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Tuberculosis Vaccine Development — Its History and Future Directions

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1. Introduction

Mycobacterium tuberculosis is the world's most successful pathogen, having survived over 70,000 years and currently infecting nearly 2 billion people worldwide [1]. With around 9 million new cases of tuberculosis (TB) each year, almost one third of the population is at risk for developing active disease [2]. In 2012 alone, an estimated 3,500 people *per day* died of TB and, in fact, human immunodeficiency virus (HIV) is the only other infectious disease responsible for more deaths each year than *M. tuberculosis* [1, 3]. Because of these deadly statistics, the World Health Organization (WHO), the Centers for Disease Control (CDC), and the Bill and Melinda Gates foundation, among many others, have committed to eradicating *M. tuberculosis* by the year 2050. A combined strategy of drug treatment, better diagnostics, and prevention (i.e. vaccine development) is the only way to reach this goal [4].

While finding a cure and treating the disease is an essential aspect of medicine, of equal importance are prevention measures to stop contracting the disease in the first place. Because extreme and totally drug resistant strains of *M. tuberculosis* are appearing with increasing frequency, it is essential that we block the spread of this pathogen by developing a vaccine that provides protection against infection.

1.1. Vaccine development

The hallmark of an effective vaccine is one that can be given to a young population, with no adverse side effects, and which will provide lifelong immunity against a particular pathogen. The basis of a functional vaccine is dependent on the immune system's ability to 'remember' an encounter with a foreign pathogen. Typically, immunological memory is established upon the first encounter with a pathogen by the creation of memory T cells. These memory T cells are specific to a particular antigen and will reside in tissues and lymph nodes until they

recognize their specific pathogen and become activated. Upon activation by a second exposure, the memory T cells will quickly and efficiently initiate a response to eliminate the pathogen and prevent disease. The creation of memory T cells and life-long immunity can be obtained either by primary exposure to the pathogen followed by disease and eventual recovery, or by being given a vaccine. Vaccines act to prime the immune system by exposing a person to a non-lethal, milder version of the pathogen's antigens so that those memory T cells can be created without causing disease in the individual. Ideally, a vaccine is made of pathogen-derived components that are critical for induction of protective immunity and consequently, are often composed of either an attenuated (non-virulent) or killed version of the bacteria, inactivated bacterial toxins, or subunits of the pathogen. The vaccines will have antigens similar to the virulent version of the pathogen but would lack factors necessary for disease. Thereafter, if a vaccinated individual is exposed to that particular set of antigens a second time, the immune system will quickly eliminate the pathogen and prevent disease. The immune system is therefore required to respond in a proper manner both to vaccination and to subsequent challenges with the pathogen.

In the case of *M. tuberculosis* infection, however, the immune response initiated upon exposure does not result in memory and instead, infection follows a more complex pathogenesis route. The main route of transmission for *M. tuberculosis* is via aerosolization of liquid droplets containing bacilli that are then breathed in by an individual. After inhalation, organisms are carried into the deep lung where it is phagocytosed by antigen presenting cells (APCs), such as macrophages or dendritic cells, which then travel to the draining lymph nodes and initiate an immune response [1, 5]. *M. tuberculosis* specific T cells then migrate to the lungs and are required for the formation of a granuloma around the site of infection [1]. In an immunocompetent individual, this granuloma will contain the infection in the lungs and prevent active disease [6] [1].

CD4+T cells are the hallmark of the immune response to *M. tuberculosis* infection and, together with CD8+T cells play significant roles in the various stages of infection and disease [6, 7]. Interferon (IFN)- γ and tumor necrosis factor (TNF)- α are essential cytokines for the induction of immunity to infection [6, 7]. Our understanding of the network of immune cells and cytokines required by the host to contain the infection, generate granulomas, and limit the extent of tissue involvement is increasing and will provide a better understanding of the requirements for generating vaccine mediated immunity.

This chapter will focus on the varied and unique aspects of *M. tuberculosis* that have made the development of a vaccine a challenging prospect. We will discuss the only vaccine currently on the market, BCG, its history, and why it has failed to prevent the spread of this pathogen. We will also discuss the various animal models and methodologies that are used by researchers to study the immune response to *M. tuberculosis* in the hopes of developing a more effective vaccine. We will also discuss a few of the vaccines that have already been developed and are being tested in clinical trials. Additionally, we will briefly discuss the various regulatory approval processes that are required to test a vaccine before it can finally be approved for release on the market.

2. The BCG vaccine

Bacillus Calmette-Guérin (BCG) is the most widely used vaccine having been delivered to nearly 3 billion people [8-10]. Despite its widespread use, BCG delivers only minimal protection and has failed to eradicate or reduce the disease burden of TB. The mechanisms by which BCG works to provide its marginal protection are incompletely understood. Even though BCG provides an imperfect defense against *M. tuberculosis* infection, it is still the best vaccine available. In this section of the chapter we will discuss the history of BCG and some possible reasons why it has failed to stem the tide of deaths due to *M. tuberculosis* infection.

2.1. History

Albert Calmette and Camille Guérin developed BCG at the beginning of the 20th century. Developed from a virulent strain of *Mycobacterium bovis*, the BCG vaccine was attenuated by serially passing the strains on potato slices supplemented with glycerol over a period of 13 years until a non-virulent strain was obtained [8-10]. Trials of the newly developed vaccine were performed in cows, monkeys, and African apes and proved to be efficacious [11] [12]. The strain was first used as a vaccine in humans in 1921 with few adverse side effects observed in the patients who received it [8, 9]. The non-virulence of this strain was then established before it was sent out to several laboratories throughout the world and used as a vaccine. The vaccine was then propagated in the various countries in different ways with varying passage numbers, resulting in the emergence of different variants of the BCG vaccine [8, 10].

2.2. World use

BCG is very cheap to produce and at only \$2-3USD per dose, it is one of the most cost effective ways to provide at least partial protection against *M. tuberculosis* to millions of children worldwide [13]. Most of the BCG given in the world is supplied by UNICEF – obtained from three major sources; producers in Denmark, Japan, and Russia [8, 9]. Most countries around the world require that the BCG vaccine be given during childhood. The US, Canada, and Italy are some of the very few countries that have never required that children be given BCG. Most of Europe and Australia did at some point in their history require BCG to be given to children, but have since changed their policies. All of Africa, Asia, and most of South America currently require BCG to be given in early childhood [14].

2.3. Safety and efficacy

A huge reason for the widespread use of BCG is that it is considered to be one of the safest vaccines on the market [15]. Side effects of BCG are very rare with the most common complication being swelling around the injection site [15]. The most significant issue to arise from BCG vaccination is in HIV infected and other immunocompromised individuals. The increased risk of dissemination in HIV infected children lead the WHO to change its policy and recommend that they not be given BCG [15, 16]. Even so, BCG is still part of the WHO's expanded program of vaccination [8].

Studies into the efficacy of BCG have revealed wide-ranging results from vaccination programs with some reports suggesting 80% efficacy and others showing no protection at all [15, 17]. BCG is thought to convey protection against dissemination of the mycobacteria to other organs during childhood, an event that is highly fatal if left untreated. BCG does not, however, provide protection against adult pulmonary disease, which is the main route by which *M. tuberculosis* is transmitted [16].

2.4. Variability in efficacy

There are several possible reasons behind the huge variability in the reported efficacy of BCG; we will discuss some of these reasons in this section. It is important to keep in mind that some of the issues surrounding BCG efficacy variability are also issues which apply to the new *M. tuberculosis* vaccines currently being developed.

2.4.1. Variation in the strain of BCG that is used for vaccination

There are at least 11 different types of BCG vaccines currently available throughout the world [8]. A major reason for the different types of BCG and the genetic variability between the strains is mainly a product of the time period when the vaccine was developed. When Calmette and Guérin first developed BCG, they sent it out to several other laboratories around the world. Those laboratories cultured, grew, and stored BCG, each in their own way. It must be remembered that this occurred in the early part of the 20th century, before the advent of current molecular techniques and storage methods that can maintain parent strain homogeneity. Instead, as BCG was grown and cultured in these various laboratories, it accumulated a series of independent mutations that continued to build upon themselves [8]. It was not until the 1960s, with the introduction of culture seed stocks that soon became the norm, that the standardization of these lines became possible. By that time, however, the various strains of BCG had diverged and modern analysis has demonstrated that there is significant variability in the genetic make-up of these strains [8, 10]. The genetic variation in BCG strains can lead to variation in how well they protect against infection due to difference in the cell surface proteins that would elicit an immunogenic response [10]. However, all of these BCG strains have remained non-virulent and a commonality among all of the strains is the loss of the ESX-1 excretion system. It is still not completely understood how the loss of the ESX-1 system blocks virulence since its re-addition to at least two separate BCG strains does not restore virulence back to wild type levels [10, 18]. Due to this variation in BCG strains, it is essential that accurate record keeping be in place when conducting efficacy studies for the vaccine [8].

2.4.2. The genetic diversity of the tested population

The genetic diversity of a population can affect the outcome of any clinical research study and this is an important factor to consider when examining vaccine efficacy. Various single nucleotide polymorphisms (SNPs) within a genome can affect a person's susceptibility to disease and how their immune system responds to a vaccine. The spectrum of immune system responses to a vaccine can vary from no response to complete activation and protection. Variations within a person's genome could prevent their immune system from properly

responding to the vaccine and therefore it would not convey protection when the person is exposed to the pathogen. For instance, it has been found that mutations in the IFN- γ receptors leads to an increased risk of developing disseminated disease from BCG vaccination rather than protection from *M. tuberculosis* infection [19]. Individuals with mutations in these susceptibility genes are also more prone to infection by non-virulent, environmental mycobacteria as well [19] [19].

Therefore, it is essential when designing a vaccine study in humans to have a large population that is statistically powerful enough that some variability in immune response will not skew the data. Additionally, no matter where a study is being conducted, the genetic diversity of the population must be considered. An ethnically homogenous population is more desired for a research study since that population will be more likely to share SNPs, and therefore more likely to respond to a vaccine in a similar manner. It also follows that it may also be useful to test multiple ethnic groups; indeed, some studies have suggested that the reason some vaccine trials show high protection with BCG is because they were conducted within certain population groups [15].

2.4.3. Pre-vaccination exposure to the pathogen

Another issue that is of huge importance when conducting vaccine efficacy tests is whether or not an individual has been pre-exposed to the pathogen. The immune response of an individual who has already been exposed may be quite different than someone without prior exposure. Especially in areas where TB is endemic, it is likely that a child will have already been exposed to *M. tuberculosis* prior to vaccination [1]. A child could be exposed to both *M. tuberculosis* in the form of infected adults, or to environmental nontuberculous mycobacteria (NTM) – both of which could alter how that child responds to a vaccine. Some studies have also suggested that pre-exposure to NTM may provide some protection that cannot be improved upon by BCG delivery [9, 15, 20]. This may indeed be the case with BCG as it has been found that vaccine programs with the highest degree of efficacy are those with more rigorous screening to only include children who had not been previously exposed to the antigen [17].

2.4.4. Inaccurate diagnostic methods

Because of the complications that result from pre-exposure to the pathogen, it is essential to have accurate diagnostic methods available when deciding which individuals to include in a study. A non-homogeneous population (a mixture of non- and pre-exposed individuals) can produce misleading results that can hinder accurate interpretation of vaccine efficacy. In the case of *M. tuberculosis*, current diagnostic methods are often slow or inaccurate. One of the most common diagnostics for *M. tuberculosis* is the tuberculin skin test (TST), a delayed-type hypersensitivity reaction to protein purified from *M. tuberculosis* and injected just below the skin [21, 22]. It takes two to three days after injection of the purified protein derivative (PPD) before the results can be read; a delay that can result in loss of study participant compliance or even exposure. Additionally, the TST, as well as many new serological tests, cannot distinguish between exposure to environmental NTM and infectious *M. tuberculosis* [23]. Newer IFN- γ release assays (IGRAs) are, however,

becoming more commonly used for research studies since they are selective for *M. tuberculosis* and are more sensitive than the TST [21, 22]. IGRAs are *in vitro* tests that stimulate T cells with *M. tuberculosis* specific antigens (such as ESAT-6), if the T cells have been previously exposed to these antigens, they will release IFN- γ [22].

3. Specific difficulties associated with *M. tuberculosis* vaccine development

The complications involved with studying *M. tuberculosis* in a laboratory are numerous and range from the practical to the scientific. Due to the complex nature of the pathogen, *M. tuberculosis* presents some very unique challenges for the researcher studying it. In this section we will go into more depth as to the specifics of these challenges and how they have delayed the development of a vaccine.

3.1. Biosafety considerations

When a researcher decides to investigate the development of a vaccine against *M. tuberculosis* one of the very first considerations that must be taken into account is the safety of those working with the pathogen. In the United States, the CDC provides a set of guidelines for the safety measures that need to be in place in order to work with various pathogenic organisms. These biosafety levels range from 1 (least likely to cause harm to an individual) to 4 (most harmful and infectious) [24]. The basis for these biosafety measures are derived from the WHO, which, in turn, base their recommendations upon the availability of effective preventive measures and treatment options [25]. As the biosafety level of a pathogen increases, it is necessary to increase the safety measures for a person working in these conditions. In the research laboratory setting, *M. tuberculosis* is considered a biosafety level 3 (BSL3) pathogen; a BSL3 pathogen is defined by the WHO as one that poses a high risk for an individual but a low risk for the community and can cause serious harm to an infected human. The various safety measures that must be put in place include laboratory practices, facility construction, security, and safety equipment. The exact nature of the measures put in place will vary slightly from facility to facility but in general, this includes separate rooms and equipment for work with the pathogen. Rooms in which the pathogen is manipulated must be under negative pressure so that airflow is directed from a clean room into a 'dirty' room to prevent it from spreading. All work done with the microbe needs to be conducted in a properly outfitted biosafety cabinet and special personal protective equipment (e.g. respirators) must be worn at all times.

These biosafety mechanisms can be a limiting factor when attempting to study *M. tuberculosis*, as they require full facility involvement and specialized equipment to be in place. Additionally, these biosafety measures, while necessary, can be relatively uncomfortable, and may therefore limit the time with which a person can work under them. While these are obviously not insurmountable obstacles, they should be taken into consideration before research of this nature is undertaken.

3.2. Slow growth of the pathogen

M. tuberculosis is a slow growing pathogen with a doubling time of approximately 24 hours [26]. Because it grows so slowly, it is necessary to let a significant amount of time pass after infection in order to resolve differences in vaccine treatments. If an unvaccinated mouse is given a low dose aerosol infection of virulent *M. tuberculosis*, it can take upwards of 3 to 4 weeks before visible granulomatous lesions will form in the lungs [6]. When conducting immunological research, mice are generally sacrificed 30 to 120 days after infection to examine short and long term immunity and determine if a vaccine has had any effect on the reduction in mycobacterial number. When the animal is sacrificed, the lungs must then be plated on selective agar media in order to enumerate the number of mycobacteria growing in the lungs. The time of plating the lungs until the formation of visible colonies is between 2-3 weeks. All told, experiments of this nature often require 6-9 months of work before any data is even collected. Therefore, when planning out experiments involving infected animals, researchers must consider the significant amount time it will take between the initiation of the experiment and the completion of the study.

3.3. Disease stages

As mentioned previously, infection with *M. tuberculosis* can be divided into distinct stages. In humans, infection with *M. tuberculosis* can be divided into four stages: infection, latency, reactivation, and transmission [5]. At each of these stages, the proteins expressed by the organism are thought to vary significantly and therefore protection in one stage may not protect in all. After primary infection, in an immunologically healthy individual, an infection will progress to a latent TB infection (LTBI). The persistence of infection despite no active disease in a clinically 'cured' individual, make end point goals difficult to define for a researcher developing a vaccine [16]. There is significant evidence that sterile clearance of *M. tuberculosis* is a rare occurrence and rather, it is the maintenance of an active immune system to contain the mycobacteria in the latent state that prevents active disease from forming [1, 27]. In most individuals, this is where the infection will remain unless there is an outside occurrence that reduces the functionality of their immune system [1]. A person with a LTBI will not have active disease, but will have developed memory cells against *M. tuberculosis* antigens. It is generally believed that when *M. tuberculosis* is in a latent stage it is slow growing or non-replicative and most likely expresses a different set of antigens than during the primary infection stage of the life cycle [1]. The exact immunological events that are required in order for the pathogen to progress from latency into an active state are incompletely understood.

The immune response that humans develop against *M. tuberculosis* infection appears to contain the bacteria and induce it into a latent stage, yet fails to sterilize the infection [5]. This leads to some interesting questions as to what types of antigens to target for vaccine development. When a person has a LTBI, their immune system is effectively containing the mycobacteria and they do not get sick. Do we therefore design vaccines to elicit an immune profile similar to someone with a latent infection even though that profile is not sufficient to eliminate the pathogen? It is interesting to note that individuals that are infected with *M. tuberculosis* and progress on to latency are not protected from re-infection. However, because detection of *M.*

tuberculosis when it is in a latent stage is quite difficult with our current diagnostic methods, it is often challenging to distinguish between reactivation of the primary (latent) infection or re-infected from a new exposure [1, 16]. Since our understanding of the possibility of lung sterilization post-*M. tuberculosis* infection is incomplete, the comparison of persons with LTBI to those with active disease is limited in what it can tell us about protective immunity.

Ideally, the best vaccine would be one that provides protection from all stages of infection, i.e. a vaccine must be effective against primary infection, latency, reactivation, and reinfection [1, 2].

3.4. Vaccination in immunocompromised individuals

Of the estimated 1.3 million people who died of TB in 2013 around 25% of them were co-infected with HIV [3]. In fact, TB is the number one killer of HIV infected individuals [7, 28-30]. The resurgence of *M. tuberculosis* infections in the 1990s has largely been attributed to the advent of the HIV epidemic [30, 31]. The reasons behind the increased susceptibility of HIV infected individuals to *M. tuberculosis* infection are not completely understood. An infection with HIV leads to a reduction in CD4+T cells and this is believed to be the cause of greater susceptibility to *M. tuberculosis* by most individuals [28, 30, 32]. One hypothesis is that HIV disrupts the function of the granuloma and allows for the replication of the organism [28]. The immune system of an HIV infected individual is, therefore, not able to control or contain the *M. tuberculosis* infection, resulting in reactivation of the disease [28, 30]. HIV infection may also disrupt the function of macrophages and *M. tuberculosis* specific T-cells that prevents the killing of the pathogen [28]. An interesting aspect of the *M. tuberculosis*-HIV co-infection is that HIV infection leads to susceptibility to *M. tuberculosis* even after anti-retroviral therapy has commenced and CD4+T cell counts have been restored to normal [33] [30, 34]. It has also been found that HIV infected infants do not produce a robust T helper (Th)1 immune response when vaccinated with BCG [30]. Additionally, HIV positive infants are at a significantly increased risk of dissemination of BCG after vaccination [7, 15, 16].

Another group of immunocompromised individuals that must be considered are those with poor living conditions and poor nutrition. TB is a disease of the poor and mainly affects those who are already at a socioeconomic disadvantage. Poor nutrition and living conditions increases a person's susceptibility to disease and dampens their immune response to a challenge. These living conditions could impair the ability of the immune system to respond properly both to a vaccination and exposure to a pathogen. Other conditions, such as diabetes or vitamin D deficiency, can also impair the ability of the immune system to produce a robust response and indeed, both conditions have been associated with an increased risk for developing TB [35-37].

3.5. Vaccination post exposure to the pathogen

In the case of TB, it is important to consider if the vaccine is aimed at pre or post-exposure to the pathogen. As mentioned in the previous section, a person's immune system will respond differently to a vaccine if they have already been exposed to the pathogen. Since the immune

system will have already been primed to respond to particular *M. tuberculosis* antigens that are not capable of protection, it is necessary to find a new method of immune activation. Because of the high prevalence of *M. tuberculosis* in endemic countries, it is likely that in order to eradicate TB, a vaccine will need to be effective post-exposure.

3.6. Laboratory strains versus clinical isolates

Mycobacteria from clinical isolates are often quite different than the strains commonly used in laboratory practice. Laboratories, by necessity for the repeatability of their experiments, must maintain an unchanging common lab strain. There are currently several strain types used in laboratories throughout the world (H37Rv, HN878, Erdman, CDC1551, etc.) that vary in their virulence and antigenic composition. Variation can also occur within a strain depending on how it is handled in the laboratory. Unfortunately, however, these strains can be significantly different than the mycobacteria isolated from infected patients. The mycobacteria found 'in the wild' is going to be constantly changing and mutating depending on various selection factors. The extent of the divergence between laboratory strains and clinical isolates will depend upon the strength of the selection factor. These selection factors can include incomplete drug treatments or the strength of a non-drug treated person's immune response to the pathogen. Additionally, there is a natural mutation rate for the genome of all organisms independent of selection factors due to DNA replication errors or un-repaired DNA damage. The selection factors and mutational changes observed in clinical isolates are most apparent with the emergence of multi, extreme, and totally drug resistant organisms. While it is hoped that there is enough similarity between laboratory strains and the clinical isolates that vaccination against one will provide protection from all forms of *M. tuberculosis*, the constant mutation of wild-type strains should be considered.

3.7. Intrinsic properties of *M. tuberculosis* which make it difficult to immunize against

Currently, almost all vaccines that have proven to be efficacious in humans against infectious pathogens convey protection through the production antibodies [1, 38]. These antibodies will coat a pathogen and signal to the immune cells that it should be removed from the system. *M. tuberculosis*, however, is intracellular, which means that the immune system must be able to recognize and then eliminate infected host cells without causing excessive damage to the host.

M. tuberculosis has established several methods by which it can evade recognition by the host immune system. Most of these mechanisms revolve around ways of keeping the pathogen in a quiescent state within the macrophage. For example, the recruitment of professional phagocytic cells to the site of infection provides an increased number of ideal environments for *M. tuberculosis* survival. Once phagocytic cells have taken up the organism, it can survive within macrophages by arresting and inhibiting the maturation of the phagosome to prevent its own clearance from the cell [37]. These blocks in phagosome maturation prevent fusion of the phagosome with the lysosome and acidification of the compartment and thus elimination from the cell [37]. This block in phagocytosis also prevents proper presentation of *M. tuberculosis* antigens [1]. Presentation of antigens is essential for recognition that an antigen is foreign

and needs to be eliminated, as well as for the creation of immune memory. *M. tuberculosis* also evades removal by the ability to regulate both the necrotic and apoptotic death of host cells [5].

All of the elimination avoidance methods developed by *M. tuberculosis* serve to maintain the pathogen within the macrophages where it can live for decades as a latent infection [1, 37]. While *M. tuberculosis* infection does produce an immune response in the host, this response fails to result in protection or complete clearance of the pathogen [1, 5]. Therefore, when designing a vaccine, it is necessary to activate the immune system in such a way that the host can overcome these evasion methods and recognize that the pathogen infected cells should be destroyed.

4. Animal models used for vaccine development

In some cases where the pathogen is non-lethal or there are readily available treatment options (such as malaria or influenza), it is possible to test the efficacy of a vaccine in a human model. In these instances, a willing volunteer is vaccinated, and then exposed to the pathogen by the researcher in a controlled manner [16]. In the case of *M. tuberculosis* however, this course of experimentation is unethical as it is still controversial if sterilization of the lungs post-infection is even possible. It is therefore imperative that we use animal models for the development of vaccines. A vaccine is considered efficacious in an animal model if it is able to reduce the number of colony forming units (CFU) in the lung. Currently, the goal for new vaccines is to reduce the number of CFU in the lung better than BCG, which consistently provides around a 1 log₁₀ CFU reduction in the mouse model of tuberculosis.

4.1. Mouse model

4.1.1. Advantages

Cost effectiveness and scalability. One of the biggest reasons for the use of mouse models in research is their cost effectiveness. With the exception of highly specialized knock-in or knock-out models, inbred mice can be purchased in large numbers at very low cost. Since mice can often be housed with multiple animals per cage, this further reduces the facilities cost by utilizing less space per animal. A researcher can therefore scale a study up to a magnitude that would be impossible with larger or more costly animals.

Inbred mouse strains and genetically modified models. Through the use of genetically identical inbred strains, researchers from all over the world have the ability to collaborate using the same set of tools and more effectively build upon previous studies. The ready availability of facilities like The Jackson Laboratories and Charles Rivers, which specifically maintain these inbred mouse strains are an invaluable resource for researchers. These facilities also make it easy to custom order genetically modified mouse models to test the importance of various proteins during vaccination or pathogen challenge. The availability of total or conditional knock-out mice, as well as knock-in models, allows researchers to effectively use *in vivo* system to ask questions that are unavailable by other methods.

Availability of immunological reagents. The use various molecular assays after completion of an animal experiment can provide vital information about the function of the immune system. An additional advantage to using the mouse model is that because it is so widely used, reagents are available for many types of immunological assays. In particular, antibodies for western blot, enzyme linked immunosorbent assays (ELISAs), flow cytometry, and many others, are commonly used on mouse models and are therefore relatively easy to optimize for a particular experiment.

Susceptibility of various strains. The strain of mouse that will be used for an experiment is of great importance when designing an experiment. There is significant variability between mouse strains as to their susceptibility to TB infection. This variance in susceptibility of a mouse strain can depend up on several important factors including the route of infection, the dosage, and the strain of *M. tuberculosis* [39]. While genetic modification is commonly done on a C57Bl/6 background, these mice are actually resistant to *M. tuberculosis* infection and aerosol infection does not reduce their lifespan [39]. Some strains such as CBA are very susceptible to infection, while others, such as the BALB/c, appear to be susceptible depending on the route of infection [39]. These last two strains, however, do have the disadvantage that there are fewer genetically modified strains available with this background. The immune systems of both the susceptible and resistant strains clearly respond differently to both vaccination and challenge infection and therefore, it is often useful for researchers to test a vaccine in more than one mouse model.

4.1.2. Disadvantages

A major disadvantage of the mouse model is that the immune system is not the same as that of a human. The underlying genomic inflammatory response to infection in a mouse has been shown to bear little correlation to what occurs in humans [40]. Many drugs that have been designed to modify inflammatory responses in mice, have failed human clinical trials [40]. While humanized mice (a mouse containing human genes, cells, or tissue) may allow for an immune response closer to that of a human, it is impossible to create an identical response. Additionally, unlike in humans, BCG vaccination of a mouse model does convey some protection from *M. tuberculosis* infection and reduces the number of CFU in the lungs. A better understanding of the critical cellular differences between the mouse and human immune system is needed before we can effectively translate studies from the bench to the clinic.

4.2. Guinea pig model

4.2.1. Advantages

Historically, the guinea pig was the first animal model used for tuberculosis research. Guinea pigs are considered the gold standard by which *M. tuberculosis* vaccines are tested before continuing to larger animal models or even humans. This is because guinea pigs have an immune response that more closely resembles that which is seen in susceptible humans. Guinea pigs are very susceptible to infection and demonstrate a tuberculosis disease progression that is similar to that seen in humans [41].

One aspect of guinea pig research that is both an advantage and a disadvantage is the lack of in-bred laboratory strains. There are only a handful of laboratory guinea pig strains available commercially and these are mostly out-bred stock that will not be genetically identical at every locus. This provides greater genetic diversity within an animal strain with which to test vaccine efficacy. Obviously when vaccines move into human trials, genetic diversity will be unavoidable (as mentioned previously), and therefore the guinea pig provides a more realistic model of what will happen in humans. However, this does limit the exact repeatability of an experiment due to the lack of genetically identical animals.

4.2.2. Disadvantages

A disadvantage of the guinea pig model is that they are very susceptible to infection, but humans are relatively resistant. While the phenotype observed during disease progression does resemble that of humans with active disease, this represents only a small fraction of people infected with *M. tuberculosis*. It is believed that most humans are resistant to infection and will not go on to active disease unless an outside force reduces their immune response and ability to contain infection in a latent state [6, 7]. The exact mechanisms behind reactivation of latent *M. tuberculosis* are not completely understood, but it is a vital step in disease pathogenesis that is missed in the guinea pig model.

The guinea pig also has the disadvantage that, because it is a larger animal, housing costs tend to be much higher than for mice. Guinea pigs require larger cages with fewer animals per cage and thus facility space limitations may reduce the number of animals included in a study. Additionally, because they are not as commonly used as the mouse model, it is more difficult to find the facilities and veterinary expertise necessary to house and care for guinea pigs.

Of great importance is the lack of genetically modified guinea pig models for the researcher to use. This can severely limit the ability of the researcher to develop and improve upon vaccine models.

4.3. Other animal models

The mouse and guinea pig are the most commonly used animal models for research into *M. tuberculosis* vaccine development. However, there are several other animal models that are also sometimes utilized for this purpose including rabbit, cattle, non-human primates (NHP) and even zebrafish [42-44].

Macaques are an NHP that are a promising model to use for the development of a tuberculosis vaccine. The pathophysiology of TB infection in a macaque closely resembles that of what occurs in human populations, including latency – an aspect of infection that has been difficult to model in other animals [43, 45]. Due to the size of the animals, their limited availability, the cost of housing and care, as well as ethical considerations, use of NHP, while highly informative, is extremely limited and generally only done after a vaccine has been tested in smaller animal models.

One of the biggest disadvantages to use of any of these non-mouse models is that there are very few reagents available for post-mortem tissue analysis. Few antibodies or other com-

monly used immunological reagents are available for the study of the immune response in many animal models. Sometimes there is enough overlap in antigens between mouse and humans and other animals that some tests can be conducted, but this is a fairly rare occurrence. While these are not limitations that preclude the use of non-mouse animal models, it does limit the information that can be acquired from using them [46]. There is therefore a huge need to expand our animal model base beyond just the mouse. Genetic evaluation of and the ability to produce inbred strains of other animal models would be an unbelievable advantage to all clinical research beyond just tuberculosis vaccine development.

We can garner important information from all of these animal models and it is clear that their use is absolutely essential for progress in medical research to be made. However, it must be remembered that these models are imperfect and there are scientific limitations to the results from studies using them. For instance, in a laboratory setting, animals tend to be exposed only once and to only one strain of pathogen. We know this will not be the case for humans since, as discussed above, *M. tuberculosis* is constantly mutating and in endemic regions a person will likely be exposed to several different strains at multiple times throughout their life [47]. Ultimately, the only way to test out if a vaccine will be effective in a human population is to actually test it in a human population. While it is certainly possible to move on to human trials, there are many steps that must be undertaken before a vaccine can be put into widespread use. This process will be discussed in the next section.

Another important question to consider when examining results from animal models is if we are missing vaccines that would be highly efficacious in a human, but discarding them because they do not act well in our animal models. This is a problem for all areas of clinical research and is, unfortunately, unavoidable given our current knowledge. The only way to overcome this limitation is for research to continue to close the gap in our understanding of how animal and human immune systems differ. The continued refinement of our animal models will provide us with the tools necessary to produce an effective vaccine. This is another reason why the use of multiple animal models is often needed in order to improve the translatability of the research. Most animal models recapitulate some, but not all, aspects of the human features of a disease. If multiple animal models are tested, it increases the likelihood of finding a vaccine that will work in humans.

5. The future of *M. tuberculosis* vaccine development

There is a profound lack of understanding about what exactly a good immune response to *M. tuberculosis* infection actually looks like. This is due, in part, to our limited information about how the immune system contains infection in a latent state as well as how it can revert to active disease. Because our knowledge is incomplete, it complicates decisions regarding which vaccine candidates to pursue in animal models. Our best vaccine, BCG, is not 100% effective in killing off *M. tuberculosis* in a mouse despite eliciting a strong Th1 immune response that is thought to be necessary to produce immunity against the pathogen [1]. In general, if a new vaccine candidate can also elicit a strong Th1 response in a mouse model, it will then move on

to challenge studies where an animal model is immunized, then infected. While it is clear that protection from infection does require strong T cell immunity, it is also clear that that form of immunity is not sufficient to prevent disease. Sometimes, even when a vaccine candidate is promising in preliminary trials, this will not lead to protection when challenged with infection. This is why many of the vaccines currently in the approval process pipeline have multiple pathways by which they induce an immune response. The hope is that one of these other pathways, or a combination of various pathways, will give us more and better information about what immunological protection would look like [1].

There are at least 12 new vaccines candidate that have entered the pipeline for efficacy and safety testing and are the first to be put through the approval process in over 100 years [2, 48]. It is encouraging to note that there are so many vaccines being tested as it increases the likelihood of finding a formulation that will be effective. The arrival of these new vaccines, even those that fail, is a promising step forward in our understanding of immunity and what protection will look like. In this section we will discuss some of the characteristics of these vaccines as well as the steps that must be gone through before they can be put into widespread use.

5.1. Common tactics taken for vaccine development

There are two main tactics for the development of a new vaccine against *M. tuberculosis* infection. The first is the prime-boost or enhancing the efficacy of the existing vaccine, BCG. In this approach, an individual can be given an adjuvanted vaccine at the same time or at a specified period of time after BCG vaccination. The other tactic is to develop a whole new vaccine that is given instead of BCG. There are advantages and disadvantages to both strategies and a discussion on the ethics of trial design for both of these can be found in the next section.

Many of the vaccines that are being developed contain one or more of a select group of antigens for the initiation of an immune response. Antigen 85A/B (Ag85A/B) and the 6kD early secreted antigenic target (ESAT-6) are proteins that are commonly included in a vaccine make up [2]. Ag85A/B are *M. tuberculosis* surface proteins that are involved in bacterial cell wall biosynthesis and elicit an immune response in animal models [23]. ESAT-6 is a secreted protein critical for virulence that is not found in BCG and can also stimulates a strong immune response [18, 49]. These proteins, along with several others that have been found to be immunogenic, have been put into various formulations and are currently being tested.

In addition to multiple combinations of proteins found to be immunogenic, there has also been testing of which delivery route will provide the best protection. For instance, some vaccines provide better immune protection if they are delivered via aerosol route rather than through intradermal injection. Therefore, many studies are being conducted looking at both multiple delivery routes as well as vaccine make-up.

5.2. Ethical considerations

There are also important ethical considerations that must be addressed when developing a new vaccine. When testing out a vaccine, it is important to consider the health and safety of

the control groups as well as those receiving the novel vaccine. For instance, if you have in your possession a vaccine (i.e BCG) that conveys at least some protection against TB, you ethically cannot withhold access to that vaccine simply because you need a placebo control group for your research study. Additionally, a researcher attempting to test out an entirely new vaccine must also consider the possibility that their vaccine will not work and therefore those test subjects are at an increased risk of developing disease. While there are ways to circumvent these issues and still produce an ethically sanctioned and scientifically sound vaccine trial, it does require additional preparation and manpower to execute. Because of these significant ethical complications, however, much of the recent research into TB vaccines has been for the development of adjuvants to boost BCG rather than to develop completely new vaccines.

5.3. MVA85A

The most notable of these new vaccines is MVA85A, which is the first modern day *M. tuberculosis* vaccine to reach an efficacy trial. MVA85A is a Modified Vaccinia virus Ankara that has been engineered to express Ag85A [1, 50]. In animal models, MAV85A was shown to produce a strong Th1 response and provide better protection against *M. tuberculosis* infection than BCG [47, 51-53]. It was designed as a prime boost, to enhance the efficacy of BCG, by giving it to infants a few months after BCG vaccination. In a Phase 2b clinical trial, MVA85A was found to be quite safe and did produce an immunogenic response, but unfortunately, did not provide better protection against infection than BCG alone [53]. This was despite earlier findings that MVA85A induces the immune responses believed to be necessary for protection [54]. In fact, this (previously assumed to be beneficial) CD4+T cell response to Ag85A is still detectable in individuals given MVA85A up to 6 years after vaccination [55]. Even though MVA85A did not provide the protection that was hoped, there are some promising indications that this vaccine may be useful in other settings. For instance, the authors suggest that this vaccine maybe beneficial in preventing the spread of disease in adults rather than in infants [53]. Clearly further testing of the MVA85A vaccine needs to be conducted. Even though initial results with MVA85A were disappointing, it does represent a promising step in vaccine development. Given the state of the field, any knowledge gained about immunity against *M. tuberculosis*, even if negative, can provide useful information and guide future studies

5.4. Vaccine approval process

The WHO offers a set of guidelines for the approval of a vaccine, and many countries adopt these standards or use them as a basis for their own set of regulations. The point of these regulations is to do as much as possible to ensure the safety and efficacy of the vaccine before it is put into widespread use. In the United States, the Federal Drug Administration (FDA) regulates and oversees the approval process for the development of all vaccines. This discussion will give a quick overview of the FDA regulations specific to the US as an example of what must be undertaken before a vaccine is approved for use. The FDA approval process involves an exploratory stage, a pre-clinical stage, an Investigational New Drug (IND) application, and

finally a three phase clinical trial in human subjects [56]. The FDA has the authority to stop a vaccine trial at any point if safety becomes a concern.

The exploratory stage is the basic science side of the vaccine development process. It is in this stage where researchers investigate the potential of various antigens to induce an immune response and are frequently done *in vitro* although some *in vivo* studies may be conducted. This is where the formulation of the vaccine is first tested and the identification of immunogenic proteins is determined.

The pre-clinical stage involves animal model testing of the various antigens that were discovered in the exploratory stage. In this stage, animals are immunized using a new antigen or vaccine make up and then challenged with *M. tuberculosis* infection. The various animal models discussed in the previous section are used in this stage of vaccine development. In order for the vaccine to move on to humans, it must first demonstrate efficacy in multiple animal models. The animal models used can vary depending on pathogen, but for *M. tuberculosis* these models generally include (in order), mouse, guinea pig, and non-human primates. Once a vaccine has been found to be effective, it must pass toxicology and distribution studies in the animal models, as well as demonstration of the ability to produce the vaccine in a lab with Good Manufacturing Practice (GMP) certification [57]. After the completion of these studies an IND application is submitted and, upon approval, human clinical trials will commence.

Phase 1 clinical trials are small studies designed to test the safety and immunogenicity of the vaccine. Phase 2 clinical trials are larger and used for further refine dosage and efficacy of the vaccine as well as determining what population it can be most effectively used on. Phase 3 clinical trials involve thousands of individuals and require additional safety documentation to complete [56].

Once all of these stages have been completed, a cross-disciplinary committee from the FDA must then approve a lengthy Biologics License Application (BLA). The BLA will include not only safety and efficacy information but also guidelines for the mass manufacture and distribution of the vaccine as well. As may be obvious from this brief description, the process of vaccine approval can be quite lengthy and will often last 10-15 years. The enormous cost associated with undertaking such an endeavor often requires the collaboration of both private and public funds at multiple institutions in order to be completed.

6. Conclusions

The development of a vaccine against *M. tuberculosis* has been one of the hardest puzzles facing immunologist for the past century. When examining an infection and an appropriate immune response, the traditional approach, at least in relation to tuberculosis research, has been to try to amplify the immune response of a healthy person in order to clear the bacteria. However, is this really the approach we should take when examining *M. tuberculosis*? In the case of *M. tuberculosis* infection, is it the body's very own defense mechanisms that is causing lung tissue damage and forcing the mycobacteria into a latent stage? Is the body's response to infection

actually a good thing or is it contributing to disease progression because it creates an environment that harbors the bacteria (a granuloma) rather than eliminating it? Should we instead be attempting to induce activation of a whole new pathway in order to sterilize the lungs?

The urgency to find of an effective vaccine is nowhere as apparent as the emergence of drug resistant tuberculosis that been appearing with increasing frequency in the last decade. The currently available drugs are expensive and require a lengthy course of treatment; increasing the likelihood of non-adherence by sick people and therefore increasing the possibility of even more resistant strains evolving. Because a person does not naturally develop immunity to the pathogen when they are infected, they can become re-infected if they are exposed to the pathogen a second time and have to endure the same drug regimen as before. Only by providing continuous, protective immunity against recurrent infection can we hope to eradicate this devastating disease from the population.

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