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## ***Mycobacterium tuberculosis* RpfE Protein Purified from H37Rv Culture Filtrate Proteins Stimulates Splenocytes**

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### **ABSTRACT**

Resuscitation promoting factor E (RpfE) proteins in *Mycobacterium tuberculosis* (*M. tuberculosis*) may be recognized by the host immune system owing to their secretory nature. In this study, the *RpfE* protein was purified using DEAE–Sepharose column chromatography from *M. tuberculosis* H37Rv extracellular proteins. An upregulated production of gamma interferon (IFN- $\gamma$ ), interleukin-10 (IL-10) and IL-12 by splenocytes suggest T cell proliferation stimulated by purified RpfE protein. The present findings imply that the RpfE protein produced extracellularly in *M. tuberculosis* H37Rv can be used as a viable subunit vaccine candidate.

### **INTRODUCTION**

It is estimated that one-quarter of the world's population suffers latent tuberculosis infection, globally in 2018 (Cohen *et al.*, 2019; Dara *et al.*, 2020). Each year, around 1% of the population contracts a new infection (Adigun and Singh 2021). In 2020, it is anticipated that 10 million people would have active tuberculosis, resulting in 1.5 million deaths, making it the second-highest cause of death from an infectious disease after COVID-19 (Chakaya *et al.*, 2021).

An effective vaccine paucity, the microbial resistance in the pathogen, and the fatal interaction of co-infection with HIV are the major causes of the present epidemic (Nunn *et al.*, 2005). For decades, *Mycobacterium bovis* (*M. bovis*) bacillus Calmette-Guérin (BCG), an inactivated *M. bovis* strain, has been the solely approved TB vaccine (Barreto *et al.*, 2014; Brandt *et al.*, 2002). The currently available vaccine is effective against juvenile forms of tuberculosis (Trunz *et al.*, 2006), however, it fails to prevent adult pulmonary symptoms of the disease (Herzmann *et al.*, 2014). Furthermore, BCG vaccination is not recommended for HIV-positive people since BCG injection might induce fatal infections in immunocompromised people (Enserink 2007; Hesseling *et al.*, 2009). For the last five decades, approximately 200 vaccine candidates have been in various animal models of primary tuberculosis (Franco and Peri 2021; Zhu *et al.*, 2018).

Recombinant BCG strains, DNA-based vaccinations, live attenuated *M. tuberculosis* vaccines, and subunit vaccines along with new adjuvants have shown efficacy in preclinical animal models (Ahsan 2015; Franco and Peri 2021; Zar and Udwardia 2013).

Many of these vaccinations are now being investigated in human clinical trials, and many more vaccine formulations are expected to enter clinical trials soon (Sable *et al.*, 2019). As a result, new or improved vaccinations are urgently needed to contain the infectious spread.

Resuscitation-promoting factor (Rpf) possessing autocrine and paracrine signaling effects is essential for the latent cell activation (Commandeur *et al.*, 2011; Rosser *et al.*, 2017). Rpf can activate dormant *M. luteus* cultures and significantly raise the viable cell count and cell growth (Mukamolova *et al.*, 2002; S Kaprelyants *et al.*, 2012). Genome sequencing shows the presence of similar genes in *M. leprae*, *M. TB*, *M. bovis*, *Streptomyces* spp., and *Corynebacterium glutamicum* (Mukamolova *et al.*, 1998).

*M. tuberculosis* Rpf-like proteins boost the development of *M. bovis* BCG extended-stationary-phase cultures (Gupta *et al.*, 2010). These findings imply that Rpf proteins can impact mycobacteria growth (Cohen-Gonsaud *et al.*, 2004). Revival inability of bacteria having numerous rpf gene deletions revealed the relevance of *M. tuberculosis* Rpf-like proteins in resuscitation (Downing *et al.*, 2005). Protein sequence analysis has shown that many of them are secreted, and all five Rpfs have extracytoplasmic properties (Sasseti *et al.*, 2003), making them possible targets for identification by the host immune system during the reactivation stage of the illness.

As a result, these proteins have the potential to be used as innovative diagnostic reagents and subunit vaccine candidates in the fight against tuberculosis. The present study elucidated the production, purification and characterization of secreted protein RpfE and its effect on splenocytes.

## MATERIALS AND METHODS

### Bacterial Strain:

*M. tuberculosis* H37Rv (ATCC-27294) ( $2 \times 10^9$  cfu/ml) was cultured in

Sauton medium with 0.2 percent glycerol, 0.05 percent Tween 80, and 10% oleic albumin dextrose catalase (OADC) until the optical density at 600 nm was reached.

### T- cells Culture:

Mouse splenocytes seeded at  $5 \times 10^6$  cells per well were cultured for 72 hours at 37°C with 5% CO<sub>2</sub> in a 96-well plate and then added 2 µg pure RpfE protein per well. The plates were then incubated for 4 hours with 20 µL of MTT (5 mg/mL, diluted with PBS, pH 7.2). Each well's supernatant was then replaced with 150 µL of DMSO before the absorbance was read at 490 nm after 10 minutes of incubation. All experiments were done in triplicate and the stimulation index was calculated using wells that were not stimulated with pure RpfE protein.

### Purification of RpfE Protein:

Using a column chromatographic technique, the protein was extracted from total culture filtrate protein. Using 10 mM Tris HCl buffer containing 3% methylcellulose, a DEAE–Sephacel CL-6B (anion exchange) packed gel was equilibrated. To allow the proteins to bind to the gel matrix, concentrated culture filtrate proteins dialyzed against Tris buffer were put onto the column and left at 4°C for 30 minutes. To obtain maximal protein binding to the column and eliminate unattached protein from the gel, the column was rinsed three times with equilibrating buffer before being washed with the equilibrating buffer. To elute the bound protein from the column, a linear gradient of 50–300mM NaCl was utilized in the equilibrating buffer. The absorbance was measured using a spectrophotometer at 280nm using equilibrating buffer as a blank. The pure protein was next concentrated using an Amicon unit with a 5kDa cut-off filter, and the Tris salt was removed overnight at 4°C by dialysis against PBS. The protein concentration of each pooled fraction was determined using the Bradford method, and the protein profile was examined using SDS-PAGE.

### Cytokine Assay:

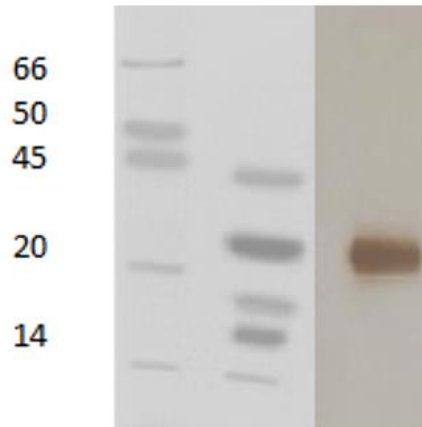
In 24-well plates, a total of  $5 \times 10^6$  splenocytes were grown per well. The supernatants from each well were taken after 48 hours of incubation and kept at  $-20^\circ\text{C}$  until testing. ELISA kits were used to detect interleukin-12 (IL-12), IL-10, and interferon-gamma (IFN- $\gamma$ ) in the culture supernatants using the standard curves

made with known concentrations of recombinant rIL-12, rIL-10, and rIFN- $\gamma$ .

### RESULTS

#### Expression and purification of RpfE protein

Before the 22-Kda protein was multicluted and tested for purity using 15% SDS-PAGE, culture filtrate proteins were purified using anion exchange chromatography (Fig. 1).

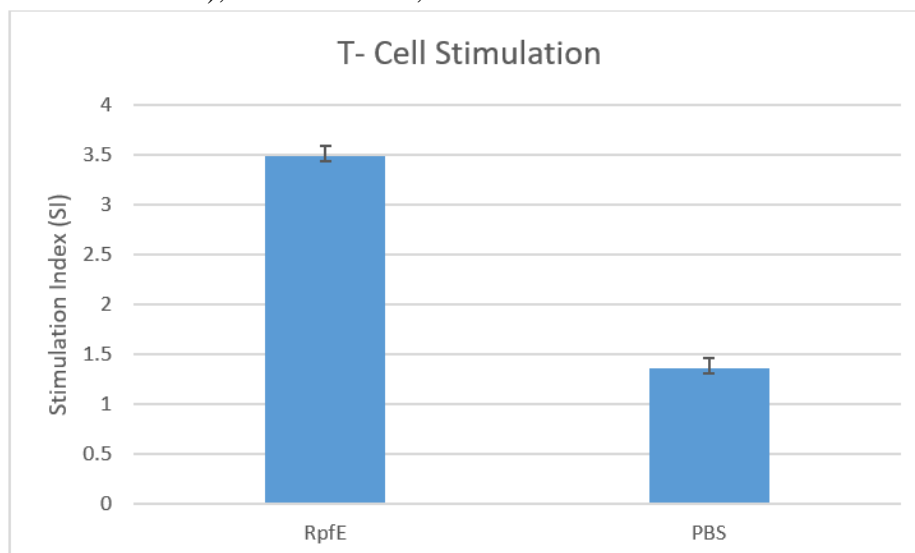


**Fig. 1.** Expression and purification of RpfE on 15% SDS-PAGE gel and Western blot analysis.

### T Cell Proliferation:

The stimulation index (SI) of splenocytes was evaluated using the MTT technique to assess the cell-mediated immune response. RpfE and control groups had SI values of  $3.49 \pm 0.10$ , and  $1.35 \pm 0.04$ ,

respectively (Fig. 2). Splenocyte proliferation is driven by RpfE protein and PBS as a negative control). The stimulation index (SI) was computed by dividing the experimental group's  $\text{OD}_{490}$  values by the controls' values.

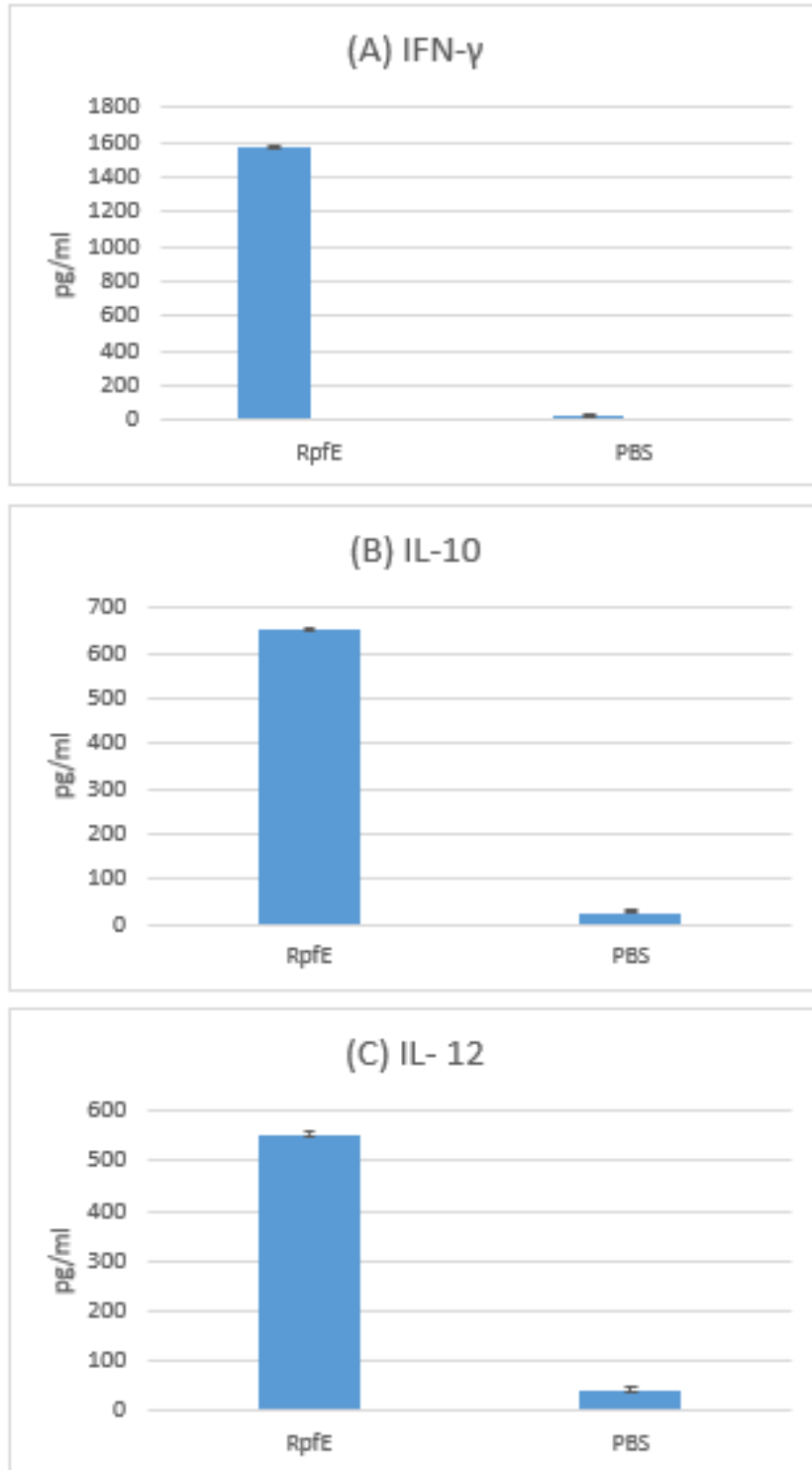


**Fig. 2.** The proliferation of splenocytes induced by RpfE protein and PBS, used as a negative control. The stimulation index (SI) was determined by dividing the experimental group's  $\text{OD}_{490}$  values by those of control group). The results are expressed as mean $\pm$ SD, and all experiments were repeated three times.

**Cytokine Production:**

Indirect ELISA was used to determine the quantities of IFN- $\gamma$ , IL-10, and IL-12 secretion triggered by RpfE antigen, which was  $1,568 \pm 4.5$ ,  $652 \pm 4$  and

$552 \pm 39$  pg/mL in the cultured supernatants of splenocytes, respectively, versus  $27 \pm 2$ ,  $27 \pm 2$  and  $8.88 \pm 2$  pg/mL in the PBS group (Fig. 3).



**Fig. 3.** Levels of IFN- $\gamma$  (A), IL-10 (B) and IL-12 (C) induced in the culture supernatant of splenocytes challenged with purified Rpf protein and PBS used as a negative control. The results are expressed as mean  $\pm$  SD, and all experiments were repeated three times.

## DISCUSSION

Resuscitation-promoting factor proteins (Rpfs) present in *Mycobacterium tuberculosis* can reactivate dormant mycobacterial growth (Kana and Mizrahi 2010; Uhía *et al.*, 2015). RpfE and RpfB interact with the cell wall hydrolase RipA, and RpfB and RipA colocalize near the septum of dividing cells, implying the central role of RpfB–RipA interactions during reactivation (Hett *et al.*, 2008; Hett *et al.*, 2010; Hett *et al.*, 2007; Nikitushkin *et al.*, 2015). Although the precise functions of all Rpfs are unknown, isolated RpfE stimulates dendritic cell maturation in an animal model (Choi *et al.*, 2015). RpfE, along with RpfA and RpfC, is found in *M. tuberculosis* culture filtrates (De Souza *et al.*, 2011; Målen *et al.*, 2007), and positioned to perform autocrine and/or paracrine signalling roles.

In vitro, deletion of a single Rpf gene has no effect on cell growth or morphology, implying that these proteins are functionally redundant, as none of the members of the family are required for cultured cell growth (Kana *et al.*, 2008; Tufariello *et al.*, 2004). In vitro, deletions of three rpf genes resulted in cell growth abnormalities showing their functional relevance in *Mtb* (Kana *et al.*, 2008). *Mtb* rpfB deletion mutants also fail to resuscitate in mice<sup>3</sup>, whereas rpfE is required for transitioning of mycobacterial cultures from slow to fast (Beste *et al.*, 2009) suggesting the importance of RpfB and RpfE proteins (Kana and Mizrahi 2010).

The reciprocal stimulation of Th1 and Th17 cellular responses is critical for the growth and development of adaptive immunity against tuberculosis (Griffiths *et al.*, 2011). In general, a robust Th1 response mediated by IFN- $\gamma$  secretion by antigen-specific CD4<sup>+</sup> T cells is central to immune response against *Mtb* infection; however, new research has highlighted the role of the Th17 response in immune elicitation against *Mtb* (Chatterjee *et al.*, 2011; Gopal *et al.*, 2012; Torrado and Cooper 2010). As a result, finding novel proteins that activate

both Th1 and Th17 immune responses simultaneously is critical for the creation of effective vaccines. Both innate and adaptive immune responses, required to induce protective immunity against *Mtb* infection (Feng *et al.*, 1999; Flesch and Kaufmann 1990), are dependent upon dendritic cells (DCs) for antigen-presentation (Megiovanni *et al.*, 2004).

Many *Mtb* antigens present within granulomas during *Mtb* infection stimulate DCs. DCs can lead to the immunopathological reaction (Suresh and Mosser 2013), however, these cells are also central to significantly heightened cellular immunological response to *Mtb* infection (Demangel and Britton 2000; Tascon *et al.*, 2000). DCs move to the draining lymphoid tissue throughout their maturation phase and prime both naive and memory T cells leading to the development of antigen-specific T cells (Dumortier *et al.*, 2005). The Th1 immune response plays a critical role in *Mtb* protection (Ottenhoff *et al.*, 1998) and despite inducing a strong Th1 response, the BCG vaccine does not appear to give adequate protection against *Mtb* infection (Majlessi *et al.*, 2006; Wozniak *et al.*, 2010).

Th17 cells, in collaboration with Th1 cells, play an important role in eliciting a protective immune response against pathogenic *Mtb* (Gopal *et al.*, 2014). Thus an effective vaccine candidate must elicit significant Th17 cell responses in addition to Th1 cell responses (Gopal *et al.*, 2014). However, few reports are available about antigens activating the Th17 cell response through vaccination. Given these findings, *Mtb* antigens that elicit both Th1 and Th17 immune responses are promising targets for the creation of novel vaccines (Gowthaman *et al.*, 2011). Moreover, an effective TB vaccine should cover targets associated with the initial infection as well as those associated with the reactivation of latent *Mtb* infection (Flynn and Chan 2001).

Resuscitation-promoting factors (Rpfs) have been recommended as excellent vaccination candidates among latency-

associated components (Romano *et al.*, 2012). However, the immunological role of Rpf antigens as vaccine candidates remains to be studied. RpfE is a potent DC activator during the antigen-DC interaction, according to a recent study. As a result, the current study looked at the involvement of RpfE in the elicitation of immunological responses. In both humans and animals, the RpfE protein has been shown to trigger strong T-cell responses.

Secreted and surface-exposed cell wall proteins are key antigens culminating in the development of a protective immune response against *M. tuberculosis*. Early reports showed that immunization with whole-culture filtrate protected mice and guinea pigs against future tubercle bacilli challenges (Derrick *et al.*, 2005; Kumar *et al.*, 2016; Tanghe *et al.*, 2001). Because RpfE is a secreted protein, the current study additionally looked at the cytokine production of splenocytes challenged with RpfE proteins in vitro. In animal models of tuberculosis, IFN- $\gamma$  has been shown to be a protective cytokine (Smith *et al.*, 2002) while IL-12 is required to develop a protective immune response against *Mtb* along with inducing IFN- $\gamma$  expression and activation of antigen-specific lymphocytes (Cooper *et al.*, 1997; Gazzinelli *et al.*, 1994).

Mycobacteria and other intracellular infections can activate IL-10, and illnesses caused by these organisms are usually linked to immunologic unresponsiveness and IFN- production failure (Gong *et al.*, 1996; Rojas *et al.*, 1999).

### Conclusion

Purified RpfE protein induced considerable IFN- $\gamma$ , IL-10, and IL-12 cytokine production in splenocytes in vitro, indicating the significant activation of the cellular immune response. The potential of RpfE protein as a multiantigenic *Mtb* subunit vaccine candidate will be determined in future in-vivo investigations using immunized mice challenged with *M. tuberculosis*.

**Conflict of Interest:** The Author declares that there is no conflict of interest.

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