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Title: Intranasal Immunization with Peptide-based Immunogenic Complex Enhances BCG Vaccine Efficacy in murine model of Tuberculosis

Introduction:

Mycobacterium tuberculosis (*M.tb*), the causative agent of tuberculosis (TB) affects about one-fourth of the global population (1). Approximately 2 million deaths globally are directly attributed to TB. Synergism between Human Immunodeficiency Virus (HIV) infections and *M.tb* along with the emergence of multidrug-resistant strains of *M.tb* has become a major concern for nations globally (2, 3). Unfortunately, cost-effective and user-friendly therapy for TB infections is long overdue. *M.tb* infections may produce varied responses between the individuals, ranging from asymptomatic infections to progressive pulmonary or extra-pulmonary TB and even death (4). The rate of progression in the severity of TB depends on the status of the host immune system.

Although world's only accepted vaccine against TB, the live attenuated strains of *Mycobacterium bovis* Bacillus Calmette-Guerin (BCG) is very effective against disseminated and meningeal TB in young children. However, its efficacy in protecting against adult pulmonary TB varies dramatically from 0-80% in different populations depending upon ethnicity and geographical regions (5-9). BCG's limited vaccine efficacy is majorly attributed to its failure to induce a significant population of central memory T cells (T_{CM}) (6, 9-11) as animal models vaccinated with BCG primarily develop antigen-specific $CD4^+$ effector memory T cells (T_{EM}). Considering the lags in BCG immunization and increased global TB burden, it is crucial to develop improved methods of immuno-prophylaxis against TB. Since, most of the world's population is vaccinated with BCG, we need an alternative therapy to improve the efficacy of BCG in terms of enhancing central memory responses leading to the induction of polyfunctional cytokine responses at the site of infection, eventually controlling the infection.

Surface antigens along with the secretome of mycobacteria have been shown to generate potent host immune responses during *M.tb* infection (12-15). Taking a cue from above findings, in this study we generated an immunogenic complex against *M.tb* which consisted of promiscuous protective T-cell epitopes along with TLR ligands adsorbed on liposomal drug delivery vehicle. These complexes, termed as PTLs (peptide-TLR agonist-liposomes) were delivered directly into the lungs through intranasal route, thereby generating a protective immune response at the site of infection.

We observed that the PTLs significantly enriched the BCG induced T_{CM} pool in the $CD4^+$ and $CD8^+$ T cells with a decrease in the T_{EM} cell pool in the lungs of mice co-immunized with BCG and PTLs as against the mice immunized with BCG or PTLs alone. The population of T_{CM} cells was maintained at elevated numbers in the spleens of co-immunized animals as well, consistent with the understanding that spleens are the potential reservoir of these cells (9). Interestingly, the frequency of immunosuppressive PD-1 expression on memory cell subsets was significantly low in the lungs and spleen of BCG and PTLs co-immunized mice as compared to other groups. Moreover, increased memory responses correlated with a remarkable reduction in bacterial burden in the lungs, spleen, and liver of the animals receiving PTLs immunization along with BCG than in other experimental groups.

Furthermore, we also noticed a significant increase in the polyfunctional cytokine secretion ($IFN\gamma^+IL17^+TNF\alpha^+IL2^+$) in $CD4^+$ and $CD8^+$T cells in the lungs of co-immunized animals as

compared to the mice vaccinated with BCG alone. A similar protective response was also observed in reactivation studies. Separate transcriptome analysis of peptide pool pulsed DCs and co-cultured T cells further sheds light on the possible multiple host protective pathways induced by PTLs.

Collectively, in our study, we report that BCG vaccinated mice when co-immunized with PTLs induced a larger pool of T_{CM} cells which may contribute to a stronger and a potent recall immune response to facilitate enhanced *M.tb* clearance. In brief, our findings suggest that PTLs co-immunization in BCG vaccinated mice significantly enhances the vaccine efficacy of BCG.

Results:

T cell peptides derived from *M.tb* induce host protective immune responses

M.tb peptides derived from ESAT6, Ag85B and MPT70 have been shown to be promising candidates for the induction of protective T cell responses during TB. Taking observation from the previous studies, we screened 14 *M.tb* peptides derived from different secretory proteins of H37Rv, for their efficacy to induce *M.tb* specific T cell activation and host protective Th1/Th17 responses (12-16). A group of mice infected with H37Rv strain of *M.tb* was subjected to 45 days of DOTS therapy starting from 15 days post-infection. After a rest period of 30 days, T cells from these infected and DOTS treated mice were isolated and co-cultured with dendritic cells (DCs) derived from the bone marrow of naïve mice and pulsed with T cell epitopes/peptides (0.2 μg/ml) or complete soluble antigen (CSA) of *M.tb* (20 μg/ml). From these set of peptides, seven peptides which induced significant T cell activation (**Figure 1A)** and enhanced IFNγ and IL17 secretion **(Figure 1B and 1C).** Next, we performed the above experiments using pool of these seven antigenic peptides (combo, 100 ng/ml of each peptide) and CSA of *M.tb* as a positive control. Combo significantly increased the expression of early surface activation marker CD69 on $CD4^+$ cells and $CD8^+$ T cells **(Figure 1D)** in comparison to CSA of *M.tb*. Furthermore, we also observed an increase in the number of IFNγ and IL17 producing $CD4^+$ and $CD8^+$ T cells **(Figure 1E & 1F)** in the T cell pool co-cultured with DCs loaded with peptide pool in comparison to the CSA.

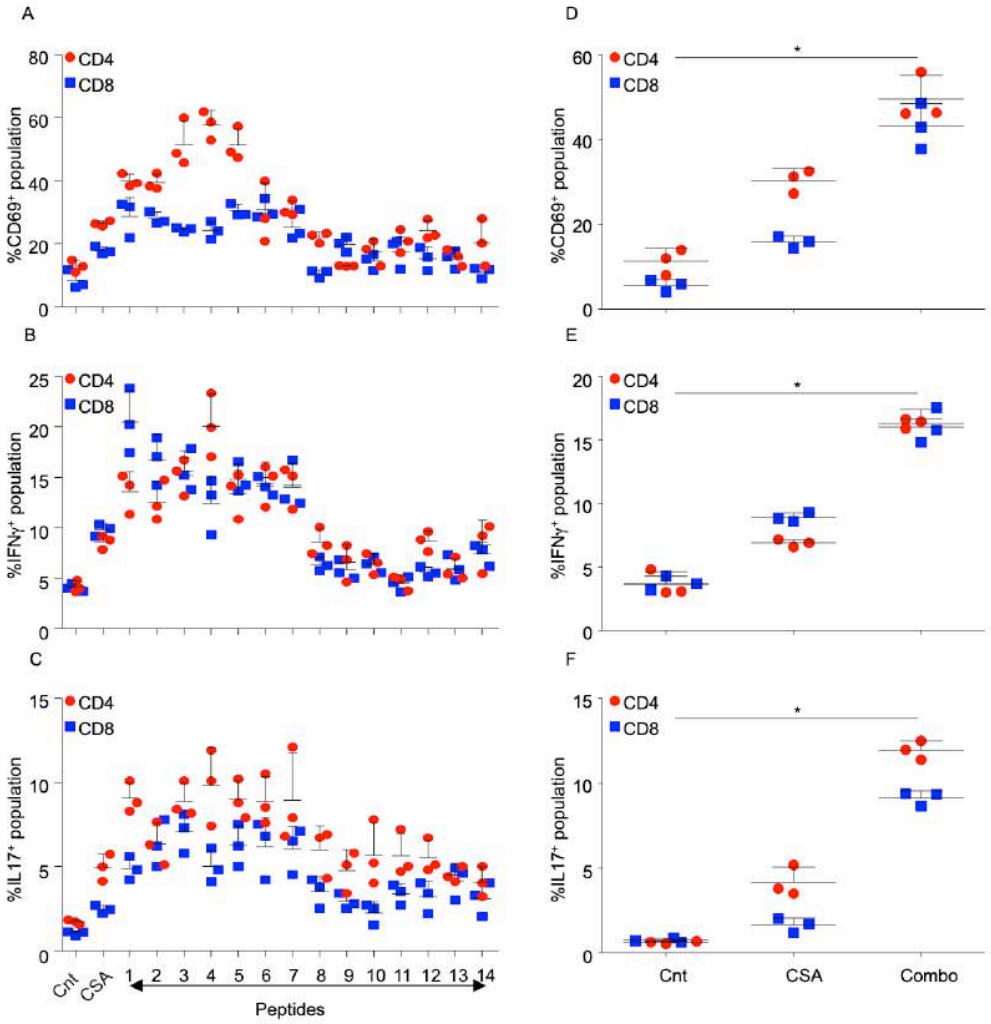

Figure 1: Mycobacterial antigens induce activation of protective T-cells responses. T cells isolated from the spleen of mice infected with H37Rv and treated with DOTS, were stimulated with DCs preloaded with the peptides for 48 h followed by surface-staining with anti-CD3, anti-CD4, anti-CD8, anti-CD69 and staining for intracellular cytokines with anti-IFNγ and anti-IL17 antibodies. Bar graphs depicting the percentage of **(A)** $CD4^+CD69^+$ and $CD8^+CD69^+$ T cells, **(B)** $CD4^+IFN\gamma^+$ and $CD8^+IFN\gamma^+$ T cells, **(C)** $CD4^+IL17^+$ and $CD8^+IL17^+$ T cells. **(D)** Activation of $CD4^+$ and $CD8^+$ T cells co-cultured with unstimulated DCs (Cnt), DCs pulsed with CSA or DCs pulsed with the peptide combo. $CD4^+$ and $CD8^+$ T cells expressing **(E)** IFNγ and **(F)** IL17 after stimulation with the combo. Each experiment was performed at least thrice in triplicates. Two-tailed student's t-test was performed for statistical analysis. Data represents mean ± SD (n=3). *$p<0.05$.

M.tb peptides induce gene expression signature required for protective immunity in DCs and T cells

To further understand the host changes at the transcription levels leading to an increased T cell activation and an augmented pro-inflammatory cytokine upon peptide pool treatment, we performed comparative transcriptome analysis of unstimulated dendritic cells DCs (Un-DC) vs peptide pool pulsed DCs (Pep-DC) and T cells co-cultured with unstimulated DCs (Un-TC) vs T cells co-cultured with peptide pool pulsed DCs (Pep-TC). Our RNAseq data revealed 1452 differentially expressed genes (1098 up-regulated, Log2FC>1 and 354 down-regulated, Log2FC<-1 with FDR ≤0.05) in the peptide pool stimulated DCs as compared to unstimulated DCs **(Figure 2A)** while T cells co-cultured with peptide pool pulsed DCs showed 2331

differentially expressed genes (1223 activated and 1108 repressed) as compared to T cells co-cultured with unstimulated DCs (Accession Number: GSE164258) **(Figure 2B)**. Differential genes in both DCs and T cells after peptide pool stimulation were highly enriched for gene sets assisting in IFNγ response and production, cytokine activity, STAT phosphorylation, ADP metabolic processes, ROS equilibrium, etc. Many pathways known to play an important role in combatting TB disease were significantly upregulated in peptides stimulated DCs and T cells as indicated by KEGG analysis **(Figure 2C and 2D)**. Differential genes in the DCs as well as the T cells majorly belonged to signaling pathways such as JAK-STAT, Tuberculosis, TNF, TLR and TGFβ signaling **(Figure 2E and 2F)**. The transcriptome data analysis revealed a very similar and indistinguishable trend of activated genes in DCs and T cells. A huge number of 724 genes (586 up-regulated and 138 down-regulated) were common between the two cell types. Moreover, these genes followed the same expression profile in both the settings. KEGG analysis indicated that these genes belonged to a number of TB related protective host signaling pathways such as NFκB, MAPK, TGFβ, TNF, IL17, etc. Taken together, our transcriptomics data strengthened our findings that peptide pool induces an intricate network of signaling pathways in DCs and T cells which leads to enhanced cytokine production and T cell activation.

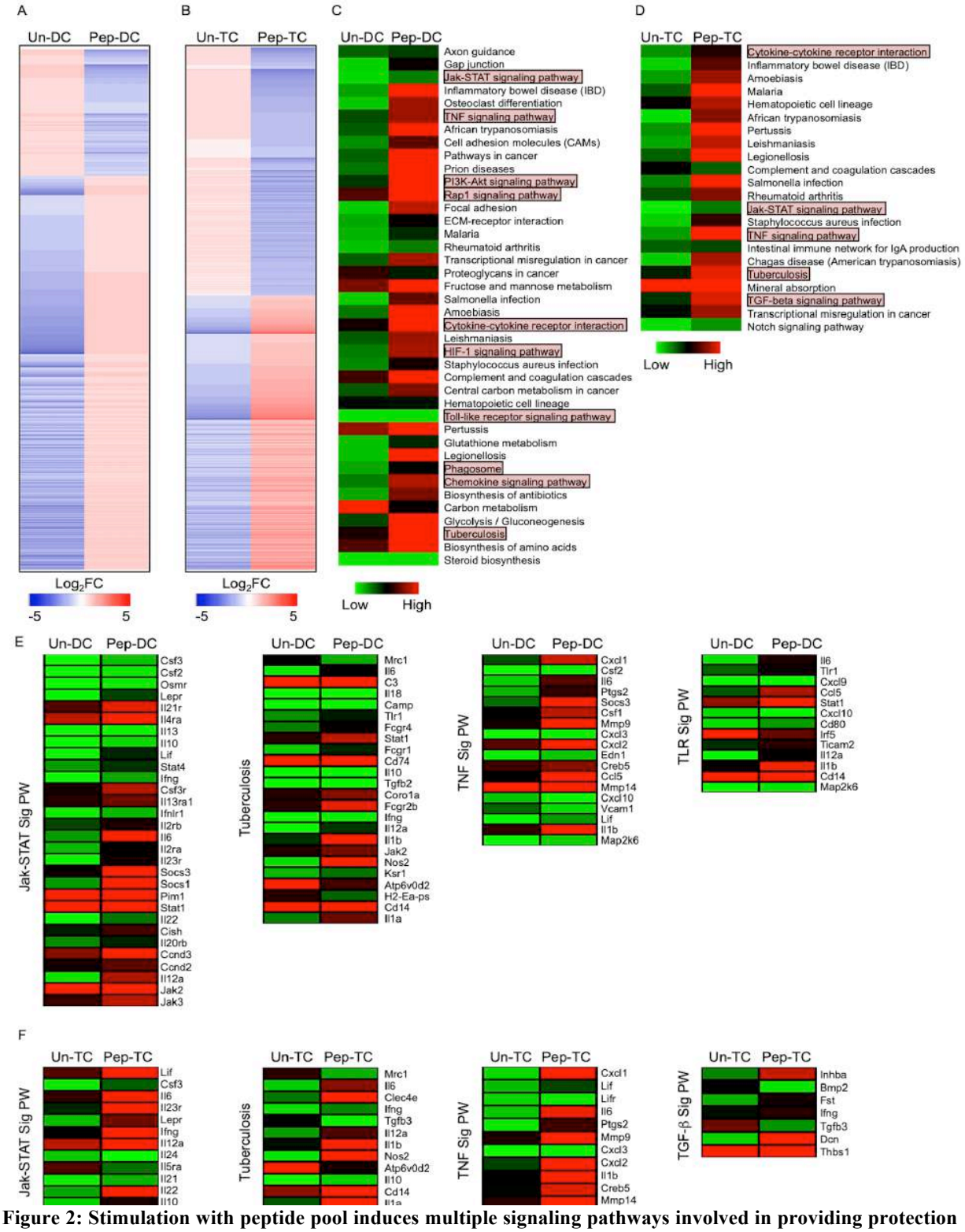

Figure 2: Stimulation with peptide pool induces multiple signaling pathways involved in providing protection during *M.tb* infection. Heatmap representation of the genes differentially expressed in **(A)** the DCs pulsed with peptide pool (Pep-DC) in comparison with unstimulated DCs (Un-DC) and **(B)** the T cells co-cultured with DCs pulsed with peptide pool (Pep-TC) vs T cells co-cultured with unstimulated DCs (Un-TC). Red depicts activation while blue represents repression. **(C)** Molecular signaling pathways majorly affected in Pep-DCs. **(D)** KEGG pathways significantly modulated in Pep-TCs. **(E)** Heatmaps representing the JAK-STAT signaling pathway,

Tuberculosis, TNF signaling pathway and TLR signaling pathways in Pep-DCs vs UN-DCs. **(F)** Heatmaps representing the JAK-STAT signaling pathway, Tuberculosis, TNF signaling pathway and TGFβ signaling pathways in Pep-TCs vs UN-TCs. RNAseq was performed once in triplicates (n=3).

Induction of immune responses in Human PBMCs by the *M.tb* peptides-TLR agonists-liposome assembly

Subject to above results, we establish here that in combination, our peptides are capable of inducing antigen-specific protective T cells; therefore we assembled these 7 antigenic peptides along with TLR2 and TLR9 agonist Pam3CysSK-4 and CpG ODN respectively, as evidenced by previous reports that TLR2 and TLR9 play an important role during *M.tb* infection (17, 18), in a liposomal delivery vehicle for the successful delivery of this cargo to the lungs through the intranasal route **(Figure 3A).** The efficacy of this assembly of peptides, TLR ligands and liposomes (PTLs) was assessed in human PBMCs derived from 10 PPD^- and 10 PPD^+ BCG vaccinated healthy individuals. The PBMCs were in vitro stimulated with *M.tb* CSA (20 µg/ml), BCG complete soluble antigen (BCG CSA, 20 µg/ml), PTLs (10 µl/ml) and BCG CSA/PTLs for 48h followed by surface/intracellular staining and FACS analysis to determine $CD4^+$ T cell activation and IFNγ production. With no significant difference in PPD^- individuals, PTLs and BCG CSA/PTLs combination induced significant expression of early activation marker CD69 and IFNγ on $CD4^+$ T cells in the PBMCs derived from PPD^+ individuals as compared to *M.tb* CSA and BCG CSA controls **(Figure 3B-E).**

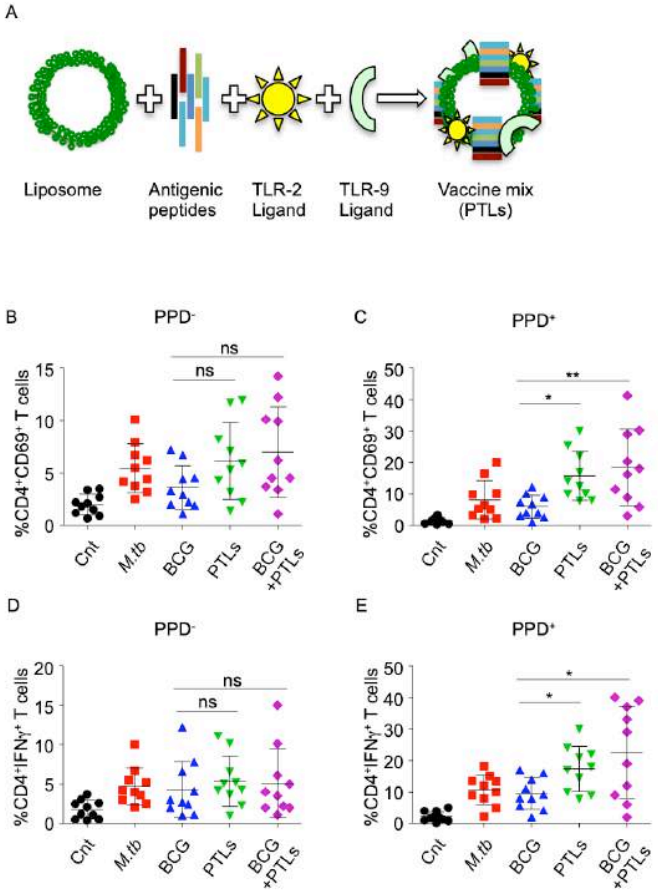

Figure 3: PTLs induce a protective immune response in human PBMCs. (A) Schematic diagram depicts the preparation of PTLs. PBMCs isolated from PPD^- and PPD^+ healthy subjects were *in vitro* stimulated with different mycobacterial antigens for 48h. **(B&C)** Expression of CD69 on $CD4^+$ T cells stimulated with mycobacterial antigens in **(B)** PPD^- and **(C)** PPD^+ individuals. **(D&E)** Percentage of $CD4^+$ T cells expressing IFNγ in the PBMCs of **(D)**

PPD$^-$ and **(E)** PPD$^+$ individuals. One-way ANOVA followed by multiple tukey tests was performed for statistical analysis. Data represents mean±SD (n=10) performed once. *p<0.05, **p<0.005.

PTLs immunization following BCG vaccination enhances host protection against TB

To confirm the successful delivery of this assembly of peptides, TLR ligand and liposomes (PTLs) into the lungs, the PTLs were labelled with PKH67 to stain the liposomes (delivery vehicle, see methods) and administered intranasally into the lungs. Two days post-delivery, mice were euthanized and lung sections were prepared for fluorescence microscopy **(Figure 4A)**. Quantification of the fluorescent images indicated the accumulation of liposomes/PTLs throughout the lungs **(Figure 4B).**

Despite its limited efficacy in adults, BCG vaccine is highly successful in young children and as a result, is administered in infants and small children in high burden countries. Keeping in mind the existing load of the global population vaccinated with BCG, we designed a strategy wherein BCG vaccinated mice were boosted with once-a-week, three-week-long PTLs boosting regimen followed by a rest period of 21 days. With no increase in the number of CD4$^+$ and CD8$^+$ T cells, we observed significant increase in the expression of CD69 on these cells in the lungs of BCG-PTLs co-immunized mice as compared to BCG/PTLs administration alone. Furthermore, co-immunized animals also showed significant increase in the CD4$^+$ T cells expressing IFNγ and IL17 as compared to single vaccinations. Similarly, CD8$^+$ T cells expressing IL17 but not IFNγ were also induced in the lungs of co-immunized mice. Similar profile was observed in the spleen of vaccinated animals. These mice were challenged with H37Rv, the laboratory strain of *M.tb* using low dose aerosol infection model (~150 CFU/mice), after which organs were harvested at different time points to look at the bacterial burden along with the elicited immune responses **(Figure 4C)**. Consistent with our expectations, co-immunized animals had fewer and smaller inflammatory lesions in their lungs than mice immunized with BCG or PTLs alone while the non-vaccinated infected mice i.e. primary infection with H37Rv *M.tb* showed a significantly higher number of inflammatory lesions **(Figure 4D)**. These results were further strengthened by histopathological analysis of lungs which confirmed the reduced lung inflammation in the co-immunized group as compared to the rest of the experimental groups **(Figure 4E)**. PTLs co-immunization significantly increased the BCG-induced TB protection, as observed by the reduced bacterial burden in various organs viz. lungs **(Figure 4F)**, spleens **(Figure 4G)**, and liver **(Figure 4H)** of infected mice. Thus, the PTLs significantly enhanced the anti-tubercular capacity of BCG immunization. Interestingly, the mice immunized with PTLs alone displayed comparable **(Figure 4F)** and enhanced **(Figure 4G and 4H)** resistance against *M.tb* as the BCG immunized group.

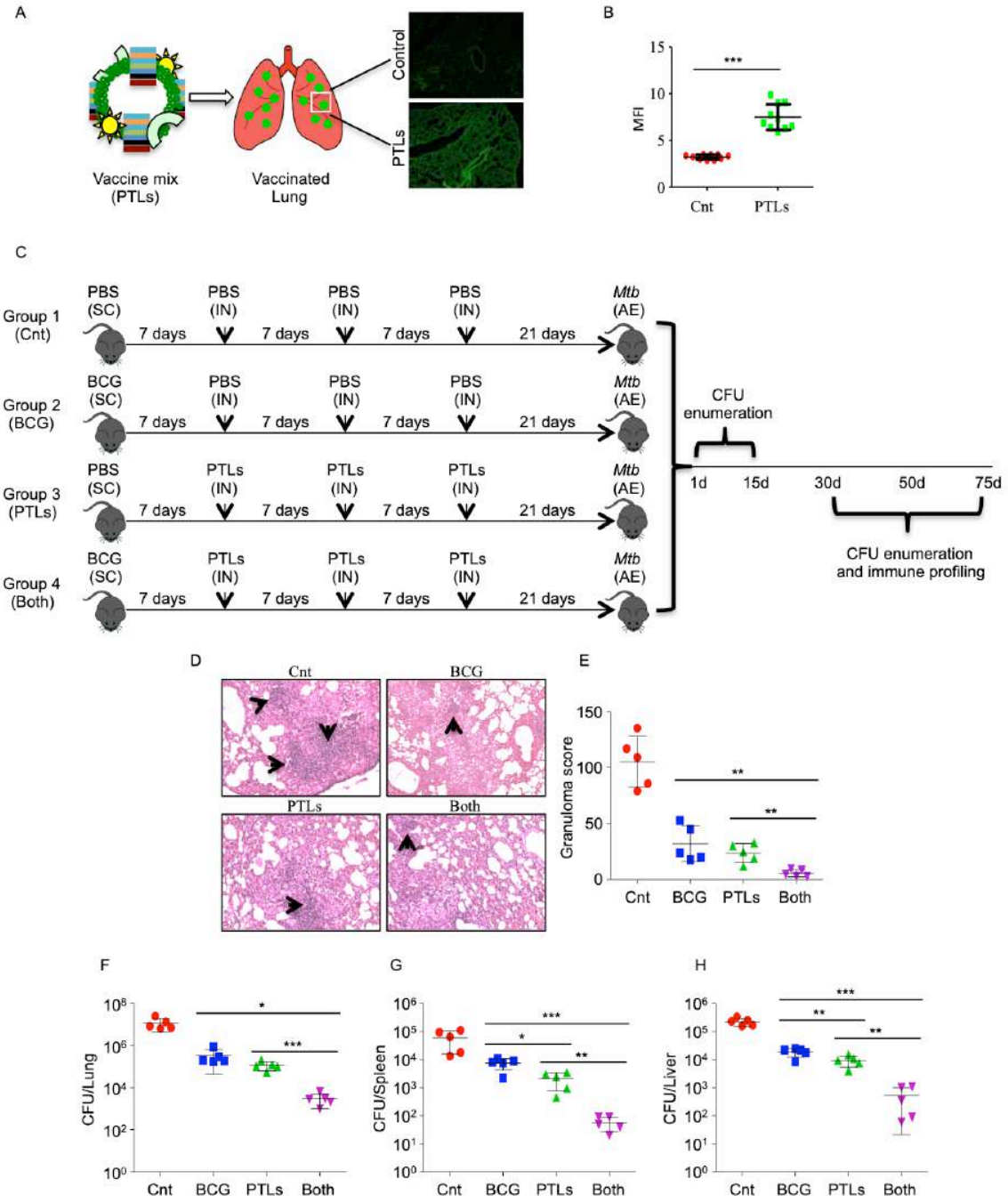

Figure 4: PTLs enhances the efficacy of BCG and protects mice against TB. (A) Lung section to show the accumulation of liposomes. Liposomes were stained with PKH67 dye. **(B)** Quantification of the fluorescent images. **(C)** Layout to show the experimental plan wherein naïve C57BL/6 mice or mice vaccinated with BCG/PTLs or a combination of both were challenged with H37Rv via the aerosol route with a low-dose inoculum of ~150 CFU/mice. Mice were sacrificed at various time points and lungs, spleen and liver were harvested to look at the bacterial burden as well as profiling of immune responses. **(D)** Lungs were harvested, preserved in 4% paraformaldehyde and processed for sectioning and staining with Hematoxylin and Eosin (H&E). **(E)** Quantification of the granuloma (inflammatory lesions) in all experimental groups. **(F)** CFU from the lung, **(G)** spleen and **(H)** liver homogenates at 50 days post infection. Two-tailed student's t-test was performed for statistical analysis. Data is representative of two independent experiments (n=5 mice/group). *p<0.05, **p<0.005, ***p<0.0005.

BCG-PTLs co-immunization elevates the adaptive immunity in the lungs and the spleen *M.tb* infected mice

The host immune system plays a pivotal role in defending against TB pathogenesis. To delve into the immunological changes involved in enhanced protection conferred by BCG-PTLs co-immunization, we profiled the immune cells in the lungs and the spleen of infected animals. **Figure 5A** describes the gating strategy employed to quantify the percentage of various T cell subsets in the lungs and spleen of infected mice. BCG-PTLs co-immunized mice showed comparable levels and activation of $CD4^+$ **(Figure 5B-D)** whereas with minimal effect on the percentage of $CD8^+$ T cells **(Figure 5E)**, BCG-PTLs co-immunization significantly enhanced the expression of early and late activation markers (CD69 and CD25 respectively) on $CD8^+$ T cells as compared to either BCG or PTLs immunization **(Figure 5F and 5G)**. Further, we observed increased frequency of $CD4^+$ T cells **(Figure 5H)** with increased expression of late activation marker CD25 in the spleen of BCG-PTLs co-immunized animals **(Figure 5I)**. CD69 expression was significantly high in splenic $CD4^+$ T cells derived from PTL immunized immunized animals as compared to BCG group **(Figure 5J)**. Percentage of $CD8^+$ T cells was significantly high in the spleen of BCG-PTLs immunized animals **(Figure 5K)** with no increase in the early and late activation markers **(Figure 5L and 5M)**.

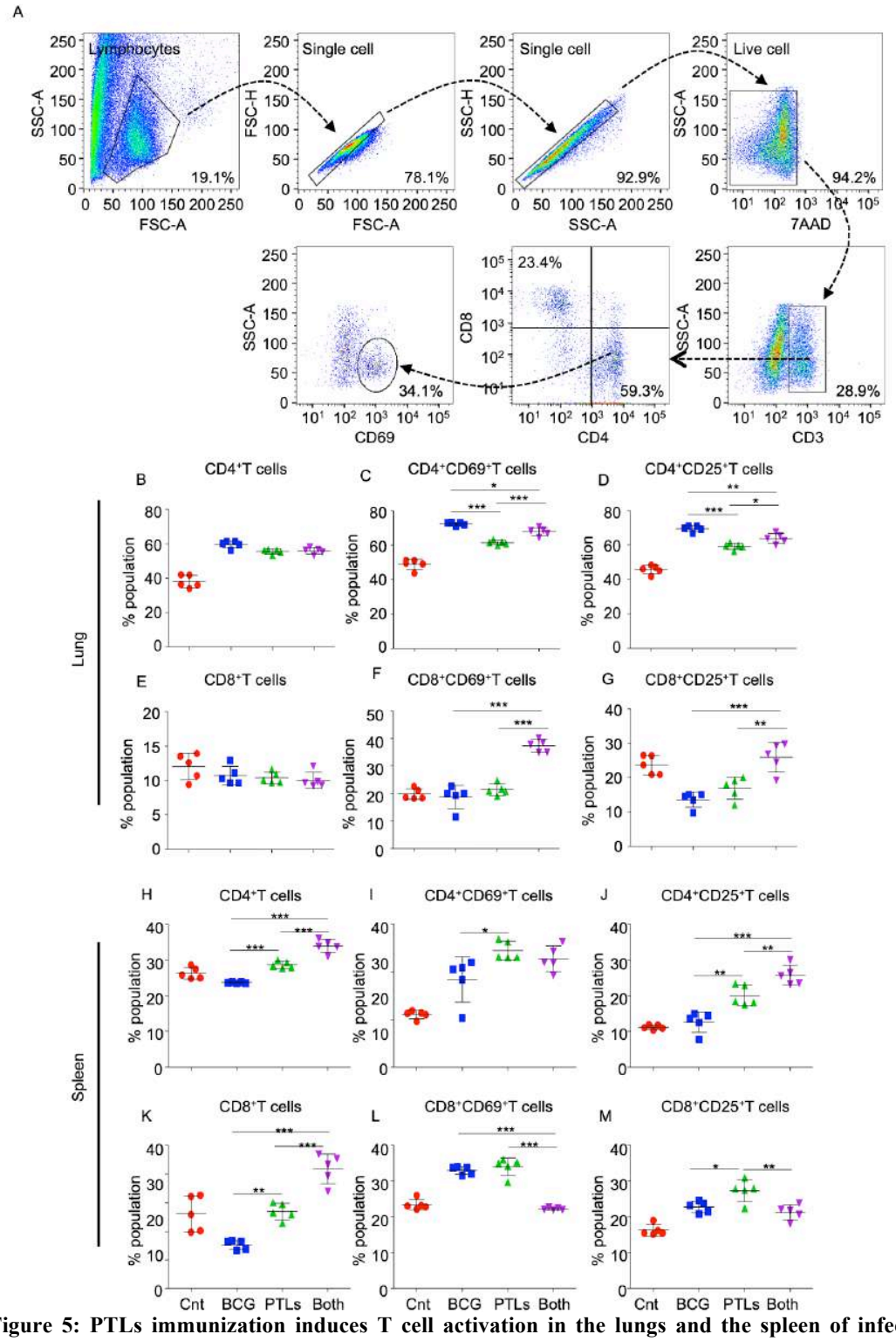

Figure 5: PTLs immunization induces T cell activation in the lungs and the spleen of infected animals. T lymphocytes were isolated from the lungs of all experimental groups and stained with 7AAD, anti-CD3, anti-CD4, anti-CD8, anti-CD25 and anti-CD69 antibodies. **(A)** Gating strategy employed to quantify the T cell activation. **(B)** Percentage of $CD4^+$ T cells and expression of **(C)** CD69 and **(D)** CD25 on $CD4^+$ T cells in the lungs of infected animals. **(E)** Percentage of $CD8^+$ T cells and expression of **(F)** CD69 and **(G)** CD25 on $CD8^+$ T cells in the lungs of infected animals. **(H-M)** T lymphocytes were isolated from the spleen of all experimental groups and stained with 7AAD, anti-CD3, anti-CD4, anti-CD8, anti-CD25 and anti-CD69 antibodies. Percentage of **(H)** $CD4^+$, **(I)**

$CD4^+CD69^+$ and **(J)** $CD4^+CD25^+$ T cells in the spleen of infected animals. Percentage of **(K)** $CD8^+$, **(L)** $CD8^+CD69^+$ and **(M)** $CD8^+CD25^+$ T cells in the spleen of infected animals. One-way ANOVA followed by multiple tukey tests was performed for statistical analysis. Data is representative of two independent experiments (n=5 mice/group). *p<0.05, **p<0.005, ***p<0.0005.

BCG-PTLs co-immunization induces polyfunctional cytokine responses in the lungs and the spleen of *M.tb* infected mice

To investigate the T cell specific cytokine responses in different experimental groups, we isolated the T cells from the lungs and the spleen of treated and control mice and estimated intracellular cytokine production. Polyfunctional T cells expressing more than two cytokines such as IFNγ, TNFα, IL17 and IL2 have been linked with enhanced protection against TB (19, 20). Moreover, the occurrence of these multi-functional T cell is highly important in the context of effective vaccines against various viruses and intracellular bacterial pathogens (21). Thus we analysed the presence of polyfunctional cells in different T cell subsets in the lungs and the spleen of all animal groups **(Figure 6A)**. Interestingly, BCG-PTLs co-immunization significantly increased the frequency of $CD4^+$ and $CD8^+$ T cells expressing single, double, triple and quadruple cytokines with a concomitant decrease in the T cell population expressing none of the four cytokines in the infected lungs **(Figure 6B and 6C)** and the spleen **(Figure 6D and 6E)** as compared to other groups. Particularly, there was an increased occurrence of 4^+ ($IFN\gamma^+TNF\alpha^+IL17^+IL2^+$), 3^+ ($IFN\gamma^+TNF\alpha^+IL17^+$) and 2^+ ($IF\gamma^+IL17^+$;$TNF\alpha^+IL17^+$) $CD4^+$ and $CD8^+$ T cells in the co-immunized group as compared to other animals **(Figure 6F and 6G)**. However, the expression of single cytokines ($IFN\gamma^+$ & $IL17^+$) was higher only in $CD4^+$ T cells **(Figure 6F and 6G)**. A similar profile was observed in the spleen of co-immunized mice where 4^+ ($IFN\gamma^+TNF\alpha^+IL17^+IL2^+$), 3^+ ($IFN\gamma^+TNF\alpha^+IL17^+$;$IFN\gamma^+TNF\alpha^+IL2^+$), 2^+ ($IFN\gamma^+IL17^+$) and 1^+ ($IFN\gamma^+$) $CD4^+$ and $CD8^+$ T cells displayed significantly enhanced frequency in BCG-PTLs co-immunized group **(Figure 6H and 6I)**. Taken together, we observed that BCG vaccination followed by PTLs immunization greatly enhanced the antigen-specific Th1 and Th17 responses as well as pro-inflammatory cytokines, TNFα and IL2. All these cytokines have been well documented to impart protection against TB.

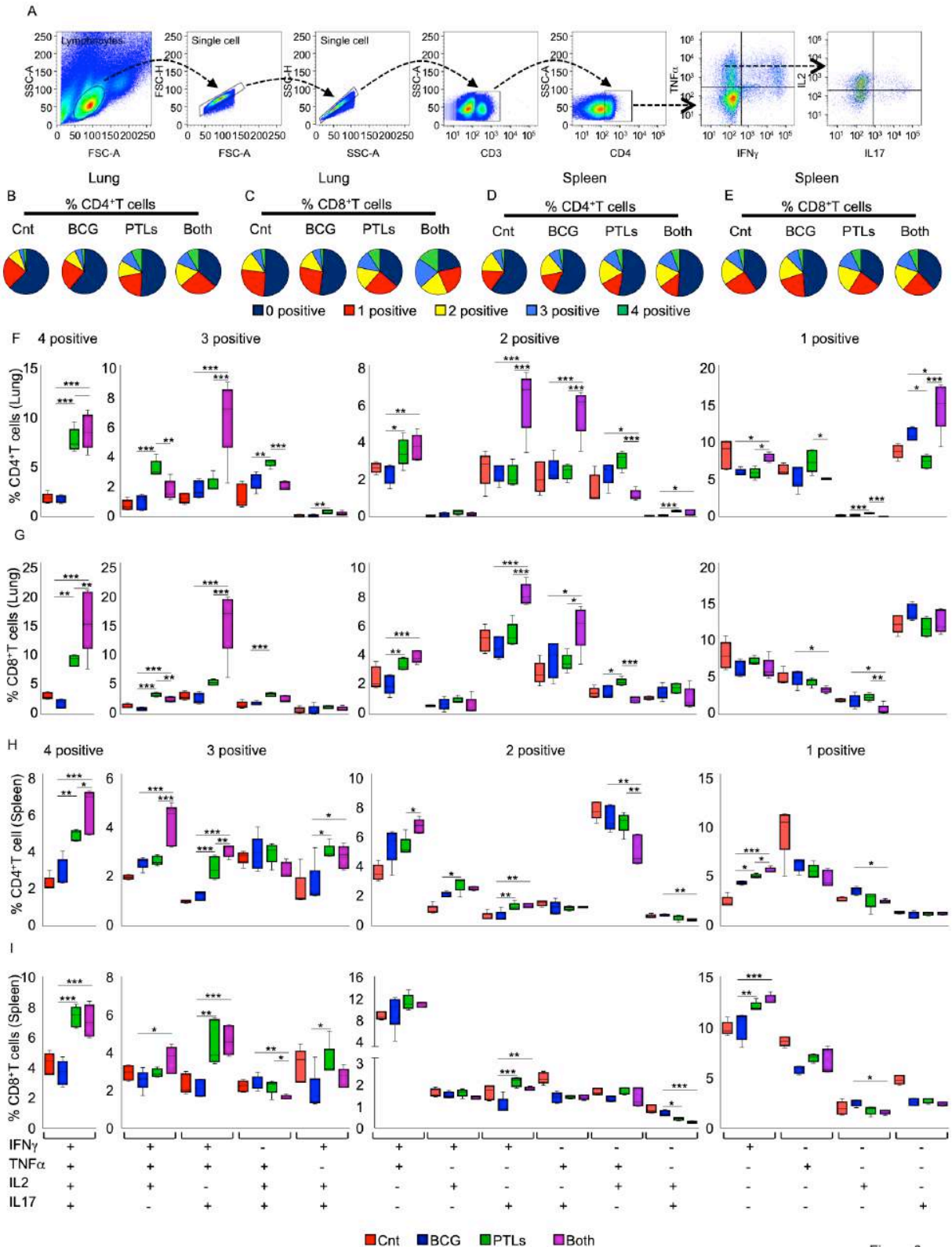

Figure 6: BCG-PTLs co-immunization induces the antigen-specific polyfunctional cytokine responses in the lungs and the spleen of infected animals. (A) Lymphocytes isolated from the lungs of infected animals were stained with anti-CD3, anti-CD4, anti-CD8, anti-IFNγ, anti-TNFα, anti-IL17 and anti-IL2 to assess polyfunctional cytokine responses. Pie charts depicting the percentage of **(B)** $CD4^+$ and **(C)** $CD8^+$ T cells expressing 4, 3, 2, 1 and 0

cytokines (IFNγ, TNFα, IL17 and IL2) in the lungs of the mice. The pie charts representing the average percentage of cytokine-producing **(D)** $CD4^+$ and **(E)** $CD8^+$ T cells producing five combinations (0^+, 1^+, 2^+, 3^+ and 4^+) of the four cytokines analyzed in the spleen of infected animals. Fifteen possible cytokine combinations are shown for **(F)** $CD4^+$ and **(G)** $CD8^+$ T cells from the lungs of infected animals. Box and whisker plots depicts fifteen combinations of responses for the four cytokines analyzed on the x-axis with the percentage of **(H)** $CD4^+$ and **(I)** $CD8^+$ responding splenic T cells on the y-axis. One-way ANOVA followed by multiple tukey tests was performed for statistical analysis. Data is representative of two independent experiments (n=5 mice/group). *p<0.05, **p<0.005, ***p<0.0005.

PTLs immunization induces central memory T cell responses critical for long lasting protection

Since recall responses are mediated by T_{CM}, these cell subtypes become a prerequisite for enhanced vaccine efficacy and superior TB protection (6, 9, 22). Thus we analysed the memory cell profile of the lung as well as the splenic T cell subsets **(Figure 7A)**. We observed that BCG-PTLs co-immunization enriched the vaccine-induced T_{CM} ($CD44^{HI}CCR7^{HI}CD62L^{HI}$) cell pool in $CD4^+$ T cells **(Figure 7B)** with a concomitant decrease in the T_{EM} ($CD44^{HI}CCR7^{LO}CD62L^{LO}$) cell pool **(Figure 7C)** in the lungs of infected mice as compared to BCG vaccinated animals. Expression of inhibitory receptors such as PD-1 and CTLA-4 is often linked with negative regulation and inhibition of activated T cells leading to T cell exhaustion (23, 24). Moreover, in non-human primates and in several human studies, increased PD-1 expression is linked with severe TB pathology and enhanced bacillary load (25, 26). Interestingly, decreased expression of PD-1 was observed on the $CD4^+$ T_{CM} and T_{EM} subsets in the lungs of mice co-immunized with BCG and PTLs as compared to BCG vaccinated group **(Figure 7D and 7E)**. A similar pattern was observed in the $CD8^+$ lung T cells **(Figure 7F-7I)** When investigated in the spleen of co-immunized animals, there was increased frequency of $CD4^+$ T_{CM} pool **(Figure 7I)** with a decrease in $CD4^+$ T_{EM} subset **(Figure 7J).** PD-1 expression was significantly less only in case of $CD4^+$ T_{CM} subset **(Figure 7L)** in the co-immunized group with no effect on $CD4^+$ T_{EM} subset **(Figure 7M)**. $CD8^+$ T_{CM}/T_{EM} cells were comparable in all the groups **(Figure 7N and 7O).** With no decrease in PD-1 expression on $CD8^+$ T_{CM} **(Figure 7P),** its expression was significantly less on $CD8^+$ T_{EM} subset in the co-immunized group **(Figure 7Q)**. Previously it has been shown that BCG vaccinated or *M.tb*-infected mice generate a profoundly expanded population of antigen-specific T_{EM} cells within the lungs whereas the T_{CM} pool is substantially smaller. However, T_{CM} is maintained in significantly larger numbers in the spleen, which is believed to be a potential reservoir for these cells (9, 22). While we found similar T_{CM}/T_{EM} profiles for infected and BCG-vaccinated controls, co-immunized mice maintained an increased pool of T_{CM} cells both in the spleen and the lungs. Nuclear FOXO1 is in an un-phosphorylated state and keeps the long-lived memory T cells enriched while the phosphorylated FOXO1 protein leaves the nucleus and is tagged for ubiquitin mediated protein degradation (27, 28). Interestingly, we also observed a significant reduction in the phosphorylation of FOXO1 transcription factor in the splenocytes of co-immunized animals in comparison to all other experimental groups. We also observed an increased activation of NFkB transcription factor in the splenocytes of BCG-PTLs co-immunized animals as compared to other groups. NFkB is believed to be the main transcription factor responsible for the expression of various pro-inflammatory cytokines such as IFNγ, TNFα, IL12, etc required for providing resistance to TB. Collectively, these data suggest that PTLs used in this study induces the activation of key transcription factors involved in generating protective immune responses inside the host.

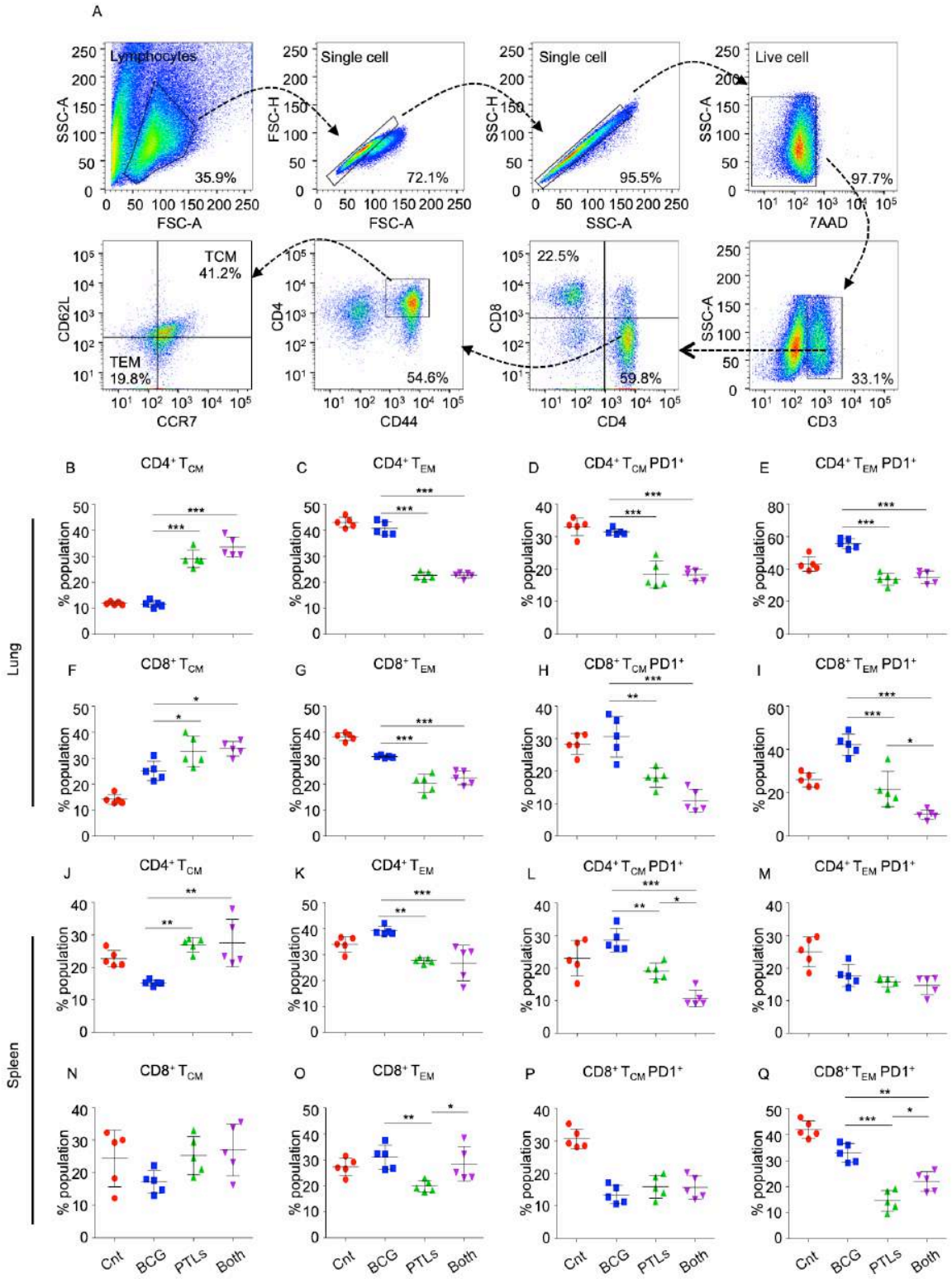

Figure 7: PTLs induces superior antigen specific T-cell memory responses in the lungs and the spleen of infected mice. T lymphocytes isolated from the lungs and the spleen of the indicated groups of experimental mice at 50 days post-infection were surface-stained with anti-CD3, anti-CD4, anti-CD8, anti-CCR7, anti-CD44, anti-CD62L and anti-PD-1 antibodies and fixed prior to acquisition by flow cytometry. **(A)** Gating strategy employed to quantify the memory T cell responses. Percentage of **(B) c**entral memory (T_{CM}: $CCR7^{HI}CD62L^{HI}CD44^{HI}$) and **(C)** effector

memory (T_{EM}: $CCR7^{LO}CD62L^{LO}CD44^{HI}$) $CD4^+$ T cells on lymphocytes isolated form the lungs of infected animals. Frequency of PD-1 expression on **(D)** central memory and **(E)** effector memory $CD4^+$ T cell subset. Percentage of **(F) c**entral memory (T_{CM}: $CCR7^{HI}CD62L^{HI}CD44^{HI}$) and **(G)** effector memory (T_{EM}: $CCR7^{LO}CD62L^{LO}CD44^{HI}$) $CD8^+$ T cells on lymphocytes isolated form the lungs of infected animals. **(H&I)** Frequency of PD-1 expression on these cell subsets. **(J-Q)** Frequency of **c**entral memory, effector memory, and PD-1 expression on these cell subsets on the lymphocytes isolated form the spleen of infected mice. One-way ANOVA followed by multiple tukey tests was performed for statistical analysis. Data is representative of two independent experiments (n=5 mice/group). *p<0.05, **p<0.005, ***p<0.0005.

Antigen-specific protective immunity induced by BCG-PTLs co-immunization can be adoptively transferred by T cells

Above results clearly demonstrate that PTLs co-immunization enhanced protective immune responses following BCG vaccination. To further reveal the antigen specificity and protective function of T cell responses generated by BCG-PTLs co-immunization, we carried out the adoptive transfer of $CD4^+$ and $CD8^+$ T cells isolated from the lungs of BCG, PTLs and BCG-PTLs co-immunized mice to check for their antigenic specific protective response in naïve mice. $CD4^+$ ($1x10^6$) and $CD8^+$ T cells ($1x10^6$) were transferred into gamma irradiated Thy1.1 mice followed by a low-dose aerosol challenge of *M.tb* H37Rv. 25 days post infection, mice were sacrificed for CFU enumeration and immune profiling **(Figure 8A)**. Fairly, we found significant decrease of bacterial load in the mice which received T cells from BCG-PTLs co-immunized animals as compared to BCG vaccinated group **(Figure 8B and 8C)**. Furthermore, the immune profiling revealed a significant increase in the percentage of INFγ producing $CD4^+$ and $CD8^+$ T cells with no difference in IL17 producing T cells in the spleen of mice which received T cells from BCG-PTLs immunized animals **(Figure 8D-8G)**. Therefore T lymphocytes ($CD4^+$ and $CD8^+$ T cells) isolated from co-immunized mice successfully transferred the protective immunity against TB in naïve animals.

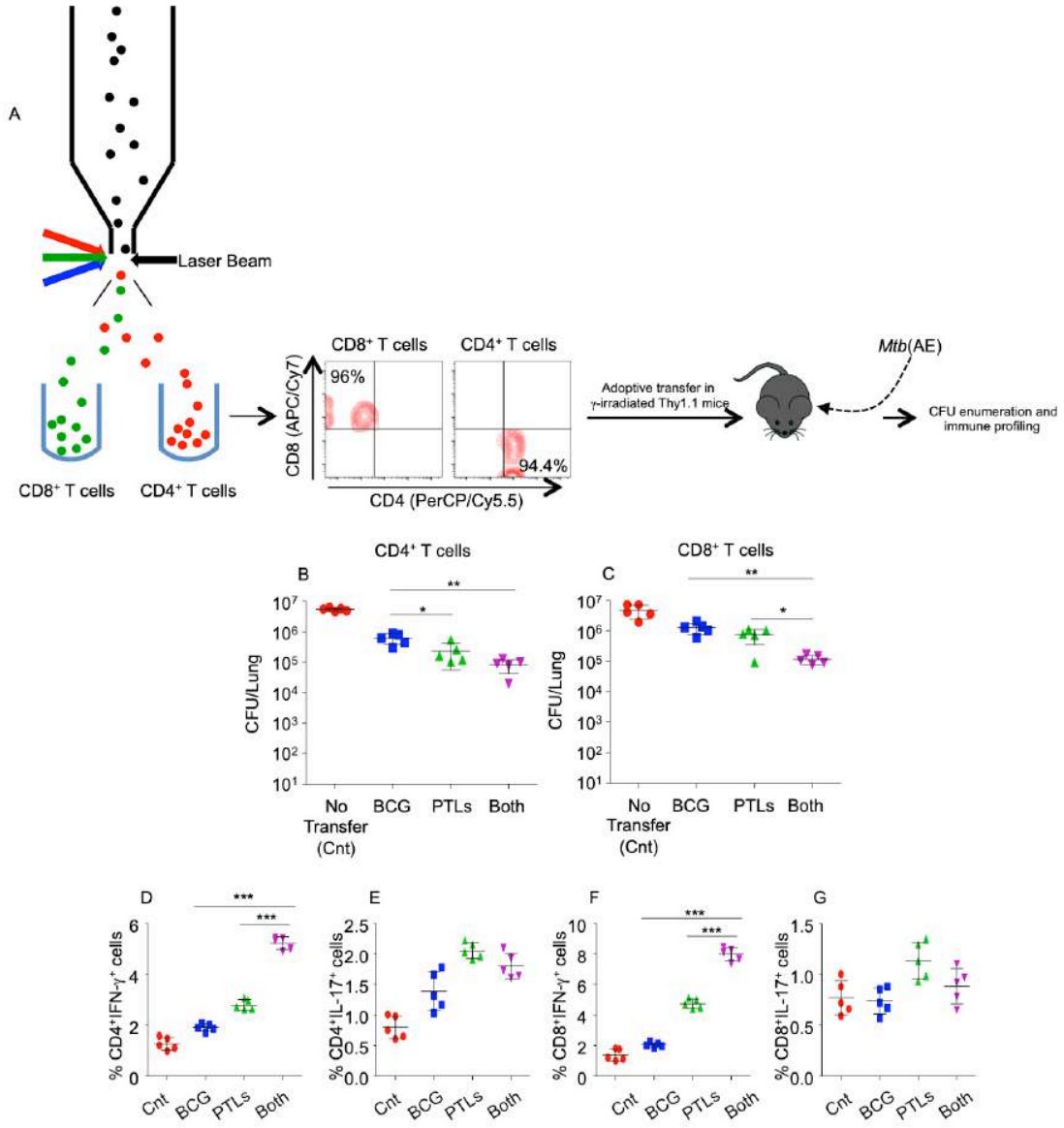

Figure 8: T cells from BCG-PTLs co-immunized mice confer improved protection against TB. (A) T lymphocytes isolated from BCG, PTLs or BCG-PTLs immunized and *M.tb* infected animals (50 days post infection) were subjected to surface staining with anti-CD3, anti-CD4 and anti-CD8 followed by sorting by FACS Aria into two distinct populations: $CD4^+$ and $CD8^+$ T cells. Sorted $CD4^+$ and $CD8^+$ T cells were cultured overnight and transferred into irradiated recipient Thy1.1 mice. Seven days after adoptive transfer, all mice were challenged with *M.tb* H37Rv through the aerosol route. At 25 days post infection, the mice were euthanized for CFU enumeration and immune profiling. CFU in the lungs of mice receiving **(B)** $CD4^+$ and **(C)** $CD8^+$ T cells. **(D-G)** Dot plots representing the percentage of INFγ and IL17 producing $CD4^+$ and $CD8^+$ T cells in the spleen of infected mice. Two-tailed student's t-test was performed for statistical analysis in **B** and **C**. One-way ANOVA followed by multiple tukey tests was performed for statistical analysis for **D-G**. Data is representative of two independent experiments (n=5 mice/group). *p<0.05, **p<0.005.

BCG-PTLs co-immunization protects antibiotic-treated animals against disease recurrence
From the above experiments, it is clear that PTLs co-immunization enhances the BCG induced host protective immunity and selectively induces central memory T-cell responses, which generally results in long-term protection against TB. To further determine the extent of long-term protection induced by PTLs co-immunization, we performed re-activation experiments in the mouse model of TB **(Figure 9A)**. Reactivation rate was calculated as the number of mice re-activated out of the total number of mice in that group. The re-activation results showed that non-vaccinated mice receiving INH and RIF treatment exhibited greater disease re-activation (7 mice out of 10, 70%) upon dexamethasone treatment while mice immunized with BCG showed around 40 % (4 mice out of 9) relapse **(Table 1)**. The relapse rate was significantly lower in mice co-immunized with BCG and PTLs (2 mice out of 10, 20%) **(Table 1)**. However, there were no differences in terms of bacterial burden in the mice which experienced re-activation **(Figure 9B)**. Since effective memory equates to enhanced protection from primary as well as secondary infections, these observations demonstrate that enhanced pro-inflammatory responses and T_{CM} responses induced by BCG-PTLs co-immunization might translate into reduced relapse incidents due to re-activation thus effectively promoting sterile immunity.

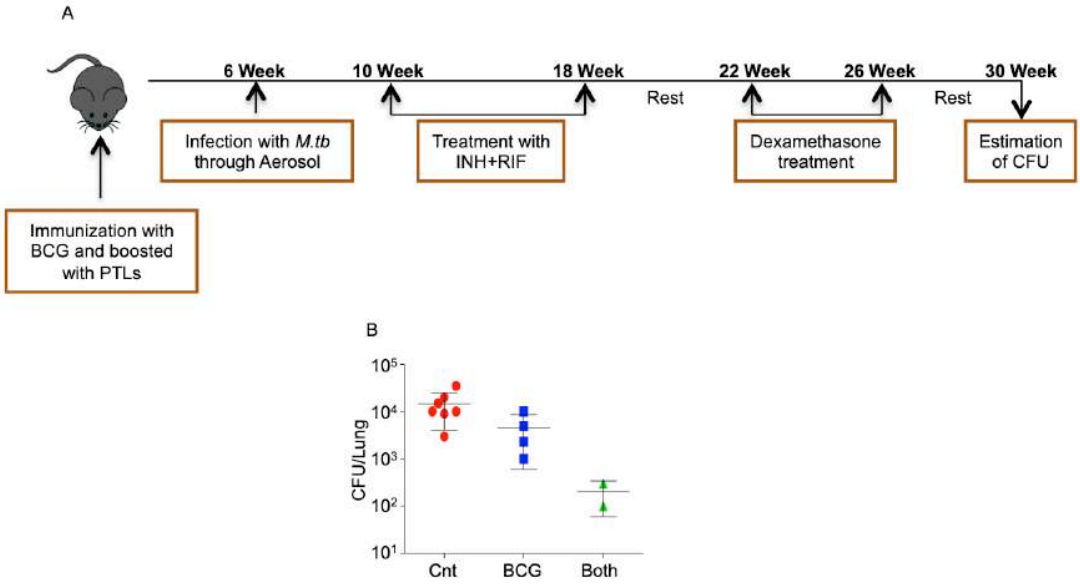

Figure 9: PTLs immunization reduces the recurrence of DOTS associated disease relapse. (A) Mice co-immunized with PTLs and BCG were infected with H37Rv *M.tb* followed by treatment with isoniazid and rifampicin for 16 weeks. After 30 days of rest, these mice were treated with dexamethasone for 30 days followed by one more period of rest for 30 days. Mice were then sacrificed for CFU estimation to determine the rate of relapse post-treatment. **(B)** CFU from the lung homogenates of the mice. The reactivation experiment was done once with 10 mice in each group.

Table 1: Determination of reactivation rate of latent *M.tb*

Group	Reactivation rate*
Control	7/10
BCG immunized	4/9
BCG immunized and boosted with mimic	2/10

*Number of mice re-activated out of total number of mice in that group.

Discussion:

The ability of host immune response to mount an activated antigenic T cell response in the case of pathogenic insult is must to decrease the pathology associated with the infection. While the knowledge about the exact kind of immune responses mounted by the host in the case of TB is still expanding, the role of IFNγ producing Th1 has been well documented(29-32). Recent work by several labs including ours has shown a synergistic role of Th1 and Th17 cells in mounting potent protective responses against TB (8, 33, 34). The mycobacterial secretory proteins play a crucial role in the induction of protective immune responses during TB infections. Mycobacterial proteins like MPT70, Ag85B and ESAT6 have already been used as candidates for antigenic vaccines against TB (14, 35, 36). Their success in T cell proliferation along with IFNγ assays have made them promising candidates to be considered in the race for novel sub-unit vaccines against TB owing to their promiscuous nature (14, 35, 36). We screened several *M.tb* antigenic peptides for their ability to induce IFNγ and IL17 and narrowed down to 7 overlapping peptides from Ag85B and ESAT6 **(Figure 1)**. The evidences gathered over a period of time indicates the inability of BCG vaccine in inducing an optimal T cell response against several T cell epitopes harbouring immunogenic antigens (37). Also, the BCG vaccine is rendered relatively ineffective in cases of adult pulmonary TB due to its inability to evoke an optimum protective T cell response in the lungs (the primary site of infection) since peripheral T cells have limited influence in the lungs (37). Thus, our rationale was to improve the vaccine's immunogenic repertoire by including relevant fragments of *M.tb* antigens which might generate an improved response vaccine against TB.

Conventionally *M.tb* produces an array of PAMPs, including lipoarabinomanan, phenolic glycolipids, phosphatidylinositol mannosidase and other lipoproteins. These molecular patterns are recognised by Toll-like receptors (TLRs). TLRs are innate cytosolic surveillance sensors found on professional antigen presenting cells (APCs) such as macrophages and DCs (18, 38). Interestingly, ligation of these PAMPs is known to trigger both protective as well as pathogenic immune responses (18, 38). Moreover, mice lacking MyD88, a major adaptor molecule required for downstream signalling events by the majority of TLR/IL1R family members, demonstrate enhanced susceptibility to aerosol infection with *Mtb* (39). Several groups have provided convincing reports that TLR2 and TLR9 both are indispensable in protection against TB (17, 40-42). Engagement of these TLRs leads to the activation of a spectrum of transcription factors that induce several pro-inflammatory cytokines including IFNγ, which confers protective immunity against TB. Therefore, we included PamCysSK-4 as TLR2 and CpG ODN as TLR9 agonist in our vaccine design. These TLR agonists along with the pool of 7 overlapping *M.tb* peptides were packaged in the liposomes for intranasal delivery into the lungs **(Figure 4A)**.. Impressively, co-immunisation of BCG and PTLs not only reduced the bacterial burden **(Figure 4)** but also led to the increase in the percentage of $CD4^+$ and $CD8^+$ T cells **(Figure 5)** that actively participate in providing protective immunity against TB. We also observed enhanced activation of these T cell subsets. In this study, we have analysed the expression of CD69 and CD25 as early and late T cell activation markers. However, $CD25^+$ T cells expressing Foxp3 represent Treg cells that have an inhibitory role in the T cell activation. This warrants the analysis of other T cell activation markers. In our study, we also observed a significant increase in the percentage of polyfunctional $CD4^+$ and $CD8^+$ T cells producing more than two cytokines in the mice co-immunized with BCG and PTLs **(Figure 6)**. Moreover, our study also provides strong evidence in favour of antigen-specific nature of these responses from adoptive transfer experiments, where T cells from co-

immunized mice were able to impart protective immunity to congenic TB unexposed naïve mice upon *M.tb* infection **(Figure 8)**.

T_{CM} cells, the perpetual source of T_{EM} cells, dictate the recall responses and are considered indispensable towards potent vaccine response providing long lasting protective immunity (22, 43). Recently, superior host protection by ΔureC::hly BCG strains has been attributed to its induction of an enhanced T_{CM} response (11). Interestingly, central memory cells were found to be elevated during co-immunization of PTLs along with BCG **(Figure 7)**. FOXO1 plays an important role in establishing long-lived T cell memory responses. BCG-PTLs co-immunization reduced the phosphorylation of FOXO1 in the splenocytes of infected animals, which leads to its increased localization in the nucleus, thereby enriching the protective T cell memory responses responsible for enhanced efficacy of vaccine (27, 28). Furthermore, the increase in NFκB activation in the co-immunized group corroborated with studies crediting NFκB activation in inducing pro-inflammatory cytokine production during TB (44-46).

Transcriptome studies on peptide pool pulsed DCs and stimulated T cells revealed that peptide pool induces differential expression of genes belonging majorly to the immune-relevant signaling pathways such as, JAKSTAT, TNF, TLR, NFκB, MAPK and TGFβ. JAK-STAT pathway is well known to regulate T cell polarization and deregulation of JAK-STAT pathway leads to increased susceptibility during TB (47, 48). Similarly, loss of TNF signaling causes increased mortality due to increased bacterial burden and necrotic death of overladen macrophages and granuloma breakdown (49, 50). Moreover, patients receiving TNF neutralizing therapy have an increased rate of reactivation of latent TB (51). NFkB has been shown to be critical for the expression of many proinflammatory cytokines required for the protection against tuberculosis (45, 52) as NFkB knockout mice succumb to *M.tb* infection (45, 53). Our data also indicated that peptide pool induces MAPK signaling pathways which have a phenomenal role during TB (54). Dephosphorylation of MAPK, ERK and P38 leads to increased susceptibility during tuberculosis (55). There are many reports suggesting that MAPK pathway is not only involved in many aspects of immune responses from initiation of innate immunity to adaptive immunity but also its termination through apoptosis and maintenance of T cell homeostasis (56-58). Moreover, MAPKs phosphorylate and activate downstream molecules, resulting in T cell activation, proliferation, and differentiation into T helper phenotypes. *M.tb*-induced production of proinflammatory cytokines also depends on MAPK activation (57, 58).

In spite of the limited ability of the BCG vaccine to provide protective immunity against adult pulmonary TB, it is quite effective in mounting a strong protective response in young children against meningeal and other disseminated TB. Considering that a large percentage of the population in countries with high TB burden are BCG vaccinated at birth, an improvised strategy like ours that accentuates BCG efficacy by selectively increasing central memory T cell pools and polyfunctional T cells which in turn provide long-lasting immune response against TB is long desired. The study warrants further validation in TB models more close to humans such as non-human primates.

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Place: New Delhi Signature of the Applicant

Date: 29th August 2023