Challenges and Future in Vaccines, Drug Development, and Immunomodulatory Therapy

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Abstract

Pulmonary diseases and infections are among the top contributors to human morbidity and mortality worldwide, and despite the successful history of vaccines and antimicrobial therapeutics, infectious disease still presents a significant threat to human health. Effective vaccines are frequently unavailable in developing countries, and successful vaccines have yet to be developed for major global maladies, such as tuberculosis. Furthermore, antibiotic resistance poses a growing threat to human health. The “Challenges and Future in Vaccines, Drug Development, and Immunomodulatory Therapy” session of the 2013 Pittsburgh International Lung Conference highlighted several recent and current

studies related to treatment and prevention of antibiotic-resistant bacterial infections, highly pathogenic influenza, respiratory syncytial virus, and tuberculosis. Research presented here focused on novel antimicrobial therapies, new vaccines that are either in development or currently in clinical trials, and the potential for immunomodulatory therapies. These studies are making important contributions to the areas of microbiology, virology, and immunology related to pulmonary diseases and infections and are paving the way for improvements in the efficacy of vaccines and antimicrobials.

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Diseases and infections of the lung are among the top contributors to global human morbidity and mortality (1). Tuberculosis, viral and bacterial respiratory infections, and chronic pulmonary diseases continue to be among the most frequent causes of death worldwide. In addition, influenza imposes a substantial economic burden, affecting 5–15% of the population each year with upper respiratory tract infections, and reassortment of the virus surface proteins has resulted in global pandemics (2).

Before the twentieth century, infectious disease had been the leading cause of death throughout global history. Vaccines and

antimicrobials are among the most effective interventions in modern medicine, with far-reaching impacts on human health and disease. The modern history of immunization began with Edward Jenner's smallpox vaccine in 1796, and since that time, vaccines have been indispensable to the control and prevention of disease. Equally as important, the discovery of penicillin by Alexander Fleming in 1928, followed by the determination of its chemical structure in 1940 by the group of Florey, Chain, Heatley, and Abraham, served to reshape modern medicine, and with it, began the golden age of antibiotics. Widespread use of antibiotics

and vaccines has had a major impact on global public health, vastly reducing morbidity and mortality rates from infections, and in turn, improving quality of life and benefiting economic development.

Despite these advances and the successful history of vaccines and antimicrobial therapeutics, infectious disease still presents a significant threat to human health. Effective vaccines are frequently unavailable in developing countries, and each year, millions of people die from vaccine-preventable and drug-treatable diseases. Furthermore, successful vaccines have yet to be

developed for major global maladies (3), such as tuberculosis. In addition, technologies used to develop and manufacture vaccines are often outdated and not easily adaptable for rapidly responding to disease outbreaks, such as influenza. It can be difficult for manufacturers to successfully predict the dominant circulating influenza strains each season, and when new strains emerge suddenly, as in the 2009 H1N1 pandemic, rapid preparation for distribution of a new vaccine can be challenging (4). Antibiotic resistance also presents a threat to human health, requiring development of novel antimicrobials, and is compounded by reduced antibiotic development by pharmaceutical companies.

The work presented here summarizes current advancements in the development of antimicrobials, therapeutics, and vaccines for pulmonary infections, presented at the 2013 Pittsburgh International Lung Conference. Researchers presented data on the development of novel antimicrobials, which may be used to treat antibiotic-resistant bacterial pneumonia and infections associated with cystic fibrosis; new vaccine formulations for the prevention of highly pathogenic influenza strains; tuberculosis vaccines currently in development and in clinical trials; therapies that may be useful for the treatment of respiratory syncytial virus (RSV) infection in infants; and novel methods of imaging tuberculosis lesions and monitoring efficacy of chemotherapeutics.

Antimicrobial Therapy: Current Threats and Responses

The development of antibiotic therapies began in 1928 with Alexander Fleming's discovery that a mold (*Penicillium*) could inhibit the growth of certain types of bacteria. By 1941, Howard Florey and his colleagues began human trials with penicillin, and soon after, pharmaceutical companies in the United Kingdom and the United States began production of penicillin on an industrial scale. The development of penicillins as antimicrobial therapeutics exemplified a shift in the drug discovery paradigm from the synthesis of small molecules to the exploitation of natural products as chemotherapeutics, leading to the "golden era" of antibiotic discovery (5). These discoveries also

transformed health care into a treatment-focused approach, ultimately saving countless lives. However, reports of *Staphylococcus aureus* resistance to penicillin began as early as the mid-1940s, and antibiotic resistance has been an increasing concern since that time, ushering us into what many have termed the "postantibiotic era."

Antibiotic resistance has been the driving force behind novel antimicrobial discovery and development since the mid-twentieth century; however, it is the substantial increase in frequency, diversity, and range of resistant microorganisms encountered in clinical care that is now contributing to the urgency of current threats (5).

Antibiotic resistance presents a significant threat to human health as well as a financial burden on the U.S. health care system, responsible for a staggering annual cost of \$20 billion (6). Methicillin-resistant *S. aureus* kills more Americans annually than emphysema, HIV/AIDS, Parkinson's disease, and homicide combined (7). Hospital-acquired infections are responsible for approximately 100,000 deaths in the United States each year, the majority of which are due to microorganisms that are at least partially antibiotic resistant, and mortality rates from infections resulting from resistant bacteria, such as carbapenem-resistant Enterobacteriaceae, can be up to 50% (8). The impact of antibiotic resistance is compounded by the fact that there are now fewer antibiotics in development; many pharmaceutical companies have withdrawn development of new antimicrobials because of increased cost for reduced return on investment, compromising the infrastructure for antimicrobial research and development (7). In 2010, the Infectious Diseases Society of America launched an initiative that set a goal of developing 10 new antibiotics by the year 2020 ($10 \times '20$) (7), but currently there have been only 2 new antibiotics approved for use since 2009 (telavancin [Theravance, South San Francisco, CA] and ceftaroline fosamil [Forest Laboratories, New York, NY]), and the number of antimicrobials in clinical phase 2 or 3 trials remains distressingly low. The future of antimicrobial chemotherapy is now uncertain, and success will likely depend on creative and novel combinations of existing antibiotics as well as the addition of new members to existing classes.

One of the major challenges to novel antimicrobial development is that the vast

majority of microbial species cannot be cultured on artificial media, and thus remain unexplored. At present, researchers are able to isolate/culture only 0.1% of existing microorganisms. To address the need for improved culture systems and organism recovery from environmental samples, Kaerberlein and colleagues (9) have developed a diffusion chamber in which the natural environment is simulated by the addition of seawater to agar, and intertidal marine sediment serves as the source of microorganisms (9). This system allowed for the isolation of colonies of pure organisms previously classified as "uncultivable." By improving microbial culture systems, researchers are better able to identify antimicrobials from a pure culture of organisms that previously could not be tested.

Novel antimicrobial therapies have been developed that neutralize bacterial virulence factors. Morbidity and mortality attributable to *Pseudomonas aeruginosa* ventilator-associated pneumonia (VAP) remain high, and antibiotic resistance further impedes efforts to control *P. aeruginosa* VAP. An alternative strategy to conventional *in vitro* assessment of antibiotic susceptibility involves targeting infection-essential functions (e.g., virulence factors), such as the type III secretion system (TTSS) of *P. aeruginosa* (10). The TTSS, capable of injecting endotoxins into host cells, is a major virulence factor for *P. aeruginosa*. The needle-like tip protein, PcrV, an essential component of the TTSS, is the target of an investigational, humanized, recombinant monoclonal antibody, KB001, which binds to PcrV and inhibits its function. This antibody demonstrates neutralization activity *in vitro*, and in a mouse model of *P. aeruginosa* infection, prevents mortality and enhances bacterial clearance. In clinical trials, reports have highlighted the potential for KB001 to reduce *P. aeruginosa* pneumonia incidence in mechanically ventilated patients (10) and to reduce airway inflammation in *P. aeruginosa*-infected patients with cystic fibrosis (11). KB001 is now in phase 1/2 trials in patients with cystic fibrosis and phase 3 in patients with VAP.

Finally, an additional alternative strategy to conventional antimicrobial development is photodynamic therapy (PDT), which uses photosensitizers in combination with visible light and

molecular oxygen to kill target cells (12–14). Organisms susceptible to PDT include both gram-negative and gram-positive bacteria, fungi, viruses, and protozoa (12). This therapy has been used *in vivo* in murine models of wound healing to treat *P. aeruginosa* infections of skin burns, with greater than 90% survival in treated groups, compared with 100% mortality in control groups (15). This system lacks specificity and has potential for host damage, which can be improved by using a photosensitizer prodrug that is activated by β -lactamase to combat antibiotic-resistant bacteria (16). Zheng and colleagues designed a construct (β -lactamase enzyme-activated photosensitizer, β -LEAP) to take advantage of quenching for a more specific effect on the target bacteria (16). β -LEAP was designed so that on cleavage of the β -lactamase, the photosensitizer is released, resulting in a more targeted design for exploiting PDT as an antimicrobial therapy.

The future of antimicrobial development is dependent on adoption of a drug development paradigm in which collaboration occurs between public and private entities, with large pharmaceutical companies at the terminus of the development chain. The supply of antibiotics currently available and in development cannot meet the current and future demands of a population that continues to acquire infections from drug-resistant microorganisms. To compete with an evolving microbial world, new targets must be identified and novel therapies should be developed to exploit those targets. To maximize their usefulness, all antimicrobial therapies must be coupled with prudent antibiotic stewardship and infection control measures.

Novel Influenza Vaccines

Each year, 10–20% of Americans are infected with influenza, with the highest rates of complication occurring in the elderly population, and children likely responsible for dissemination of the virus. In the United States, influenza is responsible for an average 36,000 deaths annually, more than 90% of which occur in the elderly population. Furthermore, highly pathogenic strains of influenza viruses are of great concern as potential pandemic threats, and improved response preparedness is vital to rapid control of virus dissemination in the human population.

Influenza is a single-stranded negative-sense, enveloped RNA virus in the Orthomyxoviridae family. Influenza A and B are human pathogens, and are subtyped by the major surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA). The segmented genome of influenza allows for gene segment exchange between two viruses that have coinfecting a cell, such as between an avian influenza and circulating human influenza strains, resulting in antigenic shift in HA and NA proteins and viral evasion of the host immune system. Three pandemics over the last 100 years have resulted from antigenic shift. The most well-known and severe of these was the 1918 Spanish influenza pandemic, responsible for an estimated 40 million deaths (2). The 1918 pandemic virus was shown to be similar to swine H1N1 viruses (17, 18) and may have originated as an avian influenza virus that gained the ability to transfer to humans through adaptive mutations. Later pandemics include those of 1957 (H2N2), 1968 (H3N2), and 2009 (H1N1). The 1957 and 1968 pandemics resulted from the incorporation of avian influenza virus genes into circulating human influenza viruses by reassortment, thus resulting in the pandemic viruses gaining novel surface antigens (19).

All subtypes of influenza A circulate in aquatic birds, and avian subtypes H5N1, H7N7, H7N9, and H9N2 are able to directly infect humans (20). The H5N1 subtype, which has 10 geographically distinct phylogenetic clades, is of greater concern than others because of high pathogenicity and demonstrated cross-species infection (World Health Organization), and although efficient human-to-human transmission of this viral subtype has not yet been demonstrated, this could be possible through mutation accumulation or reassortment with other subtypes (21).

An effective vaccine against H5N1 is complicated by a number of factors. A high level of virulence complicates traditional vaccine production because wild-type viruses are not capable of growing to high titer in chicken eggs, the traditional vessel for growing and harvesting viruses for vaccine production, and the level of pathogenicity mandates animal biosafety level 3 (ABSL-3) manufacturing facilities. H5N1 HA is also poorly immunogenic, requiring larger doses than the seasonal vaccine and inclusion of an adjuvant. Finally, there is poor cross-reactivity between clades, resulting in reduced potential for broadly protective antibodies generated from immunization.

To vaccinate against multiple influenza subtypes circulating within the human population, a polyvalent formulation, in which multiple antigens are mixed into a single formulation, is currently employed to broaden the reactivity of seasonal influenza vaccines. However, the breadth of immunity stimulated by immunization is limited to only those components included in the formulation. New-generation vaccine formulation uses sequencing efforts to capture multiple antigenic features, which are not naturally occurring, in a single molecule. Computational methods for generating centralized (synthetic) sequences for antigen design include (1) using the most recent common ancestor (“ancestral state” method), (2) using the phylogenetic point equidistant from all input sequences (“center of the tree”), and (3) consensus, which uses the most common amino acid at each position of the sequence (22). All of these methods are advantageous for reducing the average distance between a vaccine antigen and proteins from circulating viral strains (22). However, consensus-based vaccine design is limited to input sequences and geographic predominance of certain isolates, which in turn, may bias the output sequences generated by this design method. A novel method of antigen design, termed computationally optimized broadly reactive antigen (COBRA) strategies (23, 24), uses multiple rounds of consensus generation to overcome limitations of consensus-based design. The COBRA method aligns amino acid sequences from clade 2 human isolates and assembles a layered consensus, using global surveillance efforts, to produce a vaccine with enhanced potential for generating more diverse antibody responses (23, 24).

COBRA HA antigen has been expressed on virus-like particles (VLPs). These particles have a number of advantages when used in vaccine design: they are self-assembling, nonpathogenic, and produced in a variety of eukaryotic expression systems; they ensure correct three-dimensional configuration of HA and NA proteins; and VLPs have already been approved for use in a vaccination against human papillomavirus (25). COBRA HA VLPs have been used in immunization studies with mice, ferrets (23, 26), and cynomolgus macaques (27). Immunization with COBRA HA VLPs resulted in protective levels of HA-blocking antibody titers in mice and ferrets, and

VLP-immunized animals were protected from lethal challenge with H5N1 virus (23). Furthermore, the single COBRA HA antigen generated broader antibody responses and reduced morbidity and viral titers in immunized mice, compared with a polyvalent formulation of H5N1 HA antigens (26). The first nonhuman primate model for a clade 2 H5N1 influenza virus has also been reported, and protection induced by the COBRA VLP vaccine was examined (27). In cynomolgus macaques, COBRA VLP immunization elicited substantially greater breadth of HA-blocking antibodies, compared with a VLP with the HA from a single clade 2.2 isolate, and significantly reduced histopathology after influenza challenge (27). This vaccine elicited not only broadly reactive HA-blocking antibodies against clade 2 viruses, but also against multiple H5N1 clades (27). These studies strongly support the usefulness of COBRA methodology for the design of viable vaccine candidates.

The ultimate goal of preclinical models of influenza is to develop cross-reactive vaccines, improve methods of vaccine production, and elucidate improved ways to address current needs. Predicting appropriate seasonal influenza strains, to produce effective vaccines, presents a number of challenges, but these difficulties are compounded in the context of developing an effective vaccine for a highly pathogenic avian influenza, such as H5N1. Current H5N1 vaccine candidates lack cross-clade neutralization ability, although they are able to elicit neutralizing responses to viruses within a single clade. The COBRA method is designed to include the most common antigenic characteristics, while avoiding the sampling bias of consensus-based vaccine design. Vaccines produced from COBRA-based design address a number of challenges to influenza vaccine development, are safe and immunogenic in murine and nonhuman primate (NHP) models of infection, protect from H5N1 challenge, and elicit broadly reactive HA-blocking and neutralizing antibodies.

Understanding the Role of IFN- γ in an Infant Mouse Model of Respiratory Syncytial Virus

Respiratory syncytial virus (RSV) is the primary cause of infant bronchiolitis, the most

widespread cause of pediatric viral respiratory infections in the United States and the most frequent cause of viral death in infants worldwide. Disease is most severe in infants 2–4 months of age (28), and RSV accounts for nine times more deaths than influenza in children under the age of 1 year (29), yet there is no vaccine and no effective treatment.

The pathogenesis of RSV disease is not well understood. Premature birth, low birth weight, and cardiac disease are known risk for severe RSV infection; however, the majority of children that go on to develop severe disease are otherwise healthy. However, it remains unclear why most previously healthy children resolve RSV infection with little difficulty whereas others progress to severe disease. In rodent models of RSV infection, T-cell responses determine clearance and injury (30), and IFN- γ production enhances RSV clearance (31). In clinical studies, it has been described that IFN- γ production in the lung is impaired in infants with RSV bronchiolitis (32), and severe disease is characterized by inadequate adaptive immune responses and robust viral replication (33). In rodent models, administration of recombinant IFN- γ during primary RSV infection results in significantly lower lung viral loads and

protection against subsequent airway hyperresponsiveness and lung pathology (31). Similar to human infants with severe RSV infection, RSV-infected infant BALB/c mice fail to produce significant levels of IFN- γ (34), which is important for promoting CD4⁺ helper T-cell type 1 responses that have been implicated in improved clinical outcomes.

Although it is accepted that T cells are important for the control and clearance of RSV, less is understood about the innate immune responses to infection. It has been shown in both lung tissue specimens from human infants, as well as in a rodent model of RSV infection, that alveolar macrophages may be important in determining disease severity (35). In rodents with macrophage deficiencies, viral loads were increased and disease was more severe (35). Interferons facilitate destruction of infected cells; clearance of cellular debris is then completed by macrophages. In a mouse model of RSV infection, classically activated (M1) or proinflammatory macrophages, defined by MHC class II expression, and alternatively activated (M2) or reparative macrophages defined by mannose receptor (MR) expression, were examined for kinetics of age-dependent activation (Figure 1).

Figure 1. Neonatal BALB/cJ mice express an immature, age-dependent alveolar macrophage phenotype after respiratory syncytial virus (RSV) infection. Adult and pup BALB/cJ mice were infected with high-dose/high-volume RSV line 19 or with cell lysate. Cells were isolated from pup and adult bronchoalveolar lavage fluid on Days 0, 2, 4, 7, and 10 postinfection. The percentages of immune cell subtypes were analyzed by flow cytometry in (A and B) adults and (C and D) pups, including (A and C) MHC II and (B and D) mannose receptor (MR) on the CD11b⁺CD11c⁺ high gate. Mean values $\pm$ SD are depicted, and a significant difference was defined as a *P* value less than 0.05 for differences from mock-infected animals at the same time point (*); data are representative of two separate experiments. Reprinted by permission from Reference 34.

Neonatal mice exhibit delayed activation of classically activated (M1) macrophages, likely a result of insufficient IFN- γ production (Figure 1) (34), but this can be improved by administration of inhaled IFN- γ (Figure 2) (34). Furthermore, IFN- γ administration expedites RSV clearance in the airways of infant mice (Figure 3) (34). The current study expanded on previous work to examine the dose response to IFN- γ of infant mice infected with RSV line 19. In these studies, a high dose of inhaled IFN- γ induced an earlier but not greater activation of macrophages, while CD8⁺ T-cell infiltration remained impaired compared with age-matched controls. Compared with mice treated with low-dose IFN- γ , infant mice treated with high-dose IFN- γ failed to gain weight or significantly clear virus. These studies

further showed that low-dose IFN- γ was sufficient to reduce apoptosis and excess mucus production. Although all infected groups were reactive to methacholine challenge after secondary infection with RSV line 19, infant mice treated with high-dose IFN- γ during primary infection had significantly greater airway hyperresponsiveness on secondary challenge 6 weeks later compared with untreated infant mice (K. M. Empey, unpublished data).

A better understanding of age-dependent macrophage development and polarization is needed to explore the potential for alveolar macrophages as a vaccine target for neonatal RSV disease. The work by Empey and colleagues suggests that IFN- γ administration may be a viable treatment option, and these studies are

contributing to a better understanding of RSV disease progression and pathogenesis, using an infant mouse model of RSV disease to characterize the immune response to infection and to explore ways in which it can be improved.

Tuberculosis: Think Global, Act Local

Person-to-person transmission of *Mycobacterium tuberculosis* (Mtb), the etiologic agent of tuberculosis (TB), occurs when aerosolized droplets from a person with active disease are inhaled into the airways. Despite the fact that only 5–10% of those infected will develop active disease within 2 years of exposure, Mtb infections constitute a major global health problem, with approximately 9 million new cases and 1.8 million deaths from the disease in 2008 (36). The remaining 90–95% of Mtb-infected individuals develop latent infection, which is defined as immunologic evidence of infection, but without clinical signs of disease. These latently TB-infected persons, an estimated 2 billion worldwide, constitute the largest reservoir for potential reactivation to active disease and transmission. The majority of latent infections will never result in active disease, but the risk for reactivation (development of active disease from a latent infection) is greatly increased in cases of immunodeficiency, such as in HIV infection. From a clinical perspective, Mtb infection results only in a bimodal distribution of outcomes, either latent or active TB, which are defined on the basis of clinical parameters. However, there is increasing evidence to suggest this is an oversimplification of TB, which instead likely results in a spectrum of outcomes, with various degrees of severity of active disease, but also a variety of classifications of latency. It is assumed, although not proven, that bacterial burden increases on the spectrum from latent to active TB, and risk of reactivation likely increases with increased bacterial burden, as the pro- and antiinflammatory balance shifts in the host immune system (37, 38).

Establishment of infection begins when Mtb enters the host airway, where the bacteria may be phagocytosed by alveolar macrophages and resident dendritic cells (DCs). This interaction may result in clearance of the infection, although

Figure 2. Inhaled recombinant IFN- γ F020 (rIFN- γ) increases classically activated (M1) or proinflammatory macrophages in respiratory syncytial virus (RSV)-infected neonatal BALB/cJ mice. Pups (2- to 4-d-old BALB/cJ mice) received a high-dose/high-volume inoculum of RSV line 19 followed by a 16-ng/g concentration of intranasal rIFN- γ (RSV/rIFN- γ ⁺) or diluent only (RSV/rIFN- γ ⁻) on Days 1, 3, 5, and 7 postinfection. Control groups were mock infected with cell lysate followed by a 16-ng/g concentration of intranasal rIFN- γ (mock/rIFN- γ ⁺) or diluent only (mock/rIFN- γ ⁻) on Days 1, 3, 5, and 7 postinfection. Cells were isolated from bronchoalveolar lavage fluid on Days 4, 7, and 10 postinfection, and the percentage CD11b⁻CD11c⁺ (nonlymphocyte gate), and (A) MHC II and (B) mannose receptor (MR) (gated on CD11b⁻CD11c⁺ cells) were determined by flow cytometry. Points represent data for five mice per group $\pm$ SD. [†] P < 0.05 for comparisons between RSV/rIFN- γ ⁺ and RSV/rIFN- γ ⁻; [‡] P < 0.05 for comparisons between mock/rIFN- γ ⁺ and mock/rIFN- γ ⁻; * P < 0.05 for comparisons between RSV/rIFN- γ ⁺ and mock/rIFN- γ ⁺. Reprinted by permission from Reference 34.

Figure 3. Inhaled recombinant IFN- γ F020 (rIFN- γ) reduces viral load in respiratory syncytial virus (RSV)-infected neonatal BALB/cJ mice. On Days 1, 3, 5, and 7 postinfection pups received a 16-ng/g concentration of intranasal rIFN- γ or diluent only. (A) IFN- γ was measured from bronchoalveolar lavage fluid by Luminex assay. Viral titers were measured from left lung lobes by hematoxylin-eosin plaque assay on Days 2, 3, 4, 5, and 7 postinfection. (B) RSV titers were analyzed by two-way analysis of variance and graphically represented with a line graph. Mean values $\pm$ SD are depicted, and a significant difference was defined as a P value less than 0.05 for differences from mock-infected animals at the same time point (*); data are representative of two separate experiments. Reprinted by permission from Reference 34.

factors that determine whether acute infection is controlled or latent infection is established are not well understood. If acute infection is not controlled by the innate immune response, TB-infected macrophages may traffic to the lung parenchyma, spreading the bacteria. Resident DCs carry bacteria and bacterial antigens to draining lymph nodes, where they prime T cells to respond to the infection. T cells and macrophages are then recruited to the primary site of infection (lung), where they participate in granuloma formation, attempting to control the spread of Mtb. The granuloma is a collection of inflammatory cells thought to form an immunologic and physical barrier to contain Mtb infection, preventing dissemination of the bacteria. If the granuloma is effective at controlling bacterial replication, fibrosis may result (latent infection), and disease is either controlled for the lifetime of the host, or active disease develops from

reactivation, perhaps as a result of immunosuppression.

The NHP model of TB infection closely resembles human TB, making it a tremendously useful model for examining the pathogenesis and immunology of infection, providing an opportunity to better characterize latent TB, as well as for testing vaccine and chemotherapeutic candidates. Importantly, all clinical aspects of human TB have been observed in a macaque model of Mtb infection (39). In cynomolgus macaques, low-dose challenge with Mtb results in clinically active TB in approximately 40% of animals and latent infection in approximately 50% of animals (38–42). In addition, a small percentage (approximately 5%) of Mtb-infected macaques develop subclinical disease, in which animals are clinically asymptomatic and similar to latently infected macaques, but are occasionally positive for Mtb growth in airway samples, such as

bronchoalveolar lavage (42). Clinical parameters of Mtb infection of macaques have been validated by gross pathology and bacterial burden at necropsy (42) and compared with latently infected macaques; animals with active TB had a significantly higher bacterial burden, higher levels of IFN- γ production, and greater numbers of lung T cells (42). Within a single animal, either latently or actively infected, a spectrum of granulomas can be observed, suggesting a dynamic process. These observations have important implications for successful drug treatment of TB, because there is evidence to suggest that specific types of lesions may be associated with poor treatment outcomes in patients (43).

To further characterize TB disease at the granuloma level, and to better understand the variable responses to drug treatment, a method of TB lesion visualization that uses high-resolution micro-positron emission tomography (PET) with computed tomography (CT) scanning has been described (44). Serial imaging, using 2-deoxy-2-[¹⁸F]fluoro-D-glucose (FDG), a glucose analog, as the PET probe, has been performed on cynomolgus macaques during infection and to assess the efficacy of chemotherapeutic agents. Metabolically active cells, such as inflammatory cells, take up FDG, and when PET imaging is used in combination with CT scanning, anatomic localization of areas as small as 1 mm exhibiting elevated FDG uptake can be visualized. Using PET-CT, researchers are able to map TB lesions, track establishment and progression of TB infection (Figure 4), and importantly, detect resolution in response to chemotherapeutics, such as rifampicin (44). This method of evaluating individual granulomas will increase our understanding of the contributions of the immune microenvironment to either sterilization or progression to active disease. From these studies, it was also shown that granulomas are dynamic and independent from each other (even within the same animal) and respond differently to different chemotherapeutics. Data acquired from PET and CT imaging are capable of predicting successful drug treatment, which has been validated by assessing bacterial burden (44).

Further investigation of TB at the individual granuloma level has been accomplished by using individually tagged

Figure 4. Lung granulomas are dynamic and independent. (A) Serial views of two granulomas in the right lower lobe (RLL) with independent changes in size and metabolic activity (yellow brightness) during early *Mycobacterium tuberculosis* infection. (B) Granuloma size (based on the relative size of each sphere) and metabolic activity (standardized uptake values [SUV]; y axis) are shown over time postinfection (x axis). Granulomas were from the same animal whose lung granulomas are depicted in (A). Granuloma RLL 2 (purple) peaks in metabolic activity at 6 weeks postinfection, whereas granuloma RLL 1 (blue) peaks at 18 weeks postinfection. The granuloma in red increases dramatically at 18 weeks postinfection, unlike RLL 1 and RLL 2. Reprinted by permission from Reference 44.

Mtb isolates to better define lesion dynamics after Mtb infection of macaques (Figure 5) (45). Macaques were infected with a low dose of the inoculum and lesions were temporally monitored by FDG PET-CT. After necropsy, the relative abundance of each tagged strain was quantified in individual granulomas. From these studies, it appears that nearly all individual granulomas start with a single bacterium, and the variability in lesion dynamics/progress starts before 4 weeks after Mtb infection. Even in animals with active disease, bacterial numbers decrease in individual granulomas after 4 weeks of TB infection; however, chromosomal equivalents, a way of determining cumulative bacterial burden (46), are stable over time regardless of TB status (latent vs. active). Furthermore, even in active TB disease, there is an increase in the

numbers of sterilized granulomas over time, although this increase is greater in animals with latent infection (45). It also seems that bacterial killing increases as adaptive immunity is induced, with the exception of areas of severe pathology, leading researchers to the conclusion that animals developing active disease are able to kill bacteria at some sites of infection. Together these data suggest that differences in bacterial burden may be largely driven by killing efficacy, which is variable among lesions even in the same animal, and animals with active disease have a similar capacity for bacterial killing as animals with latent disease, at the individual granuloma level (45).

The NHP model of TB infection provides researchers with a unique opportunity to study the host immune responses and pathogenesis of Mtb, and is

also a useful tool for evaluating the efficacy of chemotherapeutic intervention and prophylactic immunization. Furthermore, it has illustrated the extensive variability of latent TB infection, which ultimately will help researchers to understand disparities in efficacy of various therapeutics. Modern imaging techniques, such as PET-CT, can be used in combination with this model to better characterize the dynamics of individual granulomas, which likely will aid in the design of more effective drug regimens. These studies suggest that individual granulomas are dynamic and independent from each other, thus resisting oversimplified classifications such as “active” or “latent.” Finally, even animals with active disease retain the capacity to kill Mtb at some sites of infection, suggesting a retained capability for sterilization or stabilization. Together these data contribute to a better understanding of the spectrum of TB disease, and will help in reducing the global health burden of TB.

TB Vaccines: Where Are We?

TB continues to be a major threat to human health worldwide. It is estimated that one-third of the world's population is infected with *Mycobacterium tuberculosis* (Mtb), the etiologic agent of TB. According to the World Health Organization (47), 8.6 million people developed TB and 1.3 million died of the disease in 2012 (47). Included in these were 450,000 persons who developed multidrug-resistant TB. Despite the fact that Mtb has been studied for more than a century, an effective vaccine remains to be developed. Furthermore, we still lack knowledge regarding immune correlates of protection and reliable predictors of disease progression or reactivation.

Developing an effective vaccine for TB remains an ongoing challenge for researchers. bacillus Calmette-Guérin (BCG) vaccine, an attenuated form of *Mycobacterium bovis*, was developed in 1921, and although evidence suggests that BCG protects against disseminated disease, the vaccine does not appear to be substantially affecting the global burden of TB. The BCG vaccine is typically given at or close to birth; however, protection by BCG administration to newborns is unreliable against adult pulmonary TB, which accounts for most Mtb transmission

Figure 5. (a) 2-Deoxy-2- ^{18}F fluoro-D-glucose (^{18}F FDG) positron emission tomography (PET) with computed tomography (CT) images of progressing and regressing lesions shown from the same animal, which developed active disease after low-dose infection (30, 32, 36 and 40 wk after infection). The images show a resolving lesion in the right upper lobe (*top*, *solid arrows*) at the same time that new lesions appear (*top*, *dashed arrow*). In the right lower lobe (*bottom*), lesions progress (*dashed arrows*) and new lesions coalesce to form a consolidation (*circles*). (b) ^{18}F FDG avidity (standardized uptake value ratio [SUVR]) is plotted against log(CFU) (Spearman's ρ , 0.4431). A linear regression model of SUVR versus CFU is shown (slope, 0.058 ± 0.0097 ; $P < 0.001$). Dots represent individual lesions ($n = 274$) from monkeys with active disease (*green*, $n = 15$) or latent infection (*blue*, $n = 10$). Reprinted by permission from Reference 45.

and disease burden, and it is also not known to protect against latent or reactivated TB.

Lack of BCG efficacy may be affected by background environmental mycobacteria exposure, or even the genetic variability of Mtb, of which there are six major strains, and the largest differences between strains arise from intercountry variability (48). Although historically, TB caused as many as half of all human deaths in North America and Europe, today the disease primarily burdens the rest of the world, including the BRICS countries (Brazil, Russia, India, China, and South Africa, five major emerging national economies). HIV/AIDS has also dramatically impacted TB incidence, especially in sub-Saharan Africa, and concern has arisen because of the global emergence of multidrug resistance to the pathogen (49).

Developing an effective TB vaccine is complicated by the lack of clear correlates of protection or immunity, the large expense of vaccine trials, and the diversity of human populations and environmental factors that may necessitate multiple vaccines. There are three major strategies for control of TB via immunization: (1) a preinfection vaccine for prevention, such as improved priming and/or novel booster vaccines; (2) a postinfection vaccine to prevent reactivation, aimed at extending and enhancing immune protection; and (3) an immunotherapeutic vaccine, which

may shorten the course of chemotherapy for active TB or improve the efficacy of multidrug-resistant TB treatment. According to mathematical models of vaccine strategies, even a vaccine with 50% efficacy could greatly reduce the burden of TB in highly endemic areas, especially when used in combination with drug treatment (50).

At present, there are 12 different antigens for candidate TB vaccines in clinical trials, with the largest number of candidates in phase 1/2a trials. However, interpretation of vaccine trial data has been complicated by varying subject populations and diagnostics and the struggle to define appropriate end points. The first large safety and efficacy trial (phase 2b) of a TB vaccine monitored nearly 2,800 infants in South Africa for up to 37 months (51). The vaccine, a modified vaccinia virus Ankara (MVA) encoding a single immunodominant antigen (MVA85A), induced low-level antigen-specific CD4⁺ T-cell activation and IFN- γ production. The primary objective of the trial was to assess the safety of the vaccine, but disappointingly, researchers showed little efficacy regarding either protection from development of TB or primary infection with Mtb.

Several studies are exploring novel antigens, nontraditional conjugates, immunomodulators, and novel delivery systems. Researchers have demonstrated

that immunization of macaques with an aerosolized adenoviral vector (rAd35) encoding Mtb antigens was highly immunogenic, inducing Mtb-specific CD4⁺ and CD8⁺ T-cell responses in the lungs of immunized animals (M. Roederer, personal communication). Spray-drying of an adenovirus (Ad35)-vectored TB vaccine, resulting in a dry powder formulation, has been shown to enhance stability of the viral vector, allowing for a greater range of storage conditions of the vaccine (52). Preclinical data support the potential of the aerosolized route of vaccination; however, technical and safety issues need to be addressed in NHPs to determine whether this is a viable candidate for vaccination.

There are a number of unanswered questions about the host immune response to TB infection and correlates of protection and disease progression, as well as a need for reliable biomarkers and end-point surrogate(s) that can substitute for clinical effects or immunological response surrogates, such as T-cell assays and *in vitro* mycobacterial growth inhibition assays. Even amid the challenges faced by researchers for developing an effective TB vaccine, there are reasons for optimism. There is evidence of BCG vaccine efficacy in children (53–56), and new TB vaccine candidates are being shown to be protective in animal models, including nonhuman primates. Novel TB vaccines boost cellular

immune responses in multiple clinical studies, and researchers now have more informative clinical clues to guide immunologic hypotheses. Ultimately, to effectively assess vaccine candidates, refinement and validation of the current systems are needed. There are now sufficient data to better understand the advantages and disadvantages of various patient populations and to establish more defined end points during vaccine trials.

Conclusions/Summary

Despite the current challenges to antibiotic and vaccine development, there are reasons for optimism. Antibiotic resistance is a growing threat to human health, but researchers are exploring novel microbial culture methods (9); new ways of targeting bacterial virulence factors (10, 11); and novel therapies, such as photodynamic therapy (12, 13, 16), for eliminating bacteria. Novel methods of consensus-based vaccine design have resulted in enhanced breadth of immune responses generated by immunization against highly pathogenic forms of influenza (23, 27). There are a greater variety and number of tuberculosis vaccines in development than ever before and improved animal models for evaluating their efficacy. Furthermore, because of NHP models

of tuberculosis infection, we can better understand latency and progression to active disease (38, 42, 44), and the immunological parameters that contribute to disease progression or protection. Longitudinal disease monitoring using the NHP model of tuberculosis, in combination with modern imaging techniques (44), has greatly enhanced our understanding of tuberculosis lesions and ultimately may lead to improved chemotherapeutics. Improved models of RSV infection are expected to elucidate novel treatments (34) and the potential for vaccine development, through better understanding of the infant immune response to RSV infection and how it can be improved.

Technological advances, as well as advances in microbiology and immunology, are paving the way for improved vaccine and chemotherapeutic efficacy. Traditionally, vaccines have relied on immunologic mimicry, building on observations from nature, for success. However, when correlates of protection are unclear or when microbes evade the host immune system, effective vaccines must go beyond imitating natural immune responses (57). Future successful antimicrobials will likewise go beyond traditional drugs for eliminating bacterial infections, and will depend on creative strategies using nontraditional or unusual therapies and

novel targets for inducing bacterial cytotoxicity. Enhanced understanding of human immunology, host-pathogen interactions, and the microbes themselves will allow researchers to identify and validate targets, to expand the current repository of vaccines and chemotherapeutics. Of particular consideration in vaccine and drug design should also be the immunologically vulnerable populations, including infants, the elderly, and those with immunosuppressive conditions. To face the challenges of changing human population demographics, an ever-evolving microbial world, and emerging diseases, and to ensure the continued success of modern medicine began by Edward Jenner, Alexander Fleming, and Howard Florey, we must focus on asking and addressing appropriate scientific questions, conducting informed surveillance for improved preparedness for emerging threats to human health, and strive for effective collaborations between public and private entities, thereby strengthening the infrastructure of the vaccine and chemotherapeutic development pipeline. ■

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