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Original Article

Development of DNA vaccine(s) against *Mycobacterium* specific genes and prime boost with BCG

Aeman Jilani[1], Mirza Imran Shahzad[1*], Muhammad Mohsin Zaman[1], Areeba Yousaf[1], Gildardo Rivera[2]
[1]Institute of Biochemistry, Biotechnology and Bioinformatics, The Islamia University of Bahawalpur, Pakistan
[2]Laboratorio de Biotecnología Farmacéutica, Centro de Biotecnología Genómica, Instituto Politécnico Nacional, México

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**Corresponding author email:*
mirza.imran@iub.edu.pk

Abstract

Tuberculosis (TB), the white plague of Europe is still uncontrolled and fatal in many parts of the world including Pakistan. It is a major cause of morbidity and mortality in human and domestic animals in Pakistan. No new vaccine in the last hundred years has been developed except for a few encouraging results from recombinant and DNA vaccines in the past two decades. Five Mycobacterium specific genes (Rv0379, Rv3914, Rv3006, Rv0432+SP, and Rv0432-SP) were selected to develop DNA vaccine(s). All the constructs were tested on mice using both naked DNA and prime-boost methodologies. Forty-five BALB/c mice were divided into three main groups; DNA vaccine group, BCG Prime boost group, and Control group. Post-vaccine (PV) and post-challenge (PC) immune responses were confirmed through cytokine ELISA and qRT PCR. IFN-γ was additionally checked in plasma as well. Based on cytokine ELISA PC immune responses showed significant differences in TNF-α levels for both naked DNA vaccine groups (Rv0379, Rv3006, and Rv0432-SP) and BCG primed Rv3914 group in comparison to the BCG control group ($p<0.05$). Based on qRT PCR, IL-6, TNF-α, IFN-γ, and IL-1β showed no significant difference among all the vaccines and BCG control groups (CT range 25- 30). IFN-γ levels in plasma were analyzed PC; two vaccines Rv3006/LppZ and BCG primed Rv0432/SodC-SP (highest mean value 1360.35 pg/ml) have shown significant results (cutoff value 21pg/ml) at 63 days. All the vaccine construct(s) alone or in combination have significant therapeutic effects in comparison to BCG and negative control groups.

Keywords: *Mycobacterium tuberculosis*, DNA vaccine, Tumor necrosis factor-alpha, Interleukin-6, Interferon-gamma, Interleukin-1beta, Bacille Calmette-Guérin (BCG)

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Introduction

Tuberculosis (TB) ranks among the primary contributors to human mortality caused by a single infectious agent. In 2020 only, 10 million cases and 1.5 million deaths were reported because of it (Organization, 2021). *Mycobacterium tuberculosis (M.tb)* is the pathogenic microorganism responsible for tuberculosis (TB) in humans. Pulmonary TB is the most common presentation of this disease

because it mainly affects the lungs (Terracciano et al., 2020). Other organs that are affected by this disease include the gastrointestinal system (GI), skin, central nervous system (CNS), lymph reticular system, reproductive system, musculoskeletal system, and the liver (Mbuh et al., 2019, Mathiasen et al., 2020). There have been some integrated global efforts in the past few decades for the eradication of TB. Notwithstanding the success of TB control, TB still carries a heavy burden of morbidity and mortality. The factor that poses a hindrance in the treatment is its two types including Multi-Drug Resistant Tuberculosis (MDR-TB) and Extremely Multi-Drug Resistant Tuberculosis (XDR-TB). The strains of *M.tb* that have developed resistance to the most common and effective antibiotics of this disease are referred to MDR-TB. To diagnose MDR-TB it has to show resistance to the two standard anti-tuberculosis drugs, Rifampicin or Isoniazid.

Confronted with ineffective drug therapies, effective vaccines including therapeutic vaccines are needed. Cellular immune response provides protective immunity against TB by the production of the Th1-type cytokines including interferon-gamma which predominates over Th2-type cytokines like Interleukin-4 (Cooper et al., 1993, Flynn et al., 1993, Flynn, 2004). In the direction of responses DNA vaccine has been found to boost and establish antigen-specific cellular immunity. Furthermore, in combination with chemotherapy in mice, plasmid DNA-based immunotherapy has demonstrated its effectiveness as a complementary treatment. It not only minimizes the treatment time but also gives improved therapy results in latent TB infection (Ha et al., 2003, Yu et al., 2008). Depending on the functions of various *M.tb* genes, many genes can help in different areas of diagnosis, treatment, and immunization against TB and thus, can be used as therapeutic vaccines. Rv0379 is documented as a critical protein for the virulence and survival of the bacteria as it encodes a putative transport protein SecE2 hence becoming a good candidate to be tested for DNA vaccine (Yari et al., 2015). Similarly, Rv3914 encoding Thioredoxin protein TrxC and Rv0432 encoding Periplasmic Superoxide dismutase SodC have been reported to be valuable candidate antigens for diagnosing active pulmonary TB (Xin et al., 2014). One dominant antigen in age-related immune responses over 45 years of age primed with BCG vaccine is Rv3006, which encodes a probable conserved lipoprotein LppZ (Yang et al., 2021).In this study, five genes of *M.tb* (Rv0379, Rv3914, Rv3006, Rv0432+SP, and Rv0432-SP) were investigated as therapeutic vaccines or boosters post-priming with BCG to enhance the immune response against TB. With the aim of cloning these genes in the mammalian expression vector pVAX1, the present study was designed, taking into consideration their importance in bacterial metabolism and their function in infection and protection through various means. Later, the vaccine constructs underwent *In vivo* testing for naked DNA vaccines on a mouse model, followed by a prime boost with BCG vaccination. Animals were challenged and cytokine response was observed and analyzed statistically.

Material and Methods

Preparation of recombinant plasmids/DNA vaccines

Five Mycobacterium-specific genes (Rv0379, Rv3914, Rv3006, Rv0432+SP, and Rv0432-SP) were amplified using forward primers specifically designed with *HindIII* (A^AGCTT) and Kozak sequences upstream the ATG and reverse primers with *XbaI* (T^CTAGA**)** restriction sites at 5' end. The obtained PCR product was ligated with plasmid vector pVAX1 (Thermo-Fisher Scientific) and then transformed into E.coli Top10 (Sambrook and Russell, 2006).The recombinant plasmids were analyzed by Macrogen, USA had 100% identity with the designed sequence by BLAST analysis. EndoFree plasmid purification kit (SolarBioEndofree maxi prep kit) was used to purify the clones for the *In vivo* trial.

In Vivo study

Forty-five BALB/c mice of mixed gender, aged 6-8 weeks, were purchased from the University of Veterinary and Animal Sciences Lahore. The mice were raised under controlled, pathogen-free conditions. The study procedures were approved by the ethical committee of the institute.

M.tb Strain

M.tb H37Rv for challenge study was provided by Bahawalpur Victoria Hospital, Bahawalpur.

Immunogenicity of DNA vaccines

Forty-five BALB/c mice were divided into three main groups: DNA vaccine group, Prime boost group and Control group. Three mice received five DNA vaccines each (100 µg in 100 µl saline), while a sixth

group received a cocktail of all five vaccines (20 µg of each in 100 µl saline). Prime Boost group was primed by BCG vaccine (B. No:0371G081) (0.05 ml) provided by Health Department of Bahawalpur and after twenty-one days DNA vaccine was injected to each group along with a cocktail group. The control group had nine mice: (1) saline as a negative control (100 µl saline), (2) vector pVAX1 as a negative control (100 µg in 100 µl saline) and (3) BCG (0.05 ml) positive control group.

Serum sample collection

After the mice were received, after inhabiting to the new environment, their first bleed was taken and they were then injected through the tail vein (B0). The second bleed (B1) was collected 21 days after the first immunization, and the BCG primed group received their DNA booster. After another 21 days i.e. 42 days from the first DNA vaccine dose, B2 bleed was collected.

Challenge and sacrifice

The mice were challenged right after B2 bleed was taken by aerosol spray (6.4×10^5 CFUs) with *M.tb* H37Rv in aerosol chamber. Challenged animals were kept alive for another 21 days with continuous monitoring of their vitals. After 21 days, the animals were sacrificed. Blood (B3) was collected via cardiac puncture, while the animals were disposed of via incineration through the proper channels.

Cytokine production in vitro

Sandwich ELISA was performed on all the pre challenge and post challenge serums for cytokines Tumor Necrosis Factor-α, Interferon-Ɣ and Interleukin-6, and absorption was recorded and converted to concentration in pictograms/milliliter.

Quantiferon assay

Total RNA was isolated using the Multi-type Sample DNA/RNA Extraction-Purification Kit (Sansure Biotech Inc, China). The RNA specimens were then reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) to produce cDNA. qRT-PCR was executed in a SLAN-96P Real-Time PCR System (Sansure Biotech Inc, China). The relevant cycle thresholds (Ct) of samples were compared against controls and control samples containing housekeeping genes (GAPDH).

Statistical analysis

Data has been summarized and presented as means and standard deviations. Statistical analyses were done by using one-way ANOVA by Dunnett's two tailed test with α=0.05 and p-value <0.05 was considered a significant difference while p-value>0.05 was considered as no or negligible difference. Fold change of gene expression was calculated from $2^{-\Delta\Delta Ct}$ values obtained from C_T values of the cytokines and graphs were designed in Graph Pad Prism 9.

Results

Preparation of clones

Restriction digestion confirmed the clones with two or more than two bands after digestion, higher MW bands showed the completely or partially digested vectors and the lower MW bands depicted the gene of interest. The Fig.1 shows representative results of restriction digestion of all the clones except Rv3006-pVAX1. This clone had DAM methylation at *Xba1* restriction site therefore the site was blocked and therefore it was linearized after cutting with *HindIII* and was further confirmed by sequence analysis. Sequencing results confirmed not only the presence of gene but also the orientation of insert in vector. 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.

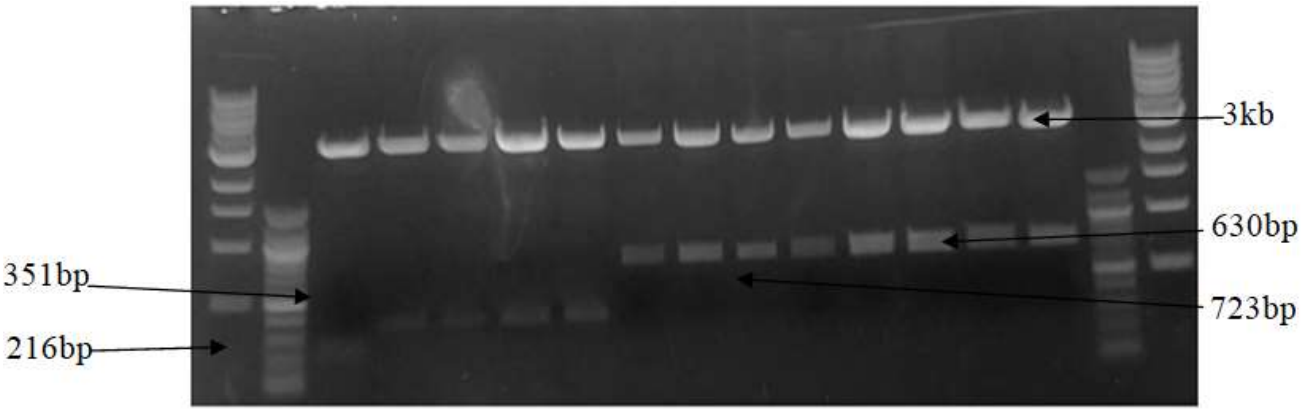


Figure-1. Gel electrophoresis results of Rv0379, Rv3914, Rv0432+SP and Rv0432-SP. lane 1 and 17: 1kb ladder (New England BioLabs *Inc.* cat #N3232L), lane 2 and 16: 100bp ladder (cat#BM301-01, TransGen Biotech Co., LTD), Lane 3: Rv0379 (216bp) + vector pVAX (3kb), Lane 4-7: Rv3914 (351bp) + vector (3kb), Lane 8-11: Rv0432+SP (723bp) + vector (3kb) and Lane 12-15: Rv0432-SP (630bp) + vector (3kb).

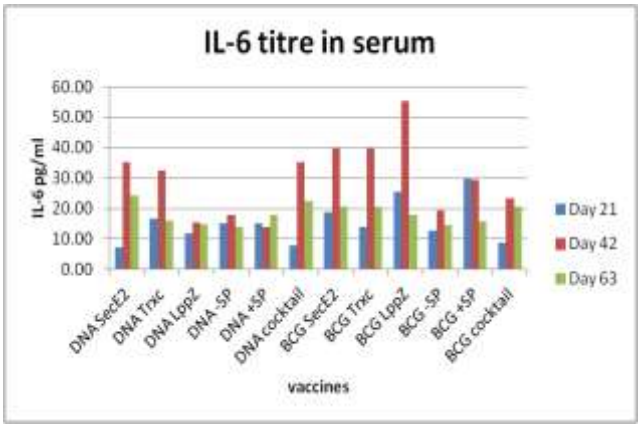
Mice immunization and survival

Animals were divided into two major groups i.e. BCG primed DNA vaccines and naked DNA vaccines. In first group BCG was administered before DNA vaccines while in second group only DNA vaccines were injected. In BCG primed DNA vaccine group 7 out of 21 animals developed fever and died in first few days after initial dose of BCG. Dead animals were replaced with healthy ones and same procedure was repeated to make the number of animals in each group constant. In group 2, none of the animals showed any fluctuation in temperature after giving the first dose of DNA vaccine and remained healthy throughout the trial with exponential weight to growth ratio. The mice were immunized with DNA vaccine by injecting it intradermally in both their legs and tails.

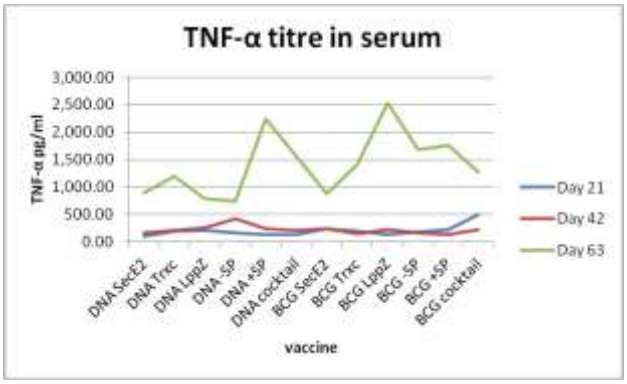
Specific cytokine production levels in serum post immunization

In the post immunization cytokine ELISA, results showed IL-6 (Graph 1) values for naked DNA Rv3914/TrxC, BCGRv0379/SecE2 (primed), BCGRv3006/LPPZ (primed) and BCGRv0432/+SP (primed) were significantly different from saline group ($p<0.05$). Cytokine ELISA values were also compared to pVAX1 vector group and significant difference was observed in BCG primed Rv3006 and Rv0432+SP (p-value<0.05). TNF-α (Graph 2) had no significant difference from commercial vaccines thus p-value>0.05 while saline and pVAX1 group resulted in p-value<0.05. BCG primed Rv0379, Rv0432 and cocktail group showed significant differences from saline group with $P<0.05$. While in comparison to the vector pVAX1 group, vaccines showed no significant difference.

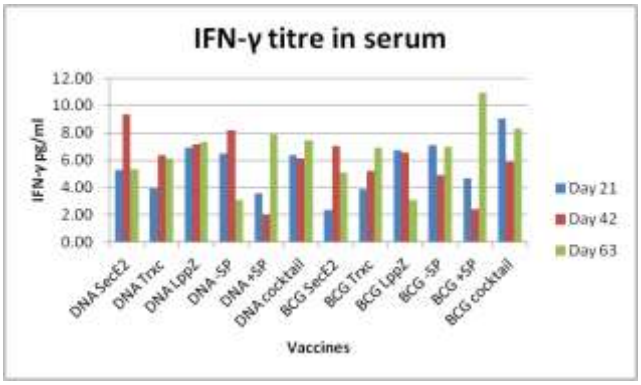
DNA vaccines either primed or naked showed no significant difference from BCG control with values of IFN-γ (Graph 3) being very close to the control ($P>0.05$). Similarly, when the vaccine induced IFN-γ values were compared with pVAX1 they showed a difference but only BCG primed Rv3006 and Rv0432+SP showed statistically different values with $P<0.05$. In case of comparison with saline group, Rv0379, Rv3914, Rv3006, Rv0432-SP, DNA cocktail had $p<0.05$ with significant difference from saline group. In BCG primed group, Rv3914, Rv3006, Rv0432+SP, and Rv0432-SP, and BCG cocktail showed significant difference from saline.



Graph-1. DNA vaccine group IL-6 titer in serum.



Graph-2. Vaccine groups mean TNF-α titer in serum.



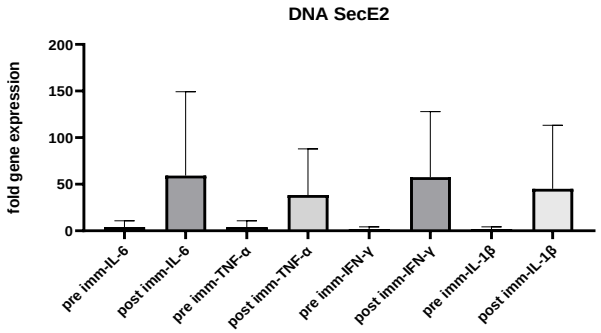
Graph-3. Vaccine groups mean IFN-γ titer in serum.

Specific cytokine production levels in serum post challenge

Although there was a difference in IL-6 levels between vaccine groups and pVAX1 groups, it was not statistically significant. However, the level was raised. In the saline group, only the DNA Rv0379/SecE2 and DNA cocktail showed a statistically significant difference with $p<0.05$ when compared to the control group. In terms of TNF-α values, when compared to the BCG control group, DNARv0432/+SP (2240.39 pg/ml) and BCG primed Rv3006/LppZ (2535.37 pg/ml) showed the maximum values of significant difference with $p<0.05$, while the rest of the test vaccines had values closer to the BCG control group with $p>0.05$. In contrast with the pVAX1 and saline group, all vaccines showed significant rise with $p<0.05$. Post-infection sera IFN-γ values were similar to BCG control values with no significant difference except for pVAX1 and saline groups which were lower.

Gene expression of IL-6, IL-1β, TNF-α, IFN-γ

Pre-immunization fold change values showed down-regulation of IL-6, while post-immunization values showed up-regulation. A similar response was observed in TNF-α, IFN-γ and IL-1β, thus, indicating macrophage activation and Th1 immune activation in response to these DNA clones. The up-regulation of gene expression for IL-6, TNF-α, IFN-γ and IL1-β after immunization confirms the capability of DNA vaccines to evoke a significant immune response. All DNA vaccines, including BCG-primed and naked DNA vaccines, showed up-regulation of all tested cytokines. Graph 4 represents the data of naked DNA Rv0379/SecE2 fold change gene expression. In the post-challenge testing of the DNA cocktail group, it was found that all cytokines exhibited up-regulated gene expression, with IL-6 and IFN-γ being particularly notable. In the pre-challenge bleeds, the fold change values were relatively low. In the BCG-primed DNA cocktail group, there was an up-regulated trend observed in IL-6 and IFN-γ, while other cytokines showed down-regulation. In the BCG control group, only IL-1β exhibited up-regulation of its gene expression, while the remaining cytokines were down-regulated in pre- and post-challenge bleed. All DNA vaccines, whether naked or BCG-primed, resulted in higher gene expression of cytokines post-challenge. BCG-primed animals showed a stronger immune response compared to those vaccinated with naked DNA vaccines.



Graph-4. Fold change of gene expression for DNA SecE2.

Interferon ELISA

Interferon levels in plasma were analyzed for statistically significant results and a mean plot was plotted for the post challenge bleed values. Based on the control values and a cutoff value of 21pg/ml, two vaccines demonstrated statistically significant results. DNA Rv3006/LppZ and BCG primed DNA Rv0432-SP had the highest mean value in the post-challenge

(63 days) bleed, with 1360.35 pg/ml.

Discussion

In this study, five genes of the bacterium *M.tb* were amplified and then cloned into the pVAX1 expression vector. This vector was used as a DNA vaccine in mice, and the cytokine levels and gene expression were measured at different time points. The results were compared to those of a commercial BCG vaccine. Cytokines are crucial in *M.tb* immunization, and upon stimulation with Mycobacterium antigen, a cascade of cytokines is released (Kellar et al., 2011).In this work, the potential of cytokines in addition to IFN-γ to provide reliable indicators of *M.tb* infection and immunization was evaluated. Comparing the measurement of various cytokine responses to the evaluation of IFN-γ alone may increase the diagnostic sensitivity for *M.tb* infection. Cytokines measured in this study have been used as an indicator of immune-enhancing activity (Liang-liang et al., 2023).

For control of TB T helper 1 (Th1) type cells are considered central in cellular immune response. TNF-α has a primordial function in TB infection as it works in unison with IFN-γ (Cavalcanti et al., 2012). IFN-γ being the important cytokine in immunity against Mycobacterium showed an increasing trend after vaccination by the clones used as DNA vaccines. TNF-α is a critical cytokine in *M.tb* infection as it increases T cell response by promoting macrophage phagolysosomal fusion as a result enhancing antigen presentation and optimizing CD4+ T cell immunity (Cavalcanti et al., 2012).In this comparative study carried out between commercial BCG and DNA vaccine, the significantly increased IFN-γ and TNF-α levels in post immunization and post challenge serum, the Th1 response abundance and the elevated ratios of Th1/Th2 cells in whole blood, specifically stimulated with antigens, were comparable between the naked DNA group and the BCG-primed DNA group, resembling the outcomes observed in the BCG control vaccine. According to a recent review IL-6 is the first cytokine produced in response to the *M.tb* infection and is responsible for creating cytokine storm (Boni et al., 2022). IL-6 levels in all DNA vaccines as well as in BCG control were not significantly different from their normal cut off value at the time of infection. Rv0432/SodC gave some high levels of TNF-α after the disease ensuring a good Th1 response. Another DNA vaccine that showed one of the highest responses was BCG primed Rv3006/LppZ. This protein is involved in the metabolism of RNA molecules and is thought to play a role in ribosome biogenesis in *M.tb* and may be involved in regulating the expression of other genes in the bacterium (Yang et al., 2021). All these antigens showed raised levels of cytokines after immunization as well as post challenge thus justifying the consequences of a stronger immune system as proven in previous studies (Domingo-Gonzalez et al., 2016).

Fold change provides a better insight into studying gene expression as it allows to analyze the data and make it more informative (Dalman et al., 2012). Naked DNA Rv0379 and DNA0432+SP showed the best fold change gene expression after immunization for all cytokines. While in BCG primed cocktail Rv3914 had the best gene expression. These cytokines provide a detailed insight into the immune system activation against *M.tb* infection (Romero-Adrian et al., 2015).

Conclusion

We accomplished the development of five Mycobacterium DNA vaccines capable of eliciting Th1-type cellular immune responses in mice and exhibiting certain immunotherapeutic effects against tuberculosis. Some of these clones may have potential use as an immunotherapeutic DNA vaccine against TB.

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Contribution of Authors

Jilani A, Zaman MM & Yousaf A: Conceived idea, conducted the research, collected, analyzed and interpreted the data and wrote the article.
Shahzad MI & Rivera G: Planned and supervised the research, edited and approved the final draft of article.

Reference

Boni FG, Hamdi I, Koundi LM, Shrestha K and Xie J, 2022. Cytokine storm in tuberculosis and IL-6 involvement. Infect Genet Evol. 97:105166.

Cavalcanti YVN, Brelaz MCA, Neves JKDAL, Ferraz JC and Pereira VRA, 2012. Role of TNF-Alpha, IFN-Gamma, and IL-10 in the Development of Pulmonary Tuberculosis. Pulm.Med. 745483.

Cooper AM, Dalton DK, Stewart TA, Griffin JP, Russell DG and Orme IM, 1993. Disseminated tuberculosis in interferon gamma gene-disrupted mice. JEM. 178:2243-2247.

Dalman MR, Deeter A, Nimishakavi G and Duan ZH, 2012. Fold change and p-value cutoffs significantly alter microarray interpretations. BMC Bioinform. 13:S11.

Domingo-Gonzalez R, Prince O, Cooper A and Khader SA, 2016. Cytokines and Chemokines in Mycobacterium tuberculosis Infection. Microbiol Spectr. 4:0018-2016.

Flynn JL, 2004. Immunology of tuberculosis and implications in vaccine development. Tuberculosis (Edinb). 84: 93-101.

Flynn JL, Chan J, Triebold KJ, Dalton DK, Stewart TA and Bloom BR, 1993. An essential role for interferon gamma in resistance to Mycobacterium tuberculosis infection. JEM. 178:2249-2254.

Global tuberculosis report 2021. Geneva: World Health Organization; 2021. Licence: CC BY-NC-SA 3.0 IGO.

Ha S, Jeon BY, Kim S, Kim D, Song M, Sung Y and Cho SN, 2003. Therapeutic effect of DNA vaccines combined with chemotherapy in a latent infection model after aerosol infection of mice with Mycobacterium tuberculosis. Gene Ther. 10:1592-1599.

Kellar KL, Gehrke J, Weis SE, Mahmutovic-Mayhew A, Davila B, Zajdowicz MJ, Scarborough R, Lobue PA, Lardizabal AA and Daley CL, 2011. Multiple cytokines are released when blood from patients with tuberculosis is stimulated with Mycobacterium tuberculosis antigens. PLoS One. 6:e26545.

Liang-liang L, Kuan-hui L, Shi-hui X, Ting L, Guang-shuang Z, Almutairi MH, Zahid M, Gondal MA, Qudratullah, Mehmood K and Wu Y, 2023. Immune enhancing activity of garlic polysaccharide, selenizing Codonopsis pilosula compounds in chickens. Asian J. Agric. Biol. 2023(4): 2023149. DOI: https://doi.org/10.35495/ajab.2023.149

Mathiasen VD, Andersen PH, Johansen IS, Lillebaek T and Wejse C, 2020. Clinical features of tuberculous lymphadenitis in a low-incidence country. Int J Infect Dis, 98:366-371.

Mbuh TP, Ane-Anyangwe I, Adeline W, Thumamo Pokam BD, Meriki HD and Mbacham WF, 2019. Bacteriologically confirmed extra pulmonary tuberculosis and treatment outcome of patients consulted and treated under program conditions in the littoral region of Cameroon. BMC Pulm Med. 19: 018-077.

Romero-Adrian TB, Leal-Montiel J, Fernández G and Valecillo A, 2015. Role of cytokines and other factors involved in the Mycobacterium tuberculosis infection. World J Immunol. 5: 16-50.

Sambrook J and Russell DW, 2006. Preparation and Transformation of Competent E. coli Using Calcium Chloride. CSH Protoc. 2006(1): pdb.prot3932. doi: 10.1101/pdb.prot3932.

Terracciano E, Amadori F, Zaratti L and Franco E, 2020. Tuberculosis: an ever present disease but difficult to prevent. Ig Sanita Pubbl. 76:59-66.

Xin P, Siliang Z, Jialin C, Bin Z, Boju P, Zhonglei P, Ying W, Ying W, Yi Z and Wei F, 2014. Screening novel diagnostic marker of Mycobacterium tuberculosis. Afr. J. Microbiol. Res. 8:776-782.

Yang QY, Zhang YT, Xiao JN, Liang YS, Ji P, Wang SJ, Wang Y and Chen Y, 2021. Age-Related Immunoreactivity Profiles to Diverse Mycobacterial Antigens in BCG-Vaccinated Chinese Population. Front. Immunol. 11:608220

Yari S, Hadizadeh Tasbiti A, Ghanei M, Shokrgozar M, Mahdian R, Fateh A, Siadat S, Vaziri F, Niknami S and Bahrmand A, 2015. Protein profiling and analysis of drug sensitive and multidrug resistant isolates of Mycobacterium tuberculosis by native polyacrylamide gel electrophoresis and mass spectrometry. Vaccine Res. 2:81-85.

Yu D, Hu X and Cai H, 2008. Efficient tuberculosis treatment in mice using chemotherapy and immunotherapy with the combined DNA vaccine encoding Ag85B, MPT-64 and MPT-83. Gene Ther. 15:652-659.