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Resuscitation-promoting factors are important determinants of the pathophysiology in *Mycobacterium tuberculosis* infection

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**ABSTRACT**

Resuscitation promoting factors (Rpf) are peptidoglycan-hydrolyzing enzymes that are pivotal in the resuscitation of quiescent actinobacteria including *Mycobacterium tuberculosis*. From the published data, it is clear that Rpf are required for the resuscitation of non-replicating bacilli and pathogenesis in murine infection model of tuberculosis, although their direct influence on human *Mycobacterium tuberculosis* infection is ill-defined. In this review, we describe the progress in the understanding of the roles that Rpf play in human tuberculosis pathogenesis and importance of bacilli dependent upon Rpf for growth for the outcome of human tuberculosis. We outline how this research is opening up important opportunities for the diagnosis, treatment and prevention of human disease, progress in which is essential to attain the ultimate goal of tuberculosis eradication.

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**Introduction**

Tuberculosis is a major global public health concern with an estimated 10.4 million incident cases and 1.4 million deaths in 2015 (WHO, 2016). Recent mathematical modeling predicted that as of 2014, 1.7 billion people were latently infected with *Mycobacterium tuberculosis* (Mtbb) (Houben & Dodd, 2016). Mtbb is transmitted primarily by respiratory droplet inhalation (Turner & Bothamley, 2015) and upon reaching the alveoli is phagocytosed by macrophages, neutrophils and dendritic cells (DCs) (Dorhoi & Kaufmann, 2014; Eum et al., 2010). Despite internalization, Mtbb can continue to replicate intracellularly (Ahmad, 2011).

Protective immunity to Mtbb requires a fine balance of innate and adaptive responses (Nunes-Alves et al., 2014). After initial infection, myeloid cells sense Mtbb through cell surface receptors and facilitate T-helper\textsubscript{1} (TH\textsubscript{1}) and T-helper\textsubscript{17} (TH\textsubscript{17}) differentiation (Dorhoi & Kaufmann, 2014). TH\textsubscript{1} cells promote inflammatory cell recruitment and enhance Mtbb killing by macrophages (Nunes-Alves et al., 2014). TH\textsubscript{17} cells are also important for protective immunity to Mtbb, particularly in the early stages of infection (Gopal et al., 2014; Prezzemolo et al., 2014; van de Veerdonk et al., 2010). Macrophages and T-cells are key to granuloma formation, which ultimately controls primary infection but in 90% of cases fails to kill Mtbb and provides a site for bacilli to latently infect the host (Ahmad, 2010; Dorhoi & Kaufmann, 2014; Ramakrishnan, 2012). At present, the physiological status of these bacteria is unknown, however it is hypothesized that conditions within granulomas may vary leading to heterogeneous populations consisting of replicating and non-replicating cells (Gideon & Flynn, 2011; Zhang et al., 2010). Moreover, differential killing of Mtbb at the onset of adaptive immunity may lead to the formation of heterogeneous lesions, some of which may be sterilized and others may progress to a maximum Mtbb burden (Lin et al., 2014). The precise fate of each lesion is probably influenced by multiple factors including the local environment, the physiological state of Mtbb and the interplay of host immune factors.

In clinical medicine, latent tuberculosis infection (LTBI) is seen as a homogenous entity, characterized by evidence of immune sensitization to mycobacterial antigens with the absence of clinical and radiological signs of disease (Barry et al., 2009). The prevailing view, however, is that LTBI is best viewed as a range of infection states extending from infection eradicated by the host immune response to subclinical active infection, at particularly high risk of reactivation (Esmail et al., 2014). It is thought that bacillary replication is curtailed by the host immune response, however, if this balance is...
perturbed by factors such as immunosuppression due to human immunodeficiency virus (HIV) infection or anti-tumor necrosis factor therapy, bacillary replication resumes leading to reactivation and clinical disease (Paige & Bishai, 2010). The mechanisms by which bacterial growth restarts are not yet resolved, although it presumed to involve cell wall alterations mediated by enzymes termed resuscitation promoting factors (Rpf) (Kana & Mizrahi, 2010).

Rpf are a family of bacterial proteins, able to resuscitate non-replicating bacteria, was discovered in Micrococcus luteus (Mukamolova et al., 1998, 2002). Mtb possesses five rpf-like genes (rpf A–E) whose products exhibit similar biological activity to the Rpf from M. luteus (Mukamolova et al., 2002). The mechanism through which Rpf resuscitate “quiescent” Mtb is poorly understood although their enzymatic activity is essential for biological effects (Cohen-Gonsaud et al., 2005; Mukamolova et al., 2006) suggesting direct cell wall remodeling or muropeptide-mediated signaling (Kana & Mizrahi, 2010; Kaprelyants et al., 2012). The importance of Rpf in Mtb infection and the reactivation of chronic TB has been shown in mice models and there is a growing body of evidence that Rpf play a central part in human tuberculosis (see Figure 1). In this review, we will discuss the current understanding of the role Rpf play in Mtb infection and how this knowledge may be applied to diagnose, treat, and prevent Mtb infection.

**Rpf are critical for Mtb replication and reactivation in vivo**

Rpf can be collectively deleted without significantly impairing Mtb growth in standard 7H9 medium (Kana et al., 2008), although this does not preclude a significant role in bacillary replication. Two studies characterizing the in vitro phenotype of rpf-deletion mutants in mononuclear phagocyte infection models produced discordant results. Russell-Goldman et al. demonstrated a ΔrpfAB (rpfA and rpfB deletion) mutant was attenuated for growth in murine macrophages (Russell-Goldman et al., 2008) whereas Kana et al. showed Mtb quadruple rpf deletion mutants were unimpaired for growth within human monocytes (Kana et al., 2008). Differences in the experimental set up between the studies may account for the discordant results. When the in vivo phenotype of rpf-deletion mutants are investigated in the mouse infection model, it is clear Rpf are important in the pathogenesis of infection (Kana & Mizrahi, 2010).

In mice, infection of the respiratory tract is characterized by an initial exponential growth phase lasting several weeks. The subsequent transition to chronic persistent infection is marked by the development of cell-mediated immunity which stabilizes bacillary replication (Hu et al., 2015; Munoz-Elias et al., 2005). Throughout this period, the expression of rpfA–rpfE can be detected (Kesavan et al., 2009; Tufariello et al., 2006). Although the precise role of Rpf is unclear, the study of rpf deletion mutants underscores the importance of Rpf in the initial stages of infection. When single rpf genes are deleted, there is no impact on the course of acute infection or the immunopathology witnessed, perhaps indicating physiological redundancy (Gupta & Srivastava, 2012). However, further deletion of rpf genes reduces Mtb virulence which manifests as reduced growth and dissemination from the site of primary infection (Biketov et al., 2007; Kana et al., 2008; Kondratieva et al., 2011; Russell-Goldman et al., 2008). Ultimately, the mortality of mice infected with

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**Figure 1.** The proposed role of Rpf and Rpf-dependent bacilli in Mtb infection and how Rpf may be clinically exploited to improve Mtb management and prevention.
attenuated rpf deletion mutants is lower (Kondratieva et al., 2011; Russell-Goldman et al., 2008).

Interestingly, when individual rpf gene(s) are deleted, the degree of attenuation varies implying Rpf are functionally distinct. In one study of mice infected with rpf double deletion mutants via the respiratory tract, only the ΔrpfAB mutant showed lower lung and spleen colony forming units (CFUs) versus the wild type Mtb strain (Russell-Goldman et al., 2008). This contrasts with an intraperitoneal model of infection where ΔrpfAC was more attenuated for growth than ΔrpfAB although differences in host, strain or experimental conditions may account for this (Biketov et al., 2007). With triple rpf deletion mutants, the loss of rpfB from a ΔrpfAC mutant was more attenuating for growth in the mouse lung than rpfD loss (Downing et al., 2005).

In chronic persistent infection, the ability of Mtb to maintain low-level replication also varies by rpf deletion. Single rpf deletion mutants do not exhibit persistence defects, however with the progressive deletion of rpf genes, replication is impaired. These mutants require more time to establish chronic infection and replicate less compared to the wild type background strain (Biketov et al., 2007; Kana et al., 2008; Russell-Goldman et al., 2008; Tufariello et al., 2006). Notably, the effect of individual rpf deletions varies with the loss of rpfD from the mutant ΔrpfACB less impactful than rpfE (Kana et al., 2008).

The diminished replication exhibited by rpf mutants during acute and chronic persistent infection has been attributed to cell wall changes (Russell-Goldman et al., 2008). In vitro studies demonstrate mutants are less resilient to stress as shown by hypersensitivity to the surfactant sodium dodecylsulfate and increased susceptibility to antibiotics (Heidrich et al., 2002; Kana et al., 2008; Wivagg & Hung, 2012). Putatively, deficiency of Rpf may alter cell wall structure, so increasing sensitivity to stresses such as those imposed by the host immune response. It may be that Rpf enable Mtb to respond to adverse conditions in vivo although the precise mechanisms and roles remain unclear.

The reactivation of LTBI can be modeled by the administration of aminoguanidine, an inducible nitric oxide synthase inhibitor, to mice with chronic persistent Mtb infection (Flynn et al., 1998). During the recrudescence of infection, the expression of rpfA–rpfE is detectable (Tufariello et al., 2004). Aside from rpfC, it has been shown that rpf expression mirrors the level of bacillary replication (Tufariello et al., 2006). When mice are treated with Rpf inhibitors, recrudescence is suppressed but active replication is unaffected suggesting Rpf are important for the initiation of Mtb replication (Kaprelyants et al., 2012). This hypothesis is supported by the impact of rpf deletion upon reactivation growth kinetics. When single rpf genes are deleted, only rpfB deficiency delays recrudescence (Tufariello et al., 2006). Progressive rpf deletion reduces the rise in CFU counts with immunosuppression with triple rpf deletion mutants exhibiting no rise at all (Biketov et al., 2007; Russell-Goldman et al., 2008).

When the available evidence is summarized, it suggests Rpf are significant virulence factors with roles in bacillary replication during acute disease and reactivation of chronic persistent infection. At this moment, the exact function and regulation of individual Rpf during infection is unknown and awaits further investigation.

**Rpf generate protective immunity**

As Rpf are secreted or cell wall attached proteins, they are potential targets for the host immune system (Commandeur et al., 2013; Kana & Mizrahi, 2010; Yeremeev et al., 2003). As previously mentioned, Rpf are key virulence factors for Mtb (Kana et al., 2008), thus responses directed to Rpf may be protective. These have been assessed both in cellular assays and mouse models.

In vitro studies have concentrated on the immune responses to RpfB and RpfE proteins. Both induce the maturation of DCs via the Toll-like receptor 4 (Choi et al., 2015; Kim et al., 2013). Subsequently, activated DCs produce IL-12p70, which polarizes T-cell proliferation toward TH1 phenotype (Choi et al., 2015; Kim et al., 2013). RpfE also induces DC IL-23p19 secretion leading to TH17 development, which in addition to TH1 is required for optimal protection against Mtb (Gopal et al., 2014). The induction of IL-23p17 secretion by RpfB was not tested. Of note, RpfE does not induce antigen reactive T regulatory cells, which can suppress T-cell proliferation in response to Mtb antigens by IL-10 production (Choi et al., 2015). These findings show RpfB and RpfE induce what are considered to be protective immune responses in vitro and support their role as potential vaccine candidates (Choi et al., 2015; Kim et al., 2013).

The character of the immune response generated in vivo by Rpf antigens and their protective efficacy against challenge with virulent Mtb has been assessed in mouse models. The studies have taken different approaches to vaccination focusing predominantly upon RpfB and RpfE. To date immunization with RpfB has been performed using a plasmid DNA (pDNA) vector approach. When C57BL/6 mice were vaccinated intramuscularly, a significant polyfunctional T-cell response, particularly in CD8+ subsets was measured, suggesting the vaccine may be protective (Lee et al., 2011; Wivagg & Hung, 2012).
2014). Administration of a similar rpfB pDNA vaccine via the intramuscular route offered some protection against challenge with virulent Mtb, as shown by a moderate reduction in pulmonary and splenic burden compared to the control group. However, the vaccine was less efficacious than the Mycobacterium bovis bacillus Calmette-Guérin (BCG) vaccine (Romano et al., 2012). At 10 weeks post infection, the rpfB pDNA vaccine offered no protection possibly related to reduced rpfB expression hampering the protective action of the RpfB-specific T-cells (Romano et al., 2012).

Different vaccine approaches have been tried for RpfE with mixed results. The vaccination of C57BL/6 mice with a construct of RpfE fused to early secretory antigen 6 (ESAT-6) was highly immunogenic generating RpfE-specific IFNγ secreting T-cells (Xin et al., 2013). However, when challenged with virulent Mtb, no reduction in lung or spleen CFUs over those receiving the phosphate buffered saline control was seen (Xin et al., 2013). In another study, C57BL/6/JCit mice were vaccinated subcutaneously with recombinant RpfE. Following exposure to virulent Mtb, they exhibited significantly prolonged survival times and lower lung and spleen CFUs compared to unvaccinated mice. The level of protection demonstrated by RpfE was comparable with ESAT-6 and Ag85B (Yeremeev et al., 2003).

The immune response to the other Rpf proteins has been less extensively studied. Immunization of C57BL/6 mice with an rpfD pDNA vaccine was disappointing eliciting only a minor IFNγ response (Romano et al., 2012). Pilot data described in a paper by Romano and colleagues suggest that rpfA and rpfC pDNA vaccines may induce significant humoral and cellular responses (Romano et al., 2012). Further study to determine the immunogenicity and protection against challenge with Mtb seems warranted to see if these Rpf are potential vaccine candidates.

In summary, RpfB and RpfE induce immune responses associated with resistance to Mtb and provide some protection in mouse models. The lack of standardization of vaccination approaches, use of different Mtb strains and mouse models complicates the interpretation of the data generated. Further human studies are required to determine whether the findings are replicable in humans and if Rpf should be considered for development as antigens in a subunit vaccine against Mtb.

**Rpf expression in human infection**

Akin to the findings from animal models, samples taken from patients with active tuberculosis demonstrate the transcription and expression of Rpf. Rachman and colleagues examined the transcriptome of Mtb isolated from human pulmonary tissue and found rpfA and rpfB transcripts (Rachman et al., 2006). The expression of Rpf in human infection was confirmed by Davies and colleagues by the application of immunohistochemistry performed on gut lymphoid tissue with anti-Rpf antibodies. The presence of Rpf in the vicinity of Mtb bacilli was established in areas of necrosis, in epithelioid/giant cells and in macrophages surrounding necrotic areas (Davies et al., 2008). However, the limitations of the method do not permit any conclusions to be drawn about the expression of specific Rpf during different phases of infection.

Studies have confirmed that Rpf are immunogenic in humans generating significant immune responses in both patients with active and latent disease (Chegou et al., 2012; Kassa et al., 2012; Riano et al., 2012). All Rpf proteins appear to be immunogenic with significant T-cell responses demonstrable in LTBI patients using interferon gamma release assays (IGRAs) (Commandeur et al., 2011; Geluk et al., 2014; Huang et al., 2013; Riano et al., 2012; Schuck et al., 2009). Whether immune responses to Rpf are protective or not is unclear. In a study of long term latently infected patients who did not develop tuberculosis, significant single and double cytokine producing CD4+ and CD8+ T-cells specific to RpfA were detected (Commandeur et al., 2011). Two studies showed that in patients with pulmonary tuberculosis, RpfA, B, D&E induced a significantly lower proportion of IFNγ producing CD4+ T-cells compared to those with LTBI (Riano et al., 2012; Schuck et al., 2009). Collectively this indirect evidence suggests that responses directed to Rpf antigens may strengthen immune surveillance in LTBI and prevent reactivation. This remains a hypothetical premise as a role of Rpf in Mtb reactivation in humans has not yet been verified.

In summary, this evidence suggests Rpf are produced during human Mtb infection and recognized by the host immune system, potentially leading to protective immune response. The data should, however, be interpreted with caution. The observed T-cell responses to Rpf antigens could simply represent ongoing exposure to environmental bacteria producing Rpf-like proteins (Commandeur et al., 2011). Further work to understand the role of Rpf in human infection may ultimately be therapeutically useful.

**Rpf-dependency phenomenon**

Rpf-dependent bacilli are those which cannot be cultured in standard media, requiring Rpf to initiate growth (Mukamolova et al., 2010; Shleeva et al., 2002). Exogenous Rpf can be provided in the form of
recombinant Rpf proteins or Rpf-containing culture supernatant (Mukamolova et al., 2010). The populations of cells recovered in culture following supplementation by either sources of Rpf may be synonymous (Turapov et al., 2016), although recently it was shown that culture supernatant from a quintuple rpf deletion mutant exhibited resuscitation activity (Chengalroyen et al., 2016), possibly related to other factors such as cAMP (Shleeva et al., 2013), muropeptides (Nikitushkin et al., 2013), and lipids (Zhang et al., 2001) which are known to modulate the growth of non-replicating mycobacteria. It is unknown whether populations dependent upon Rpf-containing culture supernatant or recombinant Rpf exhibit phenotypic differences. For the purposes of this review, they will be considered interchangeable.

In vitro Rpf-dependency can be induced by prolonged stationary growth phase culture, exposure to anti-tuberculous drugs, and gradual external acidification of culture media (Loraine et al., 2016; Mukamolova et al., 2002; Shleeva et al., 2011). Rpf-dependent mycobacteria are also generated in vivo, however the in vivo triggers for Rpf-dependency are as yet unresolved (Hu et al., 2015; Mukamolova et al., 2010; Turapov et al., 2014). Rpf-dependent mycobacteria have been grown from patients’ sputum (Chengalroyen et al., 2016; Mukamolova et al., 2010) and extrapulmonary samples (O’Connor et al., 2015). Moreover, Rpf-dependent bacilli frequently make up the vast majority of the sample population, however, some sputum samples do not contain detectable Rpf-dependent Mtb (Chengalroyen et al., 2016; Mukamolova et al., 2010).

Variation in the proportion of Rpf-dependency between individuals implicates host factors in its formation (Turapov et al., 2014). At present, the precise mechanism(s) by which Rpf-dependency is generated are unknown although recent studies have shed some light. When Rpf-dependent Mtb and M. bovis BCG were isolated from sputum and mouse lung homogenates, respectively, subsequent exposure of mycobacteria to these did not induce Rpf-dependency (Mukamolova et al., 2010; Turapov et al., 2014) so suggesting contact with the pulmonary environment itself is not the critical factor. More insight came from studying the dynamics of Rpf-dependency development, which indicates a potential role for the adaptive immune response (Turapov et al., 2014). When the lung CFU counts were measured at 24 h post infection of mice with M. bovis BCG Glaxo, no significant Rpf-dependent population was present, however, by 2 weeks Rpf-dependency had developed and persisted thereafter (Turapov et al., 2014). Notably, this correlates temporally with the development of the adaptive immune response in mice (Vallerskog et al., 2010). When mycobacteria were incubated in mouse serum, which contains complement and immunoglobulins, Rpf-dependency was not generated, indicating the cellular arm of the adaptive immune response may be responsible (Hood et al., 2005; Turapov et al., 2014). The possible link of Rpf-dependency with the cellular immune response was illustrated also by the study of Mtb cells isolated from macrophages (Biketov et al., 2000) and the observation that HIV patients with CD4 T-cell counts >200 cells mm⁻³ or who were HIV negative had comparatively higher levels of cells dependent upon Rpf-containing culture supernatant for growth (Chengalroyen et al., 2016). At present, the mechanism by which the immune system may induce Rpf-dependency remains to be elucidated although immune-related stresses placed upon tubercle bacilli during infection may play a role. This area warrants further investigation as an understanding of how the immune system may induce Rpf-dependency could allow us to modulate the response so avoiding Rpf-dependent bacilli and their potential negative consequences for patients.

Rpf-dependent bacilli appear to represent a significant hurdle to the treatment of tuberculosis as they may underlie relapse and the need for prolonged chemotherapy for clinical cure. The putative role in relapse was illustrated in a study by Hu and colleagues where the eradication of Rpf-dependent Mtb bacilli in a Cornell mouse model with high dose rifampicin prevented relapse (Hu et al., 2015). Currently, there is no human data to confirm these findings. Of late, studies on sputum have demonstrated populations of Rpf-dependent Mtb bacilli that are tolerant to the action of rifampicin, isoniazid, and streptomycin and are low to clear from sputum (Mukamolova et al., 2010; Turapov et al., 2016). It may be that clearance is required for cure although further studies are needed to verify this. If this hypothesis is born out, quantification of Rpf-dependent bacilli during therapy may have potential use as a biomarker of response to treatment and better predict the efficacy of potential drug candidates (Turapov et al., 2016). Anti-tuberculous therapy (ATT) could be personalized so minimizing treatment duration without increasing relapse risk.

**Application of Rpf to improve TB diagnosis, treatment and prevention**

An understanding of the biology of Rpf and Rpf-dependency has opened up new potential avenues to improve upon mycobacterial culture, immune based diagnostics, vaccination and treatment approaches. These areas are expounded on below.
**Immunodiagnosics**

One of the most noteworthy developments in immunodiagnostic testing for Mtb infection was the development of IGRAs. IGRAs measure the cell mediated immune response by quantifying the IFN\(\gamma\) induced by microbial antigens (Darby et al., 2014). Current commercial IGRAs such as QuantiFERON\textsuperscript{®} TB Gold In-Tube (Qiagen, Hilden, Germany) and T-SPOT\textsuperscript{®} (Oxford Immunotec, Abingdon, UK) use culture filtrate protein 10 kDa (CFP-10) and ESAT-6 to detect exposure to Mtb (Pai et al., 2014). They are able to diagnose LTBI with a sensitivity of up to 90% and are unaffected by BCG vaccination status (Darby et al., 2014). These IGRAs are however limited to this narrow remit. They are unable to differentiate active from latent TB and cannot predict patients at high risk of progression to active disease who would most benefit from chemopreventative therapy (Pai et al., 2014).

By quantifying the production of IFN\(\gamma\) in response to stimulation by Rpf antigens, authors have shown they can differentiate between Mtb disease states potentially overcoming these limitations. In a non-endemic setting, Serra-Vidal and colleagues showed RpfD could distinguish uninfected, LTBI and active Mtb cases (Serra-Vidal et al., 2014). In a high burden endemic setting, where the usefulness of commercial IGRAs is limited by high LTBI rates, Chegou and colleagues showed RpfA performed well and could distinguish those with and without active tuberculosis (Chegou et al., 2012). Interestingly, Huang and colleagues showed RpfD was a highly specific antigen, even in an endemic setting, and was able to separate household contacts of active tuberculosis cases from community exposed persons (Huang et al., 2013). This is an important finding as household contacts are at high risk, particularly within the first year of developing active Mtb (Fox et al., 2013). Collectively these studies indicate that Rpf IGRAs may overcome the limitations of current commercial IGRAs although as the response to Rpf antigens can vary by location (Sutherland et al., 2013), these studies should be replicated in different settings. Furthermore, given the exposure of individuals to Rpf through, for example, BCG vaccination or nontuberculous mycobacterial disease, the diagnostic performance of Rpf IGRAs should be verified in these populations.

**Mycobacterial culture**

The successful culture of Mtb from patient samples is an important component of clinical management (Tsara et al., 2009). It enables drug susceptibility testing, typing for outbreak investigations and advanced “omic” studies (Asmar & Drancourt, 2015; Hayer et al., 2013; Ryu, 2015). Despite advances in the molecular detection of Mtb, culture remains the gold standard for diagnosis (Asmar & Drancourt, 2015). It is more sensitive and is unencumbered by dead bacilli (Asmar & Drancourt, 2015). Nevertheless, culture sensitivity can be low, particularly for extrapulmonary samples (Sandgren et al., 2013), so improving culture performance would have undoubted clinical benefits.

The addition of Rpf to standard culture either as recombinant proteins or in culture supernatant is one potential approach. Mukamolova and colleagues showed Rpf could increase the yield of sputum culture (Mukamolova et al., 2010). In some samples, Rpf enabled the recovery of Mtb that would otherwise be negative by standard culture (Chengalroyen et al., 2016; Mukamolova et al., 2010). This was also demonstrated in one extrapulmonary tuberculosis case (O’Connor et al., 2015). Although highly preliminary, these findings suggest Rpf may improve culture performance.

One major drawback of tuberculosis culture is the slow growth of Mtb (Ghodbane et al., 2014). While recent advances have made the culture of Mtb in less than 48 h a possibility (Ghodbane et al., 2015), currently these techniques are technologically sophisticated and limited to reference laboratory/research settings. In comparison, Rpf may offer a simpler method of speeding up tuberculosis culture. This was illustrated by Wu and colleagues who demonstrated that recombinant RpfB added to mycobacterial growth indicator tubes (MGITs) improved the time-to-positivity of low inocula of quiescent Mtb H37Rv (Wu et al., 2008). Huang and colleagues showed the effect was replicable in clinical samples with both RpfB and RpfE reducing the time-to-positivity of sputum cultures that were positive after more than 20 days by standard culture methods (Huang et al., 2014).

These preliminary studies suggest Rpf supplementation of standard culture could improve time to detection and sensitivity. The veracity of these initial findings should be tested in a prospective clinical diagnostic study. As Rpf are unstable and difficult to produce (Mukamolova et al., 2010), work to produce an easy-to-use, durable form should be pursued.

**Vaccination**

The availability of an effective vaccine would greatly enhance progress toward the END TB goal of less than 10 cases per 100 000 per year by 2035 (Fletcher & Schrager, 2016). Modeling indicates that mass immunization of those with LTBI, particular adolescents and young adults who are the main sources of TB
In the era of increasing antibiotic resistance, targeting of bacterial virulence factors is being seen as a possible treatment strategy (Lynch & Wiener-Kronish, 2008). While it prevents severe disease in younger children, in older children and adults its protection is limited and further boosting with BCG is unable to extend this (da Costa et al., 2015; Doherty et al., 2007; Roy et al., 2014). One promising alternative approach is a subunit vaccine consisting of key Mtb antigens (Xin et al., 2013). Rpf are strong contenders as they are appropriately immunogenic and may generate durable responses enabling long term control of LTBI (Choi et al., 2015; Geluk et al., 2014; Kim et al., 2013). Combined with latency antigens, they could induce host immune responses that eliminate quiescent Mtb as well as inhibiting Mtb reactivation (Geluk et al., 2014; Huang et al., 2013), so reducing the reservoir of infection as well as transition to clinical disease. It should be noted that in theory the vaccination of LTBI patients with Rpf could induce reactivation (Zhao et al., 2015). This potential risk should be investigated before widespread use is considered.

Treatment

In the era of increasing antibiotic resistance, targeting of bacterial virulence factors is being seen as a possible treatment strategy (Lynch & Wiener-Kronish, 2008). Agents antagonizing virulence factors may be useful as adjuncts to antibiotics or as therapies in their own right (Hauser et al., 2016). In Mtb, Rpf represent inviting targets for intervention. They are important virulence factors, they lack human homologs ensuring a high degree of drug specificity and their extracellular nature means small molecule inhibitors do not need to enter the cell for biological activity (Kana et al., 2008; Machowski et al., 2014). Inhibitors of Rpf, such as 2-nitrophenylthiocyanates, could be given to patients with LTBI who need treatment with anti-TNF inhibitors to prevent the reactivation of tuberculosis (Demina et al., 2009; Kaprelyants et al., 2012). Contrarily, Rpf could be administered to patients with active infection, reactivating and sensitizing quiescent bacilli which are thought to be responsible for the requirement for long treatment courses so shortening the duration of treatment (Davies et al., 2008; Zhang et al., 2010). It is also possible that by interfering with the host immune response to minimize pathological host responses or to boost innate immunity (Nathan, 2012), the development of Rpf-dependency may be prevented. While these approaches are at the conceptual stage, the ability to control entry into and exit from non-replicating states could be therapeutically powerful and merit further research to determine their feasibility.

Conclusions

Substantial progress has been made understanding the biology of Rpf, illuminating the crucial role they play in tuberculosis pathogenesis (see Figure 1). Their immunogenicity and lack of human homologs make Rpf ideal targets for therapeutic and preventative interventions. The use of Rpf inhibitors as well as booster vaccines incorporating Rpf could significantly impact upon LTBI reactivation, reducing clinical cases and transmission of infection. Rpf-dependent populations appear to underlie the difficulty we have in improving TB treatment, yet their presence appears to be driven by both treatment and host factors. A greater understanding of the mechanisms underlying their genesis may yield novel approaches to improve treatment. Furthermore, quantification of the dynamics of the Rpf-dependent population during infection and treatment could predict individual patient outcomes as well as assess the efficacy of new drug candidates. Currently, this is technically demanding and would be greatly enhanced by the development of proxy biomarker. Future studies are required to test whether these scientific findings translate into successful diagnostic and therapeutic modalities to tackle our ancient scourge.

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