Bovine serum albumin promotes reactivation of viable but non-culturable *Mycobacterium tuberculosis* via the activation of protein kinase-dependent cell division processes

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**Abstract**

Objective: *Mycobacterium tuberculosis* (Mtb) H37Ra strain has been reported to rapidly enter the viable but non-culturable (VBNC) state following treatment with an NADH oxidase inhibitor (diphenyleneiodonium [DPI]) and to be reactivated by fetal bovine serum (FBS). However, the mechanism underlying FBS-induced reactivation is unclear. We tried to reveal the mechanism of FBS-induced reactivation using *M. tuberculosis* H37Rv.

Methods: First, we evaluated the effect of DPI on culturability, viability and changes of acid-fastness toward H37Rv. Secondly, we measured the reactivation-promoting effects of human serum albumin, egg-white albumin and antioxidative agents in DPI-induced VBNC cells. We also inhibited adenylly cyclase and protein kinase which is the downstream of adenylly cyclase to evaluate the influence to reactivation capacity of bovine serum albumin (BSA).

Results: DPI treatment induced VBNC state in H37Rv, resulting in a high proportion of viable cells but a low proportion of culturable cells, loss of acid-fastness and lipid-accumulation. Not only FBS but also BSA alone could reactivate H37Rv. Contrary to our expectation, only human serum albumin had a similar restorative effect to BSA. The inhibition of adenylly cyclase by SQ22536 did not have a significant effect on reactivation; however, the inhibition of mycobacterial protein kinase by H89 and staurosporine strongly suppressed the BSA-induced reactivation.

Conclusion: DPI-induced VBNC Mtb cells may be reactivated via the activation of protein kinase-dependent cell division processes through interaction with BSA.

**Introduction**

*Mycobacterium tuberculosis* (Mtb) is known to as one of the intracellular parasitic bacteria that can survive inside host macrophages. It develops a latent phenotype, dormancy, due to various stresses such as host immune response, oxygen depletion, nutritional starvation, *etc.*
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The dormant Mtb cells can persist inside the host and cause latent tuberculosis infection (LTBI), which seeks reactivation. Lowering the risk of Mtb reactivation in LTBI patients is considered to be one of the important strategies to reduce the incidence of TB (1). The dormancy phenomenon in non-sporulating bacteria has been described as two major states: the viable but non-culturable (VBNC) state and bacterial persistence (2). VBNC cells are a nonculturable population that can be induced by exposure to stress. In addition, they cannot be grown on nutrient media, even after removal of stress, and require a much longer time or specific stimulus for reactivation (2, 3). On the other hand, persistent cells are stochastically arising cells within growing cultures that are tolerant to stress, including high-dose antibiotics, and they are also growth-arrested. However, they are typically able to grow on nutrient media within a short period after the removal of stress (2).

Many pathogenic bacteria, including Mtb, can enter into the VBNC state, and it is considered to be their natural state in the environment including human body (4). Various *in vitro* models, such as models of hypoxia, nutritional starvation (including potassium-deficiency), a lipid-rich environment, and other multiple stresses are reported to induce a VBNC state in Mtb cells (5–9). A deep understanding of the mechanisms of reactivation is necessary for the prevention of active tuberculosis.

One of the major hypotheses regarding the mechanism underlying the formation of VBNC bacteria involves oxidative damage generated by harsh external conditions (10). Thus, some studies have reported that the antioxidation during culture is one of the key mechanisms through which VBNC bacteria are reactivated (11, 12). Some mycobacterial culture media contain bovine serum albumin (BSA) and BSA on mycobacterial culture has been considered to be growth-supporting agent that is involved in detoxification by the absorption of free fatty acid and other growth-suppressing agents that are spontaneously generated during culturing (13–15). For this reason, the mycobacterial culture media that are most frequently used at the present time are BSA-containing systems, such as Middlebrook 7H series or Dubos medium.
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However, few studies have focused on the effect of such media on VBNC cells.

Mukamolova et al. revealed that among patients with active TB, there is certain subpopulation for whom sputum is not culturable in conventional medium, but which becomes to be culturable in the presence of resuscitation promoting factor (Rpf), which is a peptidoglycan-hydrolyzing protein that is highly conserved in actinobacteria (16). Rpf reactivates non-culturable Mtb cells both in vitro and in vivo (17), suggesting the presence of VBNC Mtb cells in clinical specimens.

Previously, NADH oxidase inhibitor diphenyleneiodonium chloride (DPI) was reported to simply and rapidly induce a VBNC state in the Mtb H37Ra strain, and incubation with fetal bovine serum, which is a common supplement for mammalian cell cultures, could facilitate the reactivation of VBNC cells, while incubation with OADC (oleate-albumin-dextrose-catalase) supplementation could not (18). This report gave us an important clue for constructing a simple and rapid assay system to induce and reactivate VBNC Mtb cells. However, when we tried to apply this system to H37Rv, we happened to find that DPI-induced VBNC cells that could be reactivated by not only fetal bovine serum but also by the addition of BSA—this was observed for both H37Rv and H37Ra (internal data). Then, we hypothesized that BSA would act as a reactivation-promoting agent toward DPI-induced VBNC cells.

One possible mechanism that may promote reactivation of BSA is its antioxidative property, which has been well-studied (19). Another possibility is a cellular processes involving cyclic AMP, an important second messenger of the cell. Shleeva et al. showed that the presence of high amounts of cAMP provided by adenylyl cyclase is also essential for the reactivation of M. smegmatis and Mtb from the VBNC state (20, 21). Notably, the overexpression of the Rv2212 gene, which encodes adenylyl cyclase in Mtb (22), significantly affects both entry into and reactivation from the VBNC state (21).

An understanding of the detailed mechanism of reactivation from the VBNC state is crucial for reducing the risk of reactivation of Mtb cells in LTBI patients. In this study, we examined
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the effects of antioxidative agents and the inhibition of adenylyl cyclase to reveal the roles of these factors in the reactivation of VBNC Mtb cells.

**Materials and Methods**

**Bacterial strains, growth media and culture conditions**

This work was carried out using *Mycobacterium tuberculosis* H37Rv (ATCC 27294) and H37Ra (ATCC 25177). Both cells were cultured for 3–5 days in a 60 mL glycol-modified polyethylene terephthalate (PETG) bottle containing 20 mL Dubos broth (2.5 g/L Na₂HPO₄, 2.0 g/L L-asparagine, 1.0 g/L KH₂PO₄, 0.5 g/L pancreatic digest of casein, 0.2 g/L Tween 80, 0.5 mg/mL CaCl₂•2H₂O, 0.1 mg/mL CuSO₄, 0.1 mg/mL ZnSO₄•7H₂O, 0.05 g/L ferric ammonium citrate, 0.01g MgSO₄•7H₂O) with 5% (v/v) glycerol, and 10% (v/v) ADC supplementation (5.0 g bovine serum albumin fraction V (BSA), 2.0 g dextrose, 0.003 g catalase in 100 mL distilled water) at 37°C in an orbital shaker (100 rpm) until the OD₆₀₀ reached up to 0.35. All procedures were performed at a BSL-3 facility. For further analysis, we used commercially available BSA Cohn fraction V (Merck, Darmstadt, Germany), unless otherwise stated. In addition, we used molecular biology grade Tween 80 (Merck product number P5188-100ML) without any predilution.

**Counting the number of culturable cells**

The cultured bacterial suspension was serially diluted 10-fold in phosphate buffered saline (pH 6.8) with 0.1%(w/v) Tween 80 (PBS-T) and 25 µL of each diluent was inoculated onto a Middlebrook 7H10 agar plate supplemented with oleic-albumin-dextrose-catalase (OADC) (Becton Dickinson, Sparks, MD) in duplication. Colonies were counted after at least 3 weeks’ incubation at 37°C under 5% CO₂. The limit of detection was determined to be 20 CFU/mL because of the diluent factor.

**Effect of BSA on the growth of Mtb cells in Dubos medium**
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Ten milliliters of log phase culture of Mtb H37Rv that reached an OD_{600} of up to 0.35 in Dubos medium, as described previously, was sedimented at 3,000 \times g for 10 minutes at 4^\circ C and the supernatant was discarded. The sediment was washed twice with fresh Dubos broth and resuspended with 10 mL of Dubos broth. The suspension was diluted to 1:10 with Dubos broth with or without the addition of BSA solution (5.0 g BSA fraction V in 100 mL distilled water) at the final concentration of 0.1% (w/v) BSA. The diluted suspension was dispensed into 24-well plates (Sarstedt, Nümbrecht, Germany) in 1.5 mL increments and sealed with gas-permeable film (Breathe-Easy, Diversified Biotech Inc, Dedham, MA, United States). Cultures were incubated for 5, 10, 15 and 20 days at 37^\circ C under 5% CO_2. At each time-point, the number of culturable cells was measured as described above.

**Induction of the VBNC cells**

Induction of the VBNC cells were performed according to the previous report (18) with some modification. In short, the log phase culture of Mtb H37Rv or H37Ra that reached an OD_{600} of up to 0.35 in Dubos medium, as previously described, was evenly divided into 30 mL PETG bottles and 5 mg/mL diphenyleneiodonium chloride (DPI) in dimethyl sulfoxide (DMSO) solution was directly added at the final concentration of 4 µg/mL with immediate agitation. The same volume of DMSO was added to the untreated control sample. Cultures were further incubated for 24 h at 37^\circ C without shaking. After incubation, their culturability and viability were measured as described above.

**Cell viability assay**

Mtb cell viability was measured using esterase activity and membrane integrity of the cells using dual staining with 10 mg/mL carboxyfluorescein diacetate (CFDA, Dojindo, Kumamoto, Japan) and ethidium bromide (EtBr, Dojindo, Kumamoto, Japan). Briefly, 500 µL of DPI-treated or untreated Mtb culture was sedimented by centrifugation at 15,000 \times g for 5 minutes at 4^\circ C and washed twice with the same volume of PBS-T. The washed bacterial sediment was
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resuspended in 200 µL of PBS-T with CFDA and EtBr incubated at room temperature (RT) for 30 minutes in shade. The final concentration of CFDA and EtBr were 60 µg/mL and 2 µg/mL, respectively. The stained Mtb cells were washed twice with PBS-T and fixed with 3.7% formaldehyde solution at RT for at least 2 h. After fixation, 10 µL of bacterial suspension was smeared on three APS-coated 8-well slides (Matsunami glass, Osaka, Japan) and air-dried. Then the slides were mounted with DPX new non aqueous mounting medium (Merck, Darmstadt, Germany) and a 150 µm-thick cover glass (Matsunami glass, Osaka, Japan) prior to the microscopic observation. Specimens were examined with a BX53 fluorescence microscope (Olympus, Tokyo, Japan) at a magnification of 100× for green (esterase-active with intact membrane; live) or red (esterase-negative with damaged membrane; dead) fluorescing cells. The excitation source for CFDA and EtBr was generated from a mercury-arc lamp (blue beam, 470–495 nm; green beam, 530–550nm) using a corresponding band-pass filter. The green-fluorescence emission from the CFDA-positive cells was collected through a 510–550 nm band-pass filter. The red-fluorescence emission from EtBr-positive cells was collected through a 575 nm long-pass filter. At least 10 random fields were observed for each sample and the images (TIFF format) were analyzed using the Fiji/ImageJ software program (23). The live/dead ratio was calculated by direct counting of green or red fluorescing cells.

**Auramine-O/Nile Red dual staining**

Loss of acid-fastness and accumulation of neutral lipids are distinctive features of VBNC mycobacteria (24). To detect the phenotype, fluorescent acid-fast staining with auramine-O and neutral lipid staining with Nile Red (9-dimethylamino-5H-benzo-α-phenoxadine-5-one) were performed using a previously described method (9) with some modification. Briefly, 10 µL of DPI-treated or untreated culture were smeared onto an APS-coated slide (Matsunami glass, Osaka, Japan) and air-dried. Then, the slide was heat-fixed and cooled down to room temperature before staining. The smear was flooded with fluorochrome staining solution (10
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µg/mL auramine-O in 5% [w/v] phenol solution) and incubated at room temperature for 20 min in shade. Excessive dye was removed using 3% hydrochloric acid ethanol for 15 minutes. Next, the smear was covered with 10 µg/mL Nile Red in ethanol and incubated at room temperature for 15 minutes. Finally, the smear was counterstained with 0.1% (w/v) potassium permanganate solution with 1 minute. Stained slides were air-dried and mounted as described previously.

Fluorescence microscopy was performed at 100× magnification for green (acid-fastness positive, lipid accumulation negative; active) or red (acid-fastness negative, lipid accumulation positive; VBNC) fluorescing cells. The excitation source for auramine-O and Nile Red was generated from a mercury-arc lamp (blue beam, 470–495 nm; green beam, 530–550 nm) using a corresponding band-pass filter. The green-fluorescence emission from auramine-O-positive cells was collected through a 510–550 nm band-pass filter. The red-fluorescence emission from Nile Red-positive cells was collected through 575 nm long-pass filter. At least 5 random fields were observed for each sample and images (TIFF format) were analyzed by the Fiji/ImageJ software program (23) and the proportion of acid-fast-positive cells and lipid-accumulation-positive cells was calculated by direct counting of green or red fluorescing cells.

**Ziehl-Neelsen staining**

Ziehl-Neelsen staining was also performed to confirm the loss of acid-fastness resulting in the VBNC state by DPI treatment, according to the standard method (25). The slide was observed by light microscope under 100× magnification with an oil immersion lens (BX53, Olympus, Tokyo, Japan).

**Confocal super-resolution microscopy**

Detailed images of accumulated lipids were also obtained by a confocal laser-scanning microscope (LSM900 with Airyscan 2, Carl Zeiss Microscopy, Jena, Germany). Briefly, culture slides of DPI-treated cells dual stained with auramine-O and Nile Red, as described above, were observed at 63x magnification with oil immersion lens. The excitation source of
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auramine-O and Nile Red was 488 nm (blue beam) and 563 nm (green beam), from a solid-state laser unit. The green-fluorescence emission from auramine-O and red fluorescence from Nile Red were sequentially collected through a variable dichroic beamsplitter and an Airyscan 2 detector. Super-resolution images were processed with the ZEN Blue software program (Carl Zeiss, Jena, Germany).

**VBNC reactivation assay**

The VBNC reactivation assay was performed as described elsewhere (18) with some modification. DPI-treated culture was sedimented by centrifugation at 3,000 xg for 10 minutes at 4˚C and washed twice with fresh Dubos broth, then resuspended in ten-milliliters of Dubos broth. The bacterial suspension was diluted to 1:10 with fresh Dubos medium with or without 0.1% (w/v) BSA. For both BSA-containing and BSA-free series, OADC supplementation (Becton Dickinson, Sparks, MD), fetal bovine serum (FBS; Moregate BioTech, Bulimba, Queensland, Australia), or sodium pyruvate (PA; Merck) was added. The final concentration of each reagent was as follows: 10% (v/v) for OADC supplementation, 2% (v/v) for FBS, and 3 mM for PA. Then, the diluted suspension was dispensed into 24-well plates in 1.5 mL increments and sealed with gas-permeable film. Cultures were incubated for 5, 10, 15 and 20 days at 37˚C under 5% CO₂. At each time-point, the number of culturable cells was measured as described previously.

**Evaluation of the reactivation-promoting effect of human serum albumin, egg-white albumin (ovalbumin) and antioxidative agents**

To determine whether the reactivation-promoting effect of BSA is its antioxidative property, we measured the reactivation-promoting activity of other albumins and antioxidative agents toward DPI-treated *Mtbc* cells. Washed DPI-treated cells were resuspended with fresh Dubos broth without BSA and the suspension was diluted to 1:10 with Dubos broth containing BSA, human serum albumin (HSA), ovalbumin (OVA), N-acetyl-L-cysteine (NAC) or D-mannitol
BSA promotes reactivation of VBNC *M. tuberculosis* (MAN). The final concentration of each reagent was 0.1% (w/v) for BSA, HSA and OVA, 500 µM for NAC, and 50 mM for MAN, respectively. Then, the diluted suspension was dispensed into 24-well plates in 1.5 mL increments and sealed with gas-permeable film. The plate was incubated for 20 days at 37°C under 5% CO₂. At the end of incubation, the number of colonies grown was measured as described previously.

**Evaluation of the reactivation-promoting effect of fatty acid and globulin-free BSA**

To determine the effect of BSA Cohn fraction V contaminants, we measured the reactivation-promoting activity of fatty acid and globulin-free BSA toward DPI-treated VBNC Mtb cells. Washed DPI-treated cells were resuspended with fresh Dubos broth without BSA and the suspension was diluted to 1:10 with Dubos broth containing BSA Cohn fraction V or fatty acid and globulin-free BSA. Fatty acid and globulin-free BSA were acquired from Merck. The final concentration of both BSAs was 0.1% (w/v). The number of culturable cells was measured as described previously.

**Effects of adenylyl cyclase or protein kinase on BSA-induced reactivation**

Washed DPI-treated VBNC Mtb cells were resuspended with fresh Dubos broth and the suspension was diluted to 1:10 with Dubos broth with 0.1% (w/v) BSA containing adenylyl cyclase inhibitor SQ22536 (Merck, Darmstadt, Germany) or protein kinase inhibitor H89 (Abcam, Cambridge, United Kingdom) or staurosporine (Merck). The final concentration of each inhibitor was as follows: 0.1 to 10 mM for SQ22536, 1 to 30 µM for H89 and 1 to 10 µM for staurosporine. Then, the number of culturable cells were measured as described previously.

**Molecular docking simulation**

A molecular docking simulation was performed using the AutoDock Vina software program, an open-source molecular docking program designed by the Scripps Research Institute (26, 27). The 3D structures of receptor proteins, PknA and Rv2212, were obtained from the Protein Data Bank.
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Bank (28) and AlphaFold Database (29, 30), respectively. The PDB ID for PknA was 4OW8 (31) and the AlphaFold Database identifier for Rv2212 was AF-P9WMU7-F1. We removed water molecules from proteins, added polar hydrogen and charge, and adjusted the X-Y-Z coordinates and grid size to optimize molecular docking. The 3D structures of ligand molecules, H89, SQ22536 and ATP were obtained from the PubChem Database. The PubChem CIDs of each ligand were 449241, 5270 and 5957, respectively. Graphical processing was performed using the Chimera software program (32).

Statistical analysis

The statistical analysis was performed with R (33) using the EZR on R package (34). *P* values of < 0.05 were considered statistically significant.

Results

I. **BSA is not essential for the growth of MTB in Dubos medium with purified non-prediluted Tween 80**

Figure 1 shows the different effects of BSA-containing and BSA-free Dubos medium on the growth of Mt H37Rv. As shown in Fig. 1(A), the growth rates with the two conditions did not differ to a statistically significant extent. Fig. 1(B) also shows the sufficient growth of cells. The cells were more aggregative without BSA; however, the aggregation was easily dispersed by pipetting and there was no significant difference in the OD$_{600}$ value. The albumin component was inessential for Mt growth.

II. **DPI treatment induced a VBNC state in Mt H37Rv**

Figure 2 shows the DPI treatment results for the induction of VBNC state in H37Rv and H37Ra (18). As shown in Fig. 2(A), the numbers of culturable cells of untreated control
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population and DPI-treated population were significantly different: $4.43 \pm 1.43 \times 10^8$ CFU/mL for the untreated control population and $4.64 \pm 6.10 \times 10^3$ CFU/mL for DPI-treated population. Fig. 2(B) shows that the CFDA-positive cells in the untreated control and DPI-treated populations were $81.8 \pm 11.4\%$ and $65.9 \pm 11.8\%$, respectively. These results suggested that the majority of DPI-treated H37Rv cells were alive ($p = 0.169$), while their culturability was significantly reduced ($p = 0.033$). Representative images of CFDA/EtBr dual-stained cells of the untreated control and DPI-treated population also showed that there was little difference in the proportions of CFDA-positive and EtBr-positive cells. (Fig. 2[C])

Auramine-O/Nile Red dual staining showed the reduction of acid-fastness and the accumulation of the neutral lipid. As shown in Fig. 2(D), the proportion of acid-fast-negative/lipid accumulation-positive cells was $84.0 \pm 14.0\%$ in the DPI-treated population, and $12.1 \pm 11.9\%$ in the untreated control population ($p < 0.01$). Representative images of auramine-O/Nile Red dual-stained cells of the untreated control and DPI-treated populations also showed that there were distinctive differences in the proportions of acid-fast-positive cells and lipid-accumulation-positive cells (Fig. 2[E]). The loss of acid-fastness was also confirmed by Ziehl-Neelsen staining; the majority of DPI-treated population were found to have lost their acid-fastness (Suppl. Fig. 1). Interestingly, Airyscan microscopy revealed some DPI-treated cells were both acid-fast stain-positive and neutral lipid stain-positive (Fig. 2[F]). These results indicated that DPI treatment induced the VBNC state in H37Rv as well as in H37Ra.

### III. The effects of BSA, FBS, OADC and sodium pyruvate on the reactivation of DPI-treated VBNC Mtb cells

As shown in Fig. 3(A) and (B), our results partially support the previous study (18) with some differences; although the culturability of DPI-treated H37Rv cells was restored by incubation with FBS-supplemented medium, OADC-supplemented medium could also induce culturability. The addition of albumin alone into the medium could also induce reactivation.
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284 The addition of FBS in BSA-free Dubos medium slightly reduced the regrowth rate; however, cells were successfully reactivated at the end of incubation with or without FBS. These phenomena were also observed in H37Ra (Suppl. Fig 2). Thus, we used the H37Rv strain for the further analyses in this study.

Incubation with sodium pyruvate, which was reported to have a reactivation-promoting effect on VBNC cells elsewhere, led to transient regrowth by day 15 and a slight reduction at day 20. (Suppl. Fig. 3[A] and [B]).

We also confirmed the presence of a small population of intact Mtb cells (10^3 CFU/mL) that could grow normally in BSA-free Dubos medium, suggesting that the reactivation might not be due to the presence of a small number of culturable cells after DPI-treatment (data not shown).

IV. The antioxidative property or the fatty acid from BSA did not promote reactivation.

As shown in Fig. 4(A) and (B), the reactivation-promoting effect of BSA was specific to bovine and human serum albumin. Ovalbumin did not show a reactivation-promoting effect but maintained the number of culturable cells in this system. NAC (antioxidative agent) and D-mannitol (free radical scavenger) did not show any reactivation capacity.

We also checked whether the purity of albumin affects the promotion of reactivation using fatty acid and globulin-free BSA and confirmed that there was no significant difference in reactivation-promoting effects (Suppl. Fig 4). These results suggest that commercially available albumin including fatty acids do not affect the reactivation of DPI-treated Mtb.

V. The reactivation-promoting effect of albumin was canceled by treatment with eukaryotic protein kinase inhibitor.
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As shown in Fig. 5(A) and (B), SQ22536, adenylyl cyclase inhibitor, only suppressed reactivation at a very high concentration (10 mM) in this study. However, H89, a eukaryotic protein kinase inhibitor, suppressed reactivation in a dose-responsive manner. In detail, incubation with 10 µM or 30 µM H89 resulted in a CFU/mL value for $3.42 \times 10^6 \pm 7.45 \times 10^5$ CFU/mL and $3.63 \pm 1.70 \times 10^2$ CFU/mL, while incubation without H89 resulted in $3.00 \times 10^8 \pm 5.07 \times 10^7$ CFU/mL. We also confirmed that staurosporine, which is known as mycobacterial protein kinase PknB inhibitor (35), suppressed reactivation at a concentration of 10 µM and resulted in a CFU/mL value of $2.09 \times 10^3 \pm 7.62 \times 10^2$ CFU/mL, while incubation without staurosporine resulted in $3.58 \times 10^8 \pm 5.78 \times 10^7$ CFU/mL (Fig 5[A] and [B]). None of the inhibitors used in this study caused a reduction of the growth of intact MTB cells (Suppl. Fig. 5A and 5B).

We also performed a molecular docking simulation of these inhibitors toward their considerable targets on Mtb. As shown in Suppl. Table 1, both SQ22536 and H89 have sufficient affinity toward Rv2212 (adenylyl cyclase) and PknA (protein kinase), respectively.

**Discussion**

In this study, we confirmed that DPI could induce a VBNC state in H37Rv as well as H37Ra. The mechanism underlying the effect of DPI is considered to involve the inhibitory effect of NADH oxidase, which results in the inhibition of the electron transport system of Mtb. This may induce a hypoxic response in the liquid culture, resulting in a significant decrease of culturability (Fig.2[A]). However, viability was highly retained (65.9%), suggesting that the majority of the cells transformed to VBNC (Fig. 2[B]). Additionally, VBNC transformation was confirmed by auramine-O and Nile Red staining. The loss of acid-fastness and the accumulation of neutral lipids are distinctive features of VBNC mycobacterial cells.

Our data also demonstrated that the majority of DPI-treated Mtb H37Rv cells were stained with Nile Red alone (Fig. 2[D]). Airyscan super-resolution microscopy showed the presence of
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cells that were stained with auramine-O and which contained an intracellular lipid body stained with Nile Red (Fig. 2[F]). This could also reveal the distribution of the lipid body in the cell as some foci of relatively strong signals of Nile Red, suggesting the transformation from a growing state to VBNC, with drastic alteration of the lipid metabolism.

Secondly, we found that DPI-induced VBNC could facilitate reactivation not only by incubation with FBS but also with OADC supplementation and BSA alone, suggesting that albumin might act as a reactivation-promoting agent in both H37Rv and H37Ra (Fig. 3 and Suppl. Fig. 2). These findings were contrary to the findings of the previous study, which showed that only FBS can facilitate reactivation (18). We considered that the reason for the difference may be due to a contaminant in Tween 80: the presence of an excessive amount of oleic acid. We also confirmed that the reactivation was not facilitated by the use of prediluted Tween 80 for the preparation of Dubos medium (internal data) for both H37Rv and H37Ra. Thus, our results suggested that the key reactivation-promoting agent in FBS might be serum albumin, and further analyses were performed using H37Rv.

We also tested the reactivation-promoting effect of BSA in Wayne’s hypoxic culture, which is widely used for inducing a VBNC of Mtb, and found that there was no significant difference between cultures with and without BSA (data not shown).

Pyruvate, which is known to act as reactivation promoting agent for both gram-negative bacteria, such as *Escherichia, Legionella, Vibrio* and gram-positive bacteria such as *Staphylococcus* (36–39), did not show any reactivation-promoting effect in our experiment (Suppl. Fig.3). Vilhena et al. reported that their proteomic analysis of VBNC *E. coli* cells during reactivation revealed that enzymes involved in pyruvate metabolism, pyruvate formate-lyase-activating protein (PflA), phosphoenolpyruvate carboxykinase (PckA) and lactate dehydrogenase (LldD), were significantly upregulated (40), while Wagley et al. also reported that VPA1499, an LldD homologue of *Vibrio parahaemolyticus*, was significantly upregulated in VBNC *V. parahaemolyticus* and that depletion of LldD led to quicker entry to the VBNC
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state (41). Interestingly, they showed that the addition of pyruvate, the product of lactate oxidation, to LldD-depleted mutant restored the wild-type characteristics of cells in the VBNC state. Their studies might give some crew of the lack of reactivation-promoting effect toward mycobacterial VBNC. Since Mtb does not have a catalytic center of pyruvate formate-lyase (PflB), pyruvate might not be able to act as reactivation-promoting agent, although pyruvate enhances the growth of Mtb and *M. bovis* both intracellular and extracellular growth by promoting TCA cycle and other energy-producing pathways. (42, 43).

Our results showed that the reactivation promoting effect of albumin might be structure-specific because ovalbumin (OVA) did not facilitate reactivation, but bovine (BSA) and human serum albumin (HSA) did. Focusing on their amino acid sequences, BSA and HSA share 76% sequence homology (44); however, BSA and OVA share less sequence homology (16%). One of the considerable roles of albumin may be as an antioxidative agent due to its cysteine residues with free thiols (19, 45). BSA and HSA have only one cysteine residue with free thiol, while OVA has four cysteines with free thiols (46). OVA therefore seems to be more reductive than BSA and HSA. Our results were paradoxical in relation to this hypothesis; however, Davis and Dubos showed the differential growth-promoting effect of albumin toward Mtb. Their study supplied some interesting clues for our present study; ovalbumin does not show a growth-promoting effect, while bovine and human serum albumin showed the effect (15). Moreover, the antioxidative agents used in this study, NAC and D-mannitol, showed any restorative effects (Fig. 4). We also examined catalase, a component of OADC supplementation, and its reactivation promoting effect toward VBNC *Corynebacterium, Lactobacillus, Salmonella, Ralstonia* and *Vibrio* (47–52); no restorative effects were observed (data not shown). These results suggest that the effect of albumin may be due to complex functions rather than its antioxidative property.

Serum albumin is known to act as a good carrier of many kinds of biologically active substances, including free fatty acid. It is also known as a detoxifier of growth-arresting
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substances in culture medium. BSA is added to mycobacterial culture medium as both a carrier and detoxifier of growth-supporting and growth-arresting substances, respectively. Shleeva *et al.* showed that several types of unsaturated free fatty acid, including oleic, linoleic, and arachidonic acids, can promote the reactivation of VBNC *M. smegmatis* in modified Sauton’s medium and oleic acid also works for Mtb (20). In this study, we should note that impurities of albumin did not affect reactivation. Although the underlying mechanism is still unclear, both fatty acid and globulin-free BSA and BSA Cohn fraction V showed a similar reactivation-promoting effect toward DPI-treated Mtb (Suppl. Fig. 4).

The reactivation inhibition assay by SQ22536, H89 and staurosporine gave us an important clue for understanding the effect of BSA. In the present study, we could suppress the reactivation of DPI-induced VBNC cells by inhibiting adenylyl cyclase only with a very high concentration of SQ22536. Although the underlying mechanism is unclear, adenylyl cyclase may not play a key role in reactivation from a DPI-induced VBNC state, while the inhibition of protein kinase by H89 and staurosporine clearly suppressed reactivation. These results suggest that protein kinase might mainly be involved in the reactivation of VBNC Mtb cells triggered by BSA and that the contribution of adenylyl cyclase may be smaller than that of protein kinase.

*Mtb* has the eukaryotic-type Ser/Thr protein kinases PknAB and PknD-L. For example, PknA regulates major cell processes, regulates several types of proteins involved with mycobacterial cell division and morphogenesis via phosphorylation of such proteins in coordination with PknB (Manuse *et al.*, 2016; Carette *et al.*, 2018); phosphorylation of GlmU (Rv1018c) and MurD (Rv2155c), Wag31 (Rv2145c), HupB (Rv2986c) and ParB (Rv3917c), influence the production and cross-linking of peptidoglycan precursors (55–58), the spatial localization of peptidoglycan synthesis (59), DNA condensation and partitioning (60, 61), respectively. It should be noted that the phosphorylation of FtsZ and FipA by PknA controls cell division under oxidative stress (62, 63). In addition, mycobacterial protein kinase has
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cyclic AMP-dependent protein kinase domains which is very similar to eukaryotic version, which was shown in PknB (64) and staurosporine, which act as PknB inhibitor, could also inhibit reactivation of DPI-induced VBNC Mtb (Fig. 5).

Our study suggested that the inhibition of mycobacterial protein kinase by H89 and staurosporine seems to critically affect several important cellular processes, followed by reactivation (Fig. 6). To confirm the essentiality of mycobacterial protein kinase and other involved proteins toward reactivation from this DPI-induced VBNC state by albumin, further studies using experiments such as knock-down of genes using the CRISPR interference (CRISPRi) technique (65), because such proteins—with the exception of FipA (Rv0019c)—are known to be essential for *in vitro* growth (66–69); thus, manipulating their coding genes is not permissible.

**Conclusion**

Our data showed that the presence of BSA in Dubos medium not only depleted growth-suppressing substances but also could trigger the reactivation of the DPI-induced VBNC state in both H37Rv and H37Ra strains. The reactivation-promoting effect of other kinds of albumins and antioxidative agents, including NAC and D-mannitol was tested; however, only human serum albumin showed a reactivation-promoting effect. The results of the inhibition assay of reactivation using SQ22536, H89 and staurosporine suggests the possibility that the inhibition of mycobacterial protein kinase suppressed reactivation to a much greater extent than adenyllyl cyclase, indicating the presence of a reactivation-promoting pathway that is protein kinase-dependent.

To the best of our knowledge, few studies have focused on the reactivation-promoting effect of albumin toward VBNC Mtb. Taken together, our findings indicated that the reactivation of MTB from a DPI-induced VBNC state could be obtained by interaction with bovine serum albumin and the mechanism of albumin-induced reactivation may involve the activation of
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mycobacterial protein kinase, which regulates several important processes of cellular component construction and cell division. Although it remains unclear how albumin interacts with VBNC cells, our study may supply some important clues to understanding the physiology of the mycobacterial VBNC state.

**Author Contributions**

YMori, YMura and SM conceived and designed the experiments. YMori performed experiments. YMura, KC, AA, YI, YS, MH, KK, HY, AT, and SM assisted in the experiments. YMori acquired data and YMori and YMura analyzed data. YMori performed molecular docking simulation and statistical analysis. YMori and SM wrote the manuscript. All authors contributed to the article and approved the submitted version.

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**Conflict of Interest Statement**
The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.
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**Figure legends**

> Figure 1. Growth curves of Mtb H37Rv cells in Dubos medium in the presence or absence of BSA

(A) Number of culturable cells in Dubos medium in the presence (closed circle) or absence (open circle) of 0.1% BSA at the indicated days of incubation. The CFU/mL values were determined by plating cells onto 7H10 plates in duplicate. Data represent the mean ± SD from three independent experiments.

(B) Representative images of Mtb growth captured at the end of incubation.

> Figure 2. Viability analyses of DPI-treated Mtb

(A) Number of culturable cells after DPI treatment. The CFU/mL values were determined by plating cells onto 7H10 plates in duplicate. Asterisk indicates a statistically significant difference (*p* < 0.05, Welch’s *t*-test). Data represent the mean ± SD from three independent experiments.

(B) Percentages of esterase active cells after DPI treatment. Around 850 cells were directly counted under 100× objective lens. Data represent the mean ± SD from three independent experiments.

(C) Merged fluorescence micrographs of Mtb H37Rv cells stained with carboxyfluorescein diacetate (CFDA) and ethidium bromide (EtBr). Cells stained with CFDA (green) are esterase-positive; those stained with EtBr (red) are membrane-damaged. Images are representative images from three individual experiments. Scale bar: 2µm

(D) Percentage of acid-fast-positive cells (open column) or acid-fast-negative but lipid-accumulation-positive cells (gray column). Approximately 1,100 cells were directly counted under 100× objective lens. Data represent the mean ± SD from four
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independent experiments. Asterisk indicates significant difference (**p < 0.005, Welch’s t-test). 

(E) Merged fluorescence micrographs of Mtb H37Rv cells stained with auramine-O and Nile Red. Cells stained with auramine-O (green) are acid-fast-positive; those stained with Nile Red (red) are acid-fast-negative but lipid accumulation-positive. Images are representative images from three individual experiments. Scale bar: 2µm

(F) Magnified views of Airyscan confocal super-resolution microscopy observation of three different Mtb cells obtained from DPI-treated culture. Only acid-fast-positive cells (left), both acid-fast-positive and lipid accumulation-positive cells (center, counted as acid-fast-positive) and acid-fast-negative cells with only lipid accumulation-positive cells (right). Dual-positive cells probably represent the transition to a non-culturable state. Indicated foci with arrowheads represent a neutral lipid body. Scale bar: 1 µm

Figure 3. Reactivation of DPI-treated Mtb H37Rv cells by FBS and OADC supplementation

(A) Number of culturable cells at the indicated days of incubation, without supplementation or those cells supplemented with 2% FBS or 10% OADC in the presence (closed circle) or absence (open circle) of 0.1% BSA. The CFU/mL values were determined by plating cells onto 7H10 plates in duplicate. Data represent the mean ± SD from three independent experiments. U.D. means under the limit of detection (20 CFU/mL).

(B) Representative images of regrowth under the indicated conditions captured at the end of incubation. None, FBS, and OADC represent no supplementation, 2% FBS and 10% OADC supplementation, respectively.

Figure 4. Effect of human serum albumin, ovalbumin and antioxidants on the reactivation of
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DPI-treated Mtb.

(A) Number of culturable cells at the end of incubation, supplemented with albumins or antioxidative agents. The CFU/mL values were determined by plating cells onto 7H10 plates in duplicate. Data represent the mean ± SD from three independent experiments. Asterisk indicates a statistically significant difference between Day 0 and Day 20 by Student’s *t*-test. (*p<0.05)

(B) Representative images of regrowth captured at the end of incubation.

Abbreviations:
BSA, bovine serum albumin; HSA, human serum albumin; OVA, ovalbumin; NAC, N-acetyl-L-cysteine; MAN, D-mannitol.

The final concentrations of each reagent were as follows:
BSA, HSA and OVA: 0.1% (w/v), NAC: 500 µM, MAN: 50 mM.

Figure 5. The effect of SQ22536 or H89 or staurosporine on the reactivation of DPI-treated Mtb

(A) Number of culturable cells at the end of incubation, supplemented with 0.1% BSA and SQ22536 or H89 or staurosporine. Inhibitor was added at the indicated concentration.

(B) Representative images of regrowth captured at the end of incubation.

The CFU/mL values were determined by plating cells onto 7H10 plates in duplicate. Data represent the mean ± SD from three independent experiments.

Figure 6. Schematic representation of the mechanism of BSA-induced reactivation of Mtb.

Although the detailed mechanism of interaction between BSA and PknA/PknB is not yet
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clear, BSA may activate PknA/PknB and positively regulate target proteins that are involved in the localized synthesis of peptidoglycan, cell division control, and DNA condensation and partitioning through their phosphorylation, resulting in the reactivation of VBNC Mtb cells.
Fig 1.
Fig 2.

(A) Culturable cells (CFU/mL) for Control and DPI treatments.

(B) CFDA-positive cells (%) for Control and DPI treatments.

(C) Fluorescence images showing Control and DPI treatments.

(D) Acid-fast or lipid accumulation-positive cells (%) for Control and DPI treatments.

(E) Fluorescence images showing Control and DPI treatments.

(F) Magnified images highlighting specific features in Control and DPI treatments.
Fig 3.

(A) Culturable cells (CFU/mL) over regrowth (days) for different conditions: None, FBS, and OADC.

(B) Images showing regrowth for different conditions: None, FBS, and OADC with BSA (-) and BSA (+).
Fig 4.
Fig 5.
Fig. 6

Localized synthesis of peptidoglycan

Cell division control

DNA condensation and partitioning

Reactivation