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Investigating cytokine responses in rats: Genetic immunization against tuberculosis using five *Mycobacterium tuberculosis*-specific genes

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Abstract: Tuberculosis, being an infectious disease, is unchecked and still hard to wipe out in the underdeveloped countries. Despite ongoing efforts, no new TB vaccine has been successfully developed in the past century beyond BCG, although DNA-based vaccines have shown promise over the last two decades. In this study, five *Mycobacterium tuberculosis*-specific genes- *Rv1908c/KatG, Rv3418c/GroES, Rv0934/PhoS1/PstS, Rv0440/GroEL2 and Rv0350/DnaK*- were cloned into the pVAX1 expression vector to construct DNA vaccines. These constructs were evaluated in rats using naked DNA and BCG prime-boost strategies. Forty-five Wistar albino rats were divided into three major groups: DNA vaccine group, BCG prime-boost group and no vaccine control. Post-immunization responses were evaluated through cytokine ELISA for TNF-α, IFN-γ and IL-6. Among DNA vaccines, *DnaK-pVAX1* and *GroES-pVAX1* elicited the strongest cytokine responses, followed by *GroEL2-pVAX1* and *PstS-pVAX1*. The prime-boost groups (especially *BCG + DnaK-pVAX1*, *BCG + GroES-pVAX1* and BCG + cocktail) showed further enhanced responses. Statistical analysis confirmed significant cytokine elevation in vaccinated groups compared to controls ($p < 0.05$). DNA vaccines, whether used alone or in combination with BCG, show strong potential as immunogenic and therapeutic tools for TB and may help reduce treatment duration in the future.

Keywords: Bacille Calmette-Guérin (BCG); DNA vaccine; Interleukin-6 (IL-6); Interferon-gamma (IFN-γ); *Mycobacterium tuberculosis*; pVAX1; Tumor necrosis factor-alpha (TNF-α)

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INTRODUCTION

Tuberculosis (TB) is one of the ancient diseases known to humanity and is a leading infectious and communicable disease contributing to illness and mortality worldwide. In humans, *Mycobacterium tuberculosis (M. tb)*, which is part of the *Mycobacterium tuberculosis* complex (MTBC), is responsible for the development of TB (Natarajan *et al*., 2020). According to World Health Organization (WHO) Global TB Report 2023, TB was the second leading cause of death from a single infectious agent globally, following COVID-19 and resulted in nearly double the number of deaths compared to HIV/AIDS (Organization, 2023). It is estimated that over 25% of people on the planet have been exposed to TB with more than 10 million of new cases reported each year (Cords *et al*., 2021). Moreover, nearly half a million cases of Multi-Drug Resistant Tuberculosis (MDR-TB) are documented annually (Salari *et al*., 2023). Nonetheless, not all *M. tb* infected individuals exhibit disease symptoms. In fact, most infected people remain clinically asymptomatic. However, they face a serious risk of active TB development if they become immuno-compromised, leading to the reactivation of latent *M. tb (LoBue et al., 2010)*. The WHO End TB Strategy aims to decrease TB incidence by 90% and TB-related deaths by 95% by the year 2035 (Organization, 2023). This goal can only be accomplished through the development and delivery of robust diagnostic technologies, latest short-course therapies and effective vaccines (Safar *et al*., 2020).

The BCG (Bacillus Calmette-Guérin) vaccine was developed in 1921 by Albert Calmette and Camille Guérin in France. It was first used in humans in 1921 to prevent TB. Since then, it has remained the only licensed vaccine against TB, making it the sole TB vaccine for over 100 years. (Kumar, 2021, Sachdeva and Chadha, 2024). Despite its widespread use in many parts of the world, BCG has not consistently provided protective efficacy in humans, especially in the developing world. It is most effective in infants under 5 years, especially when given at birth, offering up to 40% protection against pulmonary TB. Its effectiveness declines with age and is minimal in adults, particularly in regions with high exposure to environmental mycobacteria. Prior non-tuberculous mycobacteria (NTM) exposure may interfere with BCG-induced immunity, reducing its protective efficacy (Moorlag et al., 2022, Adepoju and Onyezue, 2023). Hence, efforts have shifted towards developing new vaccines that can either complement or replace BCG, including genetic immunization, as a potential approach to inducing stronger, more targeted immune responses.

Genetic immunizations are considered as third-generation vaccines that stimulate both the cellular and humoral immune systems, making them ideal for preventing and

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treating various bacterial and viral infections (Wilkie and McShane, 2015). In the context of TB, DNA vaccines have the ability to induce a range of immune responses, including cytotoxic CD8+ T cell-mediated immunity and the production of interferon-gamma (IFN-γ). DNA vaccines offer several advantages over other vaccine types, including the ability to induce strong major histocompatibility complex (MHC) class I-restricted T lymphocyte (CTL) responses and facilitate efficient antigen presentation via MHC pathways. They also benefit from a standardized manufacturing process and low production costs. Additionally, DNA vaccines are highly effective at eliciting long-lasting memory immune responses, making them particularly appealing for vaccination strategies (Whitlow *et al*., 2020). Furthermore, DNA vaccine-based immunotherapy has proven effective as a complementary treatment when combined with chemotherapy in various animal models. This approach not only reduces the treatment duration but also enhances therapeutic outcomes in cases of latent TB infection (Wang et al., 2023, Liang *et al.,* 2023). Various *M. tb* genes, based on their functions, can contribute to different aspects of TB diagnosis, treatment and immunization. As a result, they can be utilized as preventive and therapeutic vaccines. *Rv1908c/KatG* is documented to be involved in *M. tb's* resistance to host-generated oxidative stress and is recognized by T cells in infected individuals, indicating its immunogenic potential (Ng *et al*., 2004). Similarly, *Rv0350/DnaK, Rv3418c/GroES* and *Rv0440/GroEL2* are chaperones that play crucial roles in *M. tb* survival and adaptation within host cells. Targeting these proteins with a DNA vaccine could potentially disrupt essential bacterial functions and enhance immune recognition with clearance of *M. tb (Qamra et al., 2004, Bulut et al., 2005, Fan et al., 2012). Rv0934/PhoS1/PstS* is part of the phosphate-specific transport system in *M. tb* and is involved in nutrient acquisition. It has been shown to induce strong cellular immune responses in experimental models, suggesting its potential as a vaccine antigen (Palma *et al*., 2013). This study was designed and conducted with the aim to clone the five *M. tb* specific genes *Rv1908c/KatG, Rv3418c/GroES, Rv0934/PhoS1/PstS, Rv0440/GroEL2 and Rv0350/DnaK* into mammalian expression vector pVAX1 for genetic immunization and to investigate the cytokine responses in the rat model. The selection of these specific genes for DNA vaccine development against *M. tb* is based on their immunogenicity, conservation across strains, functional roles in bacterial survival and their ability to induce robust immune responses in shape of cytokine production.

MATERIALS AND METHODS

Bacterial strains, vector and selected genes

The mammalian expression vector pVAX1™ vector and the DH5α bacterial strain used for transformation were obtained from Invitrogen, Thermo Fisher Scientific™, USA. All the selected genes in cloned pET vector form were obtained from BeiResources, National Institute of Allergy and Infectious Diseases (NIAID), USA.

Development of DNA vaccines

All the selected genes were amplified through PCR by using DreamTaq™ Green PCR–Master Mix from Thermo Scientific. Primers for each gene were designed by incorporating appropriate restriction sites into both the forward and reverse primers. In the forward primers of each gene, the Kozak sequence was also added before the restriction site to ensure proper initiation of translation. pVAX1 vector and all the PCR products were double-digested with their respective restriction endonucleases followed by ligation using T4 DNA ligase and finally transformed into competent cells. Confirmation of positive clones was done through colony PCR, restriction digestion and ultimately by DNA sequencing from BGI Tech Solutions Hong Kong. Endotoxin free plasmid preparation was made using GeneJET plasmid Maxiprep kit (Cat # K0491) of Thermo Fisher Scientific™ (Zaman *et al*., 2024).

Animal trial

Forty-five nonpathogenic six to eight weeks old female Wistar albino rats (weighing around ~160 ± 20 g) were obtained and kept at animal house of the institute in controlled conditions(temperature: 25-30 °C, humidity: 45 ± 15%, light/dark cycle: 12 h).

Immunization

Rats were divided into three major groups: A-DNA vaccine group, B-Prime boost group and C-No Vaccine group. Group A was further divided into seven sub-groups including first five groups (AI-AV) received individual DNA vaccine (200µg/200µl normal saline intramuscularly in quadriceps) and sixth group (AVI) received a cocktail of all DNA vaccines (40µg of each), while in seventh group (AVII) 200µg of pVAX1 vector was injected as negative control. Similarly, prime boost group B is also subdivided into seven groups representing the first five individual DNA vaccine groups (BI-BV) and cocktail group (BVI). All these groups (BI-BVI) were primed by BCG vaccine (B. No: 0371G081) 10^6 CFU (subcutaneously) and after twenty-one days DNA vaccine was injected, while BVII control group received only BCG vaccine. Third major group C represents the no vaccine group which received only normal saline (200µl). Each subgroup (AI–AVII, BI–BVII) and C consisted of 3 rats (n = 3).

Collection of serum samples

Upon receiving the rats and once they had acclimated to the new environment, the first blood sample (T1) was taken on day zero, prior to vaccination. After 21 days (T2), the second blood collection was performed and a DNA booster was administered in the BCG-primed groups (BI-BVI). The final blood sample (T3) was collected after 42 days and the animals were disposed of through incineration in accordance with the proper procedures.

Post vaccination cytokine analysis and Statistical Evaluation

Cytokine ELISA was performed for the estimation of Tumor Necrosis Factor-alpha (TNF-α), Interferon-gamma (IFN-γ) and Interleukin-6 (IL-6) levels in each rat serum sample by using Invitrogen™ Rat ELISA Kit for specific cytokine. TNF-α, IFN-γ and IL-6 were selected as they are key cytokines involved in the host immune response against *M. tb*. Their levels reflect the Th1-type cellular immunity and inflammatory response induced by the DNA vaccine. Following data summarization and presentation as means and standard deviations, statistical analyses were conducted using one-way ANOVA by Dunnett's two-tailed test with $\alpha=0.05$ and a p-value <0.05 indicated a significant difference, whilst a p-value >0.05 indicated no or very little difference.

RESULTS

DNA vaccine construction

All the selected PCR amplified Mycobacterium specific genes along with pVAX1 vector were double digested followed by ligation and transformation into competent cells. Single and double restriction digestion and Colony PCR confirmed the clones and successful transformation. Proper orientation of the specific gene into the vector was finally verified through sequence analysis. According to BLAST analysis of each gene, the sequence confirmed the same protein as reported in its own bacterial system. It also gave the exact gene size as was inserted into vector hence confirming the clones (Zaman *et al*., 2024).

Inoculation of rats and their survival

Three main groups of animals were created representing the A-DNA vaccination group, the B-Prime boost group and the C-No vaccination group BCG was given to the group-B prior to DNA vaccinations, whereas just DNA vaccines were injected into the group-A. Five of the twenty-one animals in the BCG primed DNA vaccine group died within a few days of receiving their first dose of BCG due to fever. To maintain a constant number of animals in each group, healthy animals were used to replace the dead ones and the process was repeated; the replaced animals survived without any adverse effects. Following the initial injection of the DNA vaccine, none of the animals in group A displayed any temperature changes and they all maintained an exponential weight-to-growth ratio and stayed healthy throughout the study.

Post immunization analysis of serum cytokines levels

The levels of particular desired cytokine (TNF-α, IFN-γ and IL-6), estimated through Cytokine ELISA are mentioned in table 1, 2 & 3. In table 1, comparative analysis of serum TNF-α level between Group A, B and C showed that there is significant increase in the concentration of TNF-α in both vaccinated groups A & B than unvaccinated groups A-VII and C. Among the DNA vaccine groups, *DnaK/pVAX1* (AII) showed highest level of TNF-α in T2 and T3, while in BCG/DNA vaccine Group (B), BCG + cocktail (BVI) and BCG + *GroES* (BV) showed highest level of TNF-α in T3 as compared to BCG only group (BVII). Similarly, table 2 presents the comparative analysis of serum IFN-γ level between all groups as significant increase in the concentration of IFN-γ in group A and C than unvaccinated groups. *PstS/pVAX1* (AIV) and *DnaK/pVAX1* (AII) showed highest level of IFN- γ in T2 and T3 among DNA vaccine group while BCG + cocktail (BVI) showed highest level of IFN-γ in T2 and T3 along with BCG + *GroES/pVAX1* (BV) group which also showed high IFN-γ level in T3. Furthermore, Comparative analysis of serum IL-6 level between all groups mentioned in table 3 showed that first three groups of DNA vaccine (AI- AIII) showed highest level of IL-6 in T2 and T3 while among the B groups, BCG + *GroES* (BV) showed highest level of IL-6 in T2 and T3 along with BII and BVI group which also showed high IL-6 level in T2 and T3.

Statistical analysis

Results of Serum TNF-α level (Fig. 1) showed that all the groups of A and B were significantly different from pVAX1 group (AVII) and no vaccine group ($p<0.05$) in T2 and T3. Similarly, Serum IFN-γ level results (Fig. 2) indicated that in T2 and T3, all A and B groups were significantly different from the pVAX1 group (AVII) and the no vaccine group ($p<0.05$). Besides, for IL-6 (Fig. 3) AI-AIII in DNA vaccine group and BII, BV & BVI in BCG primed group were found significantly different from non-vaccinated groups in T2 and T3 while AIV, AVI, BI, BIII and BIV were differed significantly only in T2. Only AV group was present with no significant difference ($p>0.05$).

DISCUSSION

The primary goals of the WHO tuberculosis eradication program are to lower the worldwide prevalence of TB and create new, more potent medications and a vaccine to prevent the disease (Srivastava *et al*., 2023). Despite the completion of *M. tb* whole genome sequencing, over 100 DNA vaccines have been developed and tested in different animal models; yet, the protective antigen for TB remains unclear. The development of TB vaccines has been hampered by this deficiency (Zhuang *et al*., 2023). In present work, five *M. tb* genes were amplified and subsequently cloned into the mammalian expression vector pVAX1. According to reports, all of the chosen genes are structural components of the cell and have major biological roles, indicating that they are both crucial for bacterial survival and for being recognized as antigens (Munir et al., 2021, Lopes et al., 2014, Vinod et al., 2021, Esparza et al., 2015, Maurya et al., 2014). As in recent investigations *Rv1908c/KatG* was chosen and employed by various groups, revealing the significance of this gene in DNA vaccination and bacterial pathogenicity (Ofori-Anyinam *et al*., 2023). In another research, TB patients were given recombinant DnaK (Rv0350) and MPT83 (Rv2873) gene constructs, which were found to be effective in inducing an immune response against *M. tb* (LI *et al*., 2019).

Table 1: Cytokine ELISA for TNF-α in Group A, B and C.

T1 Day Zero (Pre-vaccination Time)		T2 (21 days after T1)		T3 (42 days after T1)
Sample Name		Mean Conc. (pg./ml) SD	Mean Conc. (pg./ml) SD.	Mean Conc. (pg./ml) SD.
Group A	AI (KatG/pVAX1)	312.716 ±2.4	395.32 ±1.4	570.767 ±0.8
	AII (DnaK/pVAX1)	288.058 ±0.9	585.312 ±0.4	767.915 ±1.4
	AIII (GroEL2/pVAX1)	304.56 ±1.2	739.487 ±1.3	582.518 ±2.1
	AIV (PstS/pVAX1)	279.775 ±1.7	580.708 ±2.3	562.386 ±1.5
	AV(GroES/pVAX1)	289.204 ±0.8	536.915 ±1.7	515.21 ±1.9
	AVI (Cocktail)	312.457 ±1.4	529.332 ±0.8	577.776 ±2.6
	AVII (pVAX1 only)	318.445 ±2.1	342.2 ±2.1	330.563 ±3.1
Group B	BI (BCG + KatG/pVAX1)	308.985 ±1.4	546.661 ±2.1	367.232 ±2.2
	BII (BCG + DnaK/pVAX1)	306.435 ±2.4	537.87 ±2.7	450.316 ±1.6
	BIII (BCG + GroEL2/pVAX1)	282.328 ±1.5	514.457 ±1.6	565.292 ±0.9
	BIV (BCG + PstS/pVAX1)	318.714 ±1.1	523.963 ±0.4	504.591 ±2.1
	BV (BCG + GroES/pVAX1)	307.516 ±1.3	403.508 ±2.3	612.419 ±0.7
	BVI (BCG + Cocktail)	302.639 ±2.7	386.611 ±2.3	753.394 ±1.2
	BVII (BCG only)	319.554 ±2.1	434.751 ±1.4	580.519 ±1.9
Group C (No vaccine)		299.554 ±0.5	314.034 ±0.8	302.734 ±1.2

Table 2: Cytokine ELISA for IFN- γ in Group A, B and C.

T1 Day Zero (Pre-vaccination Time)		T2 (21 days after T1)		T3 (42 days after T1)
Sample Name		Mean Conc. (pg./ml) SD	Mean Conc. (pg./ml) SD.	Mean Conc. (pg./ml) SD.
Group A	AI (KatG/pVAX1)	62.646 ±1.1	161.394 ±1.7	139.83 ±1.3
	AII (DnaK/pVAX1)	61.545 ±2.4	143.783 ±1.5	196.94 ±1.7
	AIII (GroEL2/pVAX1)	76.767 ±0.4	134.724 ±0.9	133.381 ±1.2
	AIV (PstS/pVAX1)	89.876 ±1.3	145.594 ±1.4	233.978 ±0.4
	AV(GroES/pVAX1)	70.656 ±0.9	196.188 ±2.1	127.587 ±1.1
	AVI (Cocktail)	88.089 ±0.7	175.936 ±2.0	151.142 ±1.7
	AVII (pVAX1 only)	75.221 ±0.4	82.794 ±1.6	87.453 ±2.3
Group B	BI (BCG + KatG/pVAX1)	72.536 ±2.1	150.649 ±1.1	134.549 ±0.7
	BII (BCG + DnaK/pVAX1)	64.803 ±1.3	163.182 ±0.9	120.65 ±1.8
	BIII (BCG + GroEL2/pVAX1)	84.094 ±1.1	147.667 ±2.1	144.53 ±1.6
	BIV (BCG + PstS/pVAX1)	76.768 ±0.7	147.537 ±2.3	135.467 ±2.0
	BV (BCG + GroES/pVAX1)	71.719 ±0.8	118.567 ±1.9	162.531 ±1.1
	BVI (BCG + Cocktail)	84.502 ±1.4	164.776 ±1.0	186.778 ±2.1
	BVII (BCG only)	69.957 ±1.7	93.076 ±0.6	105.647 ±0.4
Group C (No vaccine)		79.554 ±0.6	84.034 ±1.1	82.734 ±0.8

Table 3: Cytokine ELISA for IL-6 in Group A, B and C

T1 Day Zero (Pre-vaccination Time)		T2 (21 days after T1)		T3 (42 days after T1)
Sample Name		Mean Conc. (ng./ml) SD	Mean Conc. (ng./ml) SD.	Mean Conc. (ng./ml) SD.
Group A	AI (KatG/pVAX1)	0.64 ±.04	10.9 ±0.1	19.1 ±.4
	AII (DnaK/pVAX1)	0.5 ±.07	11.72 ±.2	16.32 ±.3
	AIII (GroEL2/pVAX1)	0.72 ±.11	25.9 ±.3	14.98 ±.1
	AIV (PstS/pVAX1)	0.65 ±.03	11.84 ±.1	0.66 ±.03
	AV(GroES/pVAX1)	0.65 ±.09	0.75 ±.09	0.67 ±.02
	AVI (Cocktail)	0.8 ±.06	18.73 ±.13	0.65 ±.04
	AVII (pVAX1 only)	0.92 ±.12	0.75 ±.05	0.79 ±.06
Group B	BI (BCG + KatG/pVAX1)	0.65 ±.04	18.65 ±.1	0.73 ±.09
	BII (BCG + DnaK/pVAX1)	0.71 ±.06	18.61 ±.3	16.45 ±.1
	BIII (BCG + GroEL2/pVAX1)	0.89 ±.09	19.22 ±.2	0.79 ±007
	BIV (BCG + PstS/pVAX1)	0.65 ±.03	23.5 ±5	0.72 ±.06
	BV (BCG + GroES/pVAX1)	0.57 ±.1	23.21 ±.6	21.09 ±.2
	BVI (BCG + Cocktail)	0.75 ±.02	12.58 ±.11	19.38 ±.3
	BVII (BCG only)	0.56 ±.06	7.1 ±.04	6.31 ±.1
Group C (No vaccine)		0.79 ±0.6	0.79 ±0.6	0.69 ±0.02

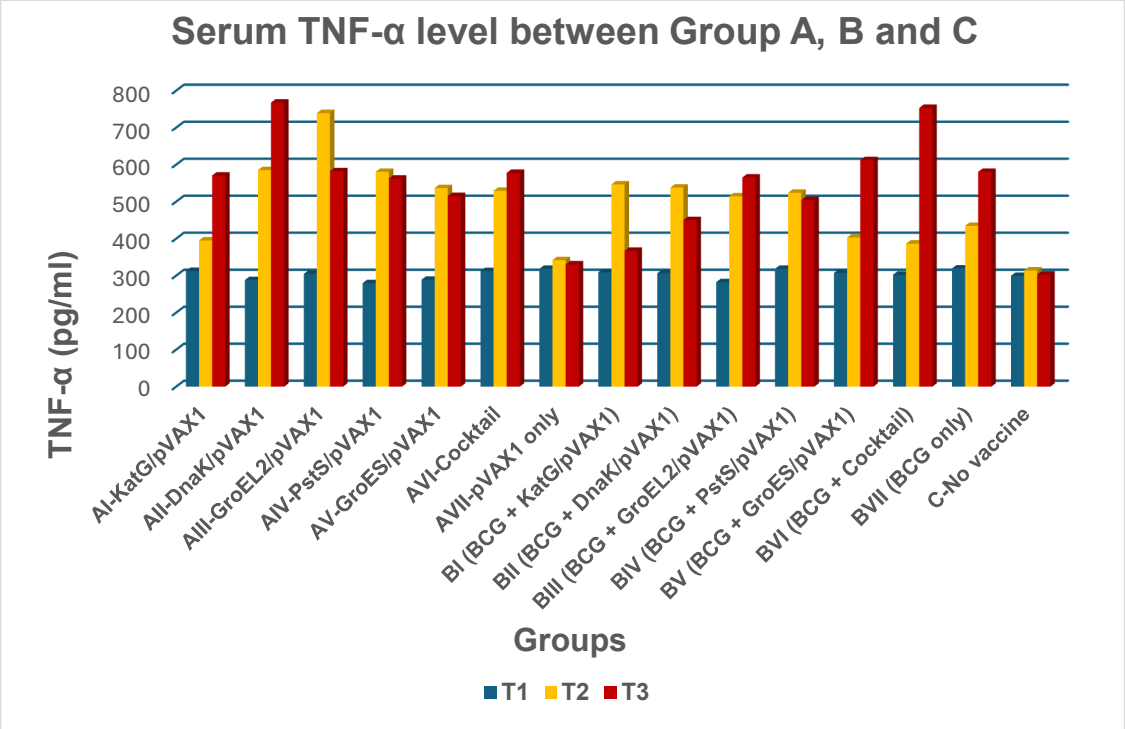

Fig. 1: Serum TNF-α levels across Group A (DNA vaccine), Group B (BCG-primed DNA vaccine), and Group C (No vaccine group) at three time points.

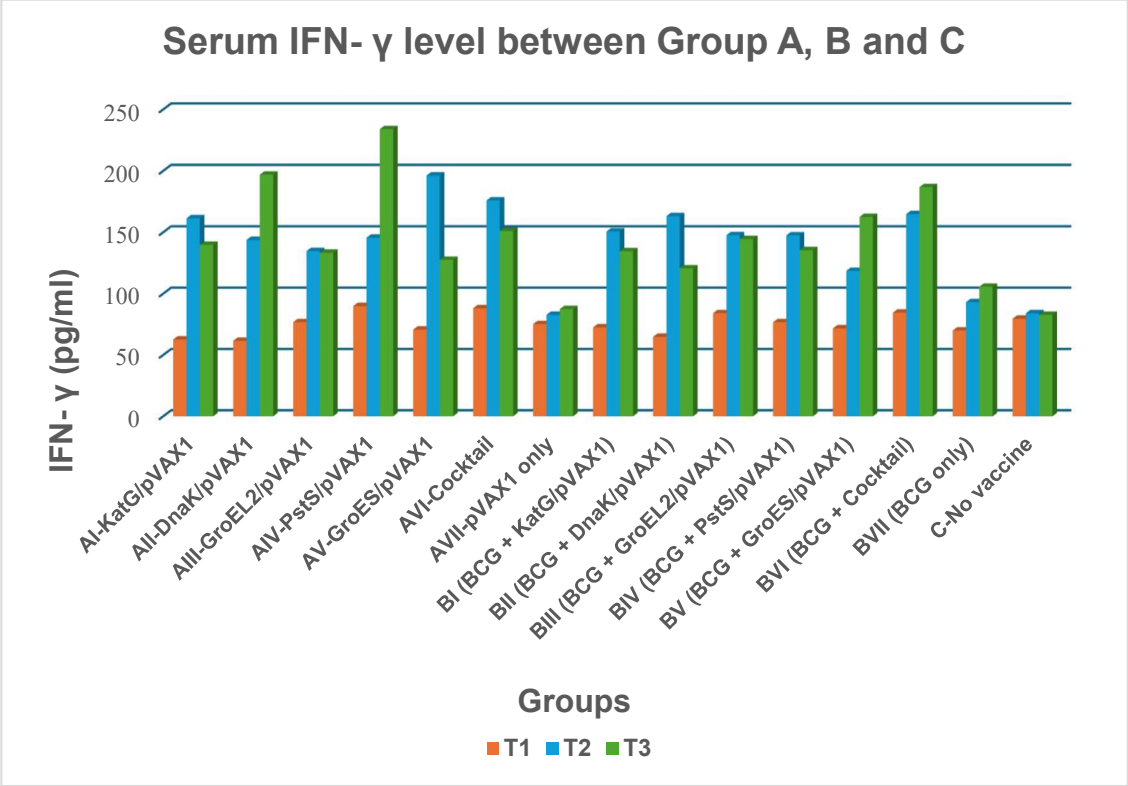

Fig. 2: Serum IFN- γ levels across Group A (DNA vaccine), Group B (BCG-primed DNA vaccine), and Group C (No vaccine group) at three time points.

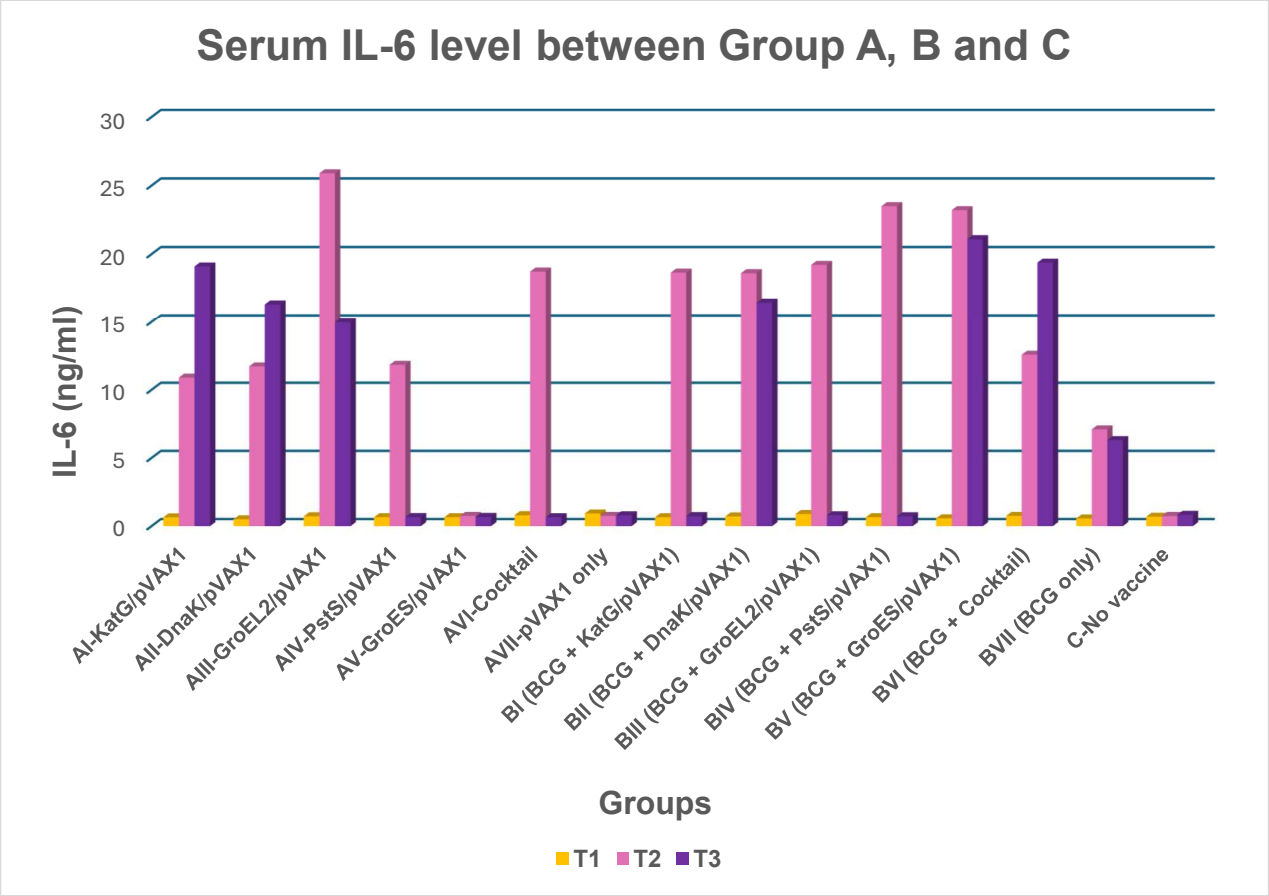

Fig. 3: Serum IL-6 levels across Group A (DNA vaccine), Group B (BCG-primed DNA vaccine), and Group C (No vaccine group) at three time points.

Cloning the genes of M. tb's immunodominant antigen, PstS and its T cell epitopes (PstS1p), is presented in another paper. The results of this study demonstrated the immunogenicity and immunity of PstS1 and its epitope PstS1p, which markedly boosted the production of antigen-specific IgG antibodies in the serum of mice, indicating an enhanced antibody response (Fan *et al*., 2021).

By measuring the level of cytokines (TNF-α, IFN-γ and IL-6), the current study investigated the effects of Mycobacterium-specific DNA vaccines and their capacity to mediate the immune system. Numerous studies have revealed the vital role of cytokines and chemokines play in the activation and control of cellular responses against *M. tb*-infected lungs. These immune mediators are crucial for controlling the degree and type of granulomatous inflammation in addition to coordinating the recruitment and arrangement of immune cells at the infection site. They are also essential in controlling the growth of germs and avoiding excessive tissue damage brought on by dispersed and unchecked immune responses (Boni *et al*., 2022). TNF-α and IFN-γ work synergistically to activate macrophages by triggering reactive nitrogen intermediates (RNIs) and promoting immune cell migration to infection sites, aiding granuloma formation. IFN-γ also plays a central role in anti-mycobacterial immunity by elevating the microbicidal activity of macrophages (Cavalcanti *et al*., 2012). Furthermore, elevated IL-6 and IFN-γ serum levels can differentiate active TB from latent infection. Effective TB diagnosis and treatment methods are thus supported by alterations in cytokine patterns (Chandrashekara *et al*., 2016).

In this comparative study, both the DNA vaccine group and the BCG-primed DNA group had considerably higher levels of cytokines in post-immunization serum, specifically activated with antigens, as compared to BCG control and no vaccine groups. Especially, *DnaK/pVAX1* and *GroES/pVAX1* present the foremost production of cytokines, while *GroEL2/pVAX1* and *PstS/pVAX1* also significantly release the cytokines. Prime boost with BCG also showed the substantial cytokine releasing potential, especially the BCG + *DnaK/pVAX1*, BCG + *GroES/pVAX1* and BCG + Cocktail.

CONCLUSION

We successfully developed five *Mycobacterium tuberculosis* DNA vaccines which were able to elicit and stimulate the immune response in shape of cytokine release

in rat models, revealing their potential protective effects when used individually, in combination, or as a prime-boost strategy with BCG. Future studies will include challenge experiments in animal models to evaluate these DNA vaccines, with continued efforts toward optimizing and validating *M. tuberculosis*-specific constructs for potential human trials.

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Authors' contributions
Muhammad Mohsin Zaman
Conceived idea, conducted the research, collected, analyzed and interpreted the data and wrote the article.
Mirza Imran Shahzad
Planned and supervised the research, edited and approved the final manuscript.
Gildardo Rivera
Edited and approved the final manuscript.
Aeman Jilani
Conducted the research, collected, analyzed and interpreted the data.
Areeba Yousaf
Conducted the research, collected, analyzed and interpreted the data.

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Data availability statement
All data supporting the findings of this study are included in the published article.

Ethical approval
All experimental procedures involving animals were conducted in strict accordance with institutional and international ethical guidelines for the care and use of laboratory animals. The study protocol was reviewed and approved by the Pharmacy Animal Ethics Committee (PAEC) of The Islamia University of Bahawalpur, under approval number PAEC/22/77. Efforts were made to minimize animal suffering, reduce the number of animals used and employ humane endpoints. All animals were housed under standard laboratory conditions with proper care, nutrition and monitoring throughout the study duration.

Conflict of interest
Authors declare no conflicts of interest.

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