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Intranasal immunization with peptide-based immunogenic complex enhances BCG vaccine efficacy in a murine model of tuberculosis

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Prime-boost immunization strategies are required to control the global tuberculosis (TB) pandemic, which claims approximately 3 lives every minute. Here, we have generated an immunogenic complex against *Mycobacterium tuberculosis* (*M.tb*), consisting of promiscuous T cell epitopes (*M. tb* peptides) and TLR ligands assembled in liposomes. Interestingly, this complex (peptide–TLR agonist–liposomes; PTL) induced significant activation of $CD4^+$ T cells and IFN-γ production in the PBMCs derived from PPD^+ healthy individuals as compared with PPD^- controls. Furthermore, intranasal delivery of PTL significantly reduced the bacterial burden in the infected mice by inducing *M.tb*-specific polyfunctional ($IFN\text{-}\gamma^+IL\text{-}17^+TNF\text{-}\alpha^+IL\text{-}2^+$) immune responses and long-lasting central memory responses, thereby reducing the risk of TB recurrence in DOTS-treated infected animals. The transcriptome analysis of peptide-stimulated immune cells unveiled the molecular basis of enhanced protection. Furthermore, PTL immunization significantly boosted the Bacillus Calmette-Guerin–primed (BCG-primed) immune responses against TB. The greatly enhanced efficacy of the BCG-PTL vaccine model in controlling pulmonary TB projects PTL as an adjunct vaccine against TB.

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 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 extrapulmonary TB — and even death (4). The rate of progression in the severity of TB depends on the status of the host immune system.

Although the 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 (Tcm) (6, 9–11), since animal models vaccinated with BCG primarily develop antigen-specific $CD4^+$ effector memory T cells (Tem). Considering the lags in BCG immunization and increased global TB burden, it is crucial to develop improved methods of immunoprophylaxis 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

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along with TLR ligands adsorbed on liposomal drug delivery vehicle. These complexes, called peptide–TLR agonist–liposomes (PTL), were delivered directly into the lungs through an intranasal route, thereby generating a protective immune response at the site of infection.

We observed that the PTL significantly enriched the BCG-induced Tcm pool in the $CD4^+$ and $CD8^+$ T cells, with a decrease in the Tem cell pool in the lungs of mice coimmunized with BCG and PTL, compared with the mice immunized with BCG or PTL alone. The population of Tcm was maintained at elevated numbers in the spleens of coimmunized 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 spleens of BCG and PTL coimmunized mice as compared with other groups. Moreover, increased memory responses correlated with a remarkable reduction in bacterial burden in the lungs, spleens, and livers of the animals receiving PTL immunization along with BCG compared with other experimental groups.

Furthermore, we also noticed a significant increase in the polyfunctional cytokine secretion ($IFN\text{-}\gamma^+IL\text{-}17^+TNF\text{-}\alpha^+IL\text{-}2^+$) in $CD4^+$ and $CD8^+$ T cells in the lungs of coimmunized animals as compared with the mice vaccinated with BCG alone. A similar protective response was also observed in reactivation studies. Separate transcriptome analysis of DCs pulsed with peptides (Pep-DCs) and cocultured T cells further sheds light on the possible multiple host-protective pathways induced by PTL.

Collectively, in our study, we report that BCG-vaccinated mice, when coimmunized with PTL, induced a larger pool of Tcm 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 PTL coimmunization 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 observations 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 (Supplemental Table 1; supplemental material available online with this article; https://doi.org/10.1172/jci.insight.145228DS1) (12–16). A group of mice infected with the H37Rv strain of *M.tb* was subjected to 45 days of DOTS therapy starting from 15 days after infection. After a rest period of 30 days, T cells from these infected and DOTS-treated mice were isolated and cocultured with DCs derived from the BM of naive 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 sets of peptides, 7 peptides — which induced significant T cell activation (Figure 1A) and enhanced IFN-γ and IL-17 secretion (Figure 1, B and C) — are indicated in Supplemental Table 2. Next, we performed the above experiments using a pool of these 7 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 with CSA of *M.tb*. Furthermore, we also observed an increase in the number of IFN-γ– and IL-17–producing $CD4^+$ and $CD8^+$ T cells (Figure 1, E and F) in the T cell pool cocultured with DCs loaded with peptide pool in comparison with the CSA.

M.tb peptides induce the 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 proinflammatory cytokine upon peptide pool treatment, we performed comparative transcriptome analysis of unstimulated DCs (Un-DCs) versus Pep-DCs and T cells cocultured with unstimulated DCs (Un-TCs) versus T cells cocultured with DCs pulsed with peptides (Pep-TCs). Our RNA sequencing (RNA-Seq) data revealed 1452 differentially expressed genes (1098 upregulated, $\log_2FC > 1$; 354 downregulated, $\log_2FC < -1$ with FDR ≤ 0.05) in the peptide pool–stimulated DCs as compared with Un-DCs (Figure 2A), while Pep-TCs showed 2331 differentially expressed genes (1223 activated and 1108 repressed) as compared with Un-TCs (accession no. GSE164258) (Figure 2B). Differential genes in both DCs and T cells after peptide pool stimulation were highly enriched for gene sets assisting in IFN-γ response and production, cytokine activity, STAT phosphorylation, ADP metabolic processes, and ROS equilibrium (Supplemental Figure 1, A and B). Many pathways known to play an important role in combating TB disease were significantly upregulated in peptide-stimulated DCs and T cells, as indicated by KEGG analysis (Figure 2, C and D). Differential genes in the DCs as well as the T cells majorly belonged to signaling pathways such as JAK/STAT, TB, TNF,

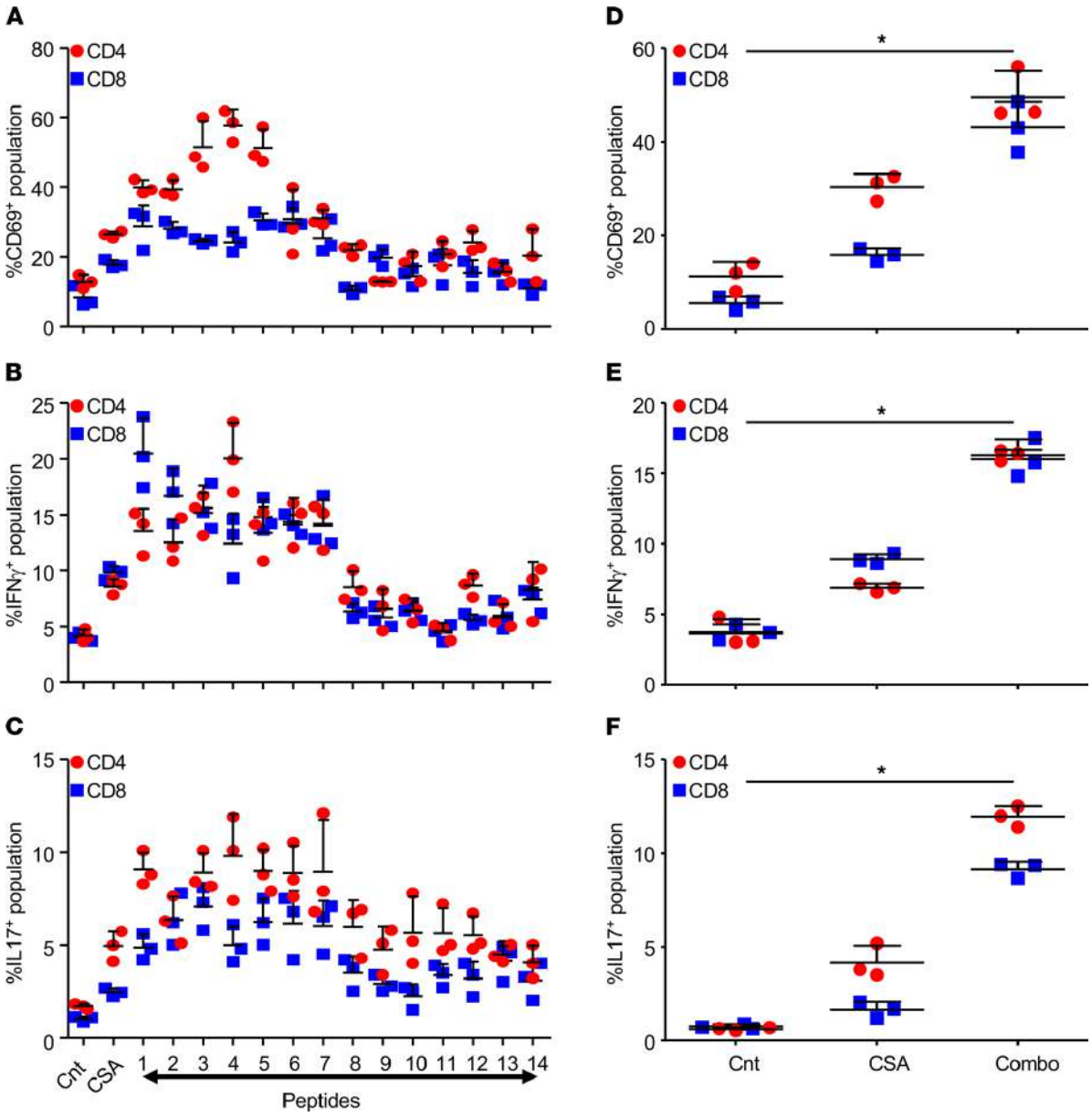
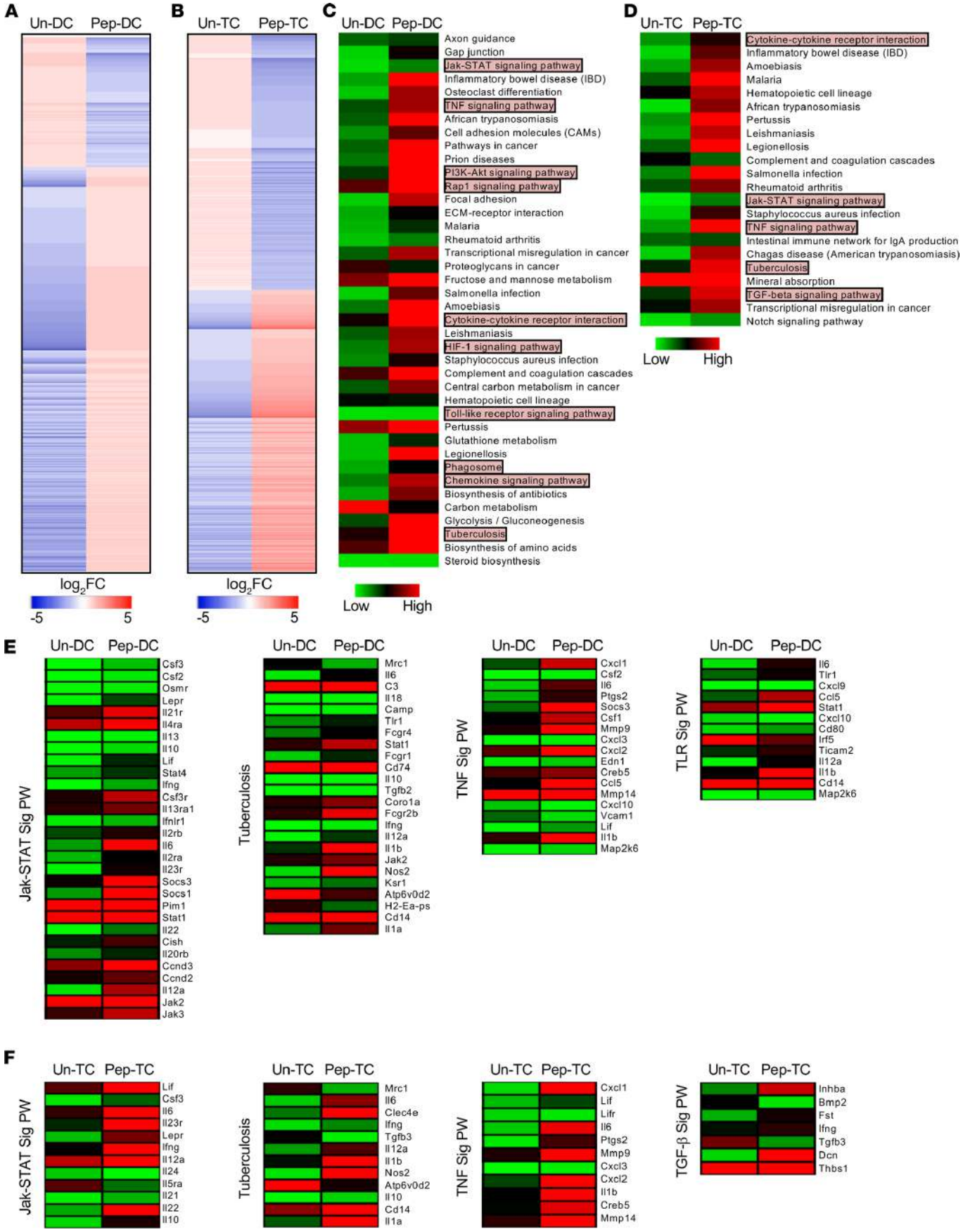


Figure 1. Mycobacterial antigens induce activation of protective T cell responses. T cells isolated from the spleens of mice infected with H37Rv and treated with DOTS were stimulated with DCs preloaded with the peptides for 48 hours, followed by surface staining with anti-CD3, anti-CD4, anti-CD8, and anti-CD69 and staining for intracellular cytokines with anti–IFN-γ and anti–IL-17 antibodies. (**A**–**C**) Bar graphs depicting the percentage of $CD4^+CD69^+$ and $CD8^+CD69^+$ T cells (**A**), $CD4^+IFN\text{-}\gamma^+$ and $CD8^+IFN\text{-}\gamma^+$ T cells (**B**), and $CD4^+IL\text{-}17^+$ and $CD8^+IL\text{-}17^+$ T cells (**C**). (**D**) Activation of $CD4^+$ and $CD8^+$ T cells cocultured with unstimulated DCs (Cnt), DCs pulsed with CSA, or DCs pulsed with the peptide combo. (**E** and **F**) $CD4^+$ and $CD8^+$ T cells expressing IFN-γ (**E**) and IL-17 (**F**) after stimulation with the combo. Each experiment was performed at least three times in triplicate. Two-tailed Student's *t* test was performed for statistical analysis. Data represent mean ± SD ($n = 3$). *$P < 0.05$.

TLR, and TGF-β signaling (Figure 2, E and F). 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 upregulated and 138 downregulated) were common between the 2 cell types (Supplemental Figure 1C). Moreover, these genes followed the same expression profile in both of the settings (Supplemental Figure 1C). KEGG analysis indicated that these genes belonged to a number of TB-related protective host signaling pathways such as NF-κB, MAPK, TGF-β, TNF, and IL-17 (Supplemental Figure 1D). Taken together, our transcriptomics data have 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.

Induction of immune responses in human PBMCs by the M.tb PTL assembly. Subject to the above results, we establish here that, in combination, our peptides are capable of inducing antigen-specific protective T cells;


A
Un-DC
Pep-DC
log2FC
-5
5
B
Un-TC
Pep-TC
log2FC
-5
5
C
Un-DC
Pep-DC
Axon guidance
Gap junction
Jak-STAT signaling pathway
Inflammatory bowel disease (IBD)
Osteoclast differentiation
TNF signaling pathway
African trypanosomiasis
Cell adhesion molecules (CAMs)
Pathways in cancer
Prion diseases
PI3K-Akt signaling pathway
Rap1 signaling pathway
Focal adhesion
ECM-receptor interaction
Malaria
Rheumatoid arthritis
Transcriptional misregulation in cancer
Proteoglycans in cancer
Fructose and mannose metabolism
Salmonella infection
Amoebiasis
Cytokine-cytokine receptor interaction
Leishmaniasis
HIF-1 signaling pathway
Staphylococcus aureus infection
Complement and coagulation cascades
Central carbon metabolism in cancer
Hematopoietic cell lineage
Toll-like receptor signaling pathway
Pertussis
Glutathione metabolism
Legionellosis
Phagosome
Chemokine signaling pathway
Biosynthesis of antibiotics
Carbon metabolism
Glycolysis / Gluconeogenesis
Tuberculosis
Biosynthesis of amino acids
Steroid biosynthesis
Low
High
D
Un-TC
Pep-TC
Cytokine-cytokine receptor interaction
Inflammatory bowel disease (IBD)
Amoebiasis
Malaria
Hematopoietic cell lineage
African trypanosomiasis
Pertussis
Leishmaniasis
Legionellosis
Complement and coagulation cascades
Salmonella infection
Rheumatoid arthritis
Jak-STAT signaling pathway
Staphylococcus aureus infection
TNF signaling pathway
Intestinal immune network for IgA production
Chagas disease (American trypanosomiasis)
Tuberculosis
Mineral absorption
TGF-beta signaling pathway
Transcriptional misregulation in cancer
Notch signaling pathway
Low
High
E
Un-DC
Pep-DC
Jak-STAT Sig PW
Csf3
Csf2
Osmr
Lepr
Il21r
Il4ra
Il13
Il10
Lif
Stat4
Ifng
Csf3r
Il13ra1
Ifnlr1
Il2rb
Il6
Il2ra
Il23r
Socs3
Socs1
Pim1
Stat1
Il22
Cish
Il20rb
Ccnd3
Ccnd2
Il12a
Jak2
Jak3
Tuberculosis
Mrc1
Il6
C3
Il18
Camp
Tlr1
Fcgr4
Stat1
Fcgr1
Cd74
Il10
Tgfb2
Coro1a
Fcgr2b
Ifng
Il12a
Il1b
Jak2
Nos2
Ksr1
Atp6v0d2
H2-Ea-ps
Cd14
Il1a
TNF Sig PW
Cxcl1
Csf2
Il6
Ptgs2
Socs3
Csf1
Mmp9
Cxcl3
Cxcl2
Edn1
Creb5
Ccl5
Mmp14
Cxcl10
Vcam1
Lif
Il1b
Map2k6
TLR Sig PW
Il6
Tlr1
Cxcl9
Ccl5
Stat1
Cxcl10
Cd80
Irf5
Ticam2
Il12a
Il1b
Cd14
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Jak-STAT Sig PW
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Csf3
Il6
Il23r
Lepr
Ifng
Il12a
Il24
Il5ra
Il21
Il22
Il10
Tuberculosis
Mrc1
Il6
Clec4e
Ifng
Tgfb3
Il12a
Il1b
Nos2
Atp6v0d2
Il10
Cd14
Il1a
TNF Sig PW
Cxcl1
Lif
Lifr
Il6
Ptgs2
Mmp9
Cxcl3
Cxcl2
Il1b
Creb5
Mmp14
TGF-β Sig PW
Inhba
Bmp2
Fst
Ifng
Tgfb3
Dcn
Thbs1

Figure 2. Stimulation with peptide pool induces multiple signaling pathways involved in providing protection during *M.tb* infection. (**A** and **B**) Heatmap representation of the genes differentially expressed in the DCs pulsed with peptide pool (Pep-DC) in comparison with unstimulated DCs (Un-DC) (**A**) and the T cells cocultured with DCs pulsed with peptide pool (Pep-TC) versus T cells cocultured with unstimulated DCs (Un-TC) (**B**). 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, TB, TNF signaling pathway, and TLR signaling pathways in Pep-DCs versus Un-DCs. (**F**) Heatmaps representing the JAK/STAT signaling pathway, TB, TNF signaling pathway, and TGF-β signaling pathways in Pep-TCs versus Un-TCs. RNA-Seq was performed once in triplicate (n = 3).

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 PTL 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 CSA (20 μg/mL), PTL (10 μL/mL), and BCG CSA/PTL for 48 hours, followed by surface/intracellular staining and FACS analysis, to determine $CD4^+$ T cell activation and IFN-γ production. With no significant difference in PPD^- individuals, PTL and BCG CSA/PTL combination induced significant expression of early activation marker CD69 and IFN-γ on $CD4^+$ T cells in the PBMCs derived from PPD^+ individuals as compared with *M.tb* CSA and BCG CSA controls (Figure 3, B–E).

PTL immunization following BCG vaccination enhances host protection against TB. To confirm the successful delivery of this assembly of PTL into the lungs, the PTL were labeled with PKH67 to stain the liposomes (delivery vehicle) and administered intranasally into the lungs. Two days after delivery, mice were euthanized and lung sections were prepared for fluorescence microscopy (Figure 4A). Quantification of the fluorescent images indicated the accumulation of liposomes/PTL 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 a once-a-week, 3-week PTL boosting regimen followed by a rest period of 21 days. Supplemental Figure 2 shows the prechallenge immune response in the lungs and the spleens of vaccinated animals. With no increase in the number of $CD4^+$ and $CD8^+$ T cells, we observed a significant increase in the expression of CD69 on these cells in the lungs of BCG-PTL–coimmunized mice as compared with BCG/PTL administration alone (Supplemental Figure 2, A–D). Furthermore, coimmunized animals also showed significant increase in the $CD4^+$ T cells expressing IFN-γ and IL-17 (Supplemental Figure 2, E and F) as compared with single vaccinations. Similarly, $CD8^+$ T cells expressing IL-17 but not IFN-γ were also induced in the lungs of coimmunized mice (Supplemental Figure 2, G and H). A similar profile was observed in the spleens of vaccinated animals (Supplemental Figure 2, I–P). These mice were challenged with H37Rv, the laboratory strain of *M.tb*, using low-dose aerosol infection model (approximately 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, coimmunized animals had fewer and smaller inflammatory lesions in their lungs than mice immunized with BCG or PTL alone, while the nonvaccinated infected mice (i.e., primary infection with H37Rv *M.tb*) showed a significantly higher number of inflammatory lesions ($P < 0.005$, Figure 4D). These results were further strengthened by histopathological analysis of lungs, which confirmed the reduced lung inflammation in the coimmunized group as compared with the rest of the experimental groups (Figure 4E). PTL coimmunization significantly increased the BCG-induced TB protection, as observed by the reduced bacterial burden in various organs, such as the lungs (Figure 4F), spleens (Figure 4G), and livers (Figure 4H) of infected mice. Thus, the PTL significantly enhanced the antitubercular capacity of BCG immunization. Interestingly, the mice immunized with PTL alone displayed comparable (Figure 4F) and enhanced (Figure 4, G and H) resistance against *M.tb* as the BCG immunized group.

BCG-PTL coimmunization elevates the adaptive immunity in the lungs and the spleens 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-PTL coimmunization, we profiled the immune cells in the lungs and the spleens of infected animals. Figure 5A describes the gating strategy employed to quantify the percentage of various T cell subsets in the lungs and spleens of infected mice. BCG-PTL–coimmunized mice showed comparable levels and activation of $CD4^+$ (Figure 5, B–D), whereas with minimal effect on the percentage of $CD8^+$ T cells (Figure 5E), BCG-PTL coimmunization significantly

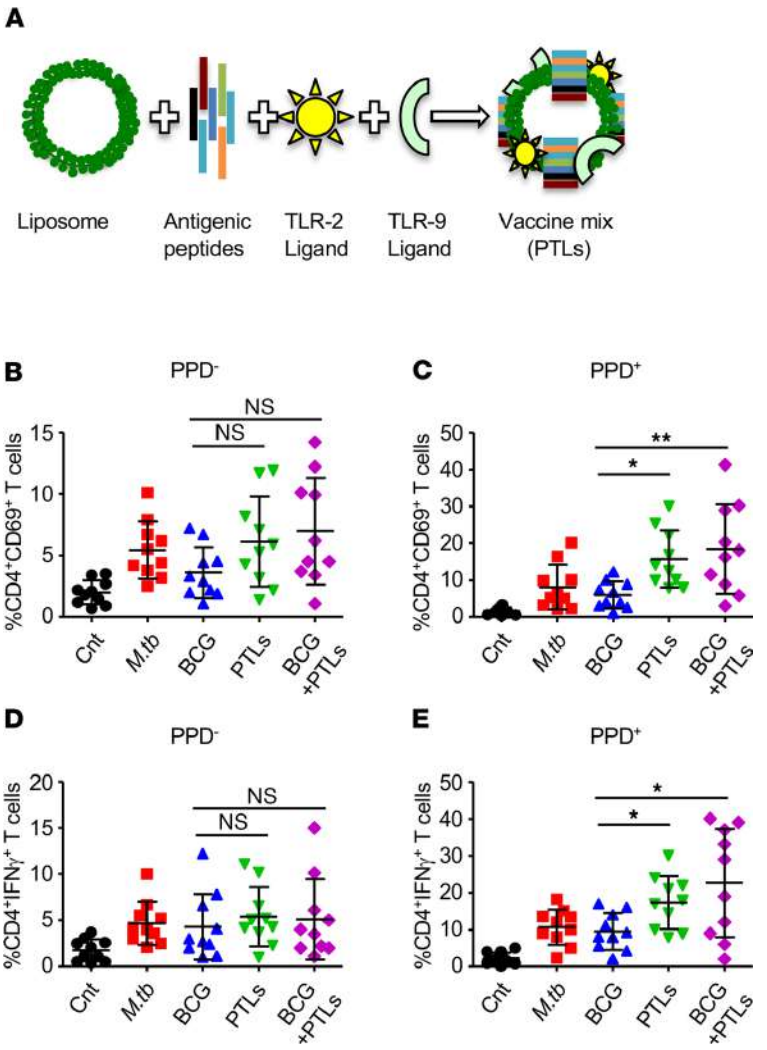

Figure 3. PTL induces a protective immune response in human PBMCs. (**A**) Schematic diagram depicts the preparation of PTL. PBMCs isolated from PPD$^-$ and PPD$^+$ healthy subjects were in vitro stimulated with different mycobacterial antigens for 48 hours. (**B** and **C**) Expression of CD69$^+$ on CD4$^+$ T cells stimulated with mycobacterial antigens in PPD$^-$ (**B**) and PPD$^+$ (**C**) individuals. (**D** and **E**) Percentage of CD4$^+$ T cells expressing IFN-γ in the PBMCs of PPD$^-$ (**D**) and PPD$^+$ (**E**) individuals. One-way ANOVA, followed by multiple Tukey tests, was performed for statistical analysis. Data represent mean ± SD (n = 10) performed once. *P < 0.05, **P < 0.005.

enhanced the expression of early and late activation markers (CD69 and CD25, respectively) on CD8$^+$ T cells as compared with either BCG or PTL immunization (Figure 5, F and G). Furthermore, we observed increased frequency of CD4$^+$ T cells (Figure 5H) with increased expression of late activation marker CD25 in the spleens of BCG-PTL–coimmunized animals (Figure 5J). CD69 expression was significantly high in splenic CD4$^+$ T cells derived from PTL-immunized animals as compared with BCG group (Figure 5I). Percentage of CD8$^+$ T cells was significantly high in the spleens of BCG-PTL–immunized animals (Figure 5K) with no increase in the early and late activation markers (Figure 5, L and M).

BCG-PTL coimmunization induces polyfunctional cytokine responses in the lungs and the spleens 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 spleens of treated and control mice and estimated intracellular cytokine production. Polyfunctional T cells expressing more than 2 cytokines, such as IFN-γ, TNF-α, IL-17, and IL-2, have been linked with enhanced protection against TB (19, 20). Moreover, the occurrence of these multifunctional T cells is highly important in the context of effective vaccines against various viruses and intracellular bacterial pathogens (21). Thus, we analyzed the presence of polyfunctional cells in different T cell subsets in the lungs and the spleens of all animal groups (Figure 6A). Interestingly, BCG-PTL coimmunization significantly increased the frequency of CD4$^+$ and CD8$^+$ T cells expressing single, double, triple,

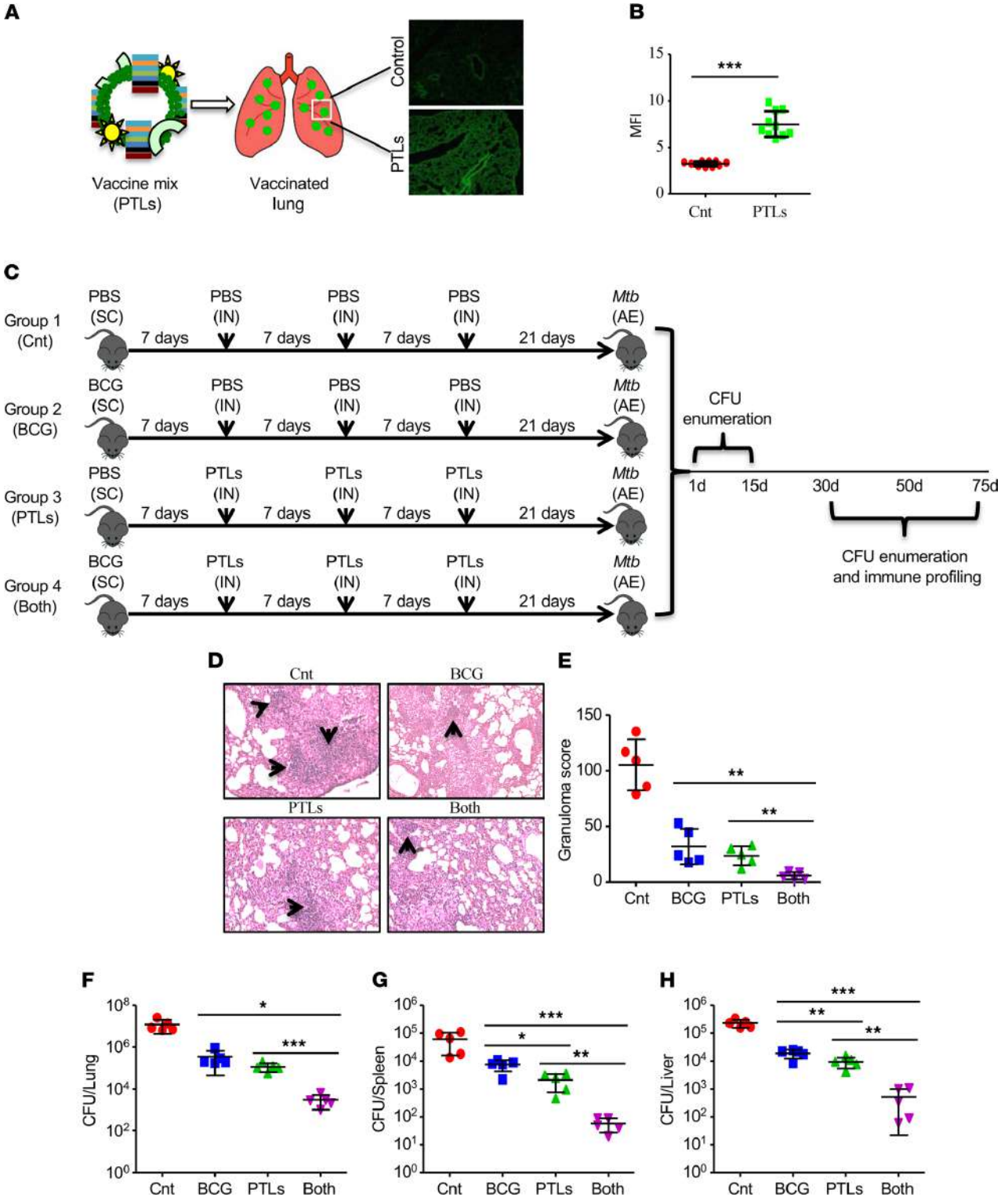

Figure 4. PTL enhances the efficacy of BCG and protects mice against TB. (**A**) Lung section to show the accumulation of liposomes. Liposomes were stained with PKH67 dye. Original magnification, ×100. (**B**) Quantification of the fluorescent images. (**C**) Layout to show the experimental plan wherein naive C57BL/6 mice or mice vaccinated with BCG/PTL 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 for observance of bacterial burden, as well as profiling of immune responses. (**D**) Lungs were harvested, preserved in 4% paraformaldehyde, and processed for sectioning and staining with H&E. Original magnification, ×100. (**E**) Quantification of the granuloma (inflammatory lesions) in all experimental groups. (**F**–**H**) CFU from the lung (**F**), spleen (**G**), and liver homogenates (**H**) at 50 days after infection. Two-tailed Student's *t* test was performed for statistical analysis. Data are representative of 2 independent experiments (n = 5 mice/group). $^{*}P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.0005$.

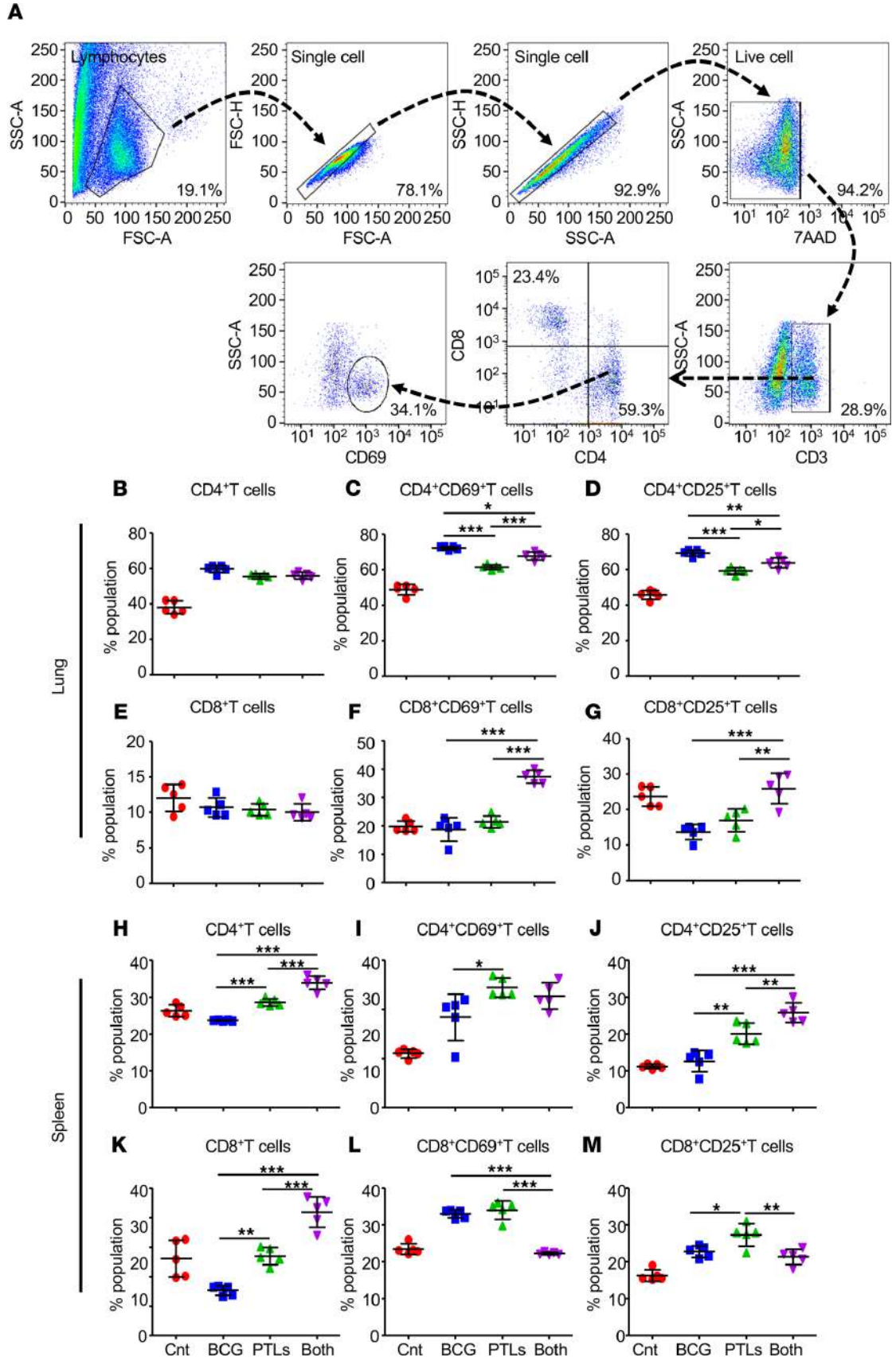

Figure 5. PTL immunization induces T cell activation in the lungs and the spleens of infected animals. T lymphocytes were isolated from the lungs of all experimental groups and stained with 7AAD, anti-CD3, anti-CD4, anti-CD8, anti-CD25, and anti-CD69 antibodies. (**A**) Gating strategy employed to quantify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

from the spleens of all experimental groups and stained with 7AAD, anti-CD3, anti-CD4, anti-CD8, anti-CD25, and anti-CD69 antibodies. (**H**–**J**) Percentage of $CD4^+$ (**H**), $CD4^+CD69^+$ (**I**), and $CD4^+CD25^+$ (**J**) T cells in the spleens of infected animals. (**K**–**M**) Percentage of $CD8^+$ (**K**), $CD8^+CD69^+$ (**L**), and $CD8^+CD25^+$ (**M**) T cells in the spleens of infected animals. One-way ANOVA, followed by multiple Tukey tests, was performed for statistical analysis. Data are representative of 2 independent experiments (n = 5 mice/group). *P < 0.05, **P < 0.005, ***P < 0.0005.

and quadruple cytokines, with a concomitant decrease in the T cell population expressing none of the 4 cytokines in the infected lungs (Figure 6, B and C) and the spleens (Figure 6, D and E) as compared with other groups. Particularly, there was an increased occurrence of 4-positive ($IFN\text{-}\gamma^+TNF\text{-}\alpha^+IL\text{-}17^+IL\text{-}2^+$), 3-positive ($IFN\text{-}\gamma^+TNF\text{-}\alpha^+IL\text{-}17^+$), and 2-positive ($IFN\text{-}\gamma^+IL\text{-}17^+$ and $TNF\text{-}\alpha^+IL\text{-}17^+$) $CD4^+$ and $CD8^+$ T cells in the coimmunized group as compared with other animals (Figure 6, F and G). However, the expression of single cytokines ($IFN\text{-}\gamma^+$ and $IL\text{-}17^+$) was higher only in $CD4^+$ T cells (Figure 6, F and G). A similar profile was observed in the spleens of coimmunized mice where 4-positive ($IFN\text{-}\gamma^+TNF\text{-}\alpha^+IL\text{-}17^+IL\text{-}2^+$), 3-positive ($IFN\text{-}\gamma^+TNF\text{-}\alpha^+IL\text{-}17^+$;$IFN\text{-}\gamma^+TNF\text{-}\alpha^+IL\text{-}2^+$), 2-positive ($IFN\text{-}\gamma^+IL\text{-}17^+$), and 1-positive ($IFN\text{-}\gamma^+$) $CD4^+$ and $CD8^+$ T cells displayed significantly enhanced frequency in BCG-PTL–coimmunized group (Figure 6, H and I). Taken together, we observed that BCG vaccination followed by PTL immunization greatly enhanced the antigen-specific Th1 and Th17 responses, as well as proinflammatory cytokines TNF-α and IL-2. All these cytokines have been well documented to impart protection against TB.

PTL immunization induces Tcm cell responses critical for long-lasting protection. Since recall responses are mediated by Tcm cells, these cell subtypes become a prerequisite for enhanced vaccine efficacy and superior TB protection (6, 9, 22). Thus, we analyzed the memory cell profile of the lung, as well as the splenic T cell subsets (Figure 7A). We observed that BCG-PTL coimmunization enriched the vaccine-induced Tcm ($CD44^{hi}CCR7^{hi}CD62L^{hi}$) cell pool in $CD4^+$ T cells (Figure 7B) with a concomitant decrease in the Tem ($CD44^{hi}CCR7^{lo}CD62L^{lo}$) cell pool (Figure 7C) in the lungs of infected mice as compared with 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 nonhuman 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 $CD4^+$ Tcm and Tem subsets in the lungs of mice coimmunized with BCG and PTL as compared with the BCG-vaccinated group (Figure 7, D and E). A similar pattern was observed in the $CD8^+$ lung T cells (Figure 7, F–I). When investigated in the spleens of coimmunized animals, there was increased frequency of $CD4^+$ Tcm pool (Figure 7J) with a decrease in $CD4^+$ Tem subset (Figure 7K). PD-1 expression was significantly less only in the case of the $CD4^+$ Tcm subset (Figure 7L) in the coimmunized group, with no effect on the $CD4^+$ Tem subset (Figure 7M). $CD8^+$ Tcm/Tem cells were comparable in all the groups (Figure 7, N and O). With no decrease in PD-1 expression on $CD8^+$ Tcm (Figure 7P), its expression was significantly less on the $CD8^+$ Tem subset in the coimmunized group (Figure 7Q). Previously, it has been shown that BCG-vaccinated or *M.tb*-infected mice generate a profoundly expanded population of antigen-specific Tem cells within the lungs, whereas the Tcm pool is substantially smaller. However, Tcm cells are 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 Tcm/Tem profiles for infected and BCG-vaccinated controls, coimmunized mice maintained an increased pool of Tcm cells both in the spleen and the lungs. Nuclear FOXO1 is in an unphosphorylated 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 coimmunized animals in comparison with all other experimental groups (Supplemental Figure 3). Furthermore, we observed an increased activation of NF-κB transcription factor in the splenocytes of BCG-PTL–coimmunized animals as compared with other groups (Supplemental Figure 3). NF-κB is believed to be the main transcription factor responsible for the expression of various proinflammatory cytokines, such as IFN-γ, TNF-α, and IL-12, required for providing resistance to TB. Collectively, these data suggest that PTL used in this study induces the activation of key transcription factors involved in generating protective immune responses inside the host.

Antigen-specific protective immunity induced by BCG-PTL coimmunization can be adoptively transferred by T cells. Above results clearly demonstrate that PTL coimmunization enhanced protective immune responses following BCG vaccination. To further reveal the antigen specificity and protective function of T cell responses generated by BCG-PTL coimmunization, we carried out the adoptive transfer of $CD4^+$ and

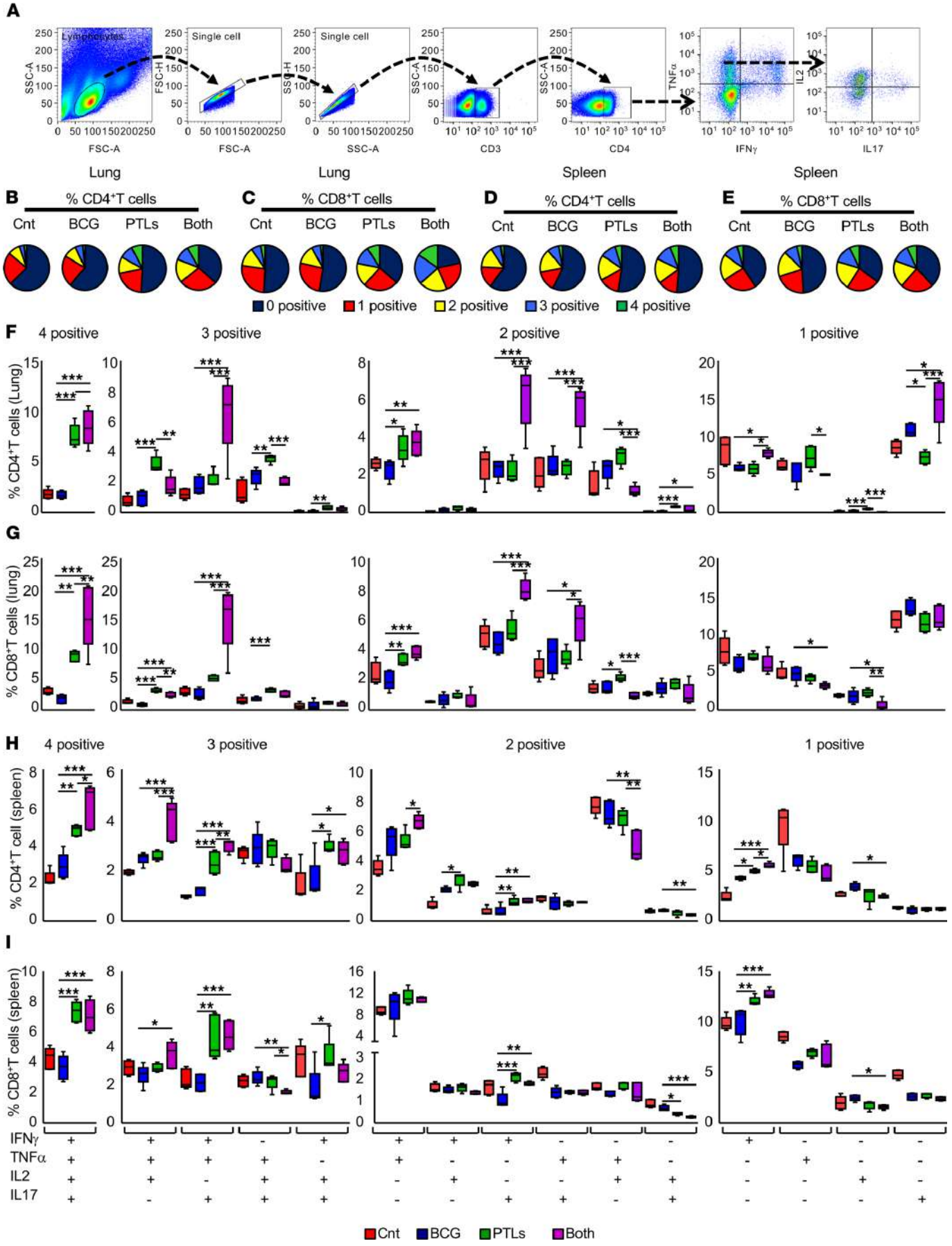
A
Lymphocytes
Single cell
Single cell
SSC-A
FSC-A
FSC-H
SSC-H
CD3
CD4
TNFα
IFNγ
IL2
IL17
Lung
Lung
Spleen
Spleen
B % CD4+T cells
C % CD8+T cells
D % CD4+T cells
E % CD8+T cells
Cnt BCG PTLs Both
0 positive 1 positive 2 positive 3 positive 4 positive
F 4 positive 3 positive 2 positive 1 positive
% CD4+T cells (Lung)
G
% CD8+T cells (lung)
H 4 positive 3 positive 2 positive 1 positive
% CD4+T cell (spleen)
I
% CD8+T cells (spleen)
IFNγ
TNFα
IL2
IL17
Cnt BCG PTLs Both

Figure 6. BCG-PTL coimmunization induces the antigen-specific polyfunctional cytokine responses in the lungs and the spleens of infected animals. (**A**) Lymphocytes isolated from the lungs of infected animals were stained with anti-CD3, anti-CD4, anti-CD8, anti–IFN-γ, anti-TNF-α, anti–IL-17, and anti–IL-2 to assess polyfunctional cytokine responses. (**B** and **C**) Pie charts depicting the percentage of $CD4^+$ (**B**) and $CD8^+$ (**C**) T cells expressing 4, 3, 2, 1, and 0 cytokines (IFN-γ, TNF-α, IL-17, and IL-2) in the lungs of the mice. (**D** and **E**) The pie charts representing the average percentage of cytokine-producing $CD4^+$ (**D**) and $CD8^+$ (**E**) T cells producing 5 combinations (0^+, 1^+, 2^+, 3^+, and 4^+) of the 4 cytokines analyzed in the spleens of infected animals. (**F** and **G**) Fifteen possible cytokine combinations are shown for $CD4^+$ (**F**) and $CD8^+$ (**G**) T cells from the lungs of infected animals. (**H** and **I**) Box and whisker plots depict 15 combinations of responses for the 4 cytokines analyzed on the *x* axis with the percentage of $CD4^+$ (**H**) and $CD8^+$ (**I**) responding splenic T cells on the *y* axis. One-way ANOVA, followed by multiple Tukey tests, was performed for statistical analysis. Data are representative of 2 independent experiments ($n = 5$ mice/group). $^*P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.0005$.

$CD8^+$ T cells isolated from the lungs of BCG, PTL, and BCG-PTL–coimmunized mice to check for their antigen-specific protective response in naive mice. $CD4^+$ (1×10^6) and $CD8^+$ T cells (1×10^6) were transferred into γ-irradiated Thy1.1 mice followed by a low-dose aerosol challenge of *M.tb* H37Rv. Twenty-five days after infection, mice were sacrificed for CFU enumeration and immune profiling (Figure 8A). We found significant decrease of bacterial load in the mice that received T cells from BCG-PTL–coimmunized animals as compared with the BCG-vaccinated group (Figure 8, B and C). Furthermore, the immune profiling revealed a significant increase in the percentage of INF-γ–producing $CD4^+$ and $CD8^+$ T cells, with no difference in IL-17–producing T cells in the spleens of mice that received T cells from BCG-PTL–coimmunized animals (Figure 8, D–G). Therefore, T lymphocytes ($CD4^+$ and $CD8^+$ T cells) isolated from coimmunized mice successfully transferred the protective immunity against TB in naive animals.

BCG-PTL coimmunization protects antibiotic-treated animals against disease recurrence. From the above experiments, it is clear that PTL coimmunization 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 PTL coimmunization, we performed reactivation experiments in the mouse model of TB (Figure 9A). Reactivation rate was calculated as the number of mice reactivated out of the total number of mice in that group. The reactivation results showed that nonvaccinated mice receiving isoniazid (INH) and rifampicin (RIF) treatment exhibited greater disease reactivation (7 of 10 mice, 70%) upon dexamethasone treatment, while mice immunized with BCG showed around 40% (4 of 9 mice) relapse (Table 1). The relapse rate was significantly lower in mice coimmunized with BCG and PTL (2 of 10 mice, 20%) (Table 1). However, there were no differences in terms of bacterial burden in the mice that experienced reactivation (Figure 9B). Since effective memory equates to enhanced protection from primary as well as secondary infections, these observations demonstrate that enhanced proinflammatory responses and Tcm responses induced by BCG-PTL coimmunization might translate into reduced relapse incidents due to reactivation, thus effectively promoting sterile immunity.

Discussion

The ability of host immune response to mount an activated antigenic T cell response in the case of pathogenic insult is a 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 IFN-γ–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 IFN-γ assays, have made them promising candidates to be considered in the race for novel subunit vaccines against TB, owing to their promiscuous nature (14, 35, 36). We screened several *M.tb* antigenic peptides for their ability to induce IFN-γ and IL-17 and narrowed it down to 7 overlapping peptides from Ag85B and ESAT6 (Figure 1). The evidence gathered over a period of time indicates the inability of the BCG vaccine in inducing an optimal T cell response against several T cell epitopes harboring 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 that might generate an improved response vaccine against TB.

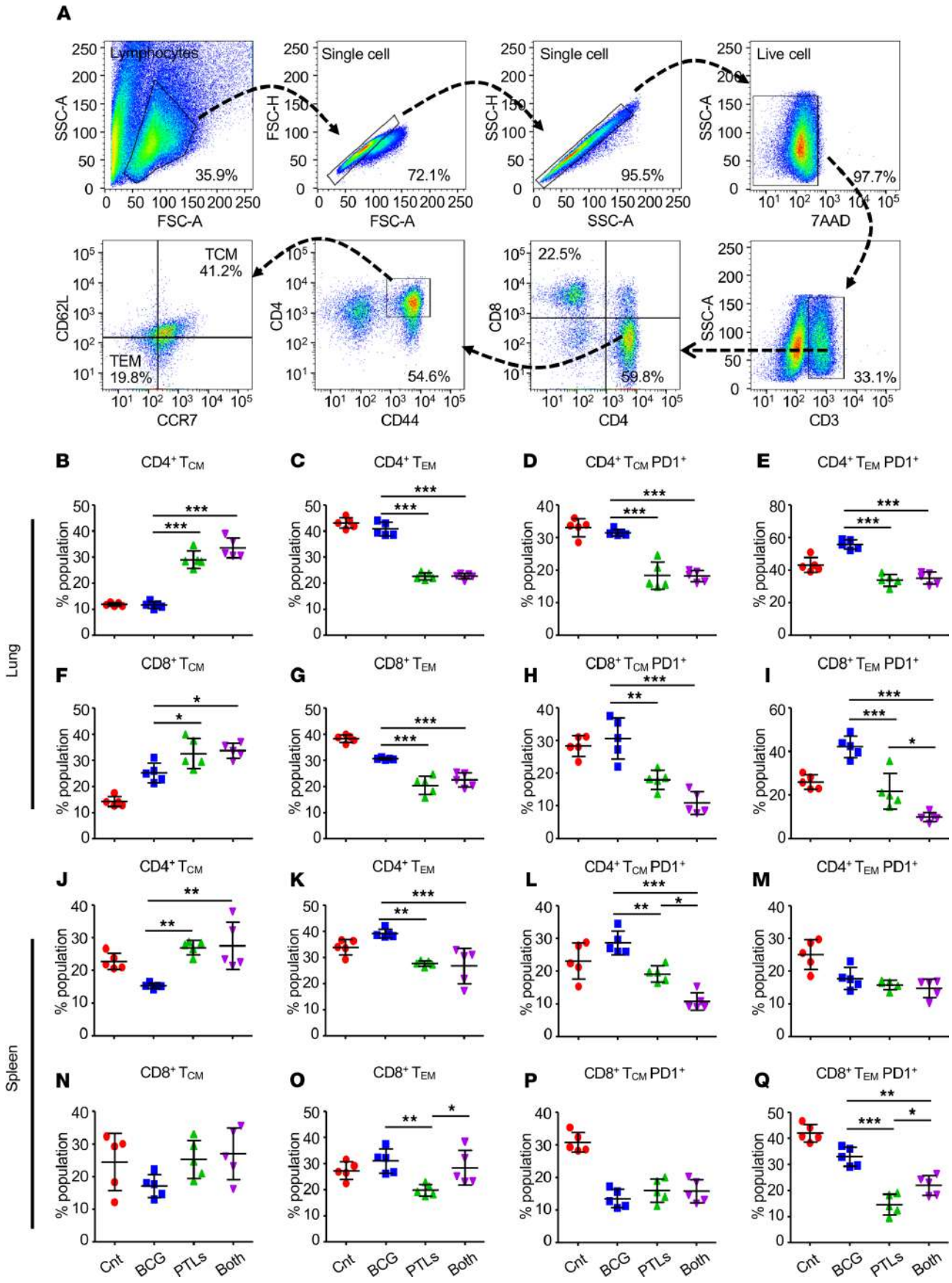
A
Lymphocytes
35.9%
SSC-A
FSC-A
Single cell
72.1%
FSC-H
FSC-A
Single cell
95.5%
SSC-H
SSC-A
Live cell
97.7%
SSC-A
7AAD
SSC-A
33.1%
CD3
22.5%
59.8%
CD8
CD4
54.6%
CD4
CD44
TCM
41.2%
TEM
19.8%
CD62L
CCR7
B CD4+ TCM
C CD4+ TEM
D CD4+ TCM PD1+
E CD4+ TEM PD1+
F CD8+ TCM
G CD8+ TEM
H CD8+ TCM PD1+
I CD8+ TEM PD1+
J CD4+ TCM
K CD4+ TEM
L CD4+ TCM PD1+
M CD4+ TEM PD1+
N CD8+ TCM
O CD8+ TEM
P CD8+ TCM PD1+
Q CD8+ TEM PD1+
% population
Lung
Spleen
Cnt
BCG
PTLs
Both

Figure 7. PTL induces superior antigen-specific T cell memory responses in the lungs and the spleens of infected mice. T lymphocytes isolated from the lungs and the spleens of the indicated groups of experimental mice at 50 days after 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. (**B** and **C**) Percentage of central memory (Tcm; $CCR7^{hi}CD62L^{hi}CD44^{hi}$) (**B**) and effector memory (Tem; $CCR7^{lo}CD62L^{lo}CD44^{hi}$) (**C**) $CD4^+$ T cells on lymphocytes isolated from the lungs of infected animals. (**D** and **E**) Frequency of PD-1 expression on central memory (**D**) and effector memory (**E**) $CD4^+$ T cell subset. (**F** and **G**) Percentage of Tcm ($CCR7^{hi}CD62L^{hi}CD44^{hi}$) (**F**) and Tem ($CCR7^{lo}CD62L^{lo}CD44^{hi}$) (**G**) $CD8^+$ T cells on lymphocytes isolated from the lungs of infected animals. (**H** and **I**) Frequency of PD-1 expression on these cell subsets. (**J**–**Q**) Frequency of central memory, effector memory, and PD-1 expression on these cell subsets on the lymphocytes isolated from the spleens of infected mice. One-way ANOVA, followed by multiple Tukey tests, was performed for statistical analysis. Data are representative of 2 independent experiments (n = 5 mice/group). $^*P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.0005$.

Conventionally *M.tb* produces an array of pathogen-associated molecular patterns (PAMPs), including lipoarabinomanan, phenolic glycolipids, phosphatidylinositol mannosidase, and other lipoproteins. These molecular patterns are recognized by 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 adapter molecule required for downstream signaling events by the majority of TLR/IL-1R family members, demonstrate enhanced susceptibility to aerosol infection with *M.tb* (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 proinflammatory cytokines, including IFN-γ, which confers protective immunity against TB. Therefore, we included PamCysSK-4 as the TLR2 agonist and CpG ODN as the 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, coimmunization of BCG and PTL 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 analyzed the expression of CD69 and CD25 as early and late T cell activation markers. However, $CD25^+$ T cells expressing Foxp3 represent Tregs 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 2 cytokines in the mice coimmunized with BCG and PTL (Figure 6). Moreover, our study also provides strong evidence in favor of the antigen-specific nature of these responses from adoptive transfer experiments, where T cells from coimmunized mice were able to impart protective immunity to congenic TB-unexposed naive mice upon *M.tb* infection (Figure 8).

Tcm cells, the perpetual source of Tem cells, dictate the recall responses and are considered indispensable toward 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 Tcm response (11). Interestingly, Tcm were found to be elevated during coimmunization of PTL, along with BCG (Figure 7). FOXO1 plays an important role in establishing long-lived T cell memory responses. BCG-PTL coimmunization 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 NF-κB activation in the coimmunized group corroborated with studies crediting NF-κB activation in inducing proinflammatory cytokine production during TB (Supplemental Figure 3) (44–46).

Transcriptome studies on Pep-DCs and stimulated T cells revealed that peptide pool induces differential expression of genes belonging majorly to the immune-relevant signaling pathways, such as JAK/STAT, TNF, TLR, NF-κB, MAPK, and TGF-β. The JAK/STAT pathway is well known to regulate T cell polarization, and deregulation of the 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). NF-κB has been shown to be critical for the expression of many proinflammatory cytokines required for the protection against TB (45, 52), since NF-κB–KO mice succumb to *M.tb* infection (45, 53). Our data also indicate that peptide pool induces MAPK signaling pathways that have a phenomenal role during TB (54). Dephosphorylation of MAPK, ERK, and P38 leads to increased susceptibility during TB (55). There are many reports suggesting

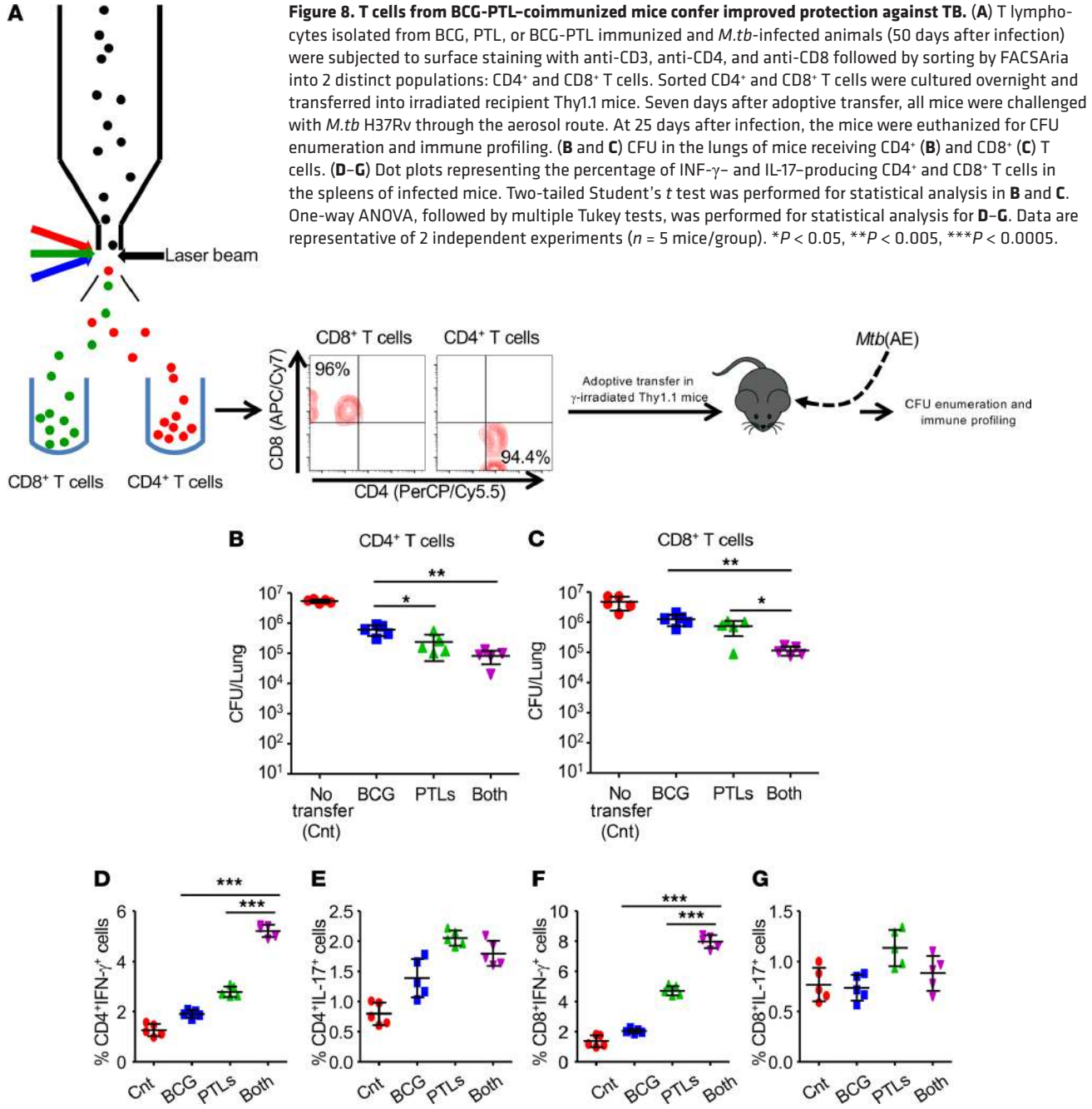

Figure 8. T cells from BCG-PTL-coimmunized mice confer improved protection against TB. (**A**) T lymphocytes isolated from BCG, PTL, or BCG-PTL immunized and *M.tb*-infected animals (50 days after infection) were subjected to surface staining with anti-CD3, anti-CD4, and anti-CD8 followed by sorting by FACSAria into 2 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 after infection, the mice were euthanized for CFU enumeration and immune profiling. (**B** and **C**) CFU in the lungs of mice receiving $CD4^+$ (**B**) and $CD8^+$ (**C**) T cells. (**D**–**G**) Dot plots representing the percentage of INF-γ– and IL-17–producing $CD4^+$ and $CD8^+$ T cells in the spleens 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 are representative of 2 independent experiments (n = 5 mice/group). $^*P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.0005$.

that the MAPK pathway is not only involved in many aspects of immune responses, from initiation of innate immunity to adaptive immunity, but also in 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 Tcm 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 similar to humans, such as nonhuman primates.

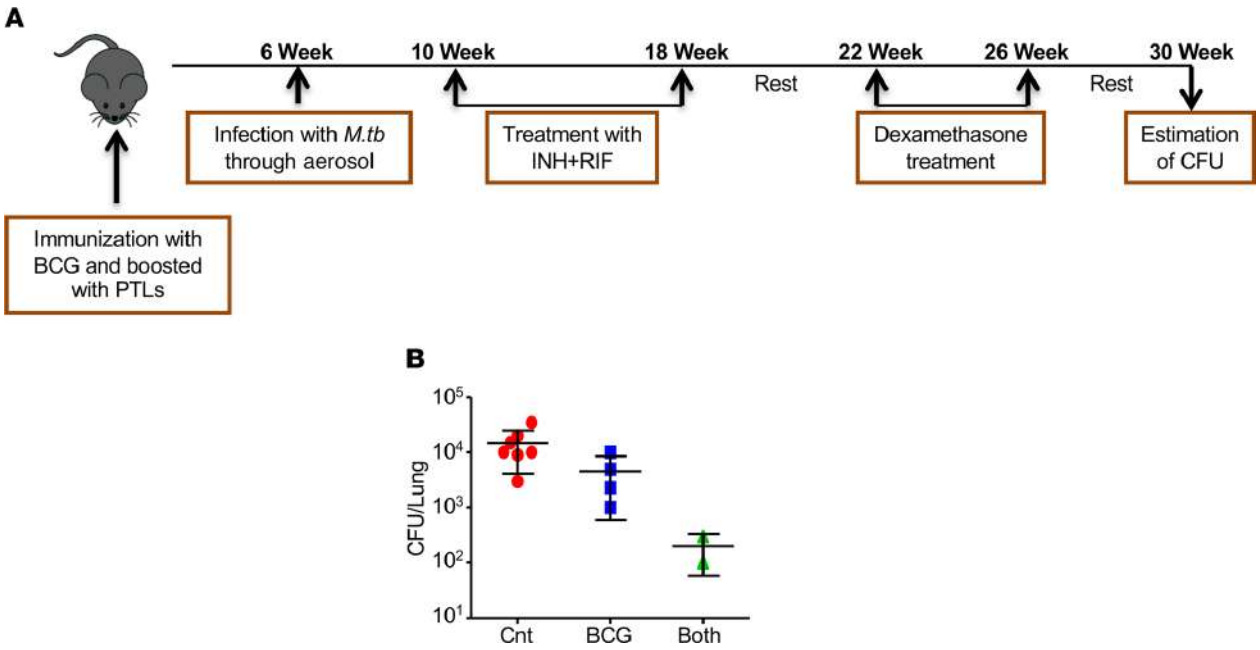

Figure 9. PTL immunization reduces the recurrence of DOTS-associated disease relapse. (**A**) Mice coimmunized with PTL and BCG were infected with H37Rv *M.tb*, followed by treatment with isoniazid and rifampicin for 16 weeks. After 30 days of rest, these mice were treated with dexamethasone for 30 days, followed by 1 more period of rest for 30 days. Mice were then sacrificed for CFU estimation to determine the rate of relapse after treatment. (**B**) CFU from the lung homogenates of the mice. The reactivation experiment was done once with 10 mice in each group.

Methods

Mice. All C57BL/6 mice (6–8 weeks of age) were maintained in the animal facility of the ICGEB and provided for experiments as and when required.

Generation of DCs. C57BL/6 mice were euthanized, and the femurs were isolated. BM was flushed out with RPMI 1640 medium using a 2.0 mL syringe (26.5 gauge). The cells were washed twice with PBS and then cultured in complete RPMI 1640 medium (Invitrogen) supplemented with GM-CSF (40 ng/mL) and IL-4 (10 ng/mL) on 12-well plates (1 million cells/mL). On the third day, 75% of the medium was replaced with fresh DC culture medium. On day fifth day, the suspended cells were removed, and the loosely adherent cells were collected as immature DCs ($CD11c^+$ cells were > 90%). For mature DCs, immature DCs were stimulated with LPS (1 μg/mL) for 24 hours. FACS analysis using anti-CD11c, -CD80, -CD86, and –MHC class II antibodies suggested that > 90% of the cells were conventional DCs. DCs were either left untreated or treated overnight with 20 μg/mL of CSA or 0.2 μg/mL of each peptide, followed by coculture with $CD3^+$ T cells isolated from *M.tb*-infected and DOTS-treated animals for 48 hours.

Human PBMC isolation. Blood samples collected from PPD^- and PPD^+ BCG–vaccinated healthy individuals were diluted in DPBS (Gibco, 14190250) at a ratio of 1:2 and layered onto Ficoll-Paque Plus (catalog GE17-1440-02) followed by centrifugation at 500*g* for 35 minutes at 25°C. Out of the 4 layers, the uppermost plasma was removed by pipette, and the second layer of the cells containing PBMCs was gently

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

Group	Reactivation rate[A]
Control	7 of 10
BCG immunized	4 of 9
BCG immunized and boosted with mimic	2 of 10

[A]Number of mice reactivated out of total number of mice in that group.

removed and suspended in complete DMEM (Invitrogen). These cells were then pelleted, counted, and seeded in the 12-well plates for further experiments.

M.tb infection of mice and estimation of CFU. *M.tb* H37Rv and BCG cultures were grown in 7H9 (Middlebrooks, Difco) medium supplemented with 10% OADC (oleic acid, albumin, dextrose, and catalase; Difco) and with 0.05% Tween 80 and 0.5% glycerol, and cultures were grown to mid-log phase. Aliquots of the cultures in 20% glycerol were preserved at –80°C, and these cryopreserved stocks were used for infections.

Mice were infected with H37Rv via the aerosol route using a Madison aerosol chamber (University of Wisconsin, Madison, Wisconsin, USA) with its nebulizer precalibrated to deposit around of 150 bacilli to the lungs of each mouse as previously described (8). Briefly, bacterial stocks were recovered from the freezer and quickly thawed and subjected to light ultrasonication to obtain a single cell suspension. A total of 15 mL of the bacterial cell suspension (10×10^6 cells per mL) was placed in the nebulizer of the Madison aerosol chamber precalibrated to deliver the desired number of CFUs to the lungs of animals placed inside the chamber, via the aerosol route. At day 1 after infection, 3 randomly selected mice were sacrificed, lungs were harvested and homogenized in 0.2 μm filtered PBS, and neat samples (without any dilution) were plated onto 7H11 Middlebrooks (Difco) plates containing 10% OADC (Difco). Neat, 10-fold diluted and 100-fold diluted lung, liver, and spleen cell homogenates were plated in triplicate on the 7H11 plates and incubated at 37°C for 21 to 28 days for the organs harvested at different time points. Colonies were counted, and CFU was calculated accordingly. Mice from various groups were euthanized at the indicated time points in various experiments; their organs were harvested for obtaining CFU counts and/or immune cell subpopulations for immunological studies as described under other subsections.

Antibiotic treatment. Thirty days after infection, groups of mice were treated with 10 mg/kg of RIF and 10 mg/kg of INH (MilliporeSigma) administered in the drinking water (changed daily) for 12 weeks. *M.tb*-infected control mice received plain drinking water.

Isolation of T cell lymphocytes from M.tb-infected animals. Lungs and spleens from infected animals were harvested and washed by swirling in PBS. They were opened up by cutting longitudinally and then cut into 0.5 cm pieces. These lung pieces were agitated in 25 mL of extraction buffer (PBS, 3% FCS, 1 mM dithiothreitol, 1 mM EDTA) for 30 minutes at 37°C. This slurry was passed through a loosely packed nylon wool column to remove the aggregates. The filtrate was layered on a discontinuous Percoll gradient (Amersham Pharmacia Biotech). This gradient was then centrifuged at 252*g* for 20 minutes at 25°C. Cells at the interface were collected and washed in staining buffer (PBS, 3% FCS). Spleen cells were homogenized and washed with RBC lysis buffer to remove RBCs. Cells from the lungs and spleens were cultured for surface and intracellular staining as described in the subsection.

Preparation of PTL. Lipid mixtures received from MilliporeSigma (catalog L4395) were used for the preparation of liposomes, which can encapsulate a broad spectrum of hydrophilic and amphipathic molecules of the low, medium, and high molecular weight (including peptides, proteins, and oligo- and polynucleotides). We mixed the *M.tb* peptides with lipid mixtures, along with TLR2 and TLR9 ligands Pam3Cys-SK-4 (catalog ALX-165-066-M002, Enzo Life Sciences) and CpG ODN (catalog ALX-746-003-C100, Enzo Life Sciences), respectively, as per manufacturer's protocol to get the homogeneous mixture of peptides and TLR ligands with liposomes (1 mL of PTL mixture contains 10 μg of each peptide, 100 μg of Pam3Cys-SK-4, and 10 μg of CpG ODN). Then, these mixtures were injected intranasally into the mice (50 μL per mice per dose). To confirm the successful delivery of the liposomes into the lungs, the liposomes were stained with PKH67 dye (MilliporeSigma) as per manufacturer's protocol. Sections of the lungs were seen under microscope for the fluorescence of the dye.

Immunization. Mice were immunized with (a) BCG (s.c.) (1×10^6 bacteria), (b) PTL (intranasal), (c) BCG (s.c.) + PTL (Intranasal), and (d) vector only. Mice were subsequently rested for 21 days and then challenged with *M.tb* strain H37Rv by the aerosol route. Organs like lungs, livers, and spleens were harvested for determination of bacterial burden and profiling of immune memory cell responses at different days after infection.

Histology. Lung tissues were fixed in formalin solution and coated with wax for sectioning. Sections were stained with H&E dyes, and slides were placed under a microscope. Granulomas were analyzed to obtain the granuloma score.

Flow cytometry. Spleens and lungs were isolated from respective mice and macerated by frosted slides in ice-cold RPMI 1640 (Invitrogen) containing 10% FBS to prepare a single cell suspension. RBCs were lysed with RBC cell lysis buffer, incubated at room temperature for 2–3 minutes, and washed with RPMI 1640

containing 10% FBS. The cells were counted, and 1×10^6 cells were used for surface staining. For intracellular staining 1×10^6, cells were cultured per well in 12-well plates (Tarsons) in the presence of H37Rv CSA overnight. Subsequently, 0.5 μg/mL Brefeldin A and 0.5 μg/mL of Monensin solution (BioLegend) were added during the last 4 hours of culture. Cells were then washed twice with FACS buffer (PBS + 3% FCS) and stained with antibodies directed against surface markers. After staining, cells were washed again with FACS buffer and fixed with 100 μL fixation buffer (BioLegend) for 30 minutes, washed, and resuspended in 200 μL permeabilization buffer (BioLegend) before being stained with fluorescently labeled anti-cytokine antibodies. FACS Verse BD was used for acquiring the cell population, and data analysis was done using Flow Jo (Tree Star Inc.).

Antibodies and reagents. We used the following BioLegend antibodies: anti-CD3 (clones 17A2 and HIT3a) Pacific-blue, FITC, PE, or APC; anti-CD4 (clones GK1.5 and A161A1) FITC, PE, PerCP-Cy5, PE/Cy7, or APC; anti-CD8 (clone 53-6.7) FITC, APC/Cy7, or APC; anti-CD44 (clone IM7) FITC; anti-CD62L (clone MEL-14) APC; anti-CD25 (clone 3C7) APC; anti-CD69 (clones H1.2F3 and FN50) FITC or PE; anti-CD197 or -CCR7 (clone 4B12) PE/Cy7; anti–IFN-γ (clones XMG1.2 and 4S.B3) APC or PE; anti–IL-12 (clone C15.6) PerCP/Cy5.5; anti–IL-17 (clone TC11-18H10.1) PE-Cy-7; anti–IL-2 (clone JES6-5H4) FITC; anti–TNF-α (clone MP6-XT22) PE or PerCP/Cy5.5; and anti–TGF-β (clone TW7-16B4) APC. Brefeldin A Solution (1000×), Monensin Solution (1000×) (catalog 420701), and Intracellular Staining Permeabilization Wash Buffer (10×) were purchased from BioLegend.

T cell adoptive transfer. For adoptive transfer experiments, T cells from lungs of the infected mice that belonged to 3 groups — (a) BCG immunized, (b) PTL immunized, and (c) BCG-PTL–coimmunized — were isolated and sorted into $CD4^+$ and $CD8^+$ T cells. $CD4^+$ (1.0×10^6 cells/mouse) and $CD8^+$ T cells (1.0×10^6 cells/mouse) were adoptively transferred into γ-irradiated Thy1.1 mice. Recipient mice were challenged with H37Rv through the aerosol route for the determination of bacterial burden and immune responses in the lungs of respective mice.

Mouse model of TB reactivation. Mice infected with *M.tb,* with low-dose aerosol infection model, were treated with 10 mg/kg of INH and RIF administered ad libitum (in the drinking water) or treated to mice coimmunized with BCG and PTL or immunized with BCG alone for 12 weeks starting at the fourth week after infection. These mice were then rested for 30 days, followed by treatment with dexamethasone (5 mg/kg, i.p.) 3 times per week for 30 days. These mice were again rested for next 30 days. Ten mice from each group were then sacrificed, and CFUs were estimated from lung homogenates to determine the reactivation rate of *M.tb*.

RNA-Seq. Total RNA was isolated from Un-DCs and DCs pulsed with peptide pool (Pep-DC), as well as from unstimulated cocultured T cells (Un-TCs) and cocultured T cells (Pep-TCs), along with DCs pulsed with peptide pool using an RNeasy RNA isolation kit (Qiagen). The raw reads generated for Un-DCs and Pep-DCs and unstimulated cocultured T cells and Pep-TCs were subjected to quality check using FastQC (version 0.11.5, http://www.bioinformatics.babraham.ac.uk/projects/fastqc/), using parameters like base quality score distribution, sequence quality score distribution, average base content per read, and GC distribution in the reads. Illumina Adapter (AGATCGGAAGAGC) was removed using Trim Galore (version 0.4.1), a wrapper script to automate quality and adapter trimming, as well as quality control. Clean reads were mapped on reference Mus-musculus_GRCm38.p6 using TopHat v2.1.0. Differential analysis was performed on counted mapped reads in a range of positions on a chromosome for Un-DC versus Pep-DC and for Un-TC versus Pep-TC combinations, predicted using htseq-count software; then, differential gene expression was found using DeSeq, a Bioconductor R package that estimates variance-mean dependence in count data and implements a range of statistical methodology based on the negative binomial distributions. Functional annotation for combination was performed using UniProt database and DAVID. The transcriptome data are available on Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE164258; accession no. GSE164258).

Western blot. Spleens were harvested from all experimental groups, homogenized, and made up the cell lysate. Whole cell lysate was prepared by using lysis buffer (50 mM Tris-HCl [pH 7.4], 5 mM EDTA, 120 mM NaCl, 0.5% Nonidet P-40, 0.5 mM NaF, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride) along with HALT phosphatase inhibitor mixture (78420, Thermo Fisher Scientific) and protease inhibitor mixture (78410, Thermo Fisher Scientific) for 1 hour. Samples were electrophoresed on a 10% SDS-polyacrylamide gel and electroblotted onto polyvinylidene difluoride membranes. Blots were

blocked for 1 hour in 5% BSA in PBS with 0.1% Tween 20. NF-κB, pNF-κB, FOXO1, and pFOXO1 proteins were detected with NF-κB (8242S), pNF-κB (3033S), FOXO1 (2880S), and pFOXO1 (9461S) monoclonal antibodies, respectively, at a dilution of 1:250 and as recommended by the manufacturer (Cell Signaling Technology). Goat anti–rabbit IgG G-conjugated horseradish peroxidase (sc-2004) (diluted 1:5000) was used as a secondary antibody (Santa Cruz Biotechnology Inc.). Immunoblotting for β-actin was carried out to confirm equal loading.

Statistics. Statistical analyses were conducted using GraphPad Prism software by performing 2-tailed Student's *t* test or 1-way ANOVA, followed by multiple Tukey tests. $P < 0.05$ was accepted as an indication of statistical significance. $^{*}P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.0005$.

Study approval. Animal experiments were performed as per ethical guidelines approved by the Institutional Animal Ethics Committee held in July 2015 at the ICGEB (New Delhi, India) and the Department of Biotechnology guidelines (Government of India) (approval ID, ICGEB/AH/2015/01/IMM-45). All mice used for experiments were ethically sacrificed by asphyxiation in carbon dioxide according to the institutional and Department of Biotechnology (DBT), Government of India, regulations. The human studies were ethically approved (approval ID, 359/SHRMU/19/2010/07) by the Institutional Human Ethics Committee, Jawaharlal Nehru University, New Delhi, India, and Regional Medical Research Centre (RMRC), Odisha, India.

Author contributions

SK and VPD initially started the study. SK, AB, CS, SRK, DKS, SC, and VPD performed in vitro experiments and analyzed data. SK, AB, and VPD performed all animal experiments. SRK also assisted in animal experiments. GP performed experiments with human PBMCs. VPD conceived the hypothesis and supervised the experiments. AB and VPD designed experiments and analyzed the data. GD provided the resources and edited the manuscript. AB and VPD wrote the manuscript.

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REVIEW
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Progressive Host-Directed Strategies to Potentiate BCG Vaccination Against Tuberculosis

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The pursuit to improve the TB control program comprising one approved vaccine, *M. bovis* Bacille Calmette-Guerin (BCG) has directed researchers to explore progressive approaches to halt the eternal TB pandemic. *Mycobacterium tuberculosis* (*M.tb*) was first identified as the causative agent of TB in 1882 by Dr. Robert Koch. However, TB has plagued living beings since ancient times and continues to endure as an eternal scourge ravaging even with existing chemoprophylaxis and preventive therapy. We have scientifically come a long way since then, but despite accessibility to the standard antimycobacterial antibiotics and prophylactic vaccine, almost one-fourth of humankind is infected latently with *M.tb*. Existing therapeutics fail to control TB, due to the upsurge of drug-resistant strains and increasing incidents of co-infections in immune-compromised individuals. Unresponsiveness to established antibiotics leaves patients with no therapeutic possibilities. Hence the search for an efficacious TB immunization strategy is a global health priority. Researchers are paving the course for efficient vaccination strategies with the radically advanced operation of core principles of protective immune responses against *M.tb*. In this review; we have reassessed the progression of the TB vaccination program comprising BCG immunization in children and potential stratagems to reinforce BCG-induced protection in adults.

Keywords: adjunct vaccination strategies against tuberculosis, vaccine, BCG, host directed therapy, immunotherapy, memory T cells

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INTRODUCTION

Tuberculosis (TB) is caused by the facultative intracellular pathogen *Mycobacterium tuberculosis* (*M.tb*). Since the 1800s, TB was the leading cause of health menace worldwide. Despite being declared a global health emergency in 1993 by World Health Organization (WHO), TB continues to be the leading cause of morbidity and mortality amongst bacterial infections (1). The majority of individuals remain asymptomatically and latently infected with *M.tb* owing to confiscation of the pathogen by immune cell populations and this does not lead to disease. Upon serious immunosuppression, around 10% of latently infected individuals develop active TB. Owing to the indefinability of the disease, explosive TB epidemics are hardly encountered which results in underestimated harm caused by *M.tb* worldwide (2). The host immune responses can restrict the pathogen but fail to accomplish complete bacterial sterility. The overwhelming progression of the

development of new therapeutics and the emergence of resistant pathogenic strains can be prevented by the enhancement of population-wide immunity against *M.tb* (3).

M.tb was originally identified in 1882 by Dr. Robert Koch as the causative agent of TB however, it has lurked among living beings since ancient times. 139 years post-discovery of this pathogen, it still endures as an eternal scourge ravaging globally. Current strategies fail to control TB, due to the upsurge of multidrug-resistant strains, increasing incidents of co-infections in immune-compromised individuals, and the emergence of TB-IRIS (Immune Responsive Inflammatory Syndrome). Globally in 2020, approximately 10 million people were disease-ridden with TB and an aggregate of 1.3 million lives were lost (together with 208000 people with HIV). Furthermore, almost one-fourth of humankind is infected asymptomatically (latently) with *M.tb*, with a 5-15% risk of progressing into clinical manifestations (1). An effective vaccine is indispensable to enhance population-wide immune protection and reduce disease burden. Vaccines operate by stimulating a cascade of immunological responses and ensuing the institution of immune memory against subsequent infections (4). Immune memory was originally described by the Greek historian Thucydides while observing survivors of the plague of Athens and comprehended that survivors have conferred life-long resistance to disease. Hence, he stated that "this disease never took any man the second time" (5). This feature of the immune system is a requisite evolutionary trait. Since historic eras for infectious diseases like smallpox, it is distinctly demonstrated that while initial infections had a fatality rate of 20% to 60% subsequently affected individuals were eternally immune to infection. Edward Jenner was the first to employ this hallmark feature of immunity to treat smallpox and provided a foundation for the development of vaccines (6). Secondary immune responses are refined protective responses mounted by sub-populations of memory cells on subsequent encounters which impart endurance to combat recurrent infections caused by pathogens in the environment. A series of events following primary exposure establishes a pool of long-lasting antigen-specific immune cells that mount quantitatively and/or qualitatively improved immune response upon reinfection. Documented from the times of ancient Greeks but still, many components of immune memory are still debatable (5). Immune memory is the cardinal property of the adaptive immune system and exclusively lymphocytes were known to mediate these responses. However, organisms that lack T and B lymphocytes similarly possess heightened proficiency to combat recurring infections caused by the same pathogen, demonstrating the existence of innate immune memory (7). Numerous studies in simpler living beings have reported that cells of the innate immune system can mount heightened secondary responses upon reinfection. Thus, the conventional characterization of immunological memory is continuously advancing (8). Better insights into the generation of immunological memory are fundamental to foster progressive vaccination strategies.

Despite pre-exposure vaccination with BCG, a large extent of latent TB infections urges the need for an efficacious complementary TB control strategy. It is evident that the most extensively used vaccine, Bacille Calmette-Guerin (BCG) which has existed for 100 years fails to impart long-term immunity and has limited efficacy against adult pulmonary TB (9). BCG immunization has a limited impact on *M.tb* transmission since it cannot inhibit primary infection or recrudescence of latent TB (10). Failure to develop a significantly effective vaccine has constrained the phasedown of the global TB burden (9). Furthermore, safety concerns regarding BCG immunization in immune-compromised individuals including HIV-TB coinfected individuals necessitate a vaccine that is safer and more efficient than BCG and can ameliorate infections (11). The efficacy of BCG against pulmonary TB is even more disappointing in tropical regions with a high TB burden (12). Escalating TB cases worldwide despite BCG administration further demands the advancement of the existing vaccination strategy. In the past decade, various research groups have utilized pioneering technologies to improve the current scenario. Progressive vaccine design strategies have been implemented and vaccine candidates have been evaluated in different clinical trials (13). It is challenging to discover more efficacious vaccine candidates for TB that can substitute BCG. It is highly critical to comprehend the shortcomings and prospects of novel vaccination strategies for better implementation and amendment of TB control measures. **Table 1** summarizes key desirable characteristics of improved vaccination strategy. In the current scenario, COVID-19 and TB are the top two causes of death from contagious diseases (1). The similarity in symptoms, risk factors, and primary organ affected further exacerbates the circumstances. TB-COVID-19 co-infection certainly intensifies the severity and threat of death. Even though both diseases primarily affect the lungs, due to disparities in modes of transmission and pathogenesis, distinct therapeutic measures are required (14). Nevertheless, with extraordinary scientific endeavour, information regarding the pathogenesis of SARS-CoV-2 was channelled to develop diagnostics, therapeutics, and vaccines

TABLE 1 | Desired characteristics for novel Tuberculosis immunization approaches.

S. No	Desired characteristics
1.	Safe to be administered in immunocompromised individuals at risk of developing active TB
2.	Expenses associated with regimen and dosage should be reasonable for high burdened developing countries.
3.	Immunization strategy must lower the risk of developing active pulmonary TB in adults previously vaccinated with BCG
4.	Must protect against *M.tb* infections for more than 10 years subsequent to immunization
5.	Minimum administrations requisite to elicit host protective responses
6.	Evaluation of protective immune correlates by employing established assays
7.	Must offer greater than 50% protective efficacy against established pulmonary TB

for COVID-19. Further, years of implementation of TB control programs were utilized to implement control strategies to constrain the pandemic (15). This prompts the necessity to retrospect and harnesses the paramount knowledge for progressive solutions to counteract the syndemic of COVID-19 and TB. Lessons learned from BCG vaccination for TB have been operative to control the COVID-19 pandemic owing to the broad-spectrum immunomodulatory potential of BCG (16). The emergence of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) and the consequent COVID-19 pandemic has negatively influenced the years of progress in tuberculosis (TB) control (17). Advancement in the direction to put an end to TB was hit hard by the ongoing COVID-19 pandemic. Reports of the World Health Organization (WHO) corroborate that advent of COVID-19 has caused a diminution in TB diagnosis and escan alation in mortality. To augment protective immune responses against TB in adults, massive scientific and economic attempts have been made globally. Aspiration to attain complete bacterial sterility to counteract active TB and restrict the transmission with next-generation vaccines has been actively trailed. Even with several vaccine candidates in different phases of clinical trials, we are yet to uncover vaccination strategies that can efficaciously restrict escalating TB burden worldwide (17). Hence, in this review, we have discussed strategies to augment the existing vaccination approach. Immune responses induced by BCG vaccination have been studied comprehensively in past and we are still uncovering new information regarding host responses stimulated by BCG that impede the establishment of effective memory responses (18). To improve the immunotherapeutic efficacy of BCG it is vital to completely understand the mechanism of BCG-induced immune responses. Modulation of host immunity *via* immunomodulators along with vaccination can be employed as a stratagem to incline immune responses to attain ever-lasting immunity against *M.tb* infections. We will reassess the radical approaches utilized by the researchers to limit the prevailing TB cases and how a better understanding of BCG is prime for progress in the TB vaccination program.

EXPEDITION FROM VIRULENT *M. BOVIS* TO THE BCG VACCINE:

Albert Calmette and Camille Guérin commenced the pursuit to develop a vaccine against TB in 1900 at the Pasteur Institute (19). They began by cultivating a virulent bovine strain of bacillus which was isolated from a tuberculous cow by Nocard. Initially, the bacilli were grown on glycerine, potato medium supplemented with ox bile to limit the clumping and attain homogenous bacterial suspension. This effort to minimize bacterial clumping additionally lowered the virulence of pathogen upon sub-culturing. This scientific observation provoked the scientists further to focus on using the attenuated strain of bacilli for the generation of TB vaccine (20). Till 1919, they successfully sub-cultured the bacilli more than 230 times. This strain failed to infect and cause TB in animals such as guinea pigs, cattle, and rabbits. Firstly named "Bacille Bilie Calmette-Guerin" this is now the most widely administered vaccine worldwide Bacille Calmette-Guerin (BCG) (20). BCG was first utilized in 1921 to immunize a new-born *via* oral route after which it was mass vaccinated to protect infants from disseminated forms of TB. To justify the escalating demand for vaccine strain worldwide, several laboratories around the world began sub-culturing BCG owing to which individuals around the world are vaccinated with characteristically distinct BCG strains (21). Based on existing knowledge, it is evident that diverse BCG strains have variable efficaciousness (22) and immunogenicity (23) but the most efficient BCG strain is yet to be established (23). As little as 1% augmented efficaciousness can rescue around 18,000 individuals and limit 83,000 TB cases in a year (24). Hence, to better understand the protective co-relates of BCG vaccination should be the top priority to upgrade the vaccination strategy.

BCG INDUCED PROTECTION AGAINST TB

Since the launch of BCG in TB immunization programs, numerous lives have been saved owing to the only existent TB vaccine (19). The percentage decline in disease that can be attributed to vaccination outlines the clinical effectiveness of a vaccine. Mainly, the BCG vaccine is administered to protect against TB. However, the protective efficacy of BCG is assessed distinctively in the case of disseminated TB in children and adult pulmonary TB on the account of colossal discrepancy (25). One of the multifaceted explanations can be the intricate biology of TB establishment and progression in humans (26). In the majority of *M.tb* infections, the immune system can proficiently restrict the progression of the pathogen to cause active TB. However, the endeavour to eliminate the bacilli completely is rarely achieved and can culminate into escalated inflammatory responses that direct distinct phases of disease such as latent TB and associated immunopathogenesis (27). The aim is to strike a balance for the resolution of *M.tb* infection exclusive of detrimental inflammation. For more than a century, researchers have attempted to ascertain correlates of BCG-induced defenses in humans through various animal models (10) and clinical trials (28). Additional to *M.tb* infections, broad-spectrum immunomodulatory characteristics of BCG are utilized to treat bladder cancer (29), asthma (30), leishmaniasis (31) and warts (32). While our understanding of key immunological aspects is continuously expanding (33), we have attained substantial knowledge regarding innate and adaptive immune responses to *M.tb* infection and BCG immunization which is briefly depicted in **Figure 1** and will be reviewed thoroughly in this section.

Innate immune system function as the first line of defense in confronting *M.tb* infections (34). The innate immune responses are the key component of the host immune responses engaged promptly at the site of infection (35). Even in the course of intradermal BCG immunization, early immune responses are elicited by resident epidermal macrophages (36), neutrophils

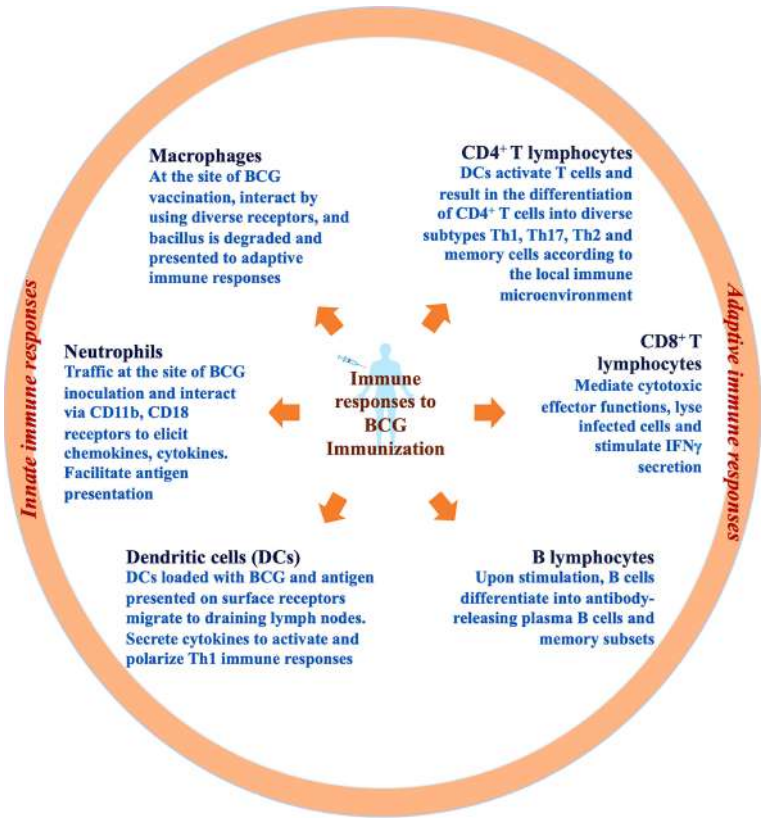

FIGURE 1 | Immune responses to BCG immunization. Immune responses to the BCG vaccine initiate at the site of inoculation by induction of innate immune cells such as resident macrophages, neutrophils, and dendritic cells. Innate immune cells internalize, degrade and present antigen of bacilli *via* surface receptors to further activate adaptive immune cells. Chiefly, DCs loaded with bacilli drain to the lymph nodes and result in lymphocyte stimulation and activation. T and B lymphocytes further differentiate into diverse subtypes including effector and memory cells.

(37), and dendritic cells (DCs) (38). BCG comprises pathogen-associated molecular patterns (PAMPs) such as cellular components (mycolic acids, peptidoglycans, and arabinogalactans) which are recognized by diverse PAMP recognizing receptors (PRRs) present on the surface of innate immune cells. Diverse PRRs abundantly expressed on innate subsets such as complement receptor 3 (CR3) (39), TLR2/4/9 (40), mannose receptor (41), Ca^{2+}-dependent lectin (MINCLE) receptors on macrophages (42), nucleotide-binding oligomerization domain (NOD)-like receptors NOD2 on monocytes, CD18, FcγRII, and FcγRIII on neutrophils and DC-SIGN, CD11c, and CD205 on dendritic cells initiate the prompt innate immune responses upon BCG immunization (43). Disparities in the cellular composition of BCG and *M.tb* has been linked with recognition by different PRRs which further influences the uptake, processing and representation of antigens to other immune cells. Since the receptor involved determines the fate of downstream signaling, the variation in receptor utilization can be further associated with relatively inefficient immune responses in the case of BCG (44). Examination of skin biopsies demonstrated that BCG blister point majorly comprises $CD15^+$ neutrophils, a small proportion of $CD14^+$ monocytes, and an infinitesimal population of $CD3^+$ T lymphocytes (45). However, in whole blood culture experiments, $CD56^+$ NK cells, γδ T cells, NKT cells, and cells from MAIT were found to be associated with BCG-induced immunity (46). In response to BCG vaccination, innate immune responses such as ROS/RNI generation by neutrophils, the release of monocytic chemokines like IL-6, TNF-α, MIP-1α, MIP-1β, IL-8, and IL-1α within 1-3 hours is initiated to direct systemic immune responses (45). BCG is known to effectively activate monocytic populations (47). In animal models, subsequent to BCG immunization mycobacterial extermination by macrophages was demonstrated independent of adaptive immune responses (48). Deficit immune responses induced by macrophages subsequent to BCG vaccination downgrade bacterial clearance. Furthermore, guinea pigs immunized with BCG upon H37Rv infection demonstrated enhancement of phagosome-lysosomal fusion with a considerable reduction in mycobacterial burden (47).

Dendritic cells (DCs) function as a nexus between innate and adaptive immune responses by presenting processed antigens to T lymphocytes post BCG immunization *via* IL-1R, MyD88 pathway (49). BCG immunization is known to enhance DC maturation and activation by upregulating the expression of markers implicated in antigen presentation such as MHC-II, CD40, CD80, and CD86 (35). Nonetheless, BCG immunization is also linked with the stimulation

of IL-10 and IL-4 cytokines by DCs which can bias the differentiation of T lymphocytes toward the T_H2 subtype and can be the cause of weakened BCG effectiveness (49). Nonetheless, the majority of information concerning the role of DCs in the case of BCG inoculation is from *in vitro* studies. The observation that *in vitro* BCG stimulation, initiates aggregation of DCs, upregulates antigen presentation with reduced endocytosis, and stimulation of TNF-α infers that DCs contribute to the initiation of immune responses. However, it is concerning that compared to *M.tb* infection these responses are inadequate to impart requisite protection (50). Apart from the major innate immune cell populations, innate lymphoid cells (ILCs) and mucosal-associated invariant T cells (MAIT) have been connected with BCG-induced innate protection (36). However, fragmentary information is accessible regarding these subsets and further exploration is necessitated.

It is now well-known that analogous to antigen-specific responses elicited by adaptive immunity, subsequent to pathogenic insults cells of innate immunity elicit heterologous memory responses (7). Distinct reports have demonstrated that natural killer (NK) cells and macrophages which have formerly encountered pathogens through epigenetic remodeling are trained to respond to distinct pathogens (51). It has been observed that epigenetic modifications such as H3K4me1, H3K4me3, and H3K27ac have been associated with the reprogramming of monocytic populations owing to unfastening of chromatin positions at the promoters of pro-inflammatory cytokines (52). BCG immunization leads to the expansion of Hematopoietic stem cells (HSCs), drives myelopoiesis and *via* epigenetic reprograming enhances host protective immune responses (53). Furthermore, it is established that macrophages interact with NK cells and bring about the refinement of innate immune responses against pathogenic insults (54). With a deeper insight into trained immunity, it was observed that BCG vaccination in healthy individuals stimulates NK cells and macrophages to uphold cytokine generation in response to *ex vivo* stimulation (55). The broad-spectrum immunomodulatory potential of BCG has been employed for the treatment of diverse ailments including the COVID-19 disease caused by SARS-CoV-2 (56). It has been experimentally proved that BCG *via* epigenetic reprogramming of immune cells exhibits cross-protection against diverse pathogens (57). The research demonstrates the impact of BCG vaccination on the induction of genome-wide histone modifications in trained monocytes which participate in IL-1β generation and the reduction of the yellow fever virus (YFV) burden (58). BCG-induced protection against YFV infection substantiates the broad-spectrum effectiveness of the vaccine against diverse viral infections such as influenza A (H1N1) virus, herpes simplex virus (HSV), and human papillomavirus (HPV) (59). Based on this information, BCG vaccination was evaluated initially during the COVID-19 pandemic. Preliminary ecological studies demonstrated that COVID-19 cases and deaths per population were fewer in countries with BCG vaccination schedules (60). The notion of inducing anti-viral immunity by employing BCG was based on the generation of heterologous immune responses (61). Since it was found that the envelope protein of the SARS-CoV-2 virus shared certain homology with strains of *Mycobacterium* species (62). It was inferred that the homology was associated with the induction of host-protective T_h1/T_h17 responses. The concept of trained immunity and heterologous responses were utilized to exploit BCG vaccination in the era of COVID-19. Since the majority of individuals are already vaccinated with BCG, it is judicious to keep BCG in reflection while developing new vaccination strategies. The fight to halt TB must continue progressively while dealing with the ongoing COVID-19 pandemic (17).

Till now adequate information is established regarding protective correlates against TB. Adaptive immune responses play a vital role in eliciting pathogen-specific immune responses with superior efficacy (26). The protective role of T lymphocytes was primarily demonstrated by the adoptive transfer of $CD4^+$ and $CD8^+$ T cells from BCG immunized mice to T and B cell-deficient (Rag1-/-) knockout mice (63). T lymphocytes contribute significantly against *M.tb* infections upon activation by components of innate immunity. The induction of T_h1/T_h17 immune responses and IFN-γ secretion is positively linked with augmented clinical outcomes in TB patients (26). Several studies have demonstrated mechanistic insights of BCG-induced defenses as a consequence of T_h1 cells through IFN-γ secretion (64). The paramount contribution of T_h1 responses was also demonstrated in infants vaccinated with BCG wherein T_h1 responses prevailed for more than a year contrary to pronounced T_h2 responses in unvaccinated infants (65). Furthermore, for the next few years, BCG-induced protection was attributable to IFN-γ releasing T lymphocytes (65). However, the outcomes of BCG immunization are still disputable and not strongly concurrent with specific immune responses. In IFN-γ deficient mice, BCG immunization demonstrated considerable protection against *M.tb* infection that vanished after depletion of $CD4^+$ T lymphocytes (66). Comparable outcomes were achieved in a study involving humans vaccinated with BCG wherein restricted *M.tb* progression and protection were linked with IFN-γ independent $CD8^+$ immunological responses (67). It is established now that polyfunctional $CD4^+$ T lymphocytes play a vital role in enhancing defenses against *M.tb* by secreting cytokines in different combinations to amend the microenvironment at the site of infection (68). It was confirmed that BCG immunization in infants does not elicit polyfunctional immune responses linked with effective protection against *M.tb* (12). However, immunization with a booster dose of MVA85A (modified vaccinia virus Ankara expressing antigen 85A) in BCG vaccinated adults confirmed induction of polyfunctional T cell responses (69). This gave rise to the hypothesis of heterologous boosting of BCG immunization for robust protective immunity against *M.tb*. Thereafter, failure of MVA85A heterologous boosting in infants to induce efficacious immune responses even with induction of polyfunctional T responses blurred the resolution of protective efficacy (70). Similarly, $CD8^+$ T cells exhibit antimycobacterial activity and are directly involved in *M.tb* killing, cytotoxic extermination of infected cell populations, and IFN-γ secretion (71). In human samples, $CD8^+$ T cells have been shown to distinctively identify and kill infected macrophages along with internalized *M.tb* with granular discharge comprising perforin and

granulysin (63). Therefore, subsiding the bacterial burden and effectively enhancing defenses against *M.tb*. The vitality of MHC class I-restricted $CD8^+$ T cells was demonstrated in β2-microglobulin (β2m) deficient mice incapable of restricting *M.tb* infection (72). It is not feasible to achieve sterilizing immunity against reinfections with mycobacteria subsequent to pathogen clearance with antimycobacterial drugs. Several studies have emphasized the significance of IL-17 generating $CD4^+$ T lymphocytes in mediating protection from reinfections and improving clinical outcomes upon vaccination (73). Consistent with these reports, in BCG immunized mice, T_h1 responses in the lungs were shown to be reliant on IL-17A and IL-23 secreted by antigen-specific T_h17 cells (73). In non-human primates (NHPs) administering a high dose of intradermal or intravenous BCG was linked with augmented protection from *M.tb* as a consequence of $CD4^+$ T lymphocytes with T_h1/T_h17 phenotypic characteristics (74). Thus, augmenting T_h1/T_h17 responses induced by BCG offer prospective solutions against TB (74). BCG immunization has been linked with the enhancement of regulatory T cells (T_{regs}) *via* alteration of immune metabolic pathways which consequently reduces the protective efficacy against TB. Depletion of T_{regs} can result in enrichment of T_h1, cytotoxic T cell responses with enhanced bacterial extermination upon infection (75). Boosting BCG with a novel vaccine candidate comprising Ag85B-Mpt64 (76–84)-Mtb8.4 (AMM) along with composite adjuvant lowers the T_{reg} population which was associated with enhanced protection in the mice model (85). Nonetheless, few studies have also demonstrated unaltered outcomes in BCG immunization upon prior T_{regs} depletion (86). Hence, further analysis is necessitated to validate the prospects for utilization in clinical operation. Regardless, the potential of the BCG vaccine to enrich T_{regs} and inhibit detrimental inflammation has been employed to treat diverse disorders including SARS-CoV-2 infection-induced cytokine storm in the COVID-19 pandemic (87). Additionally, BCG administration has been linked with increased IL-10 secretion in animal models which consequently restricts anti-mycobacterial pro-inflammatory responses induced by vaccination (88). Furthermore, obstruction of IL-10 signal transduction in the course of BCG immunization improved protective immune responses by mounting T_h1, and T_h17 responses (89). Hence, striking the immunological balance to achieve affirmative outcomes is requisite to alleviate BCG-induced protection.

APPROACHES TO AMEND INADEQUACIES OF BCG VACCINE

Despite numerous limitations of the BCG vaccine, it is still challenging to stumble upon more effective vaccine candidates for TB (90). BCG is unquestionably the most reliant vaccine for the prevention of disseminated forms of TB in children (91). Hence, it is critical to comprehend the shortcomings of BCG to extemporize protection by progressive approaches. It is widely proclaimed that wearying BCG immunity is a consequence of the non-existent T cell epitopes of *M.tb* in BCG vaccine strains (92). It is also established that expansion and differentiation of effector T cells declines in age-dependent manner upon BCG inoculation inferring toward weakened central memory responses (93). The contracted pool of antigen-specific memory populations is the basis for short-term protection against *M.tb* infections (93). Furthermore, the variable efficaciousness of BCG-induced defenses is associated with multifold factors such as ecological aspects, genetics, and differences in nutritional profiles amongst populations, listed in **Figure 2**. A major rationale for highly variable BCG efficacy in adults is exposure to environmental non-tuberculous mycobacteria (NTM). Prevalence of NTMs in tropical regions has been linked with low efficacy of BCG and consequent high TB burden due to pre-immunization exposure induced variation in protective efficacy (94). In the regions nearby the equator, UV exposure has been connected with a reduction in BCG efficacy which was further demonstrated in animal models with alterations in cytokine profiles when exposed to UV at the time of BCG immunization (60). Furthermore, variations in handling protocols and numerous passages of BCG vaccine strains have given rise to alterability in immunogenicity of BCG vaccine strains worldwide (95). Despite the wavering protective efficacy of BCG vaccine is deemed to be safe and is administered worldwide is numerous vaccination programs. However, with the raising concerns in immune-compromised HIV-TB co-infected individuals, WHO has addressed disputes regarding the utility of live vaccine in diverse risk groups. In immune-deficient children seldomly BCG vaccination can lead to systemic BCGosis. Atypical adverse reactions were detected in children with chronic granulomatous disease, Di George syndrome and severe combined immune deficiency (SCID) which can result in deadly consequences if unmanaged (96). In immunocompromised individuals especially neonates vaccination can also result in BCG lymphadenitis and disseminated BCG infection which is one of the most detrimental consequence of BCG vaccination (97). Hence, due to the overabundance of factors contributing to undermining protection elicited by BCG progressive approaches have been employed to improvise BCG against *M.tb*.

REPLACING OR RECLAIMING BCG

The major TB burden worldwide accountable for morbidity and mortality is due to adult pulmonary TB cases (1). The interval of weakening of BCG-induced defenses overlaps with an escalated incidence of *M.tb* infections in adults. On the surface foremost justification for the incompetence of the BCG vaccine appears to be immunization in the early years of life which imparts limited protection (90). The prospects of the End TB Strategy hence seem bleak without an improvised vaccination stratagem. Since BCG is the most widely utilized vaccine in the world and imparts protection in infants against disseminated forms of TB, it is judicious to reclaim BCG-induced protection rather than displacing it with a replacement. Fundamental strategy to amend BCG efficacy is by utilizing prime boost vaccination approach (98). Since one of the many desired characteristics for upcoming vaccine candidates is to efficaciously improvise the existing TB control approach i.e. prophylactic BCG immunization (99). Alternatives to strengthen the existing TB control program comprises developing a booster vaccine to augment the protective efficacy of BCG or supplementing immunotherapeutic as

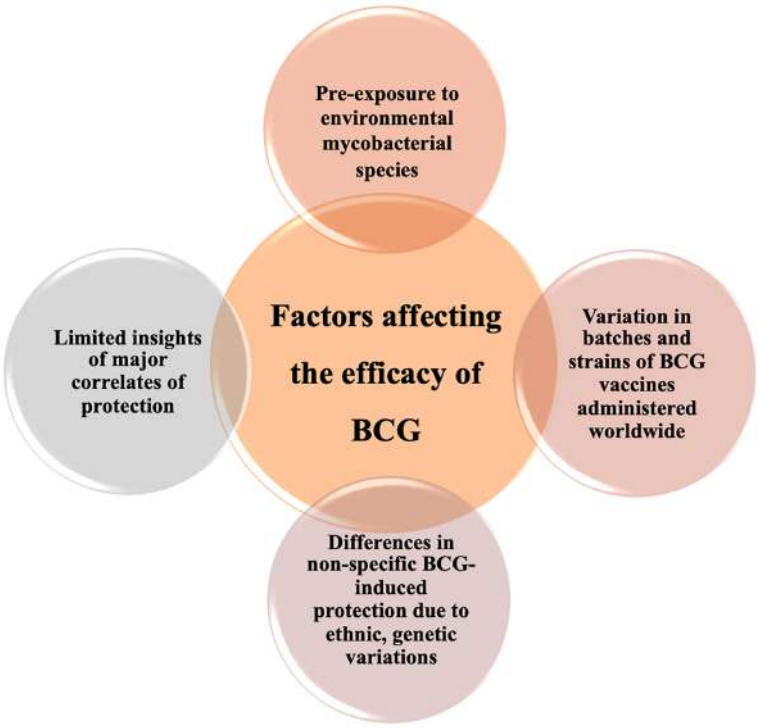

FIGURE 2 | Diverse factors associated with variable efficacy of BCG. Inconsistent efficacy of BCG vaccination can be linked to numerous host factors including genetics, geographical representation, ethnicity, and fragmentary immunological insight addition to variation in BCG vaccine strains with distinct characteristics.

an adjunct to strengthen BCG-induced immunity. Our research group has endeavored and designed a novel vaccine comprising TLR2 and TLR9 agonist along with collective of 7 overlapping immunogenic *M.tb* peptides, packed together in a liposome (PTL). We have demonstrated that intranasal immunization with PTL along with BCG drastically condensed the bacterial burden, enhanced host protective T cell responses with expansion of polyfunctional T cells as well as memory T cell subsets. Furthermore, host protective immune responses were *M.tb* specific owing to which spectrum of host protective signaling pathways critical to control TB were activated in response to PTL BCG co-immunization (100). Further assessment in higher animal models like Non-human primates (NHPs) is coveted to further validate our findings and progress the research to higher phases. We and various groups worldwide have actively devised and pursued strategies to develop new vaccine candidates, booster vaccines and immunotherapeutic to augment BCG efficacy against *M.tb* infection. However, in this review we have discussed about new TB vaccines in brief and have focused on host immunotherapeutic approaches comprehensively.

NEW VACCINES AGAINST TB

The necessity of an alternate vaccination strategy for TB control has not been overlooked and researchers around the world are actively pursuing diverse vaccine candidates to improve existing circumstances (99). On the account of immense attempts, several groups have developed vaccines by employing diverse approaches to control TB. Some classic examples include the attenuated *M.tb* bacilli strains (101) with high immunogenicity (102), the generation of genetically modified BCG strains for better immune responses (70), sub-unit vaccines incorporating immunogens absent in BCG (103), and adjuvants with enhanced potency. Major TB vaccine candidates in advanced clinial trials are tabulated in **Table 2**. A myriad of challenges is liable for slow progress in vaccine development against TB. One of which is the necessity for a vaccine that protects against adult pulmonary TB, in individuals who are presently vaccinated with BCG. Ideally, the development of vaccine candidates that can efficaciously impart protection to individuals previously exposed to mycobacteria including BCG, *M.tb* and environmental mycobacterial species would be enviable. So as to boost the immune responses thereby limiting adult pulmonary TB infections (19). Diverse studies incorporating booster vaccinations are under evaluation in animal models (104). With the advancement in technology and knowledge regarding host protective defense mechanisms, especially antigen-specific immune responses accountable for effective responses against *M.tb* we are surpassing conventional vaccination approaches. With improved understanding regarding known correlates of protection, research groups are assessing strategies to enhance long-lasting protective immune responses by employing progressive targets. What we have learned in the past decade from BCG trials as well as recent COVID-19 trials can be employed in the future to better interpret

TABLE 2 | Major TB vaccine candidates in clinical trials:.

Vaccine candidate	Composition	Clinical Trial	Clinical trial Identifier	Ref.
		Inactivated whole-cell vaccines		
DAR-901	Inactivated *Mycobacterium obusense*	Phase 2, randomized, placebo-controlled, double-blind study to evaluate the efficacy of DAR-901 TB booster to prevent TB in adolescents.	NCT02712424	(1)
MIP	Inactivated *Mycobacterium indicus pranii*	Phase 3, randomized, double-blind, interventional study to determine the efficacy and safety of MIP as an adjunct in Category I pulmonary TB patients	NCT00341328	(2)
RUTI®	Detoxified, fragmented *M.tb* contained in liposomes	Phase 2, randomized, double-blind, placebo-controlled interventional trial to assess the therapeutic vaccine, RUTI against TB	NCT01136161, NCT04919239	(3, 4)
Vaccae™	Heat-inactivated *Mycobacterium vaccae*	Phase 3, randomized, double-blind, interventional trial to assess the safety and efficacy to prevent TB in high-risk groups of TB infection	NCT01979900	(5)
		Live attenuated vaccines		
MTBVAC	Live attenuated *M.tb* vaccine with PhoP and FadD26 deletions	Phase 3, randomized, quadruple masking intervention to determine safety, efficacy and immunogenicity in newborns	NCT04975178	(6)
VPM1002	Live recombinant BCG vaccine strain with urease C deletion engineered to express listeriolysin rather than urease C	Phase 3, multicenter, double-blind, randomized, active-controlled trial to examine the safety, efficacy and immunogenicity to prevent *M.tb* infection	NCT04351685	(7)
		Subunit vaccines		
M72/ ASO1E	Fusion protein subunit vaccine based on 32A and 39A prepared in $AS01_E$ adjuvant	Phase 2, randomized, interventional clinical trial to determine the efficacy of TB vaccine candidate in Adults	NCT01755598	(8)
H56:IC31	Recombinant vaccine comprising proteins of *M.tb* (85B, ESAT6, Rv2660c) and IC31 adjuvant	Phase 2, randomized (1:1), double-blind, placebo-controlled trial to determine efficacy of H56:IC31 in preventing rate of TB recurrence	NCT03512249	(9)
GamTBvac	Recombinant subunit vaccine formulation comprising modified Ag85a and ESAT6-CFP10 *M.tb* antigens and CpG ODN adjuvant	Phase 3, randomized, multicentered, double-blind, placebo-controlled intervention to determine safety and efficaciousness of GamTBvac against pulmonary TB	NCT04975737	(10)
ID93/GLA-SE	ID93 is a recombinant fusion protein comprising 4 antigens from virulence-associated proteins in GLA-SE i.e., oil-in-water emulsion	Phase 2a, randomized, placebo-controlled, double-blind intervention to evaluate safety and effectiveness of ID93/GLA-SE in TB patients	NCT02465216	(11)

the lacunae which can be resolved for an improved TB vaccination program (16).

HOST-DIRECTED STRATEGIES TO IMPROVE BCG EFFICIENCY

The outcome of *M.tb* infection is determined not just by the action of the pathogen but also by the host response. So as to achieve the goal of the End TB strategy, progressive efforts are being made to establish efficacious therapeutics (17). In an attempt to achieve this goal, host-directed therapeutics with the potential to reprogram host defenses for better clinical outcomes are under consideration. Since it is known that in the majority of individuals, the immune system can self-reliantly eradicate the pathogen. Augmenting this phenomenon so as to achieve complete sterility can offer benefits to the existing global TB burden. TB is a chronic disease with a spectrum of pathologies (26). Hence, we need to move ahead from conventional antibiotics toward host-directed therapies for improved clinical outcomes. HDT has found a niche in the treatment of various diseases (105). However, we need more efforts to establish a standard HDT as an adjunct to conventional ATT for the augmentation of disease burden. So as to achieve this target it is a prerequisite to determine key host immune targets for better outcomes. HDTs aim at diverse pathways critical for determining the fate of the infection. HDTs work by restricting pathways exploited by pathogens or by ameliorating host protective immune responses (105). With the advancement in understanding, diverse factors contributing to the establishment of infection have been identified. The primary goal of TB drug discovery is to exterminate both active and persistent bacteria so as to attain complete sterility. Challenge is to aim at heterogenous *M.tb* populations that respond distinctly to therapeutics. It is essential to be reminiscent of the fact that *M.tb* infection can instigate a continuum of host responses owing to distinctive physiologies of heterogeneous bacterial populations. Furthermore, a profound assessment of stochastically and phenotypically drug-resistant persisting populations of *M.tb* subsequent to drug therapy is requisite to cope with TB relapse and reactivation (106). Better insight into mechanisms targeted to exterminate persistent populations is necessitated since it is not conclusive whether targeting bacterial membrane or prime respiratory components will eradicate latent bacteria. It is the need of the hour to get hold of innovative drugs to establish efficacious therapy to attain the goals of the End TB strategy. To accelerate the search for the right drugs, United States Food and Drug Administration (FDA) approved compounds are also under evaluation and operation (107).

Futuristic therapeutics should aim to shorten the duration of conventional ATT by proficient elimination of persistent bacterial populations, which also result in drug-resistant strains. With the constant expansion of drug resistance, options for treatment are continuously depleting. Chiefly, in terms of drug-resistant TB, HDTs can be employed to augment antimicrobial host defences

or to restrain detrimental inflammation instigated by infection. Some of the prospective HDTs against *M.tb* are listed in **Table 3, summarised in Figure 3** and further mechanism of protection is elaborated in different sections. Furthermore, HDTs that can limit the hepatotoxicity associated with conventional antibiotics are desired to subside unfavourable outcomes of extensive therapies. Theoretically HDTs surpass diverse issues associated with pathogen-directed therapeutics. HDTs augment host immune responses with sufficient proficiency to restrict the progression of the disease. Furthermore, targeting host components provide the advantage of reducing the generation of antibiotic resistance (129). Therapeutics targeting host components avoid the chances of drug resistance which is a global health concern. However, if not chosen wisely targeting host components can lead to off-target binding and might accelerate the chances of detrimental side effects. Thus, our knowledge regarding targeted mechanisms is vital to developing therapies to reprogram host defences for efficacious TB treatment.

ESTABLISHED HOST-DIRECTED STRATEGIES TO POTENTIATE BCG VACCINATION

In an attempt to achieve the targets of End TB strategy, diverse host-directed therapeutics with the potential to reprogram host defences for better clinical outcomes are under consideration. Since it is known that in the majority of individuals, the immune system can self-reliantly eradicate the pathogen. Augmenting host defences so as to achieve complete sterility can offer benefits in reducing existing global TB burden. To achieve superior effectiveness against *M.tb* infections, researchers have evaluated the administration of immunomodulators along with antibiotics and vaccines. This was widely employed in cancer therapies wherein inhibition of anti-inflammatory cytokines, and inhibitory signaling receptors such as PD-1 and CTLA-4 were found effective in augmenting tumor recession (130). Likewise, therapeutics known for inhibition of detrimental immune responses were found effective in improving tumor vaccine efficacies. Constructive outcomes were detected against breast cancer and pancreatic adenocarcinoma by the utilization of COX-2 inhibitors (131). In HIV-infected individuals, therapy with COX-2 inhibitor augmented effector and memory responses induced by T cell targeting vaccine; tetanus toxoid (132). Selected clinical trials that have evaluated immunomodulatory strategies adjunct to BCG immunization for improved clinical results have been listed in **Table 4** (133). Furthermore, modulation of monocytic cell populations has been exploited as a prospective strategy for augmenting efficacy of BCG vaccination. Abundance of uric acid crystals namely monosodium urate (MSU) have been linked with bone inflammation and associated immune responses (110). Presence of MSU crystals was further linked with coexisting *M.tb* joint infection in patients suffering from gout (134). MSU treatment in the THP-1 cell line brings about a generation of ROS, stimulation of phagosome-lysosome fusion, and *via* NOD-like receptor signaling enhanced BCG clearance (135). MSU alone has no anti-bacterial activity inferring potential to promote bacterial clearance by immunomodulation. MSU therapy in adjunct to BCG vaccination *in vivo* led to a reduction in bacterial burden in draining lymph nodes. However, MSU treatment did not affect the viability of BCG. As compared to BCG alone, MSU therapy significantly reduced the bacterial burden in the lungs and spleens of *M.tb* infected mice (110). Based on affirmative evidences of Vitamin D supplementation in restraint of *M.tb* infections (136), several research groups have correlated protective efficacy of BCG immunization in infants with Vitamin D levels (137). One of the research group has observed increase in Vitamin D levels in infants vaccinated with BCG and have linked the upsurge with non-specific immune responses detected subsequent to vaccination (138). In another study, infants supplemented with Vitamin D were expected to elicit protective IFN-γ responses against *M.tb* infection (137).

TABLE 3 | Potential host directed immunotherapeutic approaches against *M.tb* infection.

S. No.	Therapeutic candidates	Host protective immunological characteristics	References
1.	Ibuprofen	Inhibits neutrophil infiltration and detrimental inflammation at the site of infection	(108, 109)
2.	Acetylsalicylic acid (Aspirin)	Anti-inflammatory responses reduce detrimental pathology	(108)
3.	Monosodium Urate (MSU)	Activation of immune responses to augment antimycobacterial efficacy of BCG	(110)
4.	Calcimycin	Induction of autophagy by binding to P2X7 receptors	(111)
5.	Verapamil	Inhibits LTCC channels thereby induces autophagy by increasing Ca^{2+} levels.	(112)
6.	Clofazimine	Enrichment of stem cell memory T memory responses upon BCG revaccination	(113)
7.	Luteolin	Inhibition of $Kv_{1.3}$ K^+ channels, enhancement of antimycobacterial and T cell memory immune response	(114, 115)
8.	Rapamycin (Sirolimus)	Enhances antigen processing and presentation and directs T_h1 immunity	(116)
9.	Tat-beclin-1 fusion peptide	Autophagy induction and reduction in progression of pathogens	(117)
10.	Gefitinib	Enhances lysosomal biogenesis, action and bacterial degradation	(118)
11.	2-deoxyglucose (2-DG)	Metabolic reprograming induced reduction in pathological damage	(119)
12.	Ritonavir (Norvir)	Glucose transporter agonist induces protection against HIV as well as *M.tb*	(120)
13.	FX11	Lactate dehydrogenase inhibitor reduces oxidative stress and downgrade iNOS	(121)
14.	TEPP46	Limits inflammation by reducing PKM2 activation	(122)
15.	Metformin	Induces AMPK mediated signaling, induction of ROS and intracellular bacterial killing	(123)
16.	AICAR	Stimulate anti-microbial immune responses by *via* (PPARGC1) linked pathways	(124)
17.	C75	Inhibits lipid derived droplets biogenesis, enhances ROS, NO production and polarizes macrophages from M1 to M2	(125)
18.	Cerulenin	Inhibition of fatty acid synthase, uncouples UCP2 and promotes NLRP3 activation	(126)
19.	GW9662	PPARγ antagonist can regulate inflammation and disease progression by altering metabolism in macrophages.	(127)
20.	AGK2	Inhibits host sirtuin2 (SIRT2) and enhances bacterial clearance, host protective immune responses	(128)

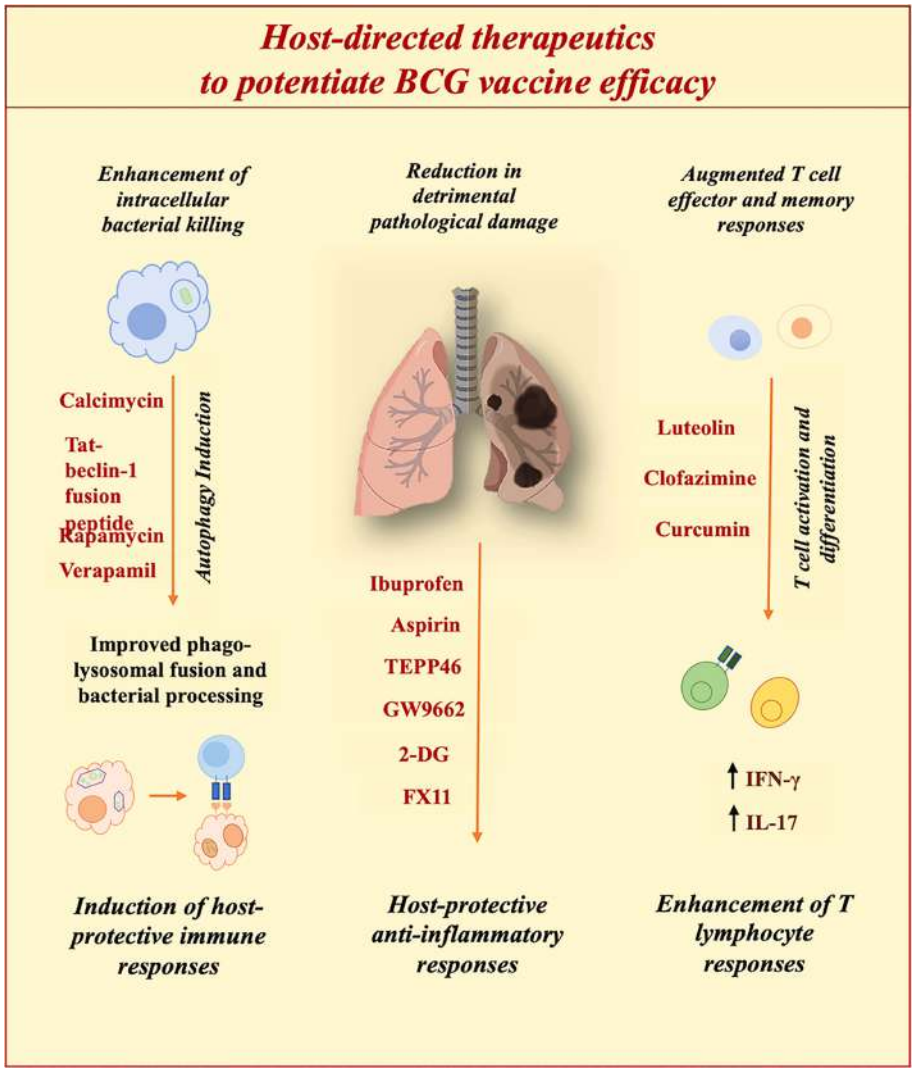

FIGURE 3 | Potential of host-directed therapies (HDTs) to improve BCG efficacy. Diverse HDTs aiming at distinct pathways are under evaluation to improve clinical outcomes. HDTs restrict pathogen-induced subversion strategies to ameliorate host defenses against *M.tb*.

TABLE 4 | List of clinical trials evaluating BCG immunization along with diverse immunotherapeutic for efficient medical utility against diverse disease conditions.

BCG vaccine and immunotherapeutic regimen	Clinical trial	Trial identifier	Diseased condition	Ref.
Intravesical hyaluronic acid (HA) with BCG	Phase 2, randomized, pilot study to examine effect of HA in reducing BCG induced local cytotoxicity	NCT02207608	Bladder urothelial cell carcinoma	(12)
Tislelizumab in Combination with BCG	Phase 2, open-label, single-arm, single center trial to evaluate safety and effectiveness of Tislelizumab along with BCG (TACBIN-01)	NCT04922047	High risk urinary bladder cancers	(13)
Vitamin D supplementation in adjunct to BCG immunization in infants	Randomized, double masked, interventional study to evaluate impact of vitamin D supplement in infants prior to BCG vaccination	NCT01288950	Tuberculosis	(14)
Vitamin A with BCG Vaccine	Phase 4, randomized, double-masked intervention to evaluate the utility of high-dose vitamin A supplementation in infants along with BCG vaccine at birth	NCT00168597	Mortality and morbidity in infants	(15)
Monoclonal Antibody A1G4 and BCG	Phase 1 intervention to evaluate the efficacy of monoclonal antibody A1G4 along with BCG in cancer patients	NCT00003023	* Neuroblastoma, Sarcoma	(16)

However, this clinical trial waned to provide evidences of Vitamin D induced protection in augmenting BCG efficacy. Further assessment is requisite to explore prospects of Vitamin D supplementation along with BCG immunization. Potential of established immunomodulators with BCG have been improvised for better clinical outcomes. One such study demonstrated induction of superior host protective T cell responses upon co-administering curcumin nanoparticles along with BCG immunization in murine model (139). Clofazimine (CLOF), an authorised therapeutic for leprosy treatment and second-line drug used in combinations against drug-resistant *M.tb* strains has also demonstrated affirmative outcomes in mice model. BCG revaccination along with CLOF administration significantly augmented T cell memory responses comprising enhancement of stem cell-like memory T cell responses (T_{SCM}) along with successive effector and memory T cell populations (113). So as to further resolve the prospects of above-mentioned strategies in enhancement of BCG efficacy, further studies in higher animal models such as non-human primates (NHPs) and clinical trials is necessitated without delay.

MODULATION OF IMMUNE RESPONSES TO IMPROVE PROTECTIVE EFFICACY

With the rise of genomics, researchers have utilized the genomic information of *M.tb* and *M. bovis* BCG vaccine strains for assessing variations that can be benefitted to develop better vaccination strategies (95). The revelation of the entire *M.tb* genome revolutionized TB research by expanding the knowledge of central immunomodulatory components (140). To achieve superior effectiveness against *M.tb* infections, researchers have evaluated the administration of immunomodulators along with vaccines. This was widely employed in cancer therapies wherein inhibition of anti-inflammatory cytokines, and inhibitory signaling receptors such as PD-1 and CTLA-4 were found effective in augmenting tumor recession (130). Likewise, therapeutics known for inhibition of detrimental immune responses were found effective in improving tumor vaccine efficacies. Constructive outcomes were detected against breast cancer and pancreatic adenocarcinoma by the utilization of COX-2 inhibitors (131). In HIV-infected individuals, therapy with COX-2 inhibitor augmented effector and memory responses induced by T cell targeting vaccine; tetanus toxoid (132). Inhibition of neutrophil infiltration at the site of *M.tb* infection by **Ibuprofen** (IBP) resulted in improved clinical outcomes and a reduction in bacterial burden in C3HeB/FeB mice (109). IBP is also known to possess specific antitubercular characteristics (141). Furthermore, IBP along with another drug; **acetylsalicylic acid** was evaluated to be repurposed as an adjunct therapy in TB patients (108). In murine model of TB, drugs were associated with enhancement of pyrazinamide (PYZ) antimycobacterial efficacy (142). This approach can be further exploited to amend BCG-induced responses. These studies indicate that treatment with immunomodulatory compounds or enriching host protective responses along with BCG might can effectively enhance protection induced by BCG vaccination.

TARGETING HOST ION CHANNELS

Another strategy is to aim at host ion channels, that orchestrate physiological features of various cell populations by operating ions facilitated currents throughout cellular and subcellular membranes (143). Intracellular calcium levels are known to regulate key immune responses in the host, directly or by directional alteration of other vital ions such as potassium (K^+), sodium (Na^+), and chloride (Cl^-) ions within immune cell populations (144). Obstruction of ion channels by employing diverse blockers has been assessed as a therapeutic target for diseases like hypertension (145). Research groups have also examined ion channel blockers for boosting anti-microbial immune responses. Intracellular calcium (Ca^{2+}) levels play a vital role in the regulation of antimycobacterial mechanisms such as autophagy, maturation of phagosome, and induction of apoptosis (146). However, the impact of Ca^{2+} levels on processes like autophagy additionally depends on the involvement of diverse ion channels contributing to the maintenance of current (147). For instance, it has been observed that Ca^{2+} currents *via* Voltage-gated calcium channels (VGCCs) impede induction of autophagy (147) while Ca^{2+} currents through P2X purinoceptor 7 (P2X7) receptor heighten autophagy induction and intracellular extermination of *M. bovis* BCG in macrophages. **Calcimycin,** an ionophore binds to P2X7 receptor which leads to rise in intracellular $Ca2^+$ levels and exerts antimycobacterial activity against *M. bovis* BCG (111) by stimulation of autophagy (148). Administering $Ca2^+$ ion channel blockers was linked with a 32% reduction in risk of progression into a diseased state, in a clinical study in TB patients with heart and cerebrovascular diseases (149). Diverse Ca^{2+} ion channel blockers evaluated in the investigation exhibited variable consequences. L-type calcium channel (LTCC) blocker – **verapamil,** an FDA approved drug is utilized to treat abnormalities in heart rhythms, angina (150), and hypertension (151). In macrophages, LTCCs attenuate Ca^{2+} discharge from the endoplasmic reticulum (ER) leading to inhibition of macrophage activation. So as to bypass host immune responses *M.tb* upregulates the expression of VGCCs in APCs. Verapamil administration inhibits LTCC currents thereby escalating Ca^{2+} concentration in the cytosol which upregulates autophagy and bacterial clearance in *M.tb* (143). Additionally, LTCC ion channel blockers can alter iron-associated metabolic pathways thereby impeding iron accessibility which lowers intracellular bacterial survival (143). Furthermore, verapamil acts synergistically with first-line anti-TB drugs- INH (152), and RIF (153) in lowering the bacterial burden in cultures, macrophages, and murine models of TB. In another study, antimycobacterial activity of verapamil was confirmed with several TB drugs including bedaquiline (BDQ) and clofazimine (CFZ) and decreased bacterial load was linked

with induction of membrane stress responses as a consequence of verapamil induced membrane function disruption (154). In the murine model, adjunctive verapamil administration augmented efficacy of recently approved MDR-TB drug; bedaquiline at lower doses and diminished the emergent resistant strains (155). Furthermore, progressive approaches such as assessment of inhalable verapamil-rifapentine particles has been assessed by researchers for utility as ATT (156). Even with significant pieces of evidence regarding anti-TB activity and negligible toxicity, verapamil has not transitioned into clinical setup. Further assessment of the anti-TB potential of verapamil along with BCG immunization is necessitated to evaluate the impact on modulating immunological responses. Similarly, Numerous K^+ ion channel blockers have been assessed as prospective anti-TB therapeutics owing to their physiochemical ability to activate macrophages. **Clofazimine** (CFZ) is a conventional first-line drug used for leprosy treatment along with RIF and dapsone (157). It was initially developed against *M.tb* but was found not as efficacious as INH and RIF. However, with emergent drug-resistant strains, it has been recently employed as a second-line anti-TB drug (158). Numerous clinical trials (BEAT-TB (159), endTB-Q (160), and TB-PRACTECAL (161)) are investigating the efficaciousness of regimens comprising clofazimine. Furthermore, phase-2 clinical trial CLO-FAST is examining 3-month ATT comprising clofazimine and rifapentine against drug-sensitive *M.tb* (162). In addition to antimycobacterial activity, clofazimine is a potent immunomodulator. It inhibits $Kv_{1.3}$ K^+ channels which are expressed in various immune cells (163). Clofazimine-induced inhibition of $Kv_{1.3}$ K^+ channels abundantly present on T effector memory cells (T_{EM}) enhances the efficacy of BCG vaccination in a murine model of TB by specifically promoting the expansion of the T central memory cell population (T_{CM}). Furthermore, CFZ enriches stem cell memory T cell responses upon BCG revaccination (113). In a similar manner, we have demonstrated the efficacy of less toxic, phytochemical namely Luteolin an established $Kv_{1.3}$ K^+ channel blocker in augmenting BCG-induced immune responses by enriching T_{CM} memory responses, improving T_{CM} : T_{EM} ratio and enhances host protective T_h1 and T_h17 immune responses against *M.tb* infection in the murine model of TB (115). Furthermore, immune-protective properties of luteolin condensed the time period of bacterial clearance with INH owing to augmented T_h1 and T_h17 immune responses and eased pathological damage and TB associated hepatotoxity *in vivo* (114).

IMPROVING ANTI-MICROBIAL IMMUNE RESPONSES

Therapeutic modulation of host immune responses to achieve complete sterility is another approach that has been pursued by several research groups (129). *M.tb* utilizes complex artillery to evade immune cell populations and associated defense mechanisms. *M.tb* owing to mycobacterial virulence factors such as cell wall component- mannose-capped lipoarabinomannan is known to inhibit phagolysosome fusion in macrophages. It is a vital phenomenon critical for curbing infection at an early stage (26). However, this can be enforced by autophagy induction, which is another cellular mechanism by which detrimental cytosolic molecules and organelles are targeted to lysosomes for degradation. Early secreted antigen 6 secretion system-1 (ESX-1) of *M.tb* is known to permeabilize the phagosome to escape degradation (164). However, this facilitates processing by components of ubiquitin-mediated autophagy mechanism and results in a reduction in *M.tb* persistence (165). Furthermore, stimulation of autophagy sequesters and degrades bacterial components and can also contribute to fostering antigen presentation and moderating pathology (166). The most widely studied autophagy inducer – **rapamycin (Sirolimus)** is known to inhibit the mammalian target of rapamycin (mTOR) which negatively regulates autophagy. It is majorly employed in organ transplantation owing to the immunosuppressive nature of the drug (167). Researchers have attempted re-purposing of Rapamycin, an autophagy inducer to heighten antigen processing and presentation in murine antigen-presenting cells (APCs) (168). It is well established that confiscation of BCG within phagosome and inability to fuse with lysosome reduces the efficacy of antigenic peptide presentation on DCs. Dendritic cells (DCs) treated with autophagy inducer, rapamycin enhanced T_h1 responses against *M.tb* (116). Improvement in DC activation and T_h1 responses against *M.* with concurrent rapamycin and BCG administration offers prospective approach to autophagy mediated enhancement of bacterial clearance (116). Similarly, the efficacy of BCG can be augmented by simultaneous treatment to direct host responses toward bacterial extermination to achieve complete sterility (116). Few piecemeal studies have questioned the immunotherapeutic strategies to enhance vaccine efficiency against TB, however a great deal is yet to be explored (169). However, side effects associated with rapamycin administration such as interstitial pneumonitis can be alarming in TB patients with substantial pathology (170). Furthermore, metabolization of rapamycin by hepatic enzyme CYP3A4 limits its utility in TB patients since CYP3A4 is intensely stimulated by standard ATT antibiotic – INH (171). Due to the mentioned limitations rapamycin has not been further evaluated as HDT against *M.tb*. **Vadimezan (also known as DMXAA)** is another prospective autophagy inducer. It is an established antitumor agent as well as in mice it triggers, a stimulator of IFN genes (STING) dependent autophagy mechanism (172). However, it was found inefficacious in humans (173). Research groups have further examined the utility of fusion peptides for the induction of autophagy. **Tat-beclin-1 fusion peptide** an autophagy inducer (174) was found to restrict the proliferation of diverse pathogenic strains and heightened survival rates in infected mice (117). Though, restrictions like regular administration by injection constrain the clinical utility of HDT. Alternatively, an inhibitor of epidermal growth factor receptor (EGFR) was found to limit *M.tb* proliferation in macrophages and reduces bacterial burden in the lungs of infected mice *via* autophagy induction (175). In *M.tb* infected macrophages treated with **Gefitinib**; tyrosine kinase inhibitor, lysosomal biogenesis, function and targeting of bacteria

to lysosome for degradation is increased thereby decreasing bacterial burden *via* EGFR signaling in macrophages (118). However, there is a need to further analyse pieces of evidence and peripheral markers of autophagy for certainty. With the advancement in technologies, sophisticated approaches to evaluate the induction of autophagy can evolve the quest for superior autophagy inducers that can be employed to enhance bacterial killing by limiting dissemination.

TARGETING HOST METABOLISM

Research focus has shifted radically in the past decade towards metabolic shifts in response to infections. Immunometabolism is an emerging field that focuses on the impact of the metabolic state of immune cell populations to provide better insight into disease progression and pathogenesis (176). In the initial course of *M.tb* infection, metabolic shift is observed to defend the host. Immune protective responses such as stimulation of pro-inflammatory cytokines, and nitric oxide (NO) release is directed *via* HIF-1-dependent glycolytic pathways (177). However, *M.tb* is known to stimulate the Warburg effect so as to inhibit anti-microbial host immune responses (178). Host metabolism is utilized by *M.tb* to survive and proliferate by escaping host protective immune mechanisms (179). This infers that metabolic reprogramming is vital for defense against *M.tb* so as to augment efficacious sterilization mechanisms. **2-deoxyglucose (2-DG)** an inhibitor of hexokinase enzyme, can limit the IL1-β generation in LPS-activated macrophages and result in succinate accumulation (180). 2-DG stimulated glycolysis inhibition can additionally result in a reduction in lung damage induced by LPS (181) *via* moderating nuclear PKM2-STAT3 signaling. Further, prospects of 2-DG to restrict pathological damage in TB cases can be assessed for augmented clinical outcomes. Similarly, **ritonavir** (Norvir), a protease inhibitor widely used as antiretroviral medication to treat HIV infections (120), is additionally known for capability to act as an glucose transporter agonist (182). Researchers have evaluated combinations of HIV drugs including ritonavir along with ATT so as to effectively counter HIV-TB coinfections (183). Strategic arrangement by utilizing characteristics of ritonavir to inhibit host glucose transporters can be assessed further in case of HIV-TB patients to better understand mechanism of protection. Inhibitor of pyruvate dehydrogenase kinase- **dichloroacetate**, is a small molecule that increases pyruvate flux into mitochondria and skews metabolism toward glucose oxidation rather than glycolysis (184). Inhibition of pyruvate dehydrogenase kinase was established as a host target to counter infection of *Salmonella enterica serovar typhimurium via* metabolic reprogramming of M1 macrophages. However, intracellular burden for *M.tb* was not reduced upon dichloroacetate treatment, alternate inhibitors can be explored with similar objective (185). Another small molecule and lactate dehydrogenase inhibitor – **FX11 (**3-dihydroxy-6-methyl-7-(phenylmethyl)-4-propylnaphthalene-1-carboxylic acid]) is known for induction of oxidative stress and reduction in tumour advancement (121). Downregulation of iNOS and cytokine generation was achieved in LPS-activated RAW 264.7 macrophages upon FX11 induced lactate dehydrogenase inhibition (186). Similarly, the inhibitor of pyruvate kinase– **TEPP46** significantly reduced PKM2 activation in LPS-induced macrophages which led to a lowering of IL-1β generation (122). Hence, small molecule inhibitors can be employed to direct metabolic flux for desired clinical outcomes to resolve immunopathology of TB by regulating host metabolism.

M.tb manipulates lipid and fatty acid metabolic pathways of the host for its persistence and proliferation (187). Foamy macrophages recruited around *M.tb* infected phagocytes, supply nutrition and support in the course of infection. *M.tb* manipulates host cells to synthesize lipids and fatty acids. Hence, components of lipid synthesis pathways manipulated by *M.tb* for survival can be targeted as HDT (188). Metabolic energy sensors such as AMP-activated protein kinase (AMPK) play a vital role in the regulation of key host protective mechanisms against infections (189). An approved type 2 diabetes drug, **Metformin** which activates the AMPK-mediated signaling mechanism has been evaluated for TB (190). Metformin induces the generation of mitochondrial reactive oxygen species (ROS) resulting in restriction in intracellular growth of *M.tb* and limiting the activation of the inflammatory gene (191). In *M.tb* infected guinea pigs, metformin acts synergistically with conventional ATT drugs – INH and ETH (192). Metformin administration causes a significant reduction in latent TB incidences in prone diabetic individuals (193). This HDT can be evaluated proficiently at advanced clinical stages. Another AMPK activator, 5-aminoimidazole-4-carboxamide-1-1-β-D-ribofuranoside **(AICAR)** stimulates anti-microbial responses by activating autophagic pathways in macrophages. AMPK activation by AICAR further controls the biogenesis of mitochondria and metabolic state in macrophages by inducing peroxisome proliferator-activated receptor gamma coactivator-1 (PPARGC1) associated pathways (124). Components of host machinery that curb the metabolism of lipids can reduce detrimental inflammation thereby establishing a balanced immune state. Fatty acid synthase inhibitors such as **C75** and **cerulenin** are prospective targets for the augmentation of efficacious immune responses. Inhibition of lipid-derived droplets by C75 can lead to polarization of macrophages from the M1 to M2 subset causing enhancement of ROS and NO production (125). C75 and cerulenin-mediated inhibition of fatty acid synthase lead to uncoupling protein-2 (UCP2) mediated NLRP3 inflammasome activation (126). PPARγ antagonist, **GW9662** is known to regulate vital processes such as metabolism, inflammation, and disease progression (127) in macrophages infected with *M. bovis* BCG (76). Link between inflammation and lipid metabolism associated PPARγ signaling can be exploited to potentiate BCG-induced protection (76). This infers that reprogramming key components of lipid metabolism can be a prospective target for progressive TB therapeutics. Sirtuins (SIRTs) are another prospective target with the potential to be targeted for the augmentation of host defences (77). SIRTs are deacetylases that regulate cellular mechanisms like inflammatory responses, regulation of lipid metabolism by modulating components of NF-κB immune signaling, and anti-inflammatory

responses by regulation of Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) (78). It has been observed that SIRT-1 expression is diminished drastically in *M.tb* infected THP-1 cells. SIRT-1 downregulates RelA/p65 unit of NF-κB so as to modulate inflammation (79). SIRT-6 is also known to diminish pro-inflammatory and anti-microbial responses in the early course of *M.tb* infection (80). Furthermore, SIRT2 has been established as an immunotherapeutic target against *M.tb* infection in mice model of TB (128). It was observed that subsequent to *M.tb* infection, SIRT2 expression increases along with translocation to nucleus to induce immune dampening epigenetic modifications. However, chemical inhibition of SIRT2 using established inhibitor, **AGK2** dramatically augmented bacterial clearance and host protective immune responses. Since, existing literature is available regarding SIRT2 induced metabolic programming (81) and signal transduction (82). Further progressive approaches can be shaped by targeting key physiological factors of host.

TARGETING MICRORNAS

miRNAs are non-coding RNAs that are involved at post-transcriptional levels to regulate array of genes decisive of immune responses (83). Over 2000 functional miRNAs are encoded by human genome (84) which regulate diverse protein-coding transcripts (194). It is now well-established that miRNAs distinctly regulate host immune responses against *M.tb* infection (195). Differential expression of miRNAs can signify the advancement of disease from latent to an active infection (196). Furthermore, miRNAs play a significant role in the moderation of apoptotic and autophagic responses during *M.tb* infection (196). Owing to advancements in technology, miRNA delivery is being employed to treat diverse diseases. This further pave way for the application of miRNAs as HDT against TB (197). Several studies have assessed mechanisms by which *M.tb* temper host immune responses for survival in an antimicrobial milieu. Diverse immune mechanisms such as phagolysosome maturation in APCs, cytokine stimulation by immune cell populations, and antigen processing and presentation are dynamically manipulated by *M.tb*. These cellular processes are strictly regulated by an assortment of miRNAs in the host (198). *M.tb* is additionally known to alter miRNA expression associated with key biological responses to escape host immune responses (199). Additionally, expectations of combined regulation of transcriptional network by miRNAs and transcription factors represent miRNAs linked with diseases as a novel category of therapeutics.

miRNAs involved in immune pathways are extensively studied for mycobacterial infections (200). It is well-known that during *M.tb* infection miR-125b inhibits TNF biosynthesis in human alveolar macrophages (201). Several research groups have observed that *M.tb* infection results in differential expression of miRNAs which determines the fate of immune responses (202). However, comprehensive knowledge is requisite for progressive considerations. Participation of miRNAs in *M.tb* induced autophagy (203) and apoptosis has provoked further interest to maneuver miRNAs for HDTs. Upon *M.tb* infection, diverse cell populations respond varyingly in conjunction with variations in miRNA expression. Upregulation of miR-155 has been observed in bone marrow-derived macrophages of mice infected with *M.tb* (204). In contrast, downregulation of miR-155 has been observed in peripheral blood mononuclear cells (PBMCs) derived macrophages upon *M.tb* infection (201). Mycobacterial infection in human monocyte-derived macrophages results in overexpression of miR-29a, miR-886-5p, and let-7e, which further target caspase 3 and caspase 7 as predicted by the integrated analysis. Differential expression of miR155, miR-146a, miR145, miR222, miR-27a, and miR-27b was observed in human macrophages infected with virulent *M.tb* H37Rv and avirulent strain *M. bovis* BCG. Downregulation of miRNA involved in the regulation of inflammation and lipid metabolism was observed. Furthermore, miR-145 known for induction of apoptosis has been reported to be downregulated upon infection with a virulent *M.tb* strain, which results in overexpression of targets which inhibit apoptosis (205). Based on microarray analysis, global changes in miRNA expression screened nine miRNA genes which were differentially expressed in *M.tb* H37Rv and *M.tb* H37Ra infected THP-1 cells. These differentially expressed miRNAs such as miR-30a, miR-30e, miR-155, miR-1275, miR-3665, miR3178, miR-4484, miR-4668-5p, and miR-4497 contribute in diverse physiological aspects. miR-155 interacts with negative regulators involved in TNF- α generation (201). Evaluation of PBMCs and pleural fluid mononuclear cells (PFMCs) linked miRNA expression with levels of IL-6 cytokine. Additionally, it has been observed that the highly-virulent Beijing/W TB strain represses plenty of miRNA in human macrophages as compared to non-Beijing/W TB strains. Alterations in miRNAs have been observed in patients with active TB. The functional assessment demonstrated that miR-144 restrains T-cell expansion and generation of key cytokines, INF-γ and TNF-α. In RAW264.7 cells, upon BCG inoculation, miRNA-144-3p overexpression is linked with inhibition of autophagy and antimycobacterial activity (206). Elevation in miR-424 and miR-365 levels has been detected in active TB patients. Diverse miRNA contributes to determining the fate of infection. Regulation of immune cell activation by miR-155, miR-146a, miR-21, and miR-9 (207), TLR signaling is positively regulated by miR155 (208). Subsequent to *M. bovis* BCG infection significant increase in miR-155 expression is observed in macrophages, which modulates diverse innate immune responses including ROS generation (209). It additionally plays role in apoptosis induction in macrophages upon *M. bovis* BCG inoculation which modulates cellular physiology and immune responses (210). In *M.tb* infected human alveolar macrophages, miR-125b inhibits TNF generation (201). miR-29 targets IFN-γ and regulate immune responses linked to *M.tb* infection (211). miR-223 targets several chemo-attractants such as CXCL2, CCL3 and contributes to directing immune response (212). It has been evaluated that endogenous block of miR-29 in transgenic mice, augmented resistance to *M.tb* infection (211). miR-27a targets IRAK4 and restrict immune response in TB (213). Another study demonstrated that BCG infection in RAW264.7 cells

upregulates miR-17-5p which was linked with augmented BCG dissemination and enhanced autophagosome related protein expression (214). As revealed diverse miRNAs are under evaluation owing to gene modulatory potentials. However, our knowledge regarding role of specific miRNA overexpression upon BCG inoculation is still fragmentary. Utilization of miRNAs to augment host immune responses, paves way for advancement in therapeutics for various diseased conditions. Differential expression of miRNAs can be moulded in such a way to achieve better clinical outcomes in TB patients. Progressive therapeutics are employing miRNA-mimics (215), antisense oligonucleotides (216) to manipulate immune responses. Although several research groups have evaluated the utility of miRNA manipulation as HDT against *M.tb*, further assessment is necessitated to establish the prominence of miRNA as therapeutic. We required more studies to comprehend the contribution of miRNA in host-pathogen interactions and a progressive strategy for augmenting conventional therapies.

CONCLUSIONS AND FUTURE PERSPECTIVE

Despite incessant debates on the variable protective efficiency of BCG, it prevails as the only vaccine for TB prevention. Owing to considerable protection in children against disseminated forms of TB, it remains a key component of TB control programs in various countries (90). Throughout our review, we have mentioned several shortcomings and lacunae linked with the failure of the TB vaccination strategy. One of which is the complexity of the disease itself, as we are envisaging resolutions that can impart complete sterility, which is seldomly accomplished in the natural course of infection (26). In this aspect, TB diverges from the diseases that are preventable *via* vaccination. Hence, progressive approaches were pursued with the utilization of known immune correlates of protection. To surpass the previously failed attempts, it is vital to redirect focus on immunological pathways that can be augmented for better clinical implications (217). Since adult pulmonary TB mainly accounts for *M.tb* transmission, advances to tackle the inadequacies of BCG to impart long-lasting immunological memory should be highlighted as a better immunization stratagem. With extraordinary scientific efforts to counteract the most fatal pathogens known to humankind, we have progressed to a situation wherein we can harness establish knowledge and immunological concepts to deal with the shortcomings of existing approaches and improvise for robust clinical trajectories. Since the most of individuals worldwide are already vaccinated with BCG, it is judicious to keep BCG in reflection while developing new vaccination strategies. Scientific communities are attempting stratagems to prevent millions of deaths from escalating infectious diseases by investing on immunotherapeutic approaches to augment immunological responses.

AUTHOR CONTRIBUTIONS

KN, VD wrote the manuscript. AB edited the manuscript. VD conceived the hypothesis. All authors contributed to the article and approved the submitted version.

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