosol-vaccinated nonhuman primate.[55]

rAd35-TBS has also been assessed clinically in phase-1 clinical trials for safety and immunogenicity in Bacillus Calmette Guerin-immunized humans. These studies show that intramuscular administration of this vaccine was safe in both infants and HIV– and HIV+ adults and induced polyfunctional CD4$^+$ and CD8$^+$ T-cell immune responses.[57–61] Again, such immune responses were only measurable following repeated high-dose administrations of the vaccine.

Recent work has shown the importance of considering the effect of TB vaccines on the innate immune responses as they can have drastic impact on the immunogenicity and efficacy of vaccines. In this regard, the AdHu35 vector was found to induce type-1 interferons which in turn suppressed T-cell activation,[62,63] providing a mechanism for the poor immunogenicity of AdHu35 vectors. Another independent study compared AdHu5 with VSV-based TB vaccines in respiratory mucosal boost vaccination. This study shows that the AdHu5-vectored vaccine-induced little type-1 IFN responses and conferred significantly enhanced protection in comparison to the VSV vaccine, which induced high levels of type-1 IFN responses, resulting in heightened IL-10 production and decreased anti-TB activities in infected APCs.[64] Type-1 IFNs are also employed by *M. tuberculosis* to dampen the Th1 immunity.[65,66] Collectively, these findings indicate the importance of careful selection of viral vectors for TB vaccine development and the detrimental activities of type-1 IFNs in anti-TB immunity. Readers are directed to ref. [11] for an excellent overview of the immunogenicity of a variety of human and nonhuman adenoviral vectors.

Chimpanzee adenovirus-based TB vaccines

With advancements in the molecular tools and developmental strategies available to generating nonhuman adenoviruses, chimpanzee adenoviruses have emerged as attractive platforms for vaccine design.[33,67] The growing popularity of chimpanzee adenoviruses as vaccine vectors is associated with their ability to bypass the negative impact of preexisting antihuman adenovirus immunity while inducing immune responses that are similar to or more potent than those elicited by their human adenoviral counterparts.[11,12,51]

Currently two chimpanzee adenovirus-based TB vaccines including AdCh68Ag85A and ChAdOx1.85A are under evaluation, both designed to express an *M. tuberculosis* antigen Ag85A.[68,69]

AdCh68Ag85A, which was developed based on chimpanzee adenovirus serotype 68, expresses the same transgene cassette as AdHu5Ag85A and has been recently assessed for its anti-TB efficacy in the murine model. The study shows that respiratory mucosal immunization with this vaccine was safe, and similar to its AdHu5 counterpart, induced minimal type-1 IFN responses.[69] AdCh68Ag85A induced significantly greater and more persisting antigen-specific T-cell responses within the lungs than AdHu5Ag85A. Although similar levels of protection were seen following immunization with AdHu5Ag85A, protection from *M. tuberculosis*-induced lung pathology varied between the two vaccines. The lungs from animals vaccinated with AdCh68Ag85A showed less pathology as indicated by a drastic reduction in the formation of granulomatous regions (a hallmark of pulmonary TB infection) in comparison to AdHu5Ag85A-vaccinated animals. Furthermore, when assessed in the context of preexisting anti-AdHu5 immunity, the protective efficacy of AdCh68Ag85A was maintained. This finding is particularly important as preexisting anti-AdHu5 immunity is globally prevalent and represents a potential roadblock to the clinical success of AdHu5-vectored vaccines.

A recent study assessing another chimpanzee adenoviral-vectored TB vaccine, ChAdOx1.85A, shows that although respiratory mucosal immunization with ChAdOx1.85A failed to significantly enhance anti-TB protection in Bacillus Calmette Guerin-primed animals, and enhanced protection when combined in a booster regimen with MVA85A.[68]

PRECLINICAL AND CLINICAL DEVELOPMENT OF ADENOVIRUS-BASED VACCINES AGAINST INFLUENZA

Seasonal epidemics of influenza remain a huge challenge to public health worldwide. It is estimated that epidemics of influenza result in three to five million severe cases and 250,000–500,000 deaths globally each year. Vaccination has been the primary prophylactic strategy against influenza infection.

Optimal protection against influenza viral infection requires both humoral and cellular-mediated immunity. Antibodies specific for the best-known antigenic determinants in influenza virus, namely hemagglutinin (HA), neuraminidase (NA), and/or matrix proteins, are critically required for blocking virus attachment to the host cell, preventing the viral release, and interfering with virus assembly, respectively.[70] T cells specific for conserved influenza viral components are required for the clearance of virally infected cells. Therefore, ideal influenza vaccines are expected to induce both neutralizing antibodies and T cellular-mediated immunity.

Conventional influenza vaccines include inactivated (intramuscular flu vaccine) and live-attenuated (nasal spray vaccine) vaccines which are derived from virulent viruses identified in previous epidemics which have proved to be protective. However, due to its error-prone polymerase, the influenza virus is characterized by its frequent antigenic drift in HA and to a less extent NA which facilitates its evasion from preexisting antibodies and memory T cells induced by prior immunizations.[70] As a result, such prediction-based flu vaccination strategy may not always be reliable and the overall protective efficacy is only about 50–60%. For these reasons, there is a need to develop further improved influenza vaccination strategies.

Novel strategies for the generation of influenza vaccines have been proposed, including recombinant proteins, virus-like particles, viral vectors, and DNA-based vaccines. These novel strategies facilitate the availability of candidate vaccines shortly after the genetic sequence of a new dominant influenza virus is determined early in a given epidemics.[70] Among these novel strategies for generating influenza vaccines, replication-deficient human and chimpanzee adenovirus vectors are promising platforms.

Human adenovirus-based influenza vaccines

In murine models, human adenovirus-vectored influenza vaccines induced both humoral and cell-mediated immune responses against influenza virus-encoded antigens.[71] One well known vaccine, a replication-defective human adenoviral-vectored vaccine expressing H5 from the avian H5N1 influenza virus (H5HA), when administered either intramuscularly or intranasally was seen to induce comparable titers of circulating neutralizing antibodies as compared with an adjuvanted recombinant H5HA protein-based vaccine. Importantly, intranasal or intramuscular immunization with this vaccine induced higher frequencies of IFN-γ^+ $CD8^+$ T cells in the spleen, as compared with the protein plus adjuvant equivalent.[71] These results suggest that adenoviral-vectored influenza vaccines might confer superior protection over protein-based ones as the former is better in $CD8^+$ T-cell priming. Importantly, intramuscular and intranasal immunization with this adenoviral-vectored vaccine comparably protected against lethal challenge with H5N1, presumably due to the preferential generation of lung resident memory T cells.

In humans, nasal and epicutaneous vaccination with a replication-defective AdHu5 vector encoding the A/PR/8/34 H1N1 influenza virus HA (referred to as AdCMV-PR8.ha) was shown to be safe and immunogenic in a graded-dose phase-1 clinical trial both following primary and booster immunization.[72] Immunogenicity of nasal vaccination was demonstrated by the induction of serum hemagglutination-inhibition antibodies. More importantly, nasal vaccination induced significantly higher frequencies of seroconversion compared with subcutaneous vaccination following both primary and booster immunization even though the dose of nasal vaccine was around 10% of the subcutaneous dose.[72] These findings suggest that respiratory mucosal immunization with human adenovirus-vectored influenza vaccines may be of superior safety and efficacy, though it is unknown in this study to which extent the T cells were activated following mucosal immunization.

A more recent phase-1 clinical trial, however, evaluated the safety and immunogenicity of a replicating human serotype 4 adenovirus-vectored influenza vaccine expressing the HA from H5N1 (Ad4-H5-Vtn) administered via oral route as a priming vaccine that was followed by parenteral H5N1 boosting vaccination.[73] The cumulative frequency of such adverse events as abdominal pain, diarrhoea, and nasal congestion was significantly higher than placebo group, though no serious treatment-related events occurred. Oral Ad4-H5-Vtn priming immunization induced cellular responses in the peripheral blood, as demonstrated by IFN-γ and interleukin-2 (IL-2) enzyme-linked immunospot assay (ELISPOT). However, the haemagglutination-inhibition seroconversion of all dose levels was only 11% following three repeated doses of the priming vaccine, as compared with that of 7% in placebo group. Following H5N1 boosting vaccination, 80% of prime-vaccinated subjects had seroconversion, which was significantly higher than that of 36% in placebo group. Preexisting anti-AdHu4 immunity dampened Ad4-H5-Vtn-induced cellular responses against H5 antigen, as well as seroconversion after boost vaccination, though only in low priming dose groups (10^7, 10^8, and 10^9 viral particles). This study suggests a potential new paradigm where adenovirus vectored priming vaccine may be used in combination with conventional seasonal influenza vaccination to enhance the immunogenicity and possibly the efficacy of the latter.

In addition to these published studies, there are recently completed or ongoing phase-1 clinical trials evaluating replication-deficient AdHu5-vectored (ClinicalTrials.gov Identifier: NCT00755703) and a replicating human Ad4-vectored influenza vaccines (ClinicalTrials.gov Identifier: NCT01806909). Notably, both adenovirus vectors were administered intranasally in both clinical trials, which together with the above published studies, suggest that the respiratory mucosal route is increasingly recognized as the preferred route to deliver human adenovirus-vectored influenza vaccines. However, the potential impact of preexisting anti-human adenoviruses immunity on the vaccine potency remains to be fully appreciated in future studies.

Chimpanzee adenovirus-based influenza vaccines

As mentioned previously, the high global prevalence of preexisting anti-human adenovirus immunity adversely impacts the efficacy of human adenoviral-based vaccines.[13] As such, chimpanzee adenovirus vectors represent more promising and efficacious platforms for the development of future clinical adenoviral-based influenza vaccines. A chimpanzee serotype 7 adenovirus-vectored vaccine expressing the NP from the H1N1 strain A/PR/8/34 (referred to as AdCh7-NP) has been assessed in a murine model.[74] Compared with an AdHu5-vectored vaccine expressing the same NP (AdH5-NP), the AdCh7-NP induced comparable T-cell immune responses against NP, and was as protective against a lethal dose of H1N1 strain A/PR/8/34. In heterosubtype challenge experiments with H5N1 strains, AdCh7-NP was as protective compared with its AdHu5 equivalent.

A recent clinical study assessed the safety and immunogenicity of a replication-deficient chimpanzee adenovirus-vectored influenza vaccine expressing NP and matrix protein 1 (ChAdOx1 NP+M1).[75] Intramuscular immunization with ChAdOx1 NP+M1 was safe and immunogenic in terms of both cellular and humoral immunity. Heterologous boosting with a vaccinia virus Ankara (MVA) expressing NP and M1 enhanced T cellular responses to NP+M1 primed by ChAdOx1 NP+M1.

Currently chimpanzee adenovirus-vectored influenza vaccines have been less studied as compared with human adenovirus vectors, though it is generally believed that chimpanzee vectors may be more immunogenic in humans than human adenovirus vectors due to the prevalence of preexisting immunity against the latter. However, due to the huge gap of knowledge on human lung mucosal immune responses induced by chimpanzee versus human adenovirus-vectored influenza vaccines, much more efforts are required to testify this potential superiority of chimpanzee adenovirus-vectored influenza vaccine.

In summary, compared with conventional egg- or cell-based influenza vaccine production, adenovirus-vectored vaccines bypass the time-consuming adaptation of influenza virus to the culture system. The adenovirus-vectored vaccine strategy is also antigen-sparing without the need of producing a large quantity of proteins required for the recombinant protein vaccination strategies. With its documented safety and immunogenicity in humans, adenovirus-vectored influenza vaccination represents a promising novel strategy in future influenza prophylaxis.

Adenoviral vectors are also useful to developing "universal" or broadly protective flu vaccines, by targeting the conserved region of HA, or internal proteins including NP and matrix proteins. In murine models, a single dose of a candidate universal influenza vaccine based on a replication-deficient AdHu5 vector expressing NP and/or matrix protein 2 (M2) provided rapid protection against subsequent infections with virulent H5N1, H3N2, and H1N1 viruses, and of importance, intranasal immunization provided superior protection over the intramuscular route.[76] In another preclinical study, a single dose of replication-defective AdHu5 vector encoding a fusion protein of humanized full-length H5 HA and the ectodomain of the M2 elicited long-lasting and antibody-dependent protection against heterosubtypic viruses (H1N1).[77] However, the efficacy clinical trials are critically required to test whether this protection observed in mice can be translated into an universal or broad protection in humans.[78]

As discussed earlier, the adenovirus vector is featured by its well-documented safety, balanced humoral and cellular immunogenicity, as well as the respiratory mucosal tropism. However, preexisting immunity against human adenoviruses may dampen the immunogenicity of human adenovirus vectors. While increased vaccine doses may overcome the impact of preexisting immunity, this could happen at the cost of safety particularly when the vaccine is delivered to the respiratory tract. Moreover, there is still a lack of solid evidence that the humoral and/or cellular immunogenicity of adenovirus-vectored influenza vaccine correlates with protection in a clinical setting, as was shown in animal models. For these reasons, it is still too early to make a direct comparison between an adenovirus-vectored influenza vaccine and the current seasonal flu vaccine shots.

PRECLINICAL AND CLINICAL DEVELOPMENT OF ADENOVIRUS-BASED VACCINES AGAINST HIV

As a major public health issue worldwide, the HIV/acquired immunodeficiency syndrome (AIDS), has claimed over three million lives globally to date. There were two million new cases of HIV infection with a total of 1.2 million AIDS-related deaths in 2014. Furthermore, as HIV-infected hosts are prone to latent TB reactivation, approximately one-third of AIDS patients succumb to TB worldwide. Although currently both the incidence of HIV infection and AIDS-related deaths per year have decreased compared with those in 2005, the total number of HIV infected people continues to increase. Better prophylactic measures including novel and effective vaccines/vaccination strategies are therefore in urgent need to protect people at high risk of HIV infection.

Based on current understandings of the early events following HIV transmission, it is believed that the viral eradication can only be achieved in the initial 5 to 10 days following HIV transmission. Neutralizing antibodies with broad antigen specificity are critically required to stop HIV from infecting host cells, while cell-mediated immunity including CD8 T cell- and NK cell-mediated killing of infected cells early after HIV transmission forms a second line of host defense to clear the founder virus if neutralizing antibodies fail to do so. Failure to eradicate HIV within the early phase of infection leads to viral spreading and generation of latent viral reservoirs which prove difficult to be cleared.[79] For this reason, HIV vaccines are developed to induce the neutralizing antibodies with broad HIV antigen specificity and HIV-specific $CD8^+$ T-cell responses.

The majority of HIV infections occur at the vaginal or rectal mucosal sites. Therefore, it is speculated that vaccination strategies with induced mucosal immune responses may confer superior protection over systemic immunization.[80] However, it is still controversial whether systemic neutralizing Abs and/or cell-mediated immune components are competent in eradicating HIV early after mucosal infection, or in other words, it is not clear whether systemic neutralizing Abs and/or cell-mediated immune response are reliable immune correlates in the protection against HIV infection via mucosal routes.

Human adenovirus-based HIV vaccines

Current preclinical evaluation of HIV vaccines relies largely on either the chimpanzee model with HIV-1 infection or the macaque model with simian immunodeficiency virus (SIV) or SIV-HIV hybrid virus (SHIV) infection.[81] Adenoviral-vectored HIV vaccines have been assessed in both models. In these studies, these vaccines were used either alone or in combination with protein or DNA vaccines in a prime-boost regimen.

One of the most inspiring preclinical studies was conducted using a replication-deficient AdHu5 vector expressing the SIV gag protein used either as a homologous booster or as a heterologous booster inoculation after priming with a SIV *gag* DNA vector plus adjuvant. In this study, the AdHu5-vectored vaccine primed $CD8^+$ T cells specific for an immunodominant SIV gag epitope p11CM and attenuated the infection of SHIV as demonstrated by reduced viral load and higher peripheral blood $CD4^+$ T-cell counts.[82] Since these promising preclinical studies, multiple clinical trials have been conducted to evaluate the efficacy of novel human adenoviral-vectored HIV vaccines.

The most extensively characterized HIV vaccine is the MERK-developed trivalent AdHu5-based vaccine expressing the HIV antigens gag, pol, and nef (referred to as MRKAd5 HIV-1 gag/pol/nef). Successful phase-1 clinical trials provided the evidence supporting its safety profile and its ability to establish robust humoral and cellular immune responses against the encoded HIV antigens,[83] thus forming the basis for its further clinical assessment in the phase-2 efficacy STEP and HVTN503/Phambili trials.[84,85]

The STEP trial, which was conducted in North and South America, the Caribbean, and Australia, unfortunately showed no efficacy and increased rates of HIV acquisition in immunized males who were either uncircumcised or AdHu5 seropositive prior to vaccination.[84] It is important to note that although there was an increase in HIV acquisition in this subcohort, a follow-up study by Duerr *et al*.[86] showed a transient nature of increased HIV infection which waned by 18 months postvaccination. Although the mechanisms still remain unclear for these observations, various studies point to the possibility of either attenuation of innate immunity in AdHu5 seropositive individuals or the transient spikes in the activation and recruitment of anti-AdHu5 CD4 T cells, the main targets of HIV, at the mucosa.[14,87] Following the STEP trial's observations, recruitment for the HVTN503/Phambili trial in South Africa was terminated. Findings from this trial also showed no vaccine-induced protection but instead, it found a nonstatistically significant increase in the rates of HIV acquisition.[85]

In another attempt carried out in the United States, a DNA prime-rAdHu5 boost vaccine regimen in the subjects at increased risks for HIV-1 infection did not lower the rate of HIV-1 infection or the viral load set point, even though this vaccination strategy primed HIV-specific $CD4^+$ T and $CD8^+$ T cell-mediated IFN-γ and/or IL-2 production in the peripheral blood as well as increased titers of neutralizing antibodies in serum.[88]

Chimpanzee adenoviral-vectored HIV vaccines

Chimpanzee adenoviral-vectored HIV vaccines have also begun preclinical evaluation, with a recent study further highlighting the potential of such vectors. In this study, chimpanzee serotypes 3 and 63 adenoviruses expressing the HIV gag, pol, and nef antigens were evaluated for T-cell immunogenicity in the murine model. The study shows that parenteral immunization with these vaccines was highly immunogenic, capable of inducing IFN-γ^+ T-cell responses to all encoded antigens.[89] Follow-up protection studies in comparison to AdHu5 vectors will be helpful to understanding the potential of such chimpanzee adenoviral vectors.

In summary, the lack of efficacy of current immunogenic adenovirus-vectored HIV vaccines highlights the importance of identifying the immune correlates of protection. Also, much more efforts should be made to decipher the relative contribution of systemic versus mucosal immune components in protection as vaginal and rectal mucosa are the primary entry site of HIV.[80] Updated knowledge on these issues will be of great value in guiding the development of novel adenovirus-vectored HIV vaccines and/or novel vaccination strategies. Furthermore, as the efficacy trial with human adenoviral-vectored HIV vaccines suggests an increased incidence of HIV infection in some AdHu5-seropositive individuals, it has been concluded that AdHu5 vectors are no longer suitable for HIV vaccine design. In this respect, chimpanzee adenoviral-vectored vaccines may represent an improved approach in future HIV vaccine development due to the very low seroprevalence of chimpanzee adenoviruses in humans. However, it remains to be seen whether the T-cell epitopes potentially shared by both AdHu5 and chimpanzee adenoviruses may still pose a limitation to the use of the latter for HIV vaccine design.[90]

CONCLUDING REMARKS

The continued success of adenoviruses as vaccine vectors is attributed not only to their ability to drive robust and sustained humoral and cellular adaptive immune responses, but to their ability to induce such responses at the mucosal sites of pathogen entry. This makes adenoviral vectors one of the most widely used platforms for the generation of mucosally deliverable vaccines. Recent advances in adenoviral vectorology, viral packaging cell lines, mucosal immunity, and vaccine immunology have expedited the development of adenoviral-vectored vaccines against a number of mucosal pathogens to which there is a lack of effective vaccination strategies. The next few years shall see the much increased clinical knowledge in the potential of mucosal vaccination with adenoviral-vectored vaccines.

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