



Heat-inactivated *Mycobacterium bovis* and P22PI protein immunocomplex: Two candidates for use as immunostimulants of innate immune response

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ABSTRACT

Tuberculosis (TB), caused by members of the *Mycobacterium tuberculosis* complex, remains a critical global health challenge, affecting humans and a wide range of domestic and wild animals. Despite the availability of anti-TB drugs, cure rates remain suboptimal, exacerbated by the rise of multidrug-resistant TB strains. The Bacille Calmette-Guérin (BCG) vaccine, the only licensed vaccine against TB, has demonstrated efficacy in reducing lesion severity and bacterial burden in animals, as well as lowering TB-related and all-cause mortality in infants. However, BCG presents several safety concerns inherent to live vaccines. To overcome these limitations, exploring alternative vaccine candidates that do not incorporate live mycobacteria is crucial. This study aimed to evaluate and compare the immunostimulatory potential of two candidates based in mycobacteria inactivated or their derivatives, heat-inactivated *Mycobacterium bovis* (HIMB) and P22PI protein immunocomplex (P22PI), in bovine foetal lung cells. To assess the expression of innate immune components, including Toll-like receptors (TLRs), cathelicidins, and cytokines, bovine foetal lung were exposed to different concentrations of HIMB and P22PI immunostimulants, starting at 7.8×10^6 CFU/ml and 10 µg/ml, respectively. These initial concentrations were subsequently diluted to 1/2 and 1/10 to evaluate dose-dependent effects. Our findings reveal that both HIMB and P22PI significantly stimulate innate immune mechanisms, as evidenced by the upregulation of TLR2 and TLR4, alongside the induction of BMAP28 cathelicidin, tumour necrosis factor alpha (TNFα) and interferons (IFNs). These results suggest their potential to orchestrate a robust innate immune response providing valuable insights into the immunological mechanisms underlying the protective effects of these immunostimulants. This underscores their potential role in *in vivo* studies as vaccine candidates. Furthermore, their ability to enhance antigen recognition via TLR and induce pro-inflammatory cytokines also indicates broader applications in immune modulation, potentially extending protection against heterologous pathogens through trained immunity.

1. Introduction

Tuberculosis (TB), caused by members of the *Mycobacterium tuberculosis* complex (MTC), is a persistent and potentially devastating

infectious disease that affects to humans and a wide range of domestic and wild animals (Balseiro et al., 2020). This airborne bacterial infection remains a global health challenge, contributing to over 1 million deaths annually worldwide (Chai et al., 2020; World Health Organization,

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2023). Despite the availability of anti-TB drugs for human use, the global cure rates remain around a concerning 56 %, particularly alarming due to the persistent emergence of drug-resistant TB cases, compelling the utilization of complex, toxic and expensive treatment regimens (Chai et al., 2020). Moreover, due to the zoonotic nature of TB and its associated public health risks, eradication programs targeting bovine TB have been established, relying on *ante-mortem* diagnostics and the culling of infected animals to protect human health (Balseiro et al., 2020). While these programs have contributed to a significant reduction in TB prevalence in developed countries in recent years, they remain socioeconomically unfeasible in most low- and middle-income countries, where the disease remains uncontrolled (Fromsa et al., 2024). Therefore, vaccination emerges as a pivotal instrument in endemic regions (World Health Organization, 2017). The bacille Calmette–Guérin (BCG) vaccine, created more than 100 years ago, is the only licensed vaccine against MTC (Balseiro et al., 2020). Numerous experimental and field trials have validated its efficacy across a wide range of wild and domestic species, demonstrating a reduction in lesion severity and bacterial burden, and consequently, lower transmission rates (Balseiro et al., 2020). Additionally, routine BCG vaccination in infants on developing countries has shown efficacy in reducing TB mortality and, under certain conditions, has also contributed to lowering all-cause infant mortality unrelated to TB (World Health Organization, 2017). This effect of BCG may be attributed to the effective stimulation of the innate and cell-mediated immune (CMI) response (Moreira-Teixeira et al., 2024). Key players in the innate immune response include Toll-like receptors (TLRs), which are actively involved in recognizing mycobacterial antigens and triggering antimicrobial activities (Faridgozar and Nikouinejad, 2017), as well as cathelicidins, small cationic peptides known for their broad-spectrum antimicrobial effects and immunomodulatory roles (Faridgozar and Nikouinejad, 2017; Hu and Spaink, 2022). These peptides, which boost TLR signalling, have been investigated as potential adjuvant therapies for TB, highlighting their promise in enhancing the overall immune response against the disease (Palacios et al., 2022). Moreover, the engagement of innate TLRs, triggers the production of pro-inflammatory cytokines and interferons (IFNs), initiating adaptive immune responses that inhibit mycobacterial replication and promote bacterial clearance (Hu and Spaink, 2022). BCG vaccination has been shown to effectively activate these innate receptors, enhancing the organism's defence mechanisms (Kleinnijenhuis et al., 2014).

Although BCG vaccination provides significant protective benefits, it is associated with several safety limitations inherent to live vaccines, such as the necessity of maintaining a cold chain, interference with TB eradication programs in animals, potential health risks due to adverse reactions and its persistence in host tissues after parenteral administration, as well as its possible environmental excretion after oral administration (Al-Hammadi et al., 2017; Garrido et al., 2011). To overcome these limitations, it would be valuable to explore vaccine candidates that do not incorporate live mycobacteria. In this context, a heat-inactivated *M. bovis* (HIMB) vaccine has been developed, providing comparable protection to BCG against TB in both domestic and wild animals (Balseiro et al., 2020). Moreover, this vaccine candidate exhibits enhanced stability under field conditions (Beltrán-Beck et al., 2014), elicits a robust CMI response in *in vivo* studies (Beltrán-Beck et al., 2014; Arrieta-Villegas et al., 2018; Vaz-Rodrigues et al., 2022) and provides protective efficacy against heterologous pathogens (Vaz-Rodrigues et al., 2022; Ferreras-Colino et al., 2023). On the other hand, a novel multi-protein complex known as P22PI, composed of immunopurified proteins from a commercial derivative of *Mycobacterium bovis* (*M. bovis*), including MPB70, MPB83, ESAT-6, and CFP-10 (Infantes-Lorenzo et al., 2017), has been developed as an important tool for TB serodiagnosis in domestic and wild animals (Infantes-Lorenzo et al., 2017; Thomas et al., 2021). P22PI complex not only stimulates antibody production (Infantes-Lorenzo et al., 2017) but also induces other cellular response mediators, such as *IFN* γ (Risalde

et al., 2017), suggesting its potential as an effective immunostimulant against TB. Despite the promising potential of P22PI and HIMB, there is a notable lack of studies that evaluate in depth their direct immunomodulation mechanisms in pathogen-free environments under conditions that closely resemble natural respiratory infections, which is crucial for understanding the role of key mediators against MTC members and other heterologous pathogens. Therefore, this study aimed to evaluate and compare the immunostimulatory effects of the vaccine candidates HIMB and P22PI in primary cultures of bovine foetal lung cells under pathogen-free conditions.

2. Materials and methods

2.1. Vaccine formulation

2.1.1. HIMB immunostimulant

The HIMB immunostimulant utilized comprised approximately 3.9×10^8 heat inactivated colony-forming units (CFU)/ml of a *M. bovis* field isolate (strain 1403; spoligotype SB0339 from a naturally infected wild boar) in sterile phosphate-buffered saline (PBS). Prior to inactivation, the bacterial concentration of HIMB was determined by assessing the turbidity of the suspension using the MacFarland scale with a VITEK® DensiCHEK® (BioMerieux, Marcy l'Etoile, France), and by plating a serially diluted aliquot onto Middlebrook 7H9 agar supplemented with glycerol (0.2 % v/v) and OADC (10 % v/v) (Becton Dickinson, Franklin Lakes, NJ, USA). The vaccine preparation followed the protocol described previously (Garrido et al., 2011), with some modifications such as extended inactivation step at 83 °C for 45 min.

2.1.2. P22PI immunostimulant

The P22PI immunostimulant is a multiprotein complex (CZ Veterinaria, S.A.) composed of several immunodominant antigens recognised by T cells, including MPB70, MPB83, ESAT-6, and CFP-10. P22PI was obtained from bovine PPD through immunopurification with monoclonal antibodies as previously described (Infantes-Lorenzo et al., 2017), and was utilized at 0.5 mg/ml.

2.2. Bovine foetal lung cell cultures

Primary culture of bovine foetal lung cells (provided by the Cell Culture Laboratory of the Institute of Virology, CICVyA, INTA Castelar) was maintained in Minimum Essential Medium (Eagle), with Earle salts (MEM-E) (Sigma- Aldrich, Saint Louis, MO, USA), 10 % foetal bovine serum (Bioser, Buenos Aires, Argentina) free of viruses and antibodies and an antibiotic-antimycotic solution (Gibco, Langley, OK, USA), containing 100 U/ml of penicillin G, 100 µg/ml of streptomycin sulphate and 0.025 µg/ml of amphotericin B. The cell cultures were incubated at 37°C in an atmosphere with 5 % CO₂.

2.3. Experimental design

Bovine foetal lung cells were seeded in 24-well plates (Greiner Bio-one, Frickenhausen, Germany) at a concentration of 3×10^5 cells/ml and cultured until they were 80–90 % confluent. Subsequently, three concentrations corresponding to 3.9×10^5 , 3.9×10^6 and 7.8×10^6 CFU/ml of HIMB and 0.5, 5 and 10 µg/ml of P22PI immunostimulants were added to separate wells. The cultures were incubated in a humidified atmosphere at 37°C with 5 % CO₂ and cells were collected at 24-hours post-treatment. Cell viability was evaluated using the trypan blue exclusion method prior to cell seeding. Additionally, monolayer integrity and confluency were microscopically assessed before and after the stimulation to ensure optimal experimental conditions.

The cells were preserved at –80 °C in Trizol for the study of the gene expression by RT-qPCR. For all the treatments evaluated, three independent experiments were carried out with three repetitions each in order to establish the effect of the different concentrations of each

immunostimulants. Untreated cultures were included as controls.

2.4. RNA extraction, DNase treatment and reverse transcription

Total RNA from bovine foetal lung cells was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol and digested with DNase I Amplification Grade (Invitrogen, Carlsbad, CA, USA) for 30 min at 37 °C to remove any contaminating genomic DNA (gDNA). The quantity of the resulting RNA were determined using an Epoch Microplate Spectrophotometer (BioTeK, Winooski, VT, USA), and the quality was assessed by determining the OD260/OD280 ratio, which ranged between 1.8 and 2. Complementary DNA (cDNA) was synthesized using a reaction mixture containing total RNA (1 µg), random hexamers (0.1 µg/µl) (Invitrogen, Carlsbad, CA, USA), and Moloney murine leukaemia virus reverse transcriptase (1 µl) (Invitrogen, CA, USA) with the following cycling: 10 min at 25 °C, 50 min at 37 °C, and 15 min at 70 °C. Negative controls, omitting the RNA or the reverse transcriptase, were included.

2.5. Transcriptional gene expression

RT-qPCR reactions for bovine TLRs (TLR2 and TLR4), bovine cathelicidin BMAP28, *IFNB1*, *TNFA* and *IFNG* were carried out using specific primers as detailed in [Supplementary Table 1](#). Expression of the reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control ([McGuire et al., 2004](#)). The qPCR reactions contained 20 µM specific forward and reverse primers, 1X PCR Master Mix (Fast-Start Universal SYBR Green Master Rox, Roche, Mannheim, Germany), and 1 µl of cDNA sample in a final volume of 20 µl. The amplification and detection of the specific products were carried out in an Applied Biosystems 7500 cycler, with the following amplification conditions: 2 min at 50 °C, 10 min at 95 °C, and 40 cycles of 20 s at 95 °C and 60 s at 60 °C. After amplification, a melting curve analysis was performed, which resulted in a single product-specific melting curve. Negative controls for cDNA synthesis and PCR procedures were included in all cases. The amplification efficiency was determined for each gene using 10-fold dilutions of the cDNA. The results are reported as the mean fold change of gene transcription levels in the in treated cells over the levels detected in untreated control cells.

2.6. Statistical analysis

The relative expression analysis of the target genes was performed using the Relative Expression Software Tool (REST, Qiagen Inc., Valencia, CA, USA) for evaluating immunostimulants and concentration differences for significance with a Pair Wise Fixed Reallocation Randomization Test© ([Pfaffl et al., 2002](#)). The RT-qPCR efficiency for each gene was determined by a linear regression model according to the equation: $E = 10^{[-1/\text{slope}]}$. P values less than 0.05 were considered statistically significant.

3. Results

3.1. HIMB and P22PI immunostimulants enhance TLR2 and TLR4 activation

After stimulating bovine foetal lung cells, it was observed that both HIMB and P22PI immunostimulants enhance TLR2 and TLR4 activation, key receptors in orchestrating innate immune responses and initiating adaptive immunity against MTC. Across all three concentrations tested in our study, it was noted a consistent increase in their expression, with a significantly higher response at 7.8×10^6 CFU/ml and 10 µg/ml for both immunostimulants ($p < 0.05$) ([Fig. 1A-B](#)). HIMB produced a dose-dependent increase in the expression of both TLRs when the immunostimulant was applied at concentrations of 3.9×10^6 and 7.8×10^6 CFU/ml. Moreover, at the higher concentration, HIMB demonstrated a fivefold

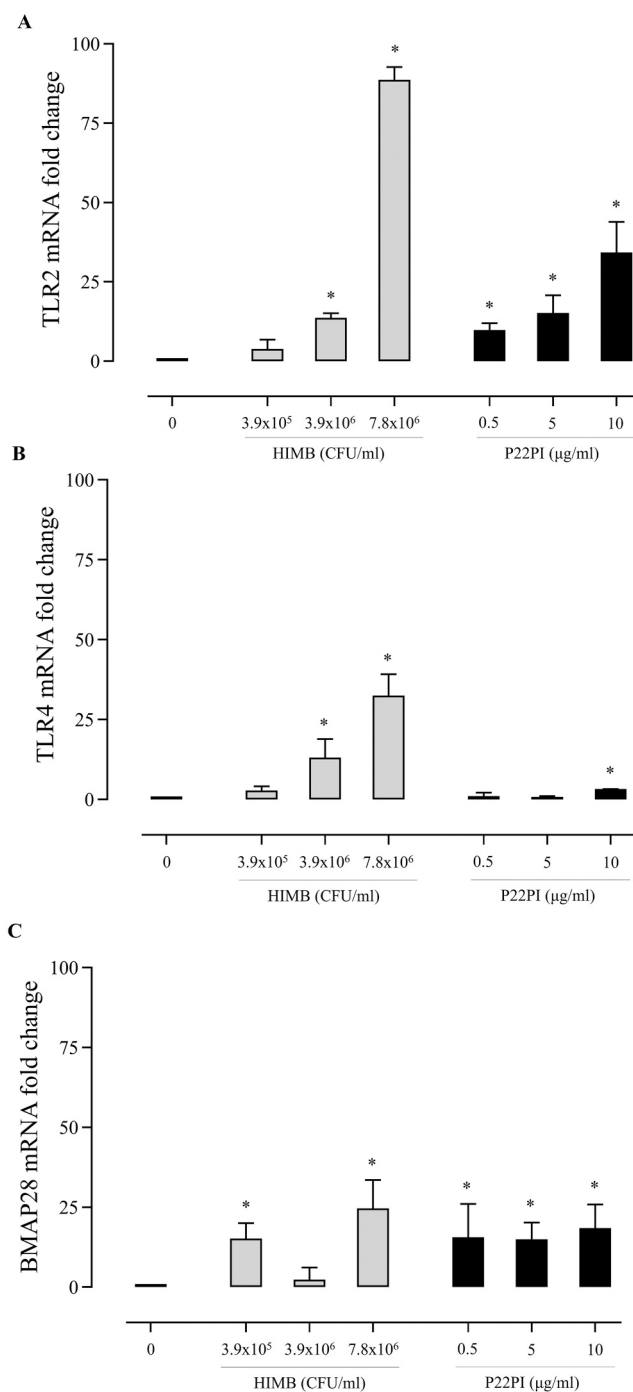


Fig. 1. Relative expression (mean fold change $\pm$ SEM) of TLRs and BMAP-28 cathelicidin in bovine foetal lung cells treated for 24 h with three concentrations of heat-inactivated *M. bovis* (HIMB) (3.9×10^5 , 3.9×10^6 and 7.8×10^6 CFU/ml) and three concentrations of P22 protein immunocomplex (P22PI) (0.5, 5 and 10 µg/ml). For all treatments, three independent experiments were conducted, each with three replicates. Statistically significant differences ($p < 0.05$) with respect to the control group.

increase in expression compared to P22PI, although this difference was not statistically significant. Regarding P22PI, this immunostimulant upregulated TLR2 at all three doses evaluated, exhibiting a dose-dependent effect. In contrast, only the highest concentration led to a slight increase in TLR4 expression ([Fig. 1A-B](#)).

3.2. BMAP28 is extensively regulated by HIMB and P22PI

As previously described in TLRs gene expression, the immunostimulants also modified the expression levels of BMAP28, an antimicrobial peptide renowned for its potent antimicrobial activity. Notably, a more pronounced response was observed for HIMB immunostimulant at 7.8×10^6 CFU/ml ($p < 0.05$) in bovine foetal lung cells (Fig. 1C). P22PI induced cathelicidin expression similarly regardless of the dose used in pulmonary cells (Fig. 1C).

3.3. HIMB and P22PI promote pro-inflammatory cytokines

HIMB and P22PI were found to stimulate pro-inflammatory cytokines in bovine foetal lung cells by upregulating *TNFA* and *IFNG*. These cytokines are crucial mediators of the innate immune response and play a key role in developing Th1-type immunity, thereby significantly enhancing host defence mechanisms (Fig. 2A-B). HIMB exhibited significantly higher expression of these markers at 3.9×10^5 CFU/ml concentration, whereas P22PI showed greater expression at 10 μ g/ml, with a dose-dependent increase in their expression observed. Furthermore, when comparing the immunostimulants, significant differences were observed in *TNFA* expression between medium and high concentrations, as well as in *IFNG* expression between the lowest and highest concentrations. Higher expression levels of both markers were detected at the highest concentrations of P22PI. Additionally, a significantly higher expression of *IFNB*, a macrophage-derived cytokine with strong regulatory and anti-inflammatory effects on the immune system, was observed at concentrations of 7.8×10^6 CFU/ml and 10 μ g/ml for the HIMB and P22PI immunostimulants, respectively (Fig. 2C).

4. Discussion

The rise of multidrug-resistant tuberculous strains in recent decades has become a serious global human health threat and a significant challenge for TB control programs, emphasizing the necessity for new strategies in both prevention and treatment, particularly in endemic regions (Chai et al., 2020; Shah et al., 2017). Extensive evidence demonstrates that BCG vaccination provides protective effects against MTC members and heterologous pathogens in both humans and animals (Netea et al., 2016; World Health Organization, 2017). Indeed, the inclusion of BCG in infant vaccination programs of developing countries has resulted in a substantial reduction in TB-related and all-cause mortality (World Health Organization, 2017). Additionally, BCG vaccination in badgers in United Kingdom has been shown to mitigate disease severity and progression, thereby decreasing bacilli excretion, in both captive and wild populations (Benton et al., 2020). However, the safety limitations associated to live vaccines underscore the need for alternative vaccination strategies. This study highlights the immunostimulatory capabilities of two promising candidates based in mycobacteria inactivated or their derivatives, HIMB and P22PI, in bovine foetal lung cells. These candidates exhibit the ability to enhance antigen recognition and induce pro-inflammatory cytokines associated with an effective Th1 immune response, under *in vitro* conditions, suggesting their potential to modulate the immune system *in vivo*.

Although the documented protective effects of HIMB against MTC bacteria and unrelated pathogens (Garrido et al., 2011; Beltrán-Beck et al., 2014; Vaz-Rodrigues et al., 2022; Ferreras-Colino et al., 2023), as well as P22PI's significant humoral and cellular immunostimulatory properties (Infantes-Lorenzo et al., 2017; Risalde et al., 2017; Ortega et al., 2024), the present work is the first study to thoroughly compare the immunomodulatory mechanisms of these immunostimulants using a model of lung immunocompetent cell lines without stimulation by live pathogens. This approach provides new insights into their immunostimulatory effects under controlled conditions.

TLRs, a family of transmembrane proteins, are essential components of the innate immune system, pivotal in recognizing and defending

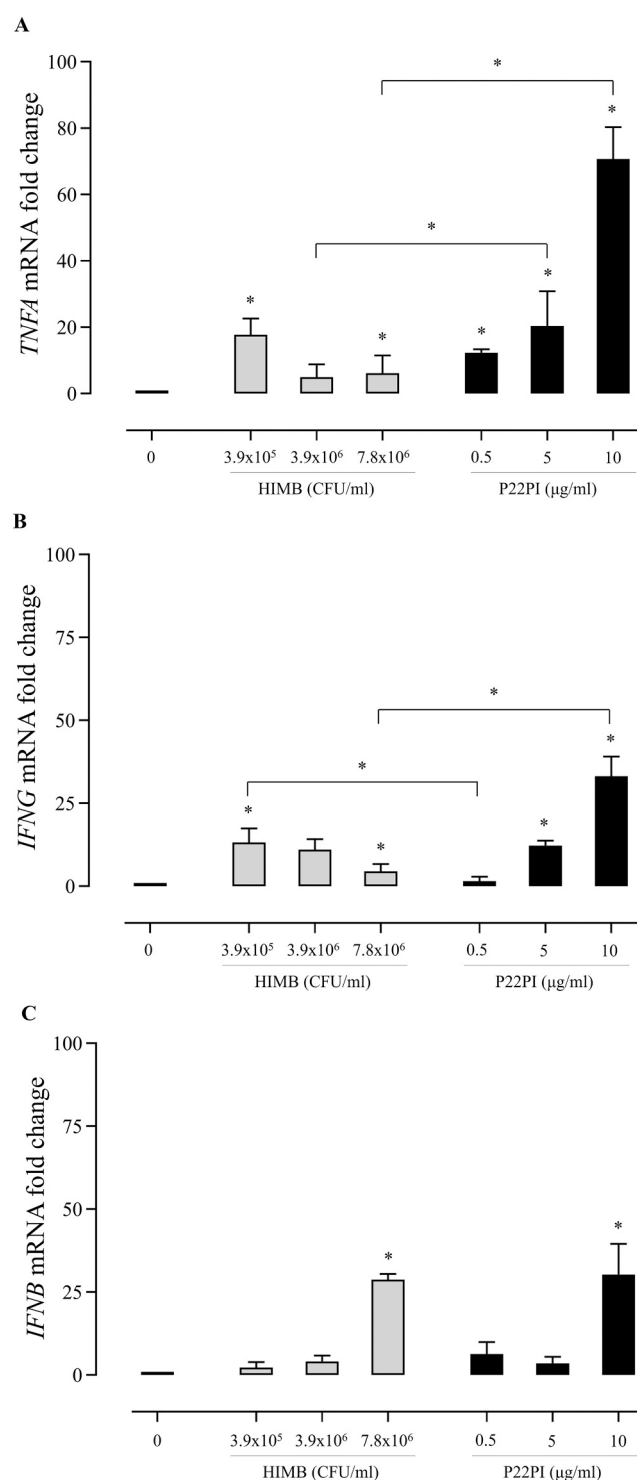


Fig. 2. Relative expression (mean fold change $\pm$ SEM) of *TNFA*, *IFNB* and *IFNG* in bovine foetal lung cells treated for 24 h with three concentrations of heat-inactivated *M. bovis* (HIMB) (3.9×10^5 , 3.9×10^6 and 7.8×10^6 CFU/ml) and three concentrations of P22 protein immunocomplex (P22PI) (0.5, 5 and 10 μ g/ml). For all treatments, three independent experiments were conducted, each with three replicates. *: statistically significant differences ($p < 0.05$) with respect to the control group.

against invading pathogens (Faridgohar and Nikouinejad, 2017). Among them, TLR2 and TLR4 are some of the most studied receptors, playing key roles in initiating innate immune responses and subsequently coordinating adaptive immunity against MTC infections and

other microorganisms (Faridgozar and Nikouejad, 2017; Ferreras-Colino et al., 2024). Our findings demonstrate that both HIMB and P22PI can boost TLR activation, potentially improving the recognition of mycobacteria. Specifically, they induced the upregulation of TLR2 and TLR4 expression in bovine foetal lung cells. Notably, P22PI primarily stimulated TLR2 rather than TLR4, while the highest overall TLR expression levels were observed in response to HIMB. These results align with previous findings showing a significant increase in TLR2 and TLR4 gene expression levels in fishes infected with *M. marinum* and immunized with inactivated BCG (Beltrán-Beck et al., 2014; Ferreras-Colino et al., 2024). The heightened TLR expression has been associated with a reduction in mycobacterial burden (Faridgozar and Nikouejad, 2017). The induction of TLR expression has also been reported in other *in vitro* studies using vaccines, with a notable increase in TLR4 expression observed in monocyte populations from healthy humans treated with the BCG Moreau vaccine (Antas et al., 2022). Indeed, studies have shown that TLR2-/- mice fail to effectively contain BCG growth in the lungs (Heldwein et al., 2003), underscoring the potential of these candidates to promote a protective immune response against mycobacteria.

TLR activation can be boosted by cathelicidins, a group of antimicrobial peptides found in several mammalian species (van Harten et al., 2018). These peptides are important components of the host's innate immune response against a broad spectrum of pathogens, such as bacteria, fungi, viruses and parasites (Khurshid et al., 2017), and they play a key role in inducing the migration of inflammatory cells such as neutrophils, monocytes/macrophages and B lymphocytes (Agier et al., 2015). Cathelicidins have demonstrated significant antimycobacterial effects in prior studies, suggesting their potential as adjunctive therapies for multi-drug-resistant mycobacteria strains (Palacios et al., 2022). Notably, BMAP 28 plays a crucial role in airway epithelial defences in cattle by exerting microbicidal activity and regulating pro-inflammatory cytokines (van Harten et al., 2018; Burucúa et al., 2020), making it a promising candidate for investigation against mycobacterial infections, although its role against tuberculosis has not been studied to date. In our study, we observed that BMAP28, a key cathelicidin in cattle (Burucúa et al., 2020), was upregulated in foetal lung cells by both HIMB and P22PI candidates. BMAP28 not only exhibits potent and spectrum *in vitro* antibacterial activity but also demonstrates efficacy in mouse models of Gram-negative bacterial infection (D'Este et al., 2012). Notably, this peptide displays cytotoxic activity and significantly reduces mortality in mouse models of bacterial infection (van Harten et al., 2018; D'Este et al., 2012). Although its antimycobacterial activity remains uncertain, its structural similarity to LL-37—a peptide known to be induced through TLR activation during the early stages of TB infection (Rivas-Santiago et al., 2008), which promotes autophagy and facilitates the intracellular killing of *M. tuberculosis* (Dhiman et al., 2023),—suggests that it may possess similar antimycobacterial properties. Both TLRs and cathelicidins, play crucial roles in regulating the expression of pro-inflammatory cytokines and cytokine receptors (Faridgozar and Nikouejad, 2017; van Harten et al., 2018). *TNFA* and *IFNG* are central players in numerous activities within both inflammatory and immune responses (Zeng et al., 2018). The production of these cytokines has been closely associated with effective T cell-mediated immunity against mycobacteria. Therefore, assessing cytokine production is a vital aspect of studying cellular immune responses to successful vaccination. We reported the upregulation of these cytokines in foetal lung cells following stimulation with HIMB and P22PI. Notably, P22PI induced a significant more robust response at higher concentrations compared to HIMB, particularly for *TNFA*. This enhanced response can be attributed to P22PI's composition, which includes several highly immunogenic antigens (Infantes-Lorenzo et al., 2017). These proteins are key to P22PI's potent immunostimulatory capacity, as evidenced by its superior sensitivity and specificity in ELISA assays compared to bPPD (Infantes-Lorenzo et al., 2017). This underscores the multiprotein complex's ability not only to be useful in studying and monitoring

humoral responses but also in inducing a robust cellular immune response *in vitro*, corroborating the findings reported previously (Risalde et al., 2017). The observed upregulation aligns with the activation of TLRs and cathelicidins described in this study. These findings are consistent with previous studies reporting a significant increase in *TNFA* and *IFNG* levels in human cell cultures treated with three attenuated *M. bovis* BCG vaccines (Ponte et al., 2018). Similarly, high levels of pro-inflammatory (*TNFA/IFNG*) profiles in BCG-infected monocytes from healthy adults vaccinated with BCG during childhood have been observed (Ponte et al., 2015). Furthermore, higher plasma levels of *TNFA* and *IFNG* in domestic (Arrieta-Villegas et al., 2018; Balseiro et al., 2017) and wild ruminants immunized with HIMB and infected with MTC members were also reported (Garrido et al., 2011; Beltrán-Beck et al., 2014). Our results indicate that HIMB and P22PI can stimulate the *in vitro* production of pro-inflammatory cytokines, which are associated with the development of the Th1 response, without exposing to MTC. However, further studies are needed to confirm whether these candidates can effectively promote a Th1 immune response *in vivo*, which, alongside the innate response, is crucial for controlling mycobacterial infections.

Exposure of foetal lung cells to HIMB and P22PI also upregulated the expression of *IFNB*, a pleiotropic cytokine that plays a crucial role in stimulating both innate and adaptive immune responses (Sabir et al., 2017). Although its significance in viral infections is well-established, its role in mycobacterial infections remains to be elucidated (Sabir et al., 2017), with both positive and negative functions observed (Moreira-Teixeira et al., 2018). Despite its late and high-level signalling has been associated with contributing to the pathogenesis of TB due to its anti-inflammatory characteristics (Moreira-Teixeira et al., 2018), the induction of *IFNB* by MTC members is a pivotal step in activating innate immunity through TLRs (Moreira-Teixeira et al., 2018). Moreover, under specific conditions, it can prime the production of pro-inflammatory cytokines (IL-12 and *TNFA*) and promote the recruitment, differentiation, and/or survival of protective myeloid cells (Moreira-Teixeira et al., 2018).

Lastly, our two immunostimulants may influence the activation and induction of immunological mediators related to innate immunity, promoting the development of heterologous memory, referred to as trained immunity. This process could be responsible for the nonspecific protective effects observed with certain vaccines, like BCG, or following certain infections (Kleinnijenhuis et al., 2014; Netea et al., 2016). In this context, HIMB has shown protective effects with other zoonotic pathogens in experimental animal models, reducing the symptoms, lesions and limited spread of unrelated bacteria (Vaz-Rodrigues et al., 2022) and intracellular parasites (Ferreras-Colino et al., 2023). While these studies highlighted the stimulation of innate immune responses after both infections, particularly involving macrophages, our findings reinforce the role of HIMB in stimulating innate immune mechanisms independent of exposure to viable pathogens, specifically through TLR activation and the production of antimicrobial peptides and pro-inflammatory cytokines.

While the findings in this study are promising, it is important to acknowledge that the use of primary cells from a single individual may not fully capture the immune variability within a population. However, to ensure the robustness of our results, three independent experiments were conducted for all treatments, each with three replicates. These technical replications enhance the reliability of the observed responses. Future studies incorporating cells from multiple individuals will be valuable to further assess potential inter-individual variability. Nevertheless, the outcomes observed in this study provide a solid foundation for future research into the development of these candidates as potential immunostimulants for broader application.

5. Conclusions

Our study demonstrates the immunostimulatory potential on innate

immune response of HIMB and P22PI under *in vitro* conditions, elucidating their mechanisms of action, including the upregulation of TLRs and cathelicidins, as well as the induction of pro-inflammatory cytokines such as *TNFA* and *IFNG*, which are integral to mounting a robust immune response against mycobacterial infections. These findings represent a significant contribution to the development of novel immunoprophylaxis strategies against TB. To fully validate the protective potential of HIMB and P22PI, it is essential that future studies focus on evaluating these candidates in *in vivo* infection models, assessing their ability to enhance antigen recognition, promote Th1 responses, and potentially modulate immunity against mycobacteria, as well as against heterologous pathogens within the framework of trained immunity

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CRediT authorship contribution statement

Martínez Remigio: Methodology. **Marin Maia S.:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Investigation, Funding acquisition, Conceptualization. **Risalde María A:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project administration, Investigation, Funding acquisition, Data curation, Conceptualization. **Ros Irene Agulló:** Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Chequepán Felipe A.:** Writing – review & editing, Methodology. **Burucúa Mercedes M.:** Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Sevilla Iker:** Writing – review & editing, Validation, Investigation, Conceptualization. **Domínguez Mercedes:** Writing – review & editing, Supervision, Investigation, Conceptualization. **Plá Natalia:** Writing – review & editing, Methodology.

Declaration of Competing Interest

None of the authors of this study has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.vetmic.2025.110527](https://doi.org/10.1016/j.vetmic.2025.110527).

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