RESEARCH WORK

TITLE:

Berberine governs NOTCH3/AKT signaling to enrich lung-resident memory T-cells during tuberculosis

INTRODUCTION:

In spite of being avertable and treatable, tuberculosis (TB) caused by *Mycobacterium tuberculosis* (*M.tb*) is the major cause of mortality and morbidity among infectious diseases. Globally 10 million people were diseased with TB and an aggregate of 1.3 million people passed away in 2020 itself [1]. Furthermore, almost one-fourth of humankind is infected asymptomatically (latently) with *M.tb*, with a 5-15% risk of progressing into clinical manifestations [1].

Existing anti-tubercular treatment (ATT) comprising of assorted anti-mycobacterial drugs can only exterminate active, drug-sensitive strains of *M.tb*. However, failure to complete the extensive TB restraint approach, directly observed treatment short-course (DOTS) frequently brings about the emergence of multi-drug resistant (MDR) and extensively-drug resistant (XDR) strains. Moreover, DOTS therapy instigates severe toxicity and impairment of host immune responses. For instance, isoniazid (INH) usage leads to the cessation of antigen-responding $CD4^+$ T lymphocytes, which results in a heightened risk of reactivation and reinfection with *M.tb* [2]. Further inefficacy of the only available vaccine *M. bovis* bacille Calmette-Guérin (BCG) to prevent adult pulmonary TB makes it a requisite to employ appropriate strategies to augment the host control of *M.tb* infection [3]**.**

Subsequent to infection, *M.tb* is phagocytosed by antigen-presenting cells (APCs) that participate in the extermination of internalized pathogens, promote activation of T lymphocytes and stimulate protective pro-inflammatory cytokines such as IFNγ and IL17 [4–6]. The immunological response in TB is extremely complex and the fate of infection is governed predominantly by subsets of T lymphocytes [7]. For instance, stimulation of T helper 2 (Th2) cells and regulatory T cells (Tregs) result in the advancement of disease by hampering protective Th1 responses [8] [9] [10] while Th1/Th17subsets are associated with host protective immune responses [11]. Nonetheless, these cytokine responses decline post *M.tb* clearance and hence, subsets of memory T cells play a crucial role in providing long-term protection in TB [11]. T cell receptor (TCR) signaling following antigen stimulation along with cytokine environment regulates and shapes host memory responses [12]. Sustained AKT activation following TCR stimulation drives terminal T cell differentiation [13] probably by targeting FOXO proteins [14,15]. JAK/STAT pathways also influence the differentiation of naïve T cells into memory subsets [16]. Further, STAT4 and Blimp1 transcription factors are known to regulate resident memory responses at the local site of infection. T effector memory (T_{EM}) cells provoke Th1 type cytokines and protect against acute *M.tb* infections whereas, T central memory (T_{CM}) can give rise to T_{EM} during disease progression, direct cell-mediated immunity for bacterial clearance and sustain long-term memory responses [17] [18]. Hence, a strengthened T_{CM} and T_{EM} population is vital for the continuation of long-term protective immune responses [19]. Apart from these, tissue-resident memory T cells (T_{RM}) localized at distinctive sites of infections like lung and spleen are linked with positive medical consequences and host protective responses [20].

Mostly host immune responses elicited in response to *M.tb* can successfully contain pathogen [4] but complete sterility is not attained. Hence, a novel immunomodulatory approach is necessitated to boost current therapeutics to ease up drug regimen, lessen therapy-induced

adversities and intensify anti-mycobacterial effects [21] [22] [23]. BBR ($C_{20}H_{18}NO_4^+$) a bioactive isoquinoline alkaloid is known for diverse therapeutic effects. Administering BBR adjunct to therapeutics can induce hepatoprotective effects by modulating inflammatory responses [24]. BBR can induce protection against INH-associated inflammation, oxidative stress and liver damage in rats [25]. BBR has shown affirmative outcomes *in vitro* against clinical drug-resistant bacterial strains [26], and *in vivo* against drug-sensitive *M.tb* with limited insight into the mechanism of protection[27]. In this study, we have investigated the anti-mycobacterial potential of BBR against pathogenic laboratory strain H37Rv and drug-resistant clinical isolates of *M.tb ex vivo* and in the murine model of TB. We observed that BBR significantly lowered the bacterial burden in the lungs and the spleen of *M.tb* infected mice in solitary or in combination with the first-line anti-TB drug INH primarily by boosting the protective host immune responses such as macrophage activation, Th1/Th17 polarization, memory T cell enhancement and pro-inflammatory cytokine responses. NOTCH mediated AKT inhibition and activation of FOXO1, STAT3, STAT4, BLIMP-1 and NFκB signaling following BBR treatment led to a profound induction of adaptive memory in human $CD4^+$ T cells and in mice model. These immunomodulatory properties of BBR were also exploited to increase the vaccine efficacy of BCG. Furthermore, induction of superior memory responses significantly lowered the recurrence of TB due to re-activation and re-infection. Overall, these results propound BBR as an attractive adjunct immunotherapeutic and immunoprophylactic against TB.

OBJECTIVES:

1. To determine the efficacy of bioactive compound in the murine model of tuberculosis
2. To examine the adjunct potential of the bioactive compound with anti-tuberculosis therapy (ATT) in providing long-term protection against drug-susceptible and drug resistant tuberculosis
3. To determine the treatment modality on the susceptibility for the reinfection and reactivation of tuberculosis
4. To determine if treatment with bioactive compound enhances BCG vaccine-induced host protection against tuberculosis
5. To understand the immuno-molecular mechanism of host-directed therapy.

MATERIALS AND METHODS:

1. Bacteria: - *M.tb* strains such as H37Rv, MDR-Jal2261and XDR-MYC 431 were revived from cryopreserved 20% glycerol stocks preserved in- 80 °C. These bacterial strains were then cultured in 10% ADC (albumin, dextrose and catalase), with 0.05% Tween 80 and 0.2% glycerol containing 7H9 media.
2. Peritoneal macrophages isolation: - 2ml of 4% thioglycolate was injected intra-peritoneally into C57BL/6 mice. Post 5 days peritoneal exudates cells were extracted in ice cold filtered PBS. And further suspend the cells in RPMI-1640 with 10% FBS and 1 % penicillin-streptomycin antibiotic for overnight incubation at 37°C and 5% CO2. Thereafter, the cells were washed with PBS to remove non-adherent cells.
3. Ex vivo, *M.tb* infection: - 0.6-0.8 O.D of bacteria was prepared using 7H9 for infection. After preparing single cell suspension we infected the macrophages with MOI (Multiplicity of Infection)- 1:10.
4. In vivo, *M.tb* infection: - The bacteria was prepared, in order to obtain a single cell suspension. Mice were then infected with different strains by the aerosol route using aerosol chamber containing nebulizer of 110 CFU for each mouse.

5. Drug administration: - By evaluating the proper toxicity, drug was dispensed to mice intra-peritoneally, along with 0.1g/liter of isoniazid dissolved in drinking water that was changed every alternative day.
6. CFU (colony forming unit) determination: - Mice from their respective groups were sacrificed and organs like lung and spleen were harvested in sterile PBS (Phosphate buffer saline). These organs were further homogenized and plated over 7H11 media containing plates. Plates were kept in 37degree incubator for 21-28 days and the colonies were subsequently counted.
7. RNA isolation: - Cells were treated with the prescribed therapeutic drug and further the Trizol was used for isolating RNA.
8. cDNA synthesis and qPCR: - After isolating RNA, we performed cDNA synthesis using kit and with the help of SYBR green that detects the expression of multiple cytokines within the samples using qPCR.
9. Protein isolation: - for protein extraction, the whole cell lysate was prepared using lysis buffer and 1X Protease inhibitor cocktail.
10. Immunoblotting: - the cell lysate was further run to 10%SDS gel electrophoresis and electroblot to nitrocellulose membrane. 5 % BSA (Bovine Serum Albumin) prepared in PBST (PBS with 0.1% Tween 20) was used to block the blots and the protein bands were detected respective antibodies.
11. Cytokine profiling using Flow cytometry: - For intracellular staining of cytokines, the cells were incubated with 500 ng/ml ionomycin along-with 10 µg/ml brefeldin A, 6 hours prior to processing. Post washing twice with filtered PBS, the cells were suspended in permeabilization buffer and further stained with their corresponding fluorescent conjugated monoclonal antibodies. The data was recorded by using Flow cytometry

RESULTS:

BBR enhances host resistance against drug-susceptible and drug-resistant strains of *M.tb*

Numerous studies have evaluated the therapeutic potential of BBR against diverse ailments. However, the effectiveness of BBR against *M.tb* infection is yet to be uncovered. Since BBR has been shown to possess weak anti-bacterial activity [28], we foremost aimed to determine its anti-mycobacterial activity against different drug-sensitive and -resistant strains of *M.tb*. Consistent with the previous studies, BBR displayed bacterial toxicity at ≤ 50 µg/ml against all the strains tested however 20 µg/ml of BBR treatment which displayed no toxicity in the mouse peritoneal macrophages significantly decreased the intracellular *M.tb* growth **(Fig1A)** indicating the immunomodulatory effects of BBR on host macrophages. With no variable effect on ROS generation , BBR treatment significantly induced apoptosis in uninfected and *M.tb* infected macrophages **(Fig1B and 1C)** and led to significant activation of transcription factors (NFkB and STAT3) which play a crucial role in combating TB **(Fig 1D).** Further, BBR treatment significantly enhanced the expression of CD11b and co-stimulatory molecules CD40 and CD86 on the surface of infected macrophages **(Fig 1E-1G)**. Moreover, BBR treated macrophages also displayed increased expression of M1 specific pro-inflammatory cytokines **(Fig 1H)**. Furthermore, immunomodulatory effect of BBR was consistent in T cells isolated from infected mice wherein the percentage of $CD69^+$ activated $CD4^+$ T lymphocytes was significantly enriched upon BBR treatment **(Fig 1I)**. Co-culturing infected macrophages with BBR primed T cells demonstrated significantly reduced intracellular bacterial burden **(Fig 1J and 1K)** as compared to infected macrophages as well as untreated T cells co-

cultured with macrophages. Few piecemeal studies have reported BBR as an efflux pump inhibitor and thereby is known to increase the intracellular concentration of the antibiotics [29]. We investigated whether BBR co-treatment increased the killing potential of INH. With no significant effect *in vitro* BBR co-treatment significantly reduced the bacterial burden in the INH treated macrophages as compared to INH treatment alone in both human **(Fig 1L)** and mice derived macrophages **(Fig 1M)**. BBR also reduced the intracellular load of MDR and XDR strains of *M.tb* **(Fig 1N)** emphasizing that the immunomodulatory potential of BBR is not restricted to drug-sensitive strain of *M.tb*. To further corroborate the outcomes of *ex vivo* experiments, C57BL/6 mice were infected with a low dose (~110 CFU) of *M.tb* H37Rv and left untreated or treated with BBR (4mg/kg) either alone or in combination with INH (100mg/L) for 45 days followed by CFU enumeration and immune profiling **(Fig 1O)**. As compared to control or INH treated infected lungs, BBR treatment significantly reduced the extent of granulomatous inflammation alone and in combination with INH **(Fig 1P and 1Q)**. Further, BBR treatment significantly reduced the bacterial burden in the lungs and the spleen of infected mice as compared to the control group **(Fig 1R and 1S)**. Interestingly, BBR co-treatment significantly enhanced the anti-tubercular potential of INH **(Fig 1R and 1S)**. These results demonstrate the adjunct potential of BBR along with INH against *M.tb* H37Rv. Since drug-resistant variants are one of the major contributors of global TB pandemic, we ascertained the efficacy of BBR against MDR and XDR TB **(Fig 1T)**. Interestingly, BBR treatment significantly lowered the bacterial burden of both the drug-resistant strains tested in the lungs and spleen of mice **(Fig 1U-1X)**.

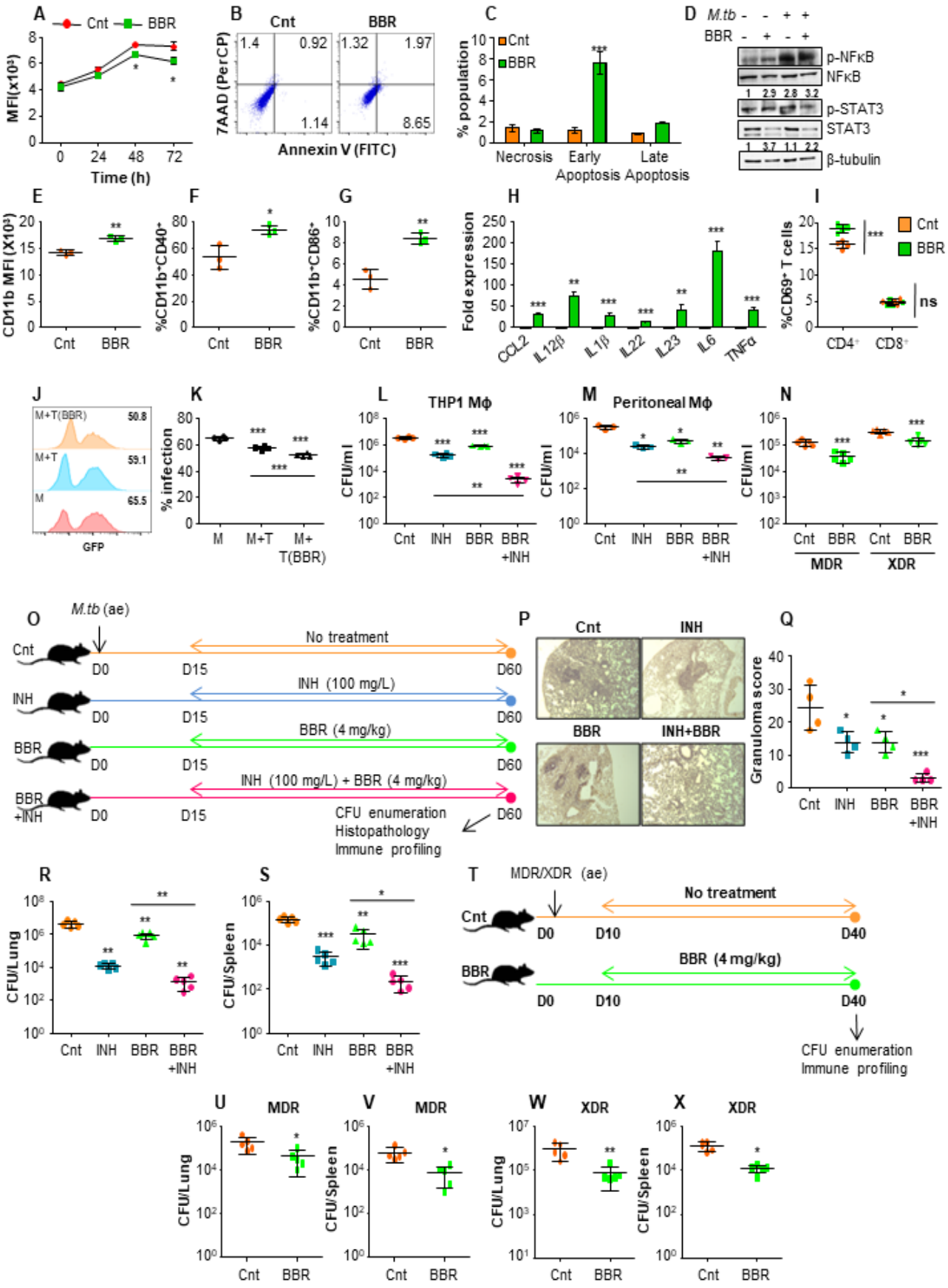

Fig 1: BBR treatment enhances host resistance against drug-sensitive and drug–resistant TB. **(A)** Mouse peritoneal macrophages were infected with GFP expressing H37Rv (Rv-GFP) at 1:10 MOI

followed by treatment with BBR (20 μg/ml). At different time points, cells were analysed by flow cytometry. Graph represents the GFP fluorescence at indicated time points with and without BBR treatment**. (B-C)** Mouse peritoneal macrophages were infected with *M.tb* at MOI of 1:10 followed by treatment with BBR (20 μg/ml) for 48 h followed by apoptosis analysis via flow cytometry. **(B)** Representative dot plots and **(C)** Percentage of apoptotic cells with and without BBR (20μg/ml) treatment. **(D)** Immunoblots depicting the phosphorylation of indicated transcription factors (NFkB and STAT3) in uninfected and infected mouse peritoneal macrophages with or without BBR treatment. **(E-G)** Infected murine peritoneal macrophages were surface stained with antibodies against CD11b (APC/Cy7), CD40 (PE) and CD86 (PerCPCy5.5) followed by flow cytometry. **(E)** Expression of CD11b on the surface of infected macrophages. Percentage of **(F)** $CD11b^{+}CD40^{+}$ and **(G)** $CD11b^{+}CD86^{+}$ infected macrophages with and without BBR treatment. **(H)** Expression of chemokines and cytokines in *M.tb* infected macrophages at 24h pi with and without BBR (20 μg/ml) treatment. **(I)** Percentage of $CD4^{+}$ and $CD8^{+}$ T cells expressing CD69 in the infected and BBR (10μg/ml) treated splenocytes. **(J)** Representative overlay plots and **(K)** percentage of RvGFP infected macrophages co-cultured with *M.tb* specific and BBR treated splenocytes. **(L)** PMA- activated THP1 macrophages were infected with H37Rv at 1:10 MOI followed by treatment with INH (1 μg/ml), BBR (20 μg/ml) or both for 48 h pi after which cells were lysed for CFU enumeration. **(M)** Experiment L was repeated in mouse peritoneal macrophages. **(N)** Mouse peritoneal macrophages were infected with MDR (Jal 2261) and XDR (MYC431) clinical strains of *M.tb* followed by treatment with 20 μg/ml of BBR. Cell lysates were plated for CFU enumeration 48 h pi. **(O)** Schematic representation of the murine model of infection. C57BL/6 mice were infected with low dose of H37Rv (~110 CFU per lung) and after 15 days of disease establishment, mice were treated with either INH (100 mg/L), BBR (4 mg/kg) or both for 45 days followed by CFU enumeration and immune profiling. **(P)** Histopathology of infected lungs with arrows indicating the granulomatous lesions. **(Q)** Immunopathology score of the infected lungs. Bacterial burden in the **(R)** lungs and the **(S)** spleen of infected animals. **(T)** Diagrammatic representation of the infection model. Bacterial burden in **(U)** the lungs and **(V)** the spleen of mice infected with MDR strain of *M.tb*. Bacterial load in **(W)** the lungs and **(X)** the spleen of mice infected with XDR strain of *M.tb*. Data is representative of two independent experiments. The data values represent mean ± SD (n = 3-5). *$p<0.05$, **$p<0.005$, ***$p<0.0005$.

BBR strengthens the host protective immune responses against TB

To comprehend the immunomodulatory properties of BBR, we profiled the innate and adaptive immune cell populations driving host protection in both the lungs and the spleen of infected mice. Increased percentage of $CD11b^{+}$ and $CD11c^{+}$ cells with enhanced expression co-stimulatory molecules CD80 and CD86 was observed in the lungs and the spleen of infected mice treated with BBR indicating significant innate cell stimulation which plays a crucial role in the activation of adaptive immune responses. Although BBR treatment did not change the percentage of $CD4^{+}$ and $CD8^{+}$ T cells **(Fig 2A-2C)**, in combination with INH, BBR convalesced INH induced reduction in percentage of $CD4^{+}$ T cells **(Fig 2B)** in the lungs of infected mice. BBR treatment significantly induced the activation of T cell subsets as the expression of early activation marker CD69 on these cells was significantly heightened in the lungs **(Fig 2D-2F)** and the spleen of infected mice.

Hence, it can be inferred that BBR treatment extensively strengthens antigen processing and presentation by APCs and consistently promotes activation of T lymphocytes to impart protection against *M.tb* infection. Furthermore, BBR treatment advanced differentiation of $CD4^{+}$ and $CD8^{+}$ into protective Th1 and Th17 subsets in the lungs of infected mice. This was evident by significant increase in IFNγ and IL17 producing T cell subsets in both the lungs **(Fig 2G-2K)** and the spleen of BBR and INH treated mice. Furthermore, BBR treatment considerably enriched host-protective chemokines and cytokines such as TNFα, IL1β, IL22 etc., and subsided the effect of INH induced anti-inflammatory cytokines such as IL10 and IL4 in co-treated mice **(Fig 2L).** We further investigated the impact of BBR treatment on the

induction of prolonged immune protection, which is mediated by memory subsets of adaptive immunity. The lungs of BBR treated mice revealed high frequency of T_{CM} cells **(Fig 2M-2O)**. Similar trend was observed in the spleen of BBR treated mice. Furthermore, the percentage of resident memory T cells (T_{RM}) which evolve from disseminating effector memory T cells (T_{EM}), remain confined to the tissues and play a key role in stimulating adaptive immune response at the tissue specific sites was considerably enhanced in the lungs **(Fig 2P-2R)** and in the spleen of infected mice. Furthermore, to strengthen our findings regarding positive immunomodulatory effects of BBR treatment on $CD4^+$ T cells, we performed adoptive transfer experiment in $Rag^{-/-}$ mice **(Fig 2S)**. Adoptive transfer of $CD4^+$ T cells from BBR treated infected mice into $Rag^{-/-}$ mice significantly reduced bacterial burden in the lungs **(Fig 2T)** and the spleen **(Fig 2U)** of $Rag^{-/-}$ mice upon *M.tb* infection concluding that BBR exerts host-protective effects by enriching *M.tb* specific $CD4^+$ T cell responses.

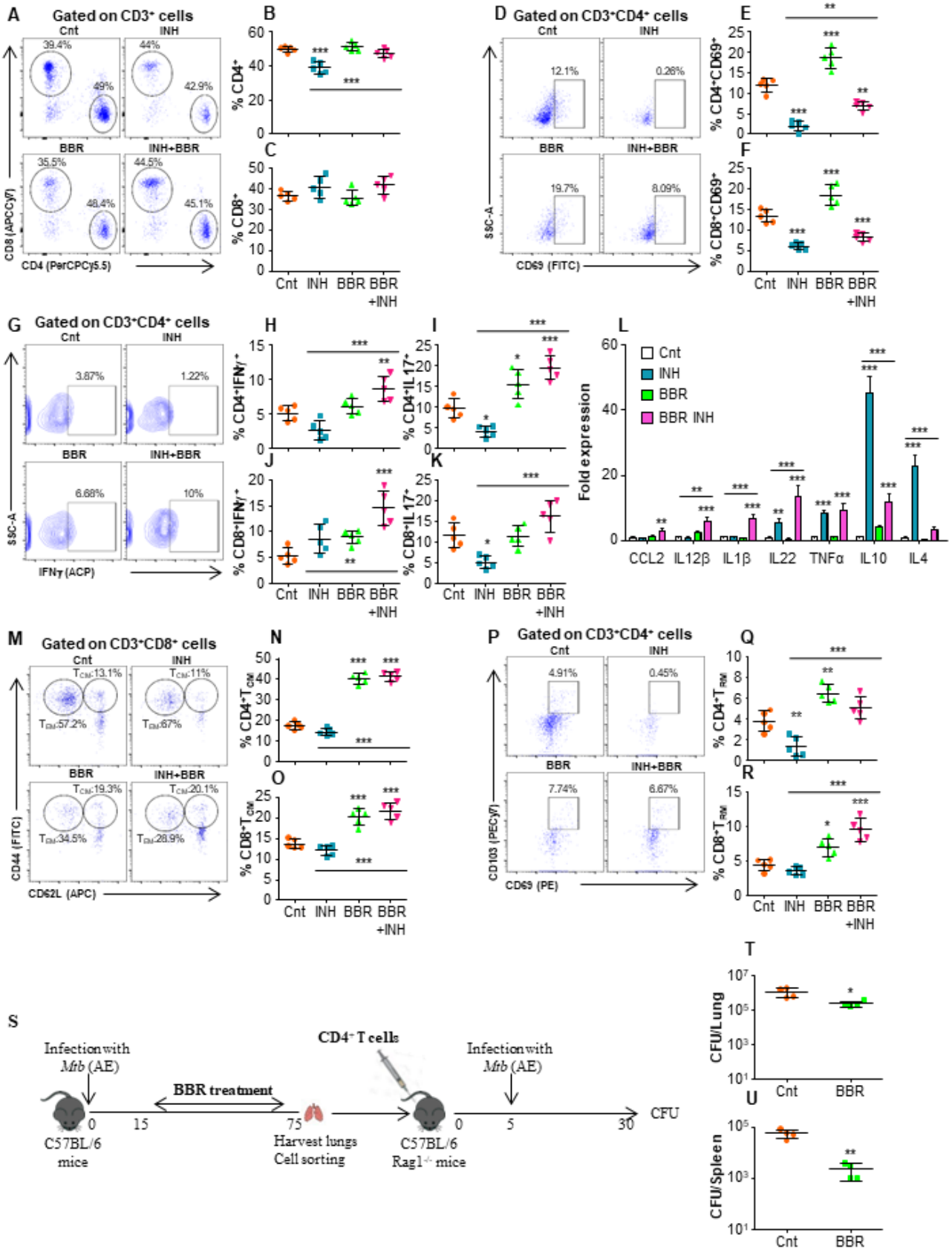

Fig 2: BBR strengthens *M.tb*-specific T cell responses during TB treatment. Single cell suspensions generated from the infected lungs were *ex vivo* stimulated with *M.tb* complete soluble antigen (CSA) for 16 h followed by surface staining with antibodies against CD3 (Pacific Blue), CD4 (PerCPCy5.5), CD8 (APCCy7) and CD69 (FITC) followed by flow cytometry. **(A)** FACS scatter dot

plots and percentage of **(B)** $CD4^+$ and **(C)** $CD8^+$ T cells in the infected lungs. **(D)** Representative FACS dot plot and the percentage of **(E)** $CD4^+CD69^+$ and **(F)** $CD8^+CD69^+$ T cells in the infected lungs. **(G-K)** After stimulation with CSA, the lung cells were treated with monensin and brefeldin A for 2 h and surface stained with α-CD3 (Pacific Blue), α-CD4 (PerCPCy5.5) and α-CD8 (APCCy7) followed by intracellular staining with α-IFNγ (APC) and α-IL17 (PECy7) and flow cytometry. **(G)** Representative dot plots and percentage of **(H)** $CD4^+IFN\gamma^+$, **(I)** $CD4^+IL17^+$, **(J)** $CD8^+INF\gamma^+$ and **(K)** $CD8^+IL17^+$ cells in the infected lungs. **(L)** Fold expression of cytokines in the lungs of infected, INH and BBR treated splenocytes. **(M-R)** To determine the frequency of central memory and resident memory T lymphocytes, *ex vivo* stimulated lung cells were surface stained with α-CD3 (Pacific Blue), α-CD4 (PerCPCy5.5), α-CD8 (APCCy7), α-CD69 (PE), α-CD103 (PECy7), α-CD62L (APC) and α-CD44 (FITC) followed by flow cytometry. **(M)** Representative FACS dot plots and **(N)** the percentage of $CD4^+T_{CM}$ ($CD62L^{HI}CD44^{HI}$) cells, T cell subset. **(O)** Percentage of $CD8^+$TCM ($CD62L^{HI}CD44^{HI}$) cells, in the infected lungs with or without drug treatment. **(P)** Representative scatter dot-plot images and percentage of **(Q)** $CD4^+$TRM and **(R)** $CD8^+$TRM cells in the lungs of infected mice. **(S)** Schematic representation of adoptive transfer experiment. CFU enumeration after 21 days of adoptive transfer in **(T)** the lungs and **(U)** the spleen of Rag-/- mice. Data is representative of two independent experiments. The data values represent mean ± SD (n = 5). *p<0.05, **p<0.005, ***p<0.0005

BBR enriches pathways associated with establishment of T_{RM} in human PBMCs

Fig 3A represents a simplistic model of T cell differentiation into different memory subsets highlighting different regulators and surface markers used to identify these cells. To understand the molecular signaling involved in the enhancement of $CD4^+$ adaptive memory after BBR treatment, we cultured PBMCs isolated from healthy PPD^+ individuals in the presence of BBR for 48h. Intriguingly, BBR treatment drove significant differentiation of $CD4^+$ T_{NAIVE} cells into T_{EM}, T_{EMRA} and T_{RM} T cell subsets with no significant increase in T_{CM} cells **(Fig 3B-3G)**. To understand the mechanistic details of heightened memory responses, we performed the whole proteome analysis of human PBMCs with or without BBR treatment. In each individual, BBR treatment induced a distinct proteome landscape with a significant number of differentially expressed proteins **(Fig 3H)**. Interestingly, BBR treatment collectively downregulated the expression of 323 proteins **(Fig 3I)** and induced the expression of 527 proteins **(Fig 3J)** in the treated PBMCs. Further analysis on these commonly expressed proteins revealed few pathways which were downregulated upon BBR treatment **(Fig 3K)**. Curiously, diverse pathways associated with key cellular processes such as cell cycle, cell adhesion, cell division and glycolysis were upregulated upon BBR treatment **(Fig 3L)**. Importantly, BBR treatment enhanced the upregulation of critical proteins of Notch signaling pathway **(Fig 3L and 3M)** which is known to stimulate the differentiation and maintenance of T_{RM} cells [30].

TCR stimulation along with downstream signalling pathways such as PI3K/AKT/mTOR play a critical role in shaping the T cell memory [12]. While activated AKT is known to phosphorylate FOXO1 triggering its nuclear exclusion, previous literature highlights the importance of FOXO1 mediated gene expression in the generation and maintenance of protective memory cells [31] [32] Further, Blimp1 transcription factor is known to regulate resident memory responses at the local site of infection [33,34]. Ironically, Notch3 transactivates PTEN which in turn inhibits the AKT signaling [35] leading to FOXO1 activation. We validated these targets by RT-PCR and observed that BBR treatment induced significant expression of genes related to notch signaling as well as the downstream targets such as Foxo1, Pten and Blimp1 **(Fig 3N)**. Moreover, BBR inhibited AKT and FOXO1 phosphorylation **(Figs 3O and 3P)** and along with AKT inhibitor (AKTi), BBR synergistically reduced T_{NAIVE} population **(S7E Fig),** increased T_{EM} and T_{EMRA} cells with no

effect on T_{CM} cells. BBR treatment also enhanced resident memory functions by modulating the BLIMP-1 expression **(Fig 3Q)**.

Cellular metabolism plays a crucial role in modulating T cell effector functions [36] while BBR is known to induce glycolysis in many cell types [37] [38]. Consistent with the previous findings and our proteomics data, BBR significantly induced glycolysis **(Fig 3R)** in human PBMCs. Furthermore, to dwell deeper into the immunological milieu induced upon BBR treatment, Bio-plex Pro Human cytokine screening Panel (48-Plex) was utilised to screen the regulation of cytokines in human PBMCs. BBR treatment consistently enhanced the expression of pro-inflammatory cytokines in human PBMCs **(Fig 3S-3V)** derived from PPD^+ individuals. Moreover, 20 pro-inflammatory cytokines and chemokines were upregulated in the PBMCs derived from at least 3 out of 4 individuals **(Fig 3W)**. Overall these results indicate that BBR enhances effector functions of $CD4^+$ T cells and upregulates critical signaling associated with T_{RM} establishment and maintenance.

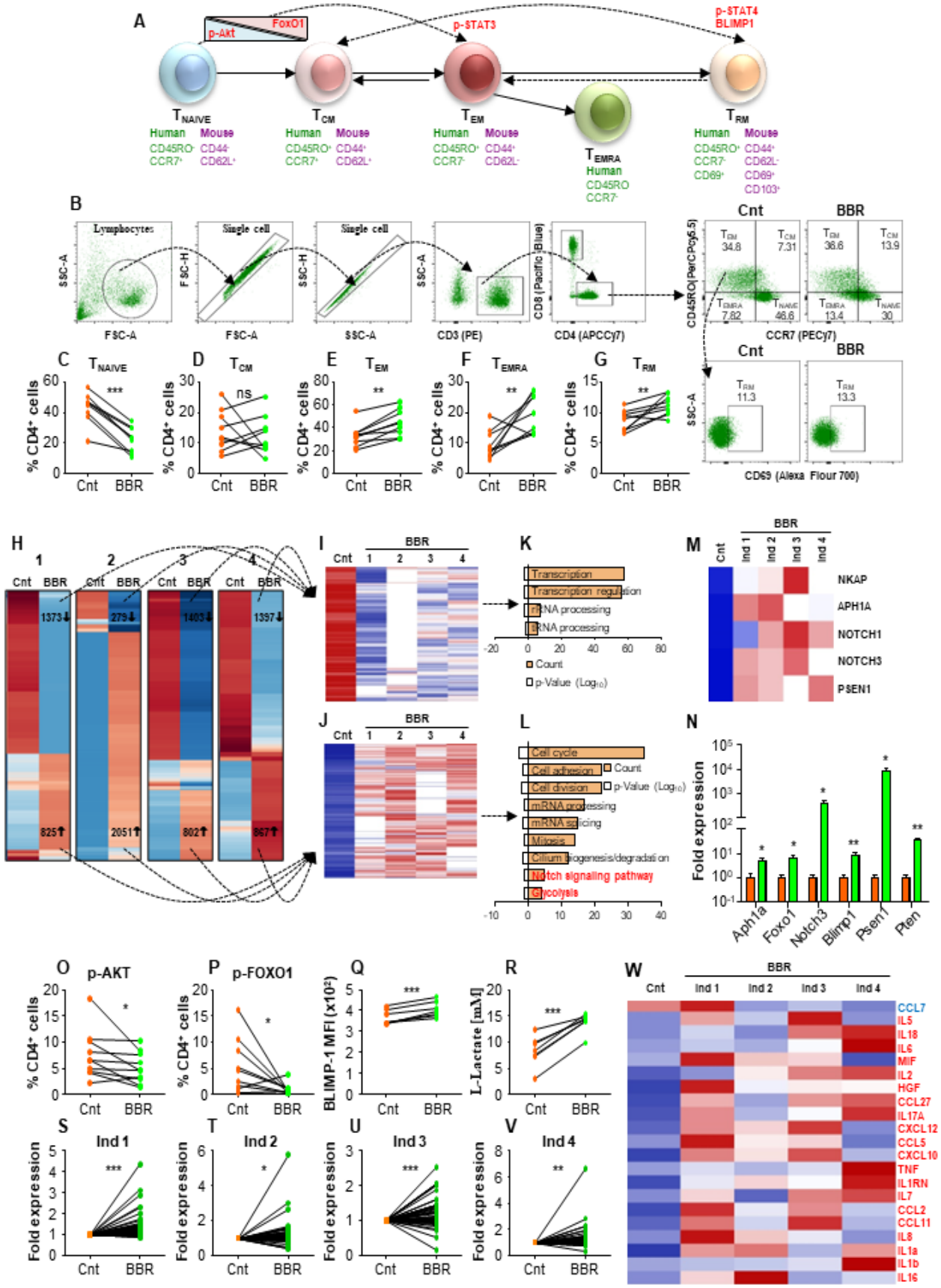

Fig 3: BBR treatment enriches human CD4⁺ memory T cells by regulating NOTCH/PTEN/Akt/FOXO1 pathway. (A) Schematic representation of T cell differentiation into

T_{EM}, T_{CM} and T_{RM} memory subsets. **(B-G)** Human PBMCs isolated from 7 PPD^+ healthy individuals were ex vivo stimulated with CSA and treated with BBR (10 μg/ml) for 48 h followed by surface staining with α-CD3 (PE), α-CD4 (APCCy7), α-CD8 (Pacific Blue), α-CD45RO (PerCPCy5.5), α-CCR7 (PECy7) and α-CD69 (Alexa Flour 700) **(B)** Gating strategy employed to depict the different memory T cell subsets. Percentage of **(C)** $CD4^+$ T_{NAIVE} cells, **(D)** $CD4^+$ T_{CM} cells, **(E)** $CD4^+$ T_{EM} cells, **(F)** $CD4^+$ T_{EMRA} cells and **(G)** $CD4^+$ T_{RM} cells. **(H)** Whole proteome profiling of untreated and BBR treated human PBMCs derived from 4 PPD^+ healthy individuals. Heat map representation of the differentially expressed proteins (Log2 fold, n=3). Common proteins in all the individuals that are **(I)** downregulated and **(J)** upregulated upon BBR treatment. Biological processes that are **(K)** downregulated and **(L)** upregulated upon BBR treatment. **(M)** Notch signalling pathway associated proteins which were upregulated in human PBMCs upon BBR treatment. **(N)** RT-PCR of genes related to Notch signaling pathway**. (O)** Human $CD4^+$ T cells expressing p-AKT and **(P)** human $CD4^+$ T cells expressing p-FOXO1. **(Q)** MFI of human $CD4^+$ T cells expressing Blimp-1. **(R)** Extracellular L-Lactate quantification in untreated and BBR treated human PBMCs. **(S-W)** represents multiplex cytokines assay upon BBR treatment in human PBMCs of derived from 4 PPD^+ healthy individuals (refer methodology). **(S-V)** Fold expression changes of 46 cytokines upon BBR treatment in different individuals. **(W)** Heat map of common differentially expressed cytokines. In **H**, **I**, **J**, **M**, and **W**, Red represents upregulation while blue represents downregulation. Data is representative of two independent experiments. The data values represent mean ± SD (n is 4 to 7). *p<0.05, **p<0.005, ***p<0.0005.

BBR drives the expansion of memory T cells by modulating key regulators of T cell development in murine T cells

To validate the molecular signaling involved in the enhancement of $CD4^+$ adaptive memory after BBR treatment, splenocytes isolated from *M.tb* infected mice were *ex vivo* stimulated with *M.tb* complete soluble antigen (CSA) and treated with BBR (10μg/ml) for 48h followed by immune profiling. Analysis of different $CD4^+$ T cell subsets via flow cytometry **(Fig 4A)** revealed that BBR treatment significantly reduced the percentage of T_{NAIVE} subset **(Fig 4B)** with a concomitant increase in the T_{EM} cells **(Fig 4C)**. While no difference was observed in the T_{CM} population **(Fig 4D)**, BBR significantly induced the T_{RM} subset **(Fig 4E)**. Consistent with the previous results, BBR treatment significantly reduced the activation of AKT **(Fig 4F and 4G)** and decreased the phosphorylation of FOXO1 **(Fig 4H and 4I)** in $CD4^+$ T cells. Inhibition of AKT signalling has been shown to promote central memory responses by increasing nuclear accumulation of FOXO1 [39]. It is also well-established that STAT3 and STAT4 play specific function in the formation of T cell memory subsets in response to infections [16] [40]. To further ascertain the influence of BBR treatment on AKT-FOXO1 axis, we repeated the *ex vivo* T cell stimulation experiment in the presence of AKTi and BBR. AKTi alone did not increase the percentage of $CD4^+$ T_{EM} and T_{RM} populations, whereas, considerable enrichment was observed upon BBR treatment alone or in combination with AKTi **(Fig 4J and 4K)**. This infers the influence of alternate signaling pathways contributing in enhancement of memory responses upon BBR treatment. We observed that BBR treatment induced the activation of STAT3, STAT4 and BLIMP-1 in $CD4^+$ T cells **(Fig 4L-4Q)** which may lead to enhanced T_{RM} response. Interestingly, heightened NFκB activation was observed in BBR treated $CD4^+$ T cells **(Fig 4R and 4S)** leading to a significant upregulation of host protective proinflammatory responses **(Fig 4T)**. Furthermore, BBR treatment led to enhanced glycolysis in $CD4^+$ T cells **(Fig 4U)**. Interestingly, 2-Deoxy-D-glucose (2DG) (glycolysis inhibitor) treatment abrogated the expression of IFNγ and IL17 in BBR treated $CD4^+$ T cells **(Fig 4V and 4W)** indicating that BBR potentiates pro-inflammatory response through metabolic reprogramming. Overall, this concludes that BBR treatment expands memory T cell subsets with proinflammatory characteristics.

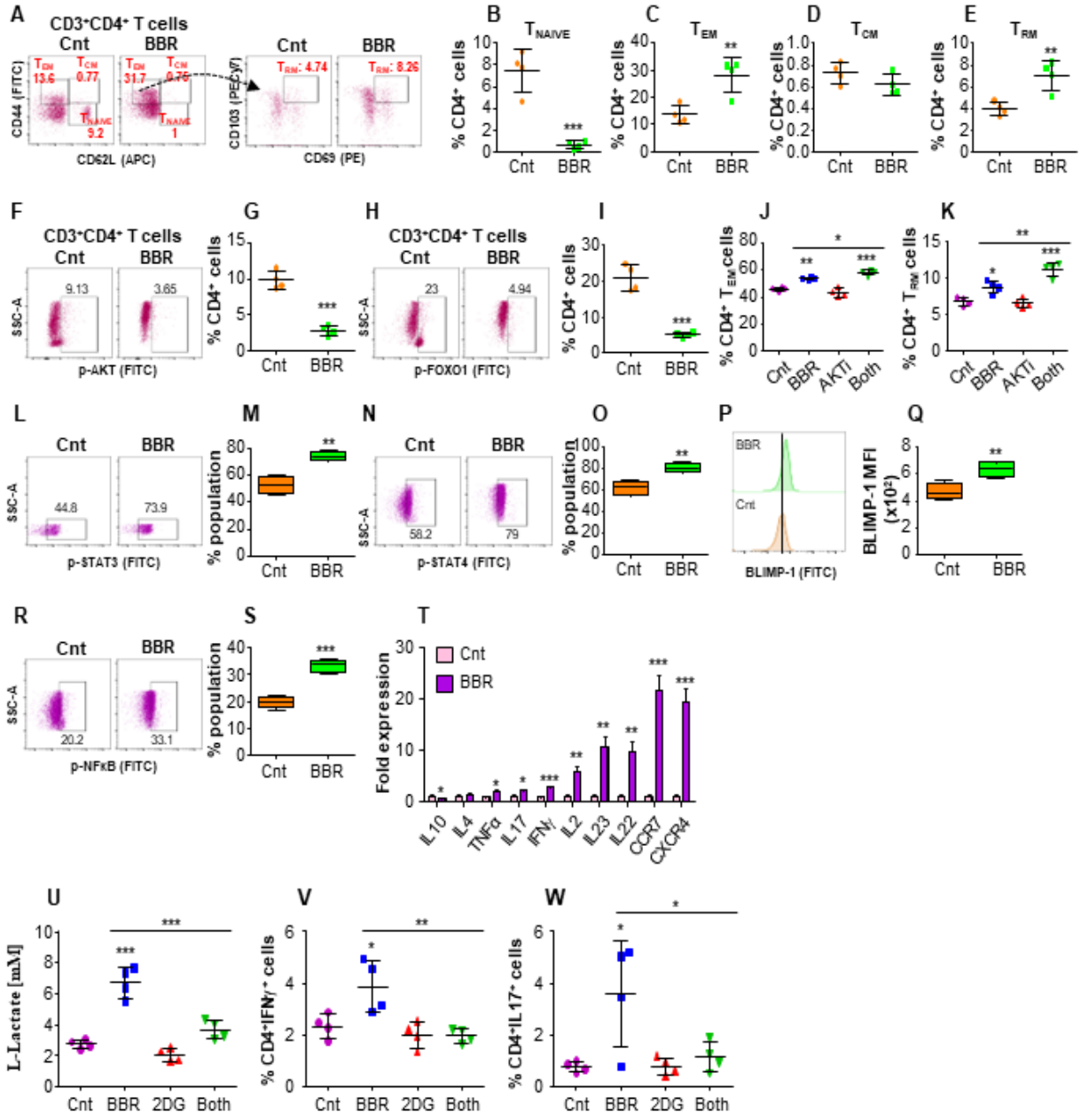

Fig 4: BBR induces expansion of antigen-specific memory T cells by targeting TCR signaling and glycolysis. Splenocytes isolated from *M.tb* infected mice were *ex vivo* stimulated with *M.tb* CSA and treated with BBR (10 µg/ml) for 48 h. *Ex vivo* stimulated splenocytes were surface stained with α-CD3 (Pacific Blue), α-CD4 (PerCPCy5.5), α-CD8 (APCCy7), α-CD69 (PE), α-CD103 (PECy7), α-CD62L (APC) and α-CD44 (FITC). **(A)** Representative dot plots and the percentage of **(B)** $CD4^+$ T_{NAIVE} cells, **(C)** $CD4^+$ T_{EM} cells, **(D)** $CD4^+$ T_{CM} cells and **(E)** $CD4^+$ T_{RM} cells after BBR treatment. **(F-I)** To analyse the activation status of key signaling molecules and transcription factors, the cells were stained with α-CD3 (Pacific Blue) and α-CD4 (PerCPCy5.5) followed by intracellular staining with antibodies against p-AKT and p-FOXO1 (see methods). **(F)** Representatives FACS scatter plots and **(G)** the percentage of $CD4^+$ T cells expressing pAKT. **(H&I)** Representative scatter plots and the percentage of $CD4^+$ T cells expressing p-FOXO1. **(J&K)** *Ex vivo* stimulated splenocytes were treated with BBR (10 µg/ml), AKTi (2.5µM) or both for 48 h followed by surface staining with α-CD3 (Pacific Blue), α-CD4 (PerCPCy5.5), α-CD69 (PE), α-CD103 (PECy7). **(J)** Percentage of $CD4^+$ T_{EM} and **(K)** $CD4^+$ T_{RM} cells. **(L-S)** Stimulation of transcription factors involved in memory responses

were examined for which the cells were stained with α-CD3 (Pacific Blue) and α-CD4 (PerCPCy5.5) followed by intracellular staining with antibodies against p-STAT3, p-STAT4, BlIMP-1 and p-NFκB (see methods). Representatives FACS scatter plots and percentage of $CD4^+$ T cells expressing **(L&M)** p-STAT3, **(N&O)** p-STAT4, **(P&Q)** Blimp-1 and **(R&S)** p-NFκB. **(T)** Expression of cytokines in *M.tb* specific T cells with or without BBR treatment. **(V-X)** *Ex vivo* stimulated splenocytes isolated from *M.tb* infected mice were treated with BBR (10 μg/ml), 2-Deoxy-D-glucose (2DG; 200 mM), both or left untreated for 24 h. **(U)** L-Lactate present in the supernatant of treated splenocytes. **(V)** Percentage of $CD4^+IFN\gamma^+$ T cells and **(W)** $CD4^+IL17^+$ T cells. The data values represent mean ± SD (n = 3-4). *p<0.05, **p<0.005, ***p<0.0005.

BBR induced adaptive memory enhances the BCG vaccine efficacy and reduces the rate of TB recurrence

Having established the potential of BBR to induce significant immunological memory against TB, we next investigated whether BBR co-treatment could enhance the BCG vaccine efficacy *in vivo*. C57BL/6 mice were divided in 3 groups: Cnt (un-vaccinated), BCG (BCG vaccinated) and BCG-BBR (BCG vaccinated and BBR treated), and were challenged with low dose of H37Rv through aerosol infection model **(Fig 5A)**. 30 days after *M.tb* infection, the mice were euthanized and analysed for bacterial burden and immune profiling. Pre-challenge immune profiling of the animals revealed increased activation of $CD4^+$ and $CD8^+$ T cells in the lungs and the spleen of the BCG vaccinated and BBR treated animals as compared to the BCG vaccinated alone. Consistent with this, BBR treatment during BCG vaccination significantly decreased the bacterial burden in the lungs **(Fig 5B)** and the spleen **(Fig 5C)** of co-treated mice as compared to only BCG vaccination. Immune analysis revealed increased percentage of $CD4^+$ and $CD8^+$ T cells in the lungs as well as in the spleen of co-vaccinated mice. BCG primarily induces effector memory responses as a result of which the anti-TB immunity induced by BCG is short lived and wanes in adults [41]. Interestingly, with no effect in the lungs, BBR treatment increased the $CD4^+$ and $CD8^+$ T_{CM} cells in the infected spleen. Furthermore, the percentage of T_{RM} cells was significantly high in the lungs **(Fig 5E & 5F)** and the spleen of infected BCG-BBR vaccinated and treated animals as compared to the BCG vaccination alone.

Memory T cells are vital for long-term immunity against disease relapse due to re-activation or re-infection. To further provide the *in vivo* evidence of the above results, we performed reactivation study in murine model **(Fig 5G)**. BBR co-therapy significantly reduced the rate of disease re-activation **(Fig 5H)** further proving that BBR treatment generates long-term *M.tb* specific protective memory responses with boosted T_{CM} and T_{RM} populations **(Fig 5I-5K)** in the lungs of infected mice. Furthermore, in re-infection murine model of TB **(Fig 5L)**, the bacterial burden was significantly reduced in the lungs **(Fig 5M)** and the spleen **(Fig 5N)** of re-infected mice previously treated with INH+RIF+BBR (BBR group) as compared with INH+RIF group (Cnt group). Immune profiling revealed increased percentage of T_{RM} cells in the lungs **(Fig 5O-5Q)** and the spleen of re-infected animals treated with INH+RIF+BBR. IL17 plays a crucial protective role in the recall protection to *M.tb* infection [6,11]. Interestingly, IL17 secreting T_{RM} population was enriched in the lungs **(Fig 5R-5T)** and the spleen of BBR treated mice. Similar trend was observed for the T_{CM} population in both the organs. Collectively, our preclinical mice and human data projects BBR as an excellent immunotherapeutic and immunoprophylactic candidate against susceptible and drug resistant TB.

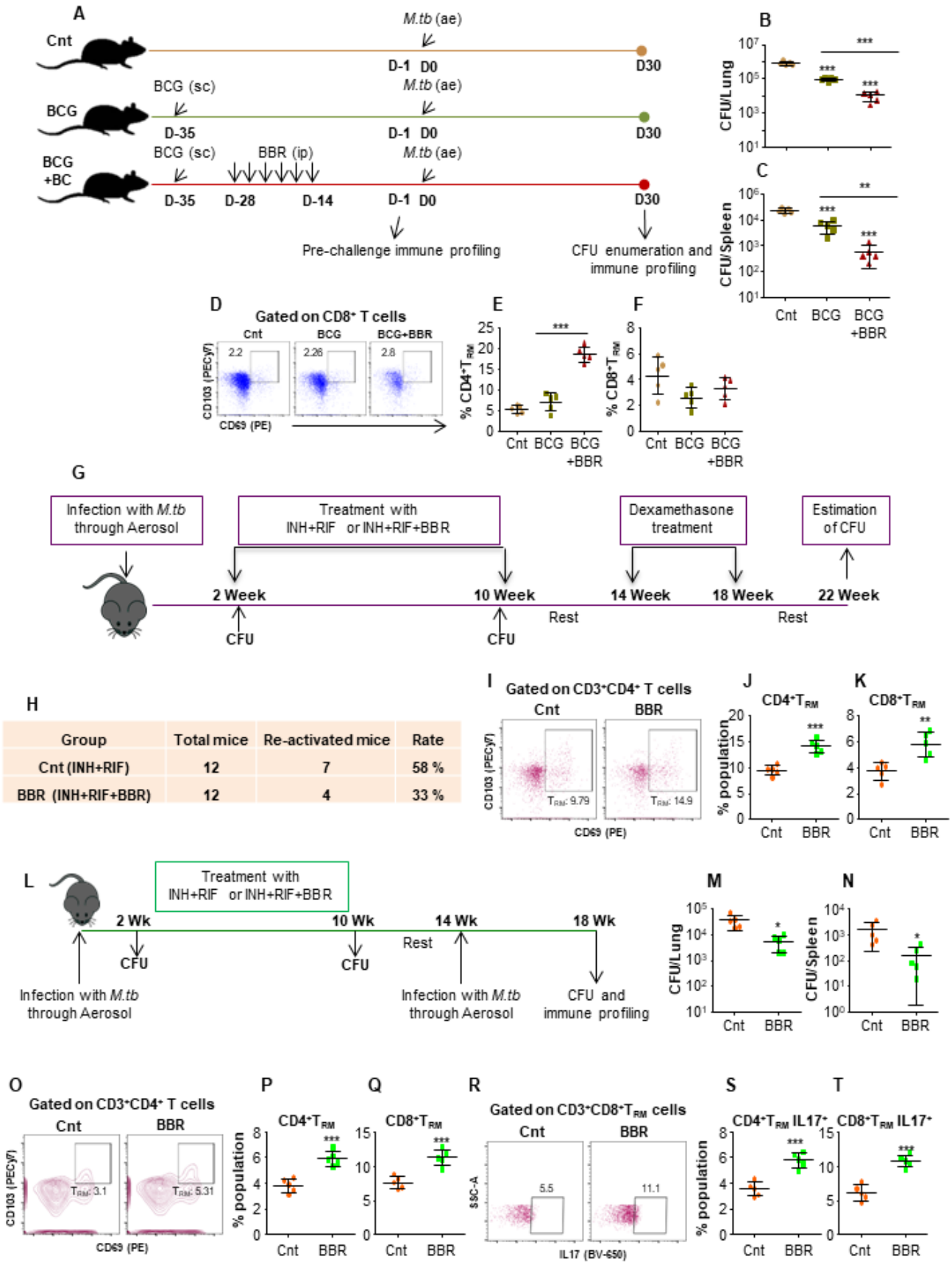

Fig 5: BBR enhances the BCG vaccine efficacy and protects against recurrent TB by inducing T cell resident memory responses in murine model. (A) Schematic representation of vaccine model used in the study. Bacterial load in **(B)** the lungs and **(C)** the spleen of infected mice. *Ex vivo*

stimulated lung cells were surface stained with α-CD3 (Pacific Blue), α-CD4 (PerCPCy5.5), α-CD69 (PE), α-CD103 (PECy7), followed by flow cytometry. **(D)** Representative FACS plots and percentage of **(E)** $CD4^+$ T_{RM} cells and **(F)** $CD8^+$ T_{RM} cells in the lung of infected animals. **(G)** Schematic diagram representing the re-activation model used in this study. **(H)** Rate of disease relapse with and without BBR treatment. **(I)** FACS plots representing CD69 and CD103 expressing $CD4^+$ T cells and **(J)** percentage of $CD4^+$ T_{RM} cells and **(K)** $CD8^+$ T_{RM} cells in the spleen of infected mice. **(L)** Diagrammatic representation of re-infection model used in the study. Bacterial burden in **(M)** the lungs and **(N)** the spleen of re-infected mice. *Ex vivo* stimulated single cell suspensions of the lungs were stained with α-CD3 (Pacific Blue), α-CD4 (PerCPCy5.5), α-CD69 (PE), α-CD103 (PECy7), α-CD62L (APC), and α-IL17 (BV650) followed by flow cytometry. **(O)** Representative FACS plots and the percentage of **(P)** $CD4^+$ T_{RM} cells and **(Q)** $CD8^+$ T_{RM} cells in the lungs of re-infected mice. **(R-T)** Percentage of resident memory T cells producing IL17. Data is representative of two independent experiments. The data values represent mean ± SD (n=5). *p<0.05, **p<0.005, ***p<0.0005.

STATISTICAL ANALYSIS:

All the experimental data was analysed using GraphPad Prism Software. Significant differences between the groups were determined by 2 tailed unpaired Student's t-test or 1-way ANOVA. Human data was analysed by 2 tailed paired Student's t-test. *p < 0.05, **p < 0.005, ***p< 0.0005.

DISCUSSION:

Immunological memory can be delineated as modification in immune responsiveness after the primary encounter to elicit prompt and robust immune responses.. Since an upsurge in incidences of drug-resistant TB along with recurrence and reactivation of *M.tb* infection is the root cause of morbidity and mortality worldwide, the utmost significance of immunological memory to combat TB remains unchanged [42]. Therefore, an efficacious immunomodulatory strategy is deemed indispensable to enhance population-wide immune protection to moderate the global TB burden. Combinatorial administration of immunotherapeutic has resolved inadequacies of numerous stratagems and was found advantageous in eliminating *M.tb* infections in several clinical trials [45]. One of the most established immunotherapeutic BBR has been known for ages for its effective therapeutic potential to treat diabetes and many other diseases [46,47]. In line with the previous literature [27], in this study we demonstrated the anti-mycobacterial potential of BBR in murine macrophages, and murine model against susceptible and drug-resistant strains of *M.tb*. Mononuclear phagocytic cells are prominent in activating T cells but during the pathogenic hijack, apoptotic inhibition occurs that restricts the presentation of bacterial antigen and further delays T cell mediated adaptive immune response [48]. BBR greatly enhanced the host defence mechanisms by modulating NF-κB and STAT-3 signaling [49].

BBR also increased the percentage of co-stimulatory molecules on mouse peritoneal macrophages and enriched the host protective chemokines, cytokines in response to *M.tb* infection which was further trailed in line with T cell responses

The therapeutic effects of BBR have been evaluated for diverse diseases in murine models, with no significant toxicity [50,51]. So, comprehensive immunological analysis was performed in the murine model of TB. BBR administration significantly reduced the bacterial load in adjunct to frontline anti-TB drug INH and resultant diminution in pathological damage in the lungs as reported recently [27]. . Our results also demonstrate the effectiveness of BBR treatment in significantly lowering the bacterial load in MDR and XDR infected animals. Hence, it can be stated decisively that BBR elicits anti-mycobacterial immune responses against a range of *M.tb* strains. Furthermore, with no prior information on BBR

induced mechanisms of protection against TB, we have comprehensively evaluated the impact of BBR on T cell signal transduction linking it with the establishment of durable immunological memory against *M.tb*.

To decipher the immunological feature of BBR induced reduction in bacterial burden, immune profiling was performed. Activation of adaptive immune cell populations was observed in compliance with an increase in the percentage and the activation of innate immune cells upon BBR treatment. IFNγ plays a vital role during *M.tb* infection by stimulating Th1 induction from naïve $CD4^+$ T cells [53] and IL17 plays a significant role by stimulating recall responses during recurrent *M.tb* infections [11,54,55] . In agreement with the previous reports, BBR treatment resulted in the increased percentage of IFNγ and IL17 secreting $CD4^+$ and $CD8^+$ T cells. Furthermore, BBR treatment enhanced expression of pro-inflammatory cytokines such as CCL2, IL12β, IL1β alone and in adjunct to INH. Furthermore, INH induced anti-inflammatory responses were countered along with BBR treatment.

Long-term memory particularly increased T_{CM} pool is vital for heightened immune responses against *M.tb* infections. Further, the T_{RM} population residing at the site of infection play a critical role in mediating diverse host protective effector functions. T_{RM} upon encountering antigen stimulates IFNγ production and recruits memory T cell and other immune cell populations to the site of infection [56,57]. Our research presents strong evidence of the augmented T_{RM} and T_{CM} responses upon BBR therapy alone and in adjunct to INH. These results were further strengthened by BCG vaccination experiments wherein we observed a striking reduction in the bacterial burden on administering BBR post BCG immunization in *M.tb* infected mice.Further, the biological evidence of long-term protective immune prophylactic effects of BBR were provided by re-infection and re-activation mice experiments wherein BBR treated animals displayed increased T_{CM} and T_{RM} cell responses leading to a significantly lower bacterial burden and reduced relapse rate.

To dwell into the immune mechanisms by which the attributes of immunological memory were enhanced upon BBR treatment, we performed *ex vivo* studies with *M.tb* specific T cells and observed that BBR treatment instigated significant differentiation of T_{NAIVE} population into T_{EM} and T_{RM} cells. *Ex vivo* BBR treatment elicited the expansion of T cell memory pool by modulating vital interconnected immune molecules such as AKT, FOXO-1, STAT-3, STAT-4, BLIMP-1 as well as NFκB. Instigation of memory establishment was in agreement with the enhancement of pro-inflammatory cytokines such as IFNγ, IL2, IL23, IL22, CCR7 and CXCR4 in BBR treated *M.tb* specific T cells. Furthermore, BBR induced metabolic flux towards glycolytic pathway thereby enhancing effector functions of $CD4^+$ T cells secreting key host protective cytokines- IFNγ and IL17.

Many times, the results obtained with mice studies poorly mimic conditions of human physiology. To ascertain the memory inducing potential of BBR in humans, we performed *ex vivo* experiments with PBMCs isolated from PPD^+ healthy donors. To our satisfaction, BBR treatment resulted in a significant reduction in $CD4^+$ T_{NAIVE} population with a concomitant increase in $CD4^+$ T_{EM}, T_{EMRA} and T_{RM} subsets. Moreover, BBR treated human PBMCs also displayed reduced phosphorylation of AKT which led to an enhanced FOXO1 activation as BBR/AKTi treatment synergistically decreased $CD4^+$ T_{NAIVE} population and increased T_{EM} and T_{EMRA} subsets. Additionally, BLIMP-1, a T_{RM} specific transcription factor was also upregulated in the BBR treated PBMCs [60]. Similar to the mice *ex vivo* experiments, BBR treatment heightened glycolytic flux in human PBMCs along with boosted pro-inflammatory cytokine profile. Furthermore, whole proteome analysis revealed upregulation of vital cellular processes in BBR treated human PBMCs including maintenance of glycolysis which is necessitated for T cell effector functions [61] and Notch signaling pathway critically known for T_{RM} establishment. Receptors, NOTCH1/3 and activators APHA1/PSEN were found to be

upregulated at the RNA as well as the protein levels in the BBR treated hPMBCs,. Convincingly, BBR treatment significantly induced the expression of PTEN and FOXO1 in human PMBCs. Based on these results; we propose a probable mechanism of action by which BBR enhances the immunological memory against TB **(Fig 6)**.

Finally, this study substantiates the prospects of BBR as a potent immunomodulator that strikingly augments protective immunological memory responses against *M.tb* infection. Further corroboration in the higher TB model that shares more similarities with humans such as non-human primates will be valuable to appraise BBR as promising host-directed therapy against susceptible and drug-resistant TB.

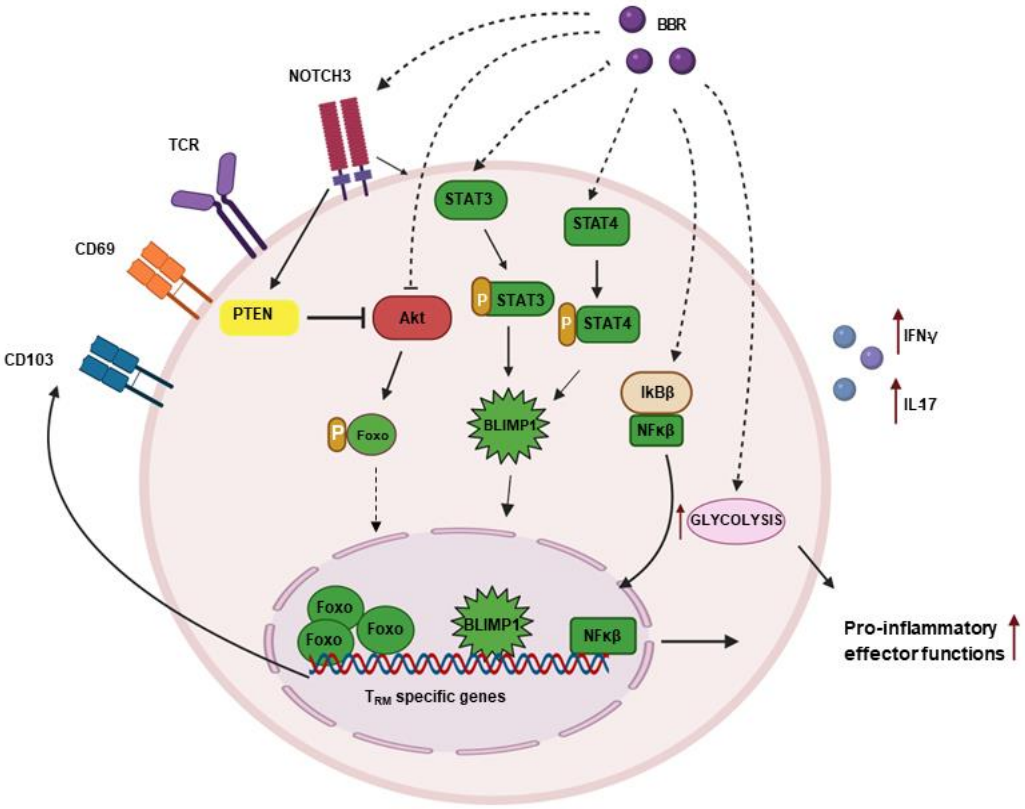

Fig6: BBR instigates host-protective immune responses against M.tb by directing key immunological signaling pathways. In response to *M.tb* infection, BBR establishes long-lived, host protective resident memory T cells (T_{RM}) at the site of infection. BBR enhances the effector functions of T lymphocytes by enhancing $CD69^{+}$ expression, directing metabolic flux towards glycolysis, activation of key host protective signaling pathways, and pro-inflammatory immune responses. BBR enriches pathways essential for the establishment and maintenance of memory T cells. BBR upregulates NOTCH3 which directs PTEN to simultaneously inhibit AKT and activate STAT signaling. AKT inhibition further decreases FOXO1 phosphorylation thereby enhancing its nuclear retention. BBR-mediated enhancement of activated STAT4 and STAT3-mediated BLIMP1 signaling axis further results in heightened expression of TRM-specific genes for long-term protection against *M.tb* infections.

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IMPACT OF THE RESEARCH IN THE ADVANCEMENT OF KNOWLEDGE OR BENEFIT TO MANKIND:

In the realm of TB research, despite extensive efforts to develop regimens targeting the4 bacteria, the available approved drugs remain limited, focusing solely on the microbial's aspect without addressing the host. Exploring immunomodulators holds significant promise in the field of TB, tapping into their considerable therapeutic potential. Notably, the WHO (World Health Organization) highlights that 80% of the global population relies on traditional medicines for their health needs. The vast reservoir of medicinal plants presents a valuable opportunity for investigating their substantial impact on combating TB. The interplay between microorganism and the immune system can disrupt immune responses, leading to prolonged illness and various immune related changes. To counteract this, immunomodulation emerges as a pivotal strategy for restoring balanced functioning within an organism's immune system. By focusing on immunomodulation, we can anticipate clinically meaningful outcomes. This approach not only minimises harm to the host but also holds the potential to influence the effects of coexisting illnesses. As we dwell deeper, our ultimate objective remains the discovery of a treatment that is concise, straightforward and economically viable.

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