Prophylaxis of *Mycobacterium tuberculosis* H37Rv Infection in a Preclinical Mouse Model via Inhalation of Nebulized Bacteriophage D29

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Running Title: Phage D29 Inhalation Provides Protection against TB

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Abstract

Globally, more people die annually from tuberculosis than from any other single infectious agent. Unfortunately, there is no commercially-available vaccine that is sufficiently effective at preventing acquisition of pulmonary tuberculosis in adults. In this study, pre-exposure prophylactic pulmonary delivery of active aerosolized anti-tuberculosis bacteriophage D29 was evaluated as an option for protection against *Mycobacterium tuberculosis* infection. An average bacteriophage concentration of approximately 1 PFU/alveolus was achieved in the lungs of mice using a nose-only inhalation device optimized with a dose simulation technique and adapted for use with a vibrating mesh nebulizer. Within 30 minutes of bacteriophage delivery, the mice received either a low dose (~50-100 CFU), or an ultra-low dose (~5-10 CFU), of *M. tuberculosis* H37Rv aerosol to the lungs. A prophylactic effect was observed with bacteriophage aerosol pre-treatment significantly decreasing *M. tuberculosis* burden in mouse lungs 24 hours and 3 weeks post-challenge ($p < 0.05$). These novel results indicate that a sufficient dose of nebulized mycobacteriophage aerosol to the lungs may be a valuable intervention to provide extra protection to health care professionals and other individuals at risk of exposure to *M. tuberculosis*.

Keywords: experimental therapeutics, global health, *in vivo* murine model, nose-only inhalation device, phage prophylaxis, vibrating mesh nebulizer

Introduction

The World Health Organization (WHO) has classified the bacterium *Mycobacterium tuberculosis* (*Mtb*) as the leading infectious killer globally for a fourth consecutive year (1). Aside from being a significant co-morbidity in persons living with human immunodeficiency virus (HIV), in both 2016 and 2017 tuberculosis (TB) was the cause of death for 1.3 million persons without HIV infection (1). Not only do low-income countries continue to suffer from endemic TB, but so do some populations in developed countries such as Indigenous peoples in Nunavut, Canada (2).

The global community is increasingly concerned about the increase of drug-resistant *Mtb* strains. The WHO estimated that, in 2017, roughly 0.5 million *Mtb* infected individuals developed rifampicin resistance and over 80% of those cases were considered multidrug-resistant (1). As global treatment success for drug-resistant *Mtb* cases remains unacceptably low - at roughly 55% -, alternative interventions that block transmission and subsequent new infections are urgently needed (1). Vaccines against *Mtb* are an active area of research (1,3-6), and indeed much of the global community receives bacille Calmette–Guérin (BCG) vaccination against TB as youth. BCG and other vaccine candidates can induce limited prophylactic protection through adolescence (7); however, BCG provides limited-to-no prophylactic effect if given to adults, does not prevent reactivation of latent TB, and does not prevent *Mtb* transmission (8,9). Indeed, there is no vaccine that effectively prevents acquisition of *Mtb* and progression to TB disease in adults (1). The lengthy, complex, multidrug treatment regimens available to target active TB often result in poor side-effects for patients. This limits adherence and perpetuates the development of drug resistance.

Epidemiologic vulnerability to TB disease correlates with proximity to an active case or intensity of exposure over time. This is evidenced in highly-exposed health care workers who have a 2-4 fold higher infection risk compared to medical students with low exposure (10). Indeed, many studies document the increased risk to health care workers (11,12), which justifies research toward the development of interventions against *Mtb* infection for this high-risk population. Bacteriophage (phage) delivery may be leveraged as an adjunct to current preventative strategies, which include personal protective equipment, administrative and environmental controls (13,14), for health care professionals who are regularly exposed to infectious active cases of TB.

Phages are diverse viruses that have co-evolved with bacteria and represent the most common biologic on earth (15). Phage strains are host- and receptor-restricted and therefore only capable of infecting a narrow-spectrum of bacteria, which notably results in minimal harm to host microbiomes such as gut flora (16). Furthermore, antibiotic-resistance does not influence bacterial susceptibility to phage lysis (16), making phages an attractive tool against drug-resistant organisms. Phages predicted to use obligatory lytic life cycles can be distinguished from temperate phages through genetic analysis (17). The lytic cycle encompasses injection of phage DNA into the cell, phage replication, and lysis of the bacterial cell wall to release the progeny. Lytic phage therapy is considered safe and regularly utilized clinically in Eastern Europe, where some phage cocktails are available without prescription (18). Indeed, studies have repeatedly demonstrated that phages are not inherently harmful to humans (19).

Phage therapy is a viable option for compassionate use in the United States, with a recent study demonstrating successful treatment of a patient with disseminated

multidrug-resistant *Acinetobacter baumannii* infection (20). In the United Kingdom, a cystic fibrosis patient with disseminated *Mycobacterium abscessus* recently showed objective clinical improvement with intravenous three-phage cocktail treatment (21). The treatment of pulmonary infections in humans using phages has been reviewed elsewhere (22). Advanced research of aerosol phage therapy has become prevalent, including *in vitro* studies evaluating phage delivery with nebulizers, dry powder inhalers, and pressurized metered-dose inhalers (23-31). As more than 80% of TB cases originate from *Mtb* infections in the lungs, aerosol delivery of phage may be an ideal mechanism for enabling activity at the primary site of *Mtb* infection (32). Note that phage aerosol delivery without the use of additional vectors is unlikely to have efficacy against *Mtb* already harboured within a granuloma; even phage infection of *Mtb* within a macrophage, the primary target cell of *Mtb*, has low efficiency (33). However, prophylactic delivery of phages to the alveoli may allow the phage to infect the mycobacteria before macrophage uptake (17,27,34). Since the lungs contain millions of alveoli, a high dose of active phage is likely required for prophylaxis. Of particular interest for prophylactic protection against TB is *Siphoviradae* mycobacteriophage D29 (Figure 1), which can effectively infect and lyse a range of mycobacteria, including *Mtb* (35).

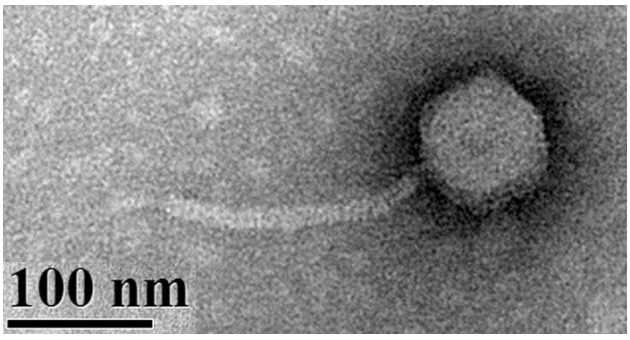

Figure 1. Transmission electron micrograph of phage D29. The icosahedral capsid contains double-stranded DNA. The tail is flexible and does not contract during infection. The method for imaging is described elsewhere (27).

Before testing the efficacy of phage D29 aerosol in humans, animal studies are of interest. Laboratory mice, commonly used for studying drug efficacy, have the advantages of low cost, short growth time, and small size, allowing for many mice to be tested simultaneously (36). Different methods for delivering aerosol to mice include a nose-only inhalation device (NOID), whole-body exposure system, nose-drip, and intranasal or intratracheal instillation. Use of a NOID is the most common exposure method for rodents as it allows for a uniform distribution of aerosol in the lungs via the nasal inhalation route, which is applicable to rodents as they are obligate nasal breathers (37-39). Whole-body exposure systems require larger doses as they are inefficient and cause aerosol deposition on the body of the mouse (36,38,40). Nose-drip methods do not simulate natural aerosol inhalation and have variable inhaled droplet size. Instillation leads to non-uniform, patchy deposition, primarily near the site of instillation, and little or no alveolar deposition (37). Furthermore, inhalation methods are preferable to injection as the aerosol is delivered directly to the site of infection, the lungs, and hence is available there at higher concentrations (41). Semler et al. (42) demonstrated that phage aerosol delivery by inhalation with a NOID was superior to intraperitoneal delivery, as evidenced by a greater reduction in bacterial burden and phage replication in the lungs.

Effective phage delivery to the lungs requires a prudent choice of aerosol delivery device to avoid phage inactivation (27). Factors that may inactivate phage are described

elsewhere, and include shear stress, osmotic shock, and thermal stress, among others (43,44). In a recent study, it was demonstrated that use of a vibrating mesh nebulizer resulted in less phage D29 inactivation and a greater active phage D29 aerosol delivery rate than use of a jet nebulizer (27). However, traditional NOID designs use jet nebulizers and have very low delivery efficiency. An adequate delivery method that allows retention of lytic capacity and ability to deliver high titers of phage are current hurdles to testing prophylactic delivery of phages to the lungs of mice. In this study, a NOID was modified for use with a vibrating mesh nebulizer to deliver high doses of active phage D29 to the lungs of mice.

In order to advance phage D29 as a candidate therapy for TB, we leveraged our well-established mouse model of low dose aerosol challenge of *Mtb.* We hypothesized that sufficient prophylactic pulmonary delivery of phage D29 would reduce *Mtb* bacterial burden 24 hours post-challenge. In order to evaluate our hypothesis, delivery parameters maximizing the inhaled dose in a repeatable fashion were experimentally simulated and subsequently used throughout the challenge studies. Measurements were then performed to quantify the number of phage reaching the lungs of mice and their clearance kinetics. Finally, phage D29 aerosol was delivered to mice prior to *Mtb* aerosol challenge to evaluate prophylactic protection afforded by this treatment.

Results

Lung Homogenization Does Not Reduce Phage Activity. Naïve mouse lungs were collected and spiked with an established titer of phage D29 and subsequently homogenized to determine if this process, used routinely to evaluate *Mtb* CFU *ex vivo*, would result in any loss of phage activity. The phage D29 titer after lung

homogenization was 12.08 ± 0.03 $\log_{10}$(PFU/mL) compared to the control titer before homogenization of 12.12 ± 0.04 $\log_{10}$(PFU/mL), with no significant difference ($p > 0.5$; n=3 each). These data indicate that the lung homogenization did not cause phage D29 inactivation, nor did any innate tissue factor influence phage activity or the properties of the plaque assay used to quantify phage in a sample. This demonstrated the viability of this approach for quantifying pulmonary phage delivery.

Nose-Only Inhalation Device - Dose Simulation Matches *In Vivo* Experiment.

Tryptophan tracer deposition at different locations within two different versions of the NOID quantified by assay of rinsate are presented in Table 1, as is the predicted dose to the lungs of a mouse, $T_{\mathrm{m/A}}$, calculated using equation {2} (see Materials and Methods). As shown, use of the smaller width of the first plenum achieved the target dose, with a $T_{\mathrm{m/A}}$ of 1.0 ± 0.1 PFU/alveolus, meaning that the total lung dose of active phage in the lungs of a mouse was predicted to be equal to the total number of alveoli in a mouse. The predicted dose of phage D29 to the lungs was 7.6 ± 0.1 $\log_{10}$(PFU/mouse). Hence, this NOID configuration was chosen for *in vivo* experiments.

Table 1. Tryptophan tracer deposition within two different versions of the NOID as a simulation of phage D29 delivery.

First plenum width (mm)	Nebulizer reservoir (%)	First plenum (%)	Mixing tube (%)	Back plenum (%)	Nosepiece & adapter (%)	Mouse filter (%)	Exit filter (%)	Unaccounted (%)	Predicted dose to a mouse $T_{\mathrm{m/A}}$ (PFU/alveolus)
95	1.0 ± 0.8	61.2 ± 7.5	1.9	5.1 ± 1.2	0.062 ± 0.008	0.013 ± 0.005	1.07 ± 0.05	30 ± 6	0.4 ± 0.2
32	1.2 ± 0.6	60.3 ± 0.4	3.2	6.3 ± 0.6	0.073 ± 0.004	0.033 ± 0.004	1.50 ± 0.023	27 ± 3	1.0 ± 0.1

Results are presented as avg ± SD from 3 replicate experiments, except for the mixing tube which was measured once. The components are labeled in Figure 5 (Materials and Methods).

It is important to note that the ratio of flow rate entering the exit filter (478 mL/min) to the flow rate entering the mouse filter and nosepiece with adapter (22 mL/min) was 22, whereas the ratio of dose on the exit filter (1.50%) to dose on the surrogate mouse filter and nosepiece with adapter (0.11%) was 14. If a uniform aerosol concentration were present the ratios would be equal. The latter ratio was lower, as expected, due to aerosol deposition in the front plenum.

A fraction of 0.044 = (22 mL/min) / (500 mL/min) of the total dose reaching all 12 noseports deposited on the single tested mouse filter and nosepiece with adapter, on which a dose of 0.11% was measured. Relative to the use of the unmodified NOID presented by Nadithe et al. (36), the amount of tryptophan reaching the mouse filter was improved by a factor of 1.8. Considering that approximately 6,000 times more active phage D29 were delivered per unit time with the vibrating mesh nebulizer than with the jet nebulizer (27), an improvement by a factor of approximately 11,000 was achieved over the unmodified NOID that used a jet nebulizer, in terms of the predicted number of active phage D29 reaching the lungs of the mice per unit time. The small standard deviation indicated that the dosing was repeatable and consistent, and provided confidence in this modified NOID setup.

We next optimized phage D29 aerosol delivery to mice in the modified NOID setup with parameters described above. As phage D29 was amplified in *Mycobacterium smegmatis*, the resulting lysate input to the nebulizer clogged the mesh and hence had to be diluted 1:1 in isotonic saline prior to delivery, i.e. 3 mL of lysate at 11.8 ± 0.1 $\log_{10}$(PFU/mL) was added to 3 mL of isotonic saline and a combined total of 6 mL was delivered. This resulted in lower delivery than the 6 mL of 12.2 ± 0.1 $\log_{10}$(PFU/mL)

191 which was assumed during dose simulation. For the lower delivery conditions, the
192 predicted dose in the lungs of mice was 6.9 ± 0.1 $\log_{10}$(PFU/mouse), which is within the
193 range of doses measured *in vivo* in the lungs of mice after phage D29 aerosol delivery,
194 shown in Table 2. This indicates that the method of dose simulation and the model
195 given by equation {2} were accurate for predicting *in vivo* phage dose to the lungs of
196 mice. This also demonstrates the accuracy and reliability of the dose simulation and the
197 NOID setup in general.

198 **Table 2**. Phage D29 dose in the lungs of mice post-NOID delivery.

Time between exposure and euthanasia (min)	Phage D29 dose in mouse lungs in $\log_{10}$(PFU/mouse)*
0	6.6 ± 0.3
30	7.3 ± 0.1
90	7.0 ± 0.4

*avg ± SD of n=3 mice per time point

199 Along with total immediate delivery, potential pulmonary clearance of phage D29 was
200 evaluated 30 and 90 minutes post-delivery (see Table 2), and no statistically significant
201 difference was observed ($p > 0.05$). Hence, phage D29 was not quickly cleared from the
202 lungs of mice, providing confidence to proceed with *Mtb* exposure.

203 ***Mtb* H37Rv is Susceptible to Phage D29.** Before commencing bacterial aerosol
204 challenge studies we confirmed prior data (45) indicating that *Mtb* strain H37Rv, a
205 common laboratory strain, was susceptible to phage D29 lysate *in vitro*. A control plate
206 (no phage added) contained 38 CFU, whereas 2 replicates with D29 addition resulted in

1 CFU and 2 CFU. Therefore, H37Rv lysis via phage D29 was 92-95% effective. The lysis may have not been 100% effective due to phage not coming into contact with every bacterium during plating, or potentially due to phage resistance. The high lysis effectiveness provided confidence to proceed with *Mtb* exposure.

Inhaled Phage D29 Provides *In Vivo* Prophylactic Protection against TB. In order to evaluate the potential application of phage D29 aerosol as a prophylactic tool against *Mtb* infection we next quantified bacterial burden (CFU) of *Mtb* H37Rv in mouse lungs 24 hours post-infection, with or without phage D29 pre-treatment less than 30 minutes prior to *Mtb* exposure. An average of 7.7 ± 0.3 $\log_{10}$(PFU/mouse) of phage D29 was delivered to the lungs in replicate 2, which corresponded to ~1 PFU/alveolus on average, indicating the target dose of phage D29 was achieved. A significant reduction ($p < 0.05$) of bacterial burden in the lungs at 24 hours post-challenge was observed (Figure 2). These data suggest that with a target dose of 1 PFU/alveolus, a significant level of prophylactic protection against inhaled *Mtb* aerosol is indeed possible.

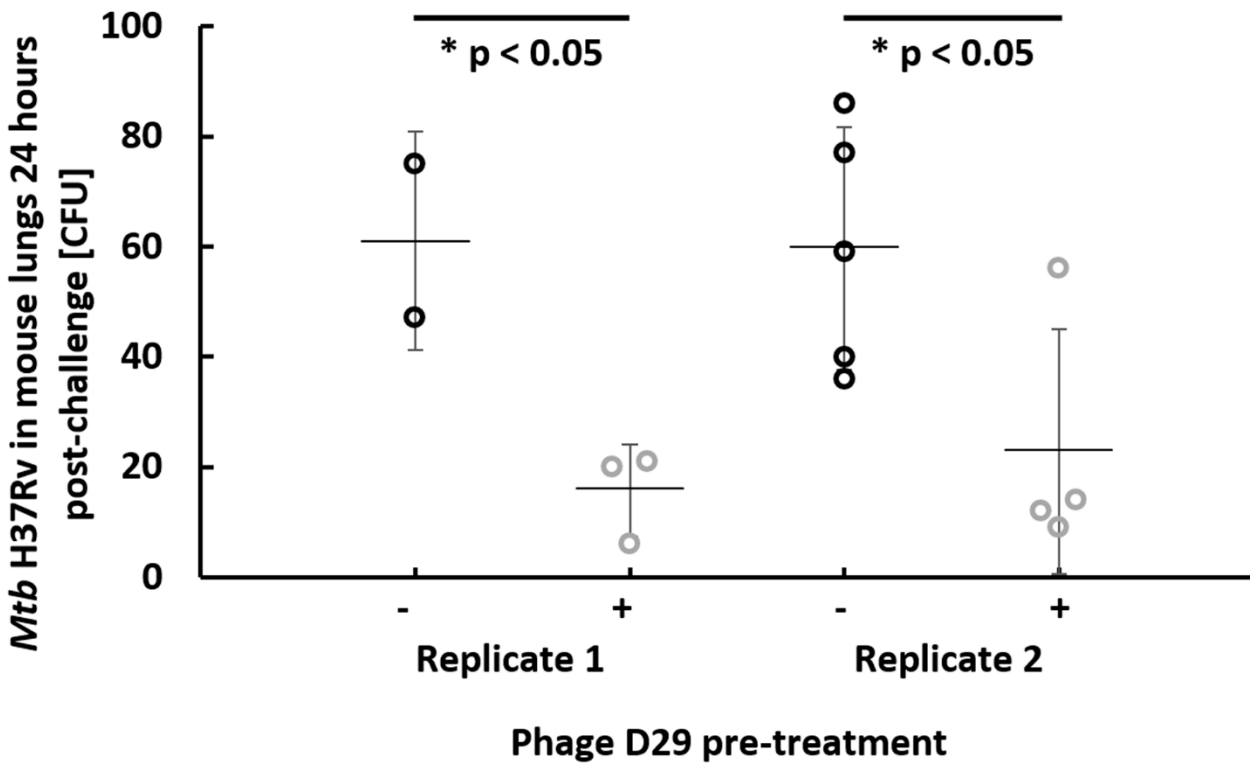

Figure 2. Pre-treatment with phage D29 aerosol delivered by nose-only inhalation significantly reduces pulmonary bacterial burden 24 hours post-challenge with low dose *Mtb* H37Rv. On the x-axis, "-" indicates no phage D29 pre-treatment and "+" indicates phage D29 pre-treatment. Each circle represents a single mouse and error bars span the standard deviation around the mean indicated by the horizontal line.

A separate cohort of mice from replicate 1 were followed out to 3 weeks post-challenge to determine if effects of prophylactic phage application would persist over time. Bacterial burden was measured in the lungs and the spleen 3 weeks post-challenge (Figure 3). Interestingly, at 3 weeks phage D29 pre-treated mice sustained a significantly lower bacterial burden than mice that did not receive phage pre-treatment in the lungs ($p < 0.05$), although bacterial burden was not significantly different in the spleen ($p > 0.1$). The bacterial burden at 3 weeks was of a high magnitude.

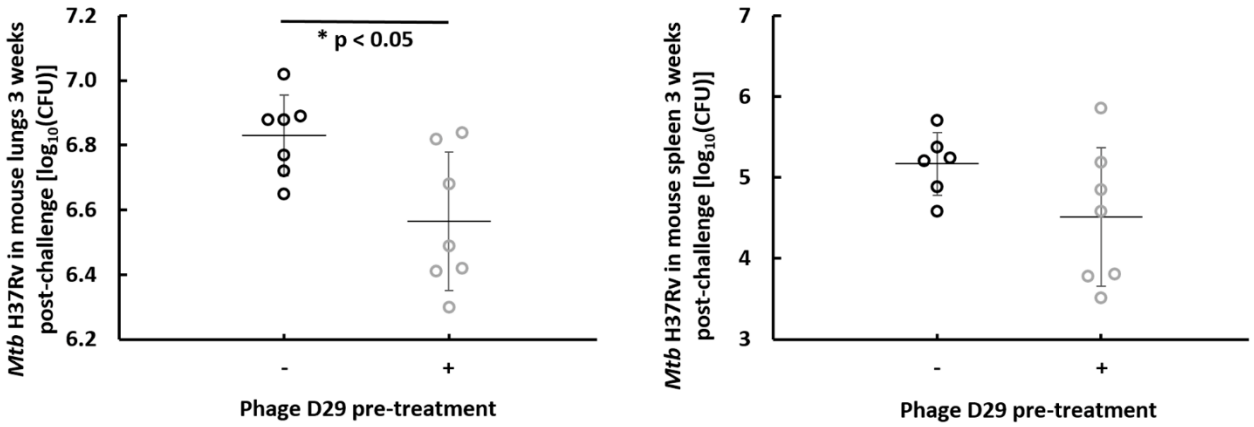

Figure 3. Log_{10} of bacterial burden 3 weeks post-challenge in the lungs (left) and spleen (right), without and with phage D29 pre-treatment. On the x-axis, "-" indicates no phage D29 pre-treatment and "+" indicates phage D29 pre-treatment. Each circle represents a single mouse and error bars span the standard deviation around the mean indicated by the horizontal line.

In order to more closely simulate *Mtb* infections in humans we next optimized and utilized an ultra-low dose aerosol challenge of H37Rv calibrated to deliver 5-10 CFU of bacteria. An average of 7.4 ± 0.1 log_{10}(PFU/mouse) of phage D29 was delivered to the lungs. At 24 hours post-challenge, a significant reduction ($p < 0.05$) of *Mtb* in the lungs was observed in the group that received phage D29 aerosol pre-treatment, relative to the group that did not (Figure 4), providing important further evidence of prophylactic efficacy.

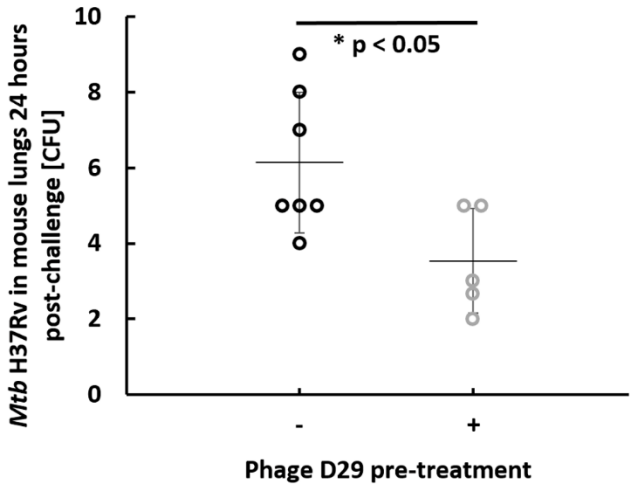

Figure 4. Pre-treatment with phage D29 aerosol delivered by nose-only inhalation significantly reduces pulmonary bacterial burden 24 hours post-challenge with ultra-low dose *Mtb* H37Rv. On the x-axis, "-" indicates no phage D29 pre-treatment and "+" indicates phage D29 pre-treatment. Each circle represents a single mouse and error bars span the standard deviation around the mean indicated by the horizontal line.

Discussion

The results of this study demonstrate that inhalation of phage D29 aerosol prior to challenge with *Mtb* aerosol can significantly decrease the pulmonary bacterial burden in mice 24 hours post-infection. These data suggest that inhaled mycobacteriophage aerosol resulted in *Mtb* lysis in the lungs prior to macrophage uptake and granuloma formation. This proof-of-principle study may have implications for the development of prophylactic aerosol treatments for health care professionals exposed to patients with active TB and reduction in *Mtb* transmission rates in this setting. This is important because many health care professionals are relatively unwilling to work in areas of hospitals with high-risk of tuberculosis transmission (46). Additionally, more protection could potentially be offered to individuals in areas in which TB is endemic, or to other

individuals at high-risk, such as household contacts and visiting family members at hospitals. This treatment is intended to complement normal precautions, which includes use of administrative controls, environmental controls, and personal respiratory protection (13,14).

The authors are only aware of one other study regarding prophylactic inhalation of phages prior to inhalation of bacteria (47), and that study demonstrated 4-day prophylaxis against multi-drug-resistant (MDR) *Pseudomonas aeruginosa* in mice*,* but used intranasal instillation rather than an aerosol delivery device. Instillation results in localized deposition and is not as representative of natural inhalation to the different regions of the lungs as nose-only inhalation (37,48). Therefore, we believe this work, the first study demonstrating prophylactic protection with phage using nose-only inhalation of aerosol, represents a significant advancement in this area of research. Additionally, to the authors' knowledge, a much higher titer of phage was delivered to the lungs of mice by nose-only inhalation in this study than in any previous study. Relative to the use of a jet nebulizer in a previous NOID design (36), approximately 11,000 times more active phage D29 could be delivered to the lungs of mice, and with repeatable results, indicating a major improvement of dosing to mice was achieved with this novel approach. Furthermore, Liu et al*.* (41) only delivered 10^2–10^3 PFU of phage D29 to the lungs of mice using a Collison jet nebulizer with a NOID, which is orders of magnitude lower than in this study, in which > 10^7 PFU was delivered to the lungs of mice. This improvement allowed, for the first time, an average dose of ~1 PFU/alveolus to be achieved. It is important to consider that some alveoli are poorly perfused, and hence > 1 PFU per accessible alveolus may have been achieved on average. Another

factor to consider is that phage progeny released after *Mtb* lysis would offer further protection in their vicinity, but are unlikely to be transferred to nearby alveoli, as they are non-motile. As *Mtb* is also non-motile, it is unlikely to move between alveoli and come into contact with phage in that manner prior to uptake by immune cells.

The prophylactic delivery of a calculated average of 1 PFU/alveolus allowed for a significant reduction of *Mtb* levels in the lungs, demonstrating a prophylactic effect. Whether such a decrease in *Mtb* levels is sufficient to decrease mortality rates is not known. While it is difficult to deliver more than 1 PFU/alveolus on average to a mouse using a NOID, such levels may be necessary to achieve complete bacterial eradication. Poisson statistics can be used to estimate the probability of a specific random occurrence during an interval of interest knowing the average number of occurrences in that interval (49). Using this method, the probability, P, that an alveolus will contain a certain number of phage, x, can be predicted knowing the average number of PFU/alveolus, λ, according to

$$P = \frac{e^{-\lambda} \cdot \lambda^{x}}{x!} \qquad \{1\}$$

Using the values $\lambda = 1$ and $x = 0$ gives $P = 0.37$. This indicates that if on average 1 PFU/alveolus is delivered to the lungs, the probability that an alveolus does not contain a phage is still 37%. Hence, the chance that a bacterium encounters at least one phage in an alveolus is about 63%. In prophylactic phage administration experiments (see Figure 2) the average reduction in bacterial burden was from approximately 60 CFU to approximately 20 CFU, or about 67%. This is in close agreement with the theoretically

calculated prediction of 63% reduction in bacterial burden that assumes that all bacteria that deposit in an alveolus in the presence of at least one phage are inactivated.

To achieve at least one phage in 99% of the alveoli of a mouse, the average number of phage per alveolus would need to be $\lambda = 4.6$. For 99.9% coverage, it is $\lambda = 6.9$. A potential indicator of the dose required for complete prophylaxis is when the probability that an alveolus contains no phage becomes less than the inverse of the number of alveoli in the lungs, which for a mouse occurs with $\lambda > 17.5$.

Achieving a high dose of active phage in human lungs is not as difficult as in mice because deposition losses in the NOID would not be present and humans do not require equally small droplets for efficient lung deposition. Human lungs contain ~4.8×10^8 alveoli (50), ~10 times the number that mouse lungs have. An average number of phage per alveolus of approximately 20 may be required for complete prophylaxis in a human according to the above indicator. A recent study suggested that in humans, a respirable dose of active phage D29 of ~1.3×10^9 PFU could be achieved with delivery of 6 mL of diluted lysate using a vibrating mesh nebulizer (27). This corresponds to a dose to human lungs of ~2.7 PFU/alveolus on average, or 93% alveolar coverage. In that study, the phage D29 lysate was diluted 1:100 in isotonic saline prior to aerosolization as without dilution the lysate purification level was not sufficient to prevent mesh clogging. Potentially, without dilution, i.e. with better purification techniques, orders of magnitude higher titers may reach the lungs, on the order of 10^2 PFU/alveolus on average. This could be sufficient for complete prophylaxis according to the above described Poisson statistics argument. The chance of non-contact between phage and bacteria would be substantially decreased at this dose

level. Additionally, with daily prophylactic doses, the likelihood that a specific alveolus receives a sufficient number of phages to eradicate intruding bacteria increases. As it may take days for granulomas to form (51), it is possible that phages delivered soon after *Mtb* exposure may still offer protection, but this depends on macrophage uptake dynamics (33), and awaits further exploration.

The development of regulatory-approved, commercial phage formulations for inhalation will likely require the use of cocktails containing various phages that target different receptors to ensure the *Mtb* does not become phage-resistant (17). The development of anti-TB cocktails is an active area of research (17). Phage D29 and others that may be included in a therapeutic cocktail would be applicable for prevention of drug-sensitive or drug-resistant strains of *Mtb.* Further development of phage cocktail therapy will require efficacy testing against clinical and drug-resistant *Mtb* strains and, importantly, measuring potential phage-induced host immune responses. It should be determined whether daily prophylactic doses of phage cocktails lead to an immune response that inactivates the phages, or to the development of bacteria which are resistant to all of the phages in the cocktail, although it is important to note that phages are capable of mutating to overcome bacterial resistance and new phages targeting different receptors can be isolated relatively rapidly. Some clinical applications of phage therapy have reported humoral immune responses to phage (anti-phage IgG and IgM antibodies), however the magnitude of the anti-phage antibody response did not correlate with a decrease in clinical efficacy (52). This suggests that even if an adaptive immune response to phage is induced it may not be detrimental to the host nor inhibit phage efficacy. Although recent evidence suggests that mucosal delivery (aerosol) does not

induce a robust or detrimental anti-phage immune response (53), future work should specifically examine the effects of repeated phage exposure to determine feasibility of protection in high risk populations likely to regularly encounter *Mtb*.

In therapeutic TB treatment, for example, with antibiotics, drug-resistant *Mtb* persisters may arise if not all bacteria are eradicated (54). Persisters may arise to some extent in prophylaxis using phages if there are bacteria that are less likely to be lysed by phages and hence have a higher probability to establish an infection. However, therapeutic treatment typically requires eradicating a large population in which a small proportion may be resistant, whereas prophylactic treatment typically requires eradicating a much smaller population, perhaps even as low as a single bacterium. Therefore, the probability of establishing resistant *Mtb* is expected to be smaller for prophylaxis as compared to therapy.

For future development of phage cocktails it will be important to ensure that each type of phage is capable of surviving the nebulization process and can effectively reach the lungs. In this study, experimental dose simulation of phage delivery to mice using a tryptophan tracer, simulated breathing, and filter aerosol capture with a NOID, resulted in accurate prediction of phage dose reaching the lungs of mice *in vivo.* Hence, assuming the same droplet size distribution, it is possible that one could simply measure the phage activity retention from the nebulizer by aerosolization to a filter as described elsewhere (27), and predict the *in vivo* dose reaching the lungs of mice in the present NOID setup using the modelling approach (see equation {2}) and aerosol delivery efficiency results (Table 1) presented in this study. This would allow the lung dose of different phages in a cocktail to be predicted prior to *in vivo* experiments, saving

time and resources, reducing the risk of failed animal work, and expediting the development process.

In summary, inhalation of anti-TB mycobacteriophage D29 aerosol is a promising and novel approach to provide prophylactic protection against primary infection of inhaled *Mtb* aerosol. Significant reduction in bacterial burden was achieved with prophylactic delivery of an average dose of active phage D29 of ~1 PFU/alveolus to the lungs. Complete prophylaxis may be achievable with larger or repeated doses of active phages. The number of active phages reaching the alveoli can be predicted prior to animal studies with a NOID by aerosolizing a tracer, simulating breathing through a filter, assay of the recovered aerosol on the filter, and applying the developed mathematical modeling approach. The development of a high titer phage cocktail against TB is recommended over monophage therapy. The cocktail may provide extra protection to health care professionals regularly exposed to patients with active TB and to individuals in areas with high rates of TB transmission whilst limiting the likelihood of resistance to phages. Additionally, countries with a high burden of TB and MDR-TB are often involved in military engagement with resulting additional risk of exposure. Given that the risk of *Mtb* transmission is higher in congregate settings, a single individual with TB disease exerts an immediate and disruptive impact upon patients' lives, military operations, and daily functioning at military and civil treatment facilities. Delivery of high doses of active phages to human lungs for prophylactic purposes appears achievable and proceeding to human clinical trials is of interest and compliance to delivery of high doses of phage aerosol on a daily basis should be studied.

Materials and Methods

Mice. The mice used in this study were female C57BL/6 mice 4-6 weeks of age, weighing 14-16 grams, purchased from Charles River Laboratories (Wilmington, MA, USA). Mice were housed at the Infectious Disease Research Institute (IDRI) biosafety level 3 animal facility under pathogen-free conditions and were handled in accordance with the specific guidelines of IDRI's Institutional Animal Care and Use Committee. The reported minute ventilation rate for CD-1 mice, similar to the C57BL/6 strain used here, was 1.46 mL/gram of body weight (55). For an average mass of 15 grams, this corresponds to an average minute ventilation of ~22 mL/min per mouse, and this value was used for calculations for nose-only aerosol delivery.

Phage D29 Amplification, Shipping, and Plaque Assay. Phage D29 was prepared to a titer of 1.6×10^{12} PFU/mL via replication with *M. smegmatis* strain $mc^{2}155$ using solid media, sterile filtration, centrifugation, and pellet resuspension in buffer, as described elsewhere (https://phagesdb.org/workflow/, 27). The amplified phage lysate was shipped to IDRI (Seattle, WA, USA) from the University of Alberta (Edmonton, AB, Canada) using cold packs and a Styrofoam container. This shipment did not result in titer reduction of the lysate. The titer of phage D29 was measured using full-plate plaque assay, as described elsewhere (https://phagesdb.org/workflow/, 27).

Lung Homogenization. It was necessary to homogenize the lungs of the mice to generate a representative liquid sample to assay and to determine the number of active phage and bacterial burden in the lungs. To verify the phage remained active after the high-shear homogenization process, a 20-µL sample of phage lysate was spiked in lung tissue within 2 mL of buffer, in a 15-mL Eppendorf tube, homogenized (Omni Prep Multi-Sample Homogenizer, Omni International; Kennesaw, GA, USA) for 1 minute, and

centrifuged for 2 minutes at 1800 rpm. The titer after homogenization was compared to the titer before homogenization to determine if the homogenization process inactivated the phages.

Nose-Only Inhalation Device – Device Design and Dose Simulation vs. *In Vivo* Experiment. A schematic of the developed NOID, a modified version of the device described by Nadithe et al. (36), set up for dose simulation experiments, is shown in Figure 5. Nadithe et al. (36) reported that their NOID design had substantial losses of aerosol. Only 0.108 ± 0.027% of the dose input to the jet nebulizer reached the mice, and only 8.19 ± 3.56% of that amount reached the lungs of the mice. This corresponded to 0.0087 ± 0.0021% (870 ppm) of the input dose reaching the lungs of all 12 mice combined. Much of this loss was attributed to the compressor of the jet nebulizer delivering 4.5 L/min of air flow into the system, resulting in a substantial amount of aerosol convecting by the noses of the mice and exiting the back of the device unused. This is because the combined total minute volume for 12 mice is only 0.264 L/min, and hence about 4.2 L/min of air flow, or 93% of aerosol available at the nosepieces, bypassed the mice. Therefore, in our modified design the minimum air flow rate into the system that could safely be used without developing a hypoxic environment (38), i.e. 0.5 L/min, was used. The negative pressure induced by a vacuum pump (Model UN726FTP, KNF Neuberger, Inc.; Trenton, NJ, USA) past the exit of the device caused the air flow into the NOID. The pressure difference between the front plenum, back plenum, and atmosphere was measured with manometers to rule out leaks in the system. A rotameter (Catalogue No. 5079K63; McMaster-Carr, Elmhurst, Il, USA), calibrated with a thermal mass flow meter (TSI 4043; TSI Incorporated, Shoreview, MN,

USA), was used to measure the air flow rate into the NOID, which was controlled with valves past the exit filter and in the rotameter. An inlet air filter (Respirgard II 303; Vital Signs Incorporated, Englewood, CO, USA) prevented foreign virus or bacterial contamination from the air flow into the device.

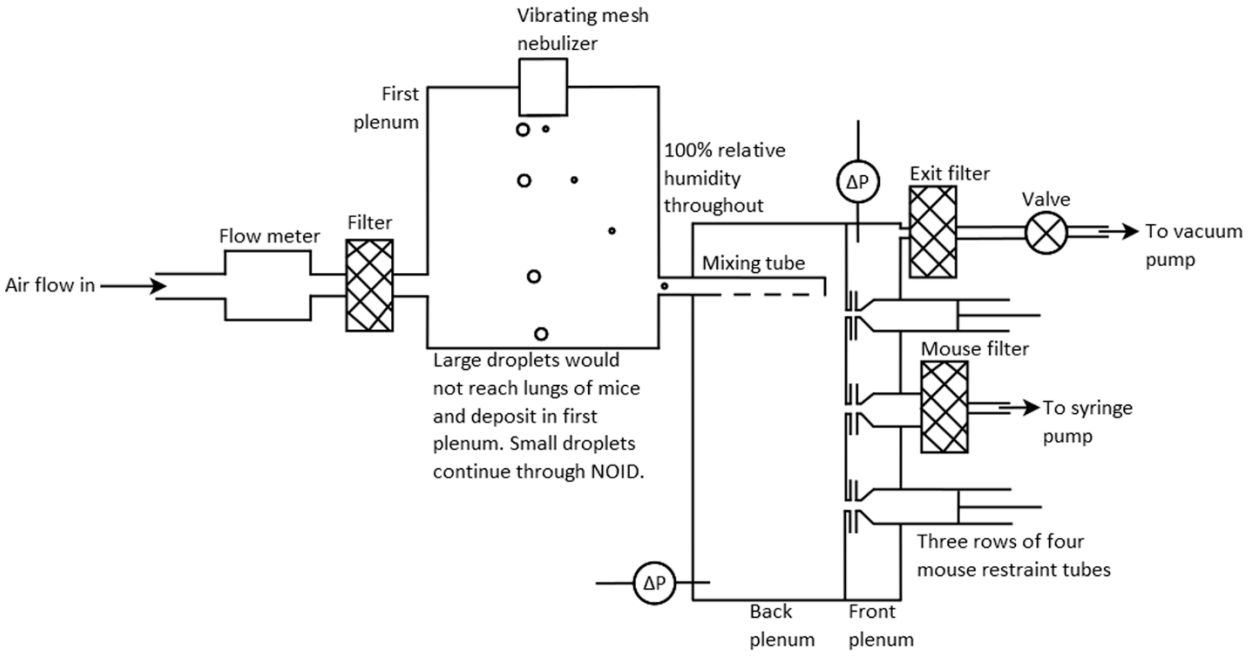

Figure 5. Schematic of a modified NOID adapted for use with a vibrating mesh nebulizer.

The NOID was modified to incorporate a vibrating mesh nebulizer (Aerogen Solo with Pro-X Controller; Aerogen Ltd., Dangan, Galway, Ireland) to produce the aerosol. The aerosol entered the first plenum, which was developed as preliminary experiments demonstrated that use of the commercial wye connector for the vibrating mesh nebulizer resulted in a large extent of aerosol recirculation, droplet coalescence, and deposition losses. This is because the small-volume wye connector was designed for use of > 3 L/min air flow rate whereas a lower air flow rate, 0.5 L/min, was used in this study.

The operation of the vibrating mesh nebulizer has been described by Carrigy et al. (27). The mesh consists of ~1000 orifices, each with a diameter of ~3 µm. The generated aerosol has been measured at the exit of a T-piece by laser diffraction to have a volume median diameter of ~5.5 µm and geometric standard deviation of ~1.8 (56). The vibrating mesh nebulizer has a liquid droplet production rate of about 0.36 mL/min (27), and hence with the chosen air flow in of 500 mL/min, liquid entered the system at a concentration of 720 g/m^3. Considering that room temperature air can hold ~17.3 g/m^3, the nebulizer produces approximately 42 times the amount of liquid required to fully saturate the air flow in, assuming it is initially dry. Hence, at a maximum, only about 2% of the mass of the droplets, corresponding to about 1% of the diameter assuming sphericity, is required to fully saturate the NOID. Therefore, the droplets essentially maintained their size after atomization as they transited through the device, neglecting any coalescence. However, mice require small droplets for deposition in the lungs (36,57). In the present NOID system, the large droplets were filtered out by the first plenum and the smaller droplets produced by the nebulizer, recalling that the geometric standard deviation is ~2, followed the air flow streamlines, due to a lower Stokes number, into the ~12-mm diameter mixing tube. This mixing tube ensured a uniform aerosol concentration entered near the top of the back plenum. Mice inhale through nosepieces attached to mouse restraint tubes, which used an airtight plunger to hold the mice in place with their noses at the noseports. The mice inhaled the aerosol from the back plenum through the noseports, and the excess air flow and exhaled air from the mice entered the front plenum and subsequently were filtered and exited the device.

Before proceeding to *in vivo* experiments, the dose was simulated to verify a biologically-relevant amount of aerosol would reach the lungs of mice. For dose simulation experiments a mouse restraint tube was replaced with a filter and attached to the nosepiece by an adapter, rapid-prototyped out of an acrylic compound (Objet VeroGray RGD850; Eden Prarie, MN, USA) using a PolyJet 3D printer (Objet Eden 350 V High Resolution 3D Printer, Stratsys, Ltd.; Eden Prarie, MN, USA). This filter was termed a mouse filter due to its surrogate mouse function and location within the set-up and was attached to a syringe pump (PHD ULTRA Syringe Pump with Push/Pull Mechanism; Model no. 70-3008; Harvard Apparatus, Hollison MA, USA). The syringe pump initiated a constant flow rate of 22 mL/min through the mouse filter, which is equivalent to the average minute volume for a mouse, as described previously. Only one port was evaluated because previous data with the unmodified NOID demonstrated a reasonably even distribution of aerosol between noseports (36). Due to the > 10 mL filter dead volume, the steady flow equivalent of tidal flow was used, and the effect of exhalation on lung deposition was neglected. L-Tryptophan (Cat #93659; Sigma Aldrich, St. Louis, MO, USA) tracer in isotonic saline was atomized and captured on the mouse filter, as well as at various points within the NOID (nebulizer reservoir, first plenum, mixing tube, back plenum, nosepiece and adapter, exit filter) to determine where deposition occurred. The tracer concentration was assayed using ultraviolet-visible (UV-Vis) spectrophotometry (8452A Diode Array Spectrophotometer; Hewlett-Packard, Mississauga, ON, Canada). The deposition was quantified for two different versions, where different widths of the developed first plenum were tested: 32 mm and 95 mm. The length of the first plenum was kept fixed at 152 mm and the depth was kept fixed at

211 mm, as this was approximately the distance at which bulk aerosol flow emitted from the nebulizer stopped after horizontal spray. Three replicate dose simulation experiments were performed for each of the two first plenum widths. As the interior volume of the NOID was ~4 L, the air flow through the system was allowed to continue for ~8 minutes after nebulization was complete to allow time for the aerosol to transit through the system.

A model was developed to predict the average number of active phage per alveolus reaching the lungs of one mouse in the NOID, $T_{\mathrm{m/A}}$, from the tryptophan experiments and literature data, and is given by,

$$T_{\mathrm{m/A}} = \frac{T_0 \cdot f_{\mathrm{n}} \cdot f_{\mathrm{i}} \cdot f_{\mathrm{m}} \cdot f_{\mathrm{l}}}{A_{\mathrm{m}}} \quad \{2\}$$

where T_0 is the initial titer of the lysate input into the vibrating mesh nebulizer in PFU, f_{n} is the fraction of the phage not inactivated by the nebulizer measured in a previous study to be 0.319 (27), f_{i} is the fraction of the breathing cycle spent inhaling approximated as 0.5, f_{m} is the fraction of the aerosol emitted from the nebulizer that is inhaled by a single mouse from tryptophan tracer dose simulation experiments, f_{l} is the fraction of aerosol that is inhaled by a mouse that reaches its lungs taken as 0.08 (36), and A_{m} is the number of alveoli per mouse taken as 4×10^7 (58).

For comparison to dose simulation, phage D29 was delivered to mice and the lungs of the mice were removed following euthanasia and homogenized within 5 mL of buffer. The number of active phage in the homogenate was determined by plaque assay.

Three mice were taken down at each of 0, 30, and 90 minutes after phage exposure to obtain a preliminary measure of lung clearance.

Host Susceptibility. Prior to performing *in vivo* experiments it was confirmed that *Mtb* H37Rv is susceptible to phage D29 lysis. A volume of 100 µL of 10^{11} PFU/mL of phage D29 lysate was added to a sample of *Mtb* H37Rv and subsequently plated on agar plates. Bacterial CFU was determined after incubating plates at 37°C and 5% CO_2 for 21 days. Plates with or without D29 application were compared.

***In Vivo* Prophylactic Protection.** The mice were acclimatized to remain calm in the restraint tubes of the NOID as per previous methods (57), therefore retaining a normal breathing pattern to maximize peripheral lung deposition. Briefly, mice were 'trained' in the NOID tubes without treatment 3-5 times for 5 minutes each in the week leading up to phage delivery. This training significantly reduced visible stress-induced changes in breathing. Within 30 minutes of receiving phage D29 aerosol with the NOID, mice were challenged with *Mtb* H37Rv aerosol using a previously described Wisconsin-Madison aerosol chamber (6,59), calibrated to deliver ~50-100 bacteria (low dose) or ~5-10 bacteria (ultra-low dose). The dose nomenclature was based on previous studies (6,60-62). After *Mtb* aerosol inhalation and euthanasia, the lung tissue was isolated and homogenized in 5 mL of PBS + Tween-80 (Sigma-Aldrich, St. Louis, Missouri, USA) buffer and the entire lung homogenate sample was plated on Middlebrook 7H10 agar plates and subsequently incubated at 37°C and 5% CO_2 for 3 weeks before colonies were counted. The bacterial burden of *Mtb* was evaluated 24 hours (n=2 experiments) and 21 days (n=1 experiment) post-challenge for the low dose model and at 24 hours (n=1 experiment) for the ultra-low dose model. The ultra-low dose bacterial challenge is

expected to better reflect human infection conditions where relatively few bacteria are able to establish a pulmonary infection in the host. The NOID was disassembled and components were disinfected with ethanol between all experiments. Following aerosol challenge with *Mtb* H37Rv, the Wisconsin-Madison aerosol chamber was thoroughly sprayed with Lysol aerosol and after 10 minutes cleaned with 70% ethanol and paper towels. Nebulizer components were sterilized between runs with 10% bleach.

Statistics. Significance was evaluated using Student's t-tests assuming equal variance at a significance level of 0.05. Two-sided t-tests were used to determine if results were significantly different, and one-sided t-tests were used to determine whether a result was significantly greater than or less than another result. Experimental results are generally represented as mean ± standard deviation.

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