



Multifaceted remodeling by vitamin C boosts sensitivity of *Mycobacterium tuberculosis* subpopulations to combination treatment by anti-tubercular drugs

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ABSTRACT

Bacterial dormancy is a major impediment to the eradication of tuberculosis (TB), because currently used drugs primarily target actively replicating bacteria. Therefore, decoding of the critical survival pathways in dormant tubercle bacilli is a research priority to formulate new approaches for killing these bacteria. Employing a network-based gene expression analysis approach, we demonstrate that redox active vitamin C (vit C) triggers a multifaceted and robust adaptation response in *Mycobacterium tuberculosis* (Mtb) involving ~ 67% of the genome. Vit C-adapted bacteria display well-described features of dormancy, including growth stasis and progression to a viable but non-culturable (VBNC) state, loss of acid-fastness and reduction in length, dissipation of reductive stress through triglyceride (TAG) accumulation, protective response to oxidative stress, and tolerance to first line TB drugs. VBNC bacteria are reactivatable upon removal of vit C and they recover drug susceptibility properties. Vit C synergizes with pyrazinamide, a unique TB drug with sterilizing activity, to kill dormant and replicating bacteria, negating any tolerance to rifampicin and isoniazid in combination treatment in both in-vitro and intracellular infection models. Finally, the vit C multi-stress redox models described here also offer a unique opportunity for concurrent screening of compounds/combinations active against heterogeneous subpopulations of Mtb. These findings suggest a novel strategy of vit C adjunctive therapy by modulating bacterial physiology for enhanced efficacy of combination chemotherapy with existing drugs, and also possible synergies to guide new therapeutic combinations towards accelerating TB treatment.

1. Introduction

Asymptomatic latent tuberculosis infection affects ~ 2 billion individuals worldwide [1]. It is believed that in this condition, tubercle bacilli persist in a dormant, low metabolic state, sometimes even through the lifetime of the individual, with very slow or no replication, that renders them resistant to killing by host defense mechanisms and antibiotics [2,3]. There is ample evidence of bacterial survival in a 'viable but non-culturable' (VBNC) state in sputum from treatment-naïve patients [4,5] and as drug-induced persisters in-vitro [6], which underscores the complex behavior of tubercle bacteria during adversity. Their presence is clinically relevant as reversal of the VBNC state may lead to the recurrence of tuberculosis (TB) in patients thought to be

successfully treated on the basis of sputum culture conversion [7,8]. VBNC bacteria do not exhibit growth on solid medium but grow in liquid media, which poses a challenge, as the determination of colony-forming units (Cfu) on solid medium is the gold standard for bacterial enumeration and by this method, the presence of VBNC organisms would have allowed for a misdiagnosis. Replication-restricted dormant tubercle bacilli display the property of phenotypic tolerance, which is considered to be a major determinant of drug resistance in TB. These phenotypically tolerant bacteria include an important subclass of VBNC bacteria that are generally not considered during conventional testing of anti-mycobacterial activity [9]. Therefore, there is a definite need for better understanding of their phenotype through the use of appropriate models. In-vitro dormancy models that replicate individual host

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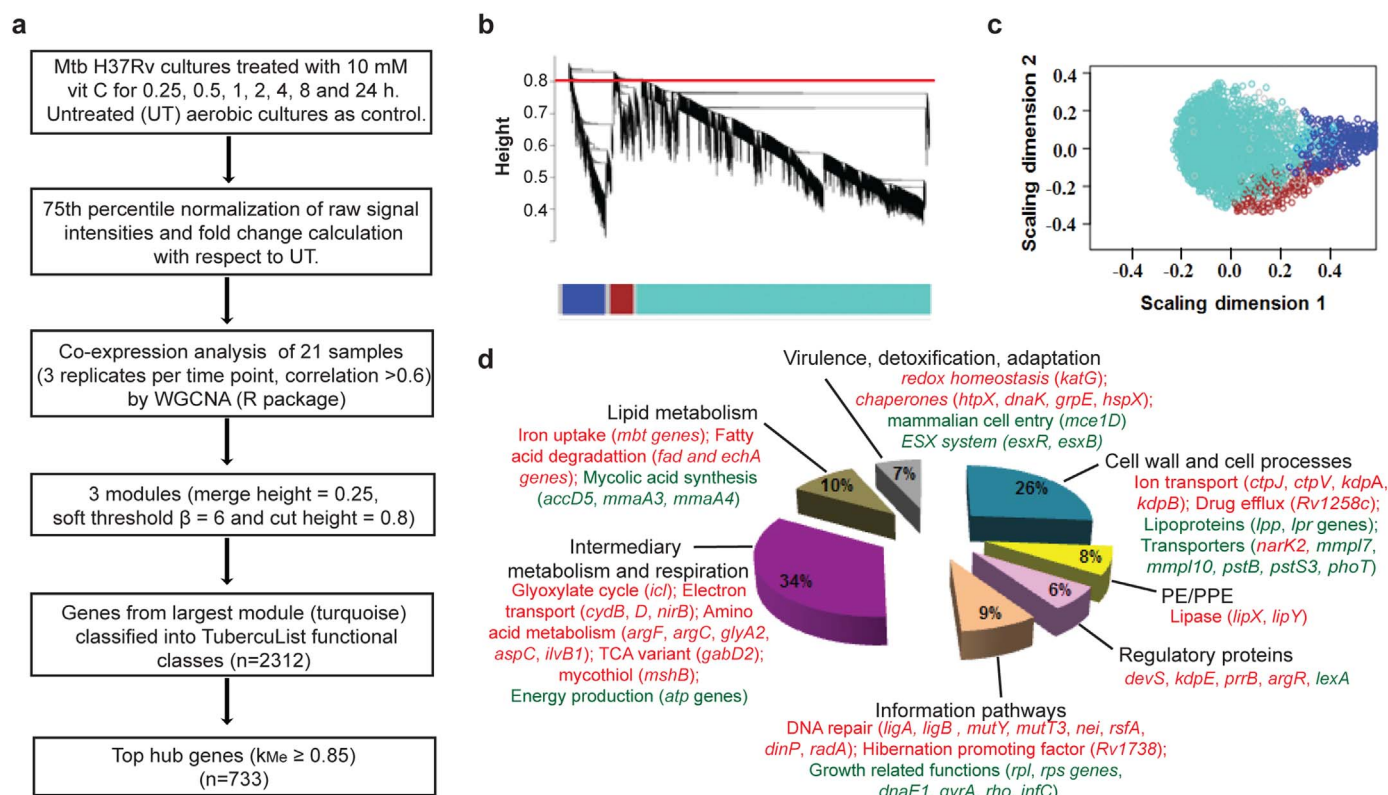


Fig. 1. Network analysis of gene expression of vit C-treated Mtb identifies modules of co-expressed genes. a) Analysis workflow of temporal gene expression and co-expression in Mtb cultures treated with vit C in-vitro. b) Dendrograms produced by average linkage hierarchical clustering of 4025 genes. The red line in the dendrogram indicates the cut tree height (0.8) to obtain modules denoting co-expressed genes that were assigned colors as indicated in the horizontal bar beneath the dendrogram. c) CMD plot (color-coded as in (b)) depicts the relative size and cohesion of modules. d) Distribution of turquoise module genes (n = 2312) into TubercuList functional categories (<http://tubercuList.epfl.ch/>). The percentage of the genes in each category is shown with top connected ($k_{ME} \geq 0.85$) up- and down-regulated genes in red and green, respectively.

conditions such as hypoxia, nutritional deficiency and acidic stresses have provided insights into bacterial strategies during dormancy [10]. However, these models have generally not examined the VBNC state or the impact of redox stresses, underscoring the need for appropriate setups that model a relevant combination of host conditions in-vitro. Also desirable are suitable infection models to assess phenotypic drug tolerance and therapeutics in the context of host for better clinical management of TB.

The link between TB disease and vitamin C (vit C) is placed in this background. Several studies, some going back several decades into the pre-antibiotic era, have documented the beneficial effect of vit C supplementation on treatment and reducing the risk of TB in patients and guinea pigs [11–14]. While these studies focused on the impact of vit C on disease outcomes, its effect on the TB pathogen was not examined until quite recently. We showed for the first time that vit C exposure induces dormancy in Mtb [15,16], whereas a subsequent report suggested that vit C manifests bactericidal activity against Mtb in-vitro [17]. This debate regarding its dormancy-inducing or bactericidal activity indicates a significant knowledge gap regarding the link between vit C and Mtb.

Here we employed transcriptomic, biochemical and microbiological approaches along with studies in the THP-1 infection model for deriving mechanistic insights into the dormancy inducing properties of vit C. We demonstrate that Mtb mounts a cohesive and multifaceted response to vit C which is protective, growth-restrictive and enables bacterial progression to a VBNC state. The survival mechanisms adopted by vit C-adapted bacteria include down-regulating growth functions, dissipating reductive and oxidative stresses, redirecting metabolism and acquiring tolerance to TB drugs. A hallmark of the vit C models described here is existence of replicating, dormant and VBNC bacterial subpopulations, as reported in the in vivo scenario [18]. For obtaining insights that are

more relevant to the human disease condition, we extended our primary findings to elucidate drug tolerance mechanisms in vit C adapted bacteria. Vit C synergizes with pyrazinamide (PZA) and boosts the sensitivity of Mtb subpopulations to combination treatment by TB drugs. Our findings suggest a novel strategy based on modulating bacterial physiology using vit C for enhancing the efficacy of existing TB drugs. Finally, the vit C-based axenic culture and infection models offer unique assays to identify compounds/combinations that simultaneously target replicating and non-replicating/slowly replicating bacteria.

2. Methods

2.1. Strains, culture conditions and staining procedures

Mtb H37Rv or Mtb 1886/13 *katG* mutant clinical isolate were cultured in DTA medium (Dubos medium containing 0.5% BSA, 0.75% Dextrose and 0.085% NaCl plus 0.1% Tween-80) with shaking at 220 rpm at 37 °C till $OD_{595} \sim 0.1$ to 0.2. Cultures were treated with 10 mM vit C for specified time periods for all experiments. For Cfu analysis, bacteria were thoroughly vortexed and plated on Middlebrook 7H11 agar containing 10% OADC (Difco MB agar) and Cfus were enumerated after 5 weeks incubation at 37 °C. Live-dead vital staining of mycobacteria was performed using the Fluorescein diacetate (FDA)/Ethidium bromide (EB) staining technique, and lipids were stained with Nile Red (Auramine-O counter stain) as described previously [19]. All reagents were from Sigma Aldrich unless mentioned otherwise.

2.2. Whole genome transcriptome analysis

RNA was isolated from Mtb H37Rv cultures in triplicate of $OD_{595} \sim$

0.1 to 0.2 treated with 10 mM vit C for 0.25, 0.5, 1, 2, 4, 8 and 24 h and untreated control culture (UT) as described [20] and subjected to microarray analysis at Genotypic India Pvt. Ltd., Bengaluru using Agilent custom 8 × 15 K Mtb arrays (60-mer probes). Briefly, RNAs were labeled with Cy3 and the labeled samples were hybridized to Mtb arrays, scanned and data were extracted using Feature Extraction Software. The schema for data analysis is shown in Fig. 1a. The raw data is deposited at NCBI (GEO accession number: GSE101048). Standard preprocessing and normalization steps, i.e. log₂transformation, 75th percentile intensity normalization were performed using Agilent's GeneSpring Software. The final gene expression matrix consisted of 21 samples (seven time points in triplicate) and 4025 genes. Weighted Gene Co-expression Network Analysis (WGCNA package in R software) was applied to the normalized data. WGCNA constructs scale-free network of weighted, soft-thresholded pairwise gene correlations followed by unsupervised clustering of these relationships into modules [21]. The soft thresholding power $\beta = 6$ was selected by the visualization of scale-free log-log plot (Fig. S1). Hierarchical clustering with a branch cut height of 0.8 was then used to identify modules. The genes for the largest module were classified into TubercuList functions and genes with high module membership were identified on the basis of $k_{ME} \geq 0.85$ [21]. Though the transcriptome analysis could not be performed beyond 24 h due to the accumulation of precipitate in the media that hindered the isolation of good quality RNA, the phenotypic responses over longer time periods provided mechanistic insights into the survival strategies of Mtb.

2.3. Re-growth/resuscitation of cultures and measurement of membrane potential

Mtb H37Rv cultures were pelleted at 4 and 8 days post vit C exposure, washed and revived in 3 ml fresh Dubos media supplemented with 10% OADC (Difco) under shaking conditions for a period of 15 days. A subset of the revived cultures was treated with 4 µg/ml INH or 80 µM tetrahydrolipstatin (THL). Culture aliquots (200 µl) were withdrawn at 5 day intervals to monitor the growth recovery of bacteria by measuring absorbance at 595 nm. At 10 days post revival, membrane potential of the bacterial culture was measured using BacLight™ Bacterial Membrane potential kit (Molecular Probes, Invitrogen) using CCCP protonophore as a negative control.

2.4. Preparation of Mtb cell lysates

Vit C-treated and untreated time matched Mtb cultures (3–4 independent cell cultures) were harvested and washed thrice with PBS. Cell pellets were resuspended in 0.5 ml PBS containing 1 mM PMSF and 20% glycerol and lysed with 0.1 mm Zirconium beads at 4800 rpm for 3 min in bead beater with 1 min intervals on ice. Cleared lysates were obtained by centrifugation at 36,000g for 5 min and filtered through 0.45 µm filter. Clarified filtrates were used for performing enzyme assays. Protein concentration was estimated in lysates by Bradford method.

2.5. Enzyme assays

All enzyme assays were performed at 37 °C except catalase, and peroxidase assays that were performed at 30 °C. All measurements were taken in spectrophotometer (Spectramax M2e, Molecular devices). The specific activity of all enzymes is expressed as nmoles/min/mg protein unless stated otherwise.

2.5.1. Lipase

TAG lipase activity was performed in a 96 well plate using Fluorescent lipase assay kit (Marker Gene Technology Inc., USA). Tetrahydrolipstatin (THL) lipase inhibitor was added at 80 µM final concentration to untreated (UT) and vit C-treated cultures.

2.5.2. Catalase

Reaction mixture (0.2 ml) contained 25 µl cell lysate, 5 mM H₂O₂ and 50 mM sodium phosphate buffer pH 7.0. The decrease in the absorbance was monitored at 240 nm for 10 min and linear part was considered for determining the rate of decrease of H₂O₂. Extinction coefficient 43.6 M⁻¹cm⁻¹ was used to calculate the reaction rate [22].

2.5.3. Peroxidase

Reaction mixture (0.2 ml) contained 25 µl cell lysate, 50 mM potassium phosphate pH 7.0, 0.1 mM O-dianisidine and 23 mM *t*-butyl hydroperoxide. Absorbance was monitored at 460 nm and the reaction rate was calculated using an extinction coefficient of 1.13 × 10⁴ M⁻¹ cm⁻¹ for O-dianisidine [22].

2.5.4. Superoxide dismutase (SOD)

Reaction mixture (1.050 ml) contained 50 µl cell lysate, 0.1 ml of pyrogallol solution (2 mM in 10 mM HCl solution), 0.9 ml of 55.6 mM Tris-Cl buffer pH 8.2 and 1.0 mM EDTA. The absorbance was monitored at 420 nm at 25 °C. The autoxidation rate of pyrogallol (control) was determined from the slope of the absorbance curve during the initial 1 min of the reaction. The absorbance change of the control was 0.02/min [23].

2.5.5. Aconitase (Acn)

Reaction mixture (0.2 ml) contained 25 mM HEPES pH 8.0, 100 mM NaCl and 25 µl cell lysate. The reaction was initiated by addition of 0.1 mM *cis*-Aconitate. Disappearance of *cis*-Aconitate was monitored at 240 nm. An extinction coefficient of 3500 M⁻¹ cm⁻¹ was used to calculate enzyme activity [24].

2.5.6. Isocitrate dehydrogenase (Idh)

Reaction mixture (0.2 ml) contained 50 mM Hepes (pH 8.0), 0.25 mM NAD⁺/NADP⁺, 10 mM MgCl₂ and 50 µg lysates. The reaction was initiated by addition of 1 mM isocitrate. The reaction rate was monitored by measuring production of NADH at 340 nm using an extinction coefficient of 6223 M⁻¹ cm⁻¹ [24].

2.5.7. Isocitrate lyase (Icl)

Reaction mixture (0.2 ml) contained 50 mM MOPS pH 7.5, 5 mM MgCl₂, 4 mM phenylhydrazine HCl and 20 µl of lysate. The reaction was started by addition of 2 mM isocitrate and rate of reaction was monitored at 324 nm. An extinction coefficient of 1.7 × 10⁴ M⁻¹ cm⁻¹ was used to calculate specific activity of Icl [25].

2.6. Estimation of hydrogen peroxide in culture supernatants

Mtb culture supernatants (0.45 µm filtered) from vit C-treated and untreated time matched control cultures were used for estimating H₂O₂ concentrations using the Pierce™ Quantitative Peroxide Assay Kit (Thermo Scientific). Briefly, culture filtrates were diluted by the addition of freshly made Dubos medium (1:1, v/v) and 10 µl of this was added to 100 µl of Xylenol Orange-based reagent and incubated at room temperature for 4 h. This dilution nullified the observed quenching of H₂O₂ in presence of 10 mM vit C (data not shown) as described by Siddique et al. [26]. An aliquot each of the filtrate was treated with 10 units of catalase for 5 mins at room temperature before H₂O₂ measurement. The intensity of the purple product was measured at 580 nm. The concentration of H₂O₂ was calculated from the respective standard curves generated over a concentration range of H₂O₂ (0–120 µM) prepared in DTA medium (with or without 5 mM and 10 mM vit C).

2.7. ROS assessment

The generation of ROS was measured using the fluorescent dye 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA). Mtb cells were harvested and washed twice with PBS. The resulting pellet was re-suspended in PBS to obtain 5 × 10⁸ bacterial cells per ml for the assay.

ROS was measured using the fluorescent dye 2',7'-dichlorodihydro-fluorescein diacetate (H₂DCFDA) in 96-well black/clear bottom plates and incubated at 37 °C for 1 h. The plate was read in a spectrophotometer (Spectramax M2e, Molecular devices) at Ex 480 nm/Em 520 nm. The final values (post PBS background subtraction) were represented as relative fluorescence units per OD of the culture (RFU/OD).

2.8. Assessment of the sensitivity of *Mtb* to drugs in axenic cultures

Mtb cultures (A₅₉₅ 0.1–0.2) were adapted to 10 mM vit C for 24 h in parallel with time matched untreated cultures. They were then exposed to 2-fold serial dilutions of antibiotics as follows: Isoniazid (INH, 0.03–4 µg/ml), Rifampicin (RIF, 0.015–8 µg/ml), Streptomycin (STR, 0.03–8 µg/ml), Ethambutol (EMB, 0.25–64 µg/ml) and pyrazinamide (PZA, 6.25–400 µg/ml). PZA controls were: Con 1, DTA buffered to pH 5.5 with acetic acid, Con 2, Vit C + DTA buffered to pH 6.5 with sodium hydroxide and Con 3, unbuffered DTA medium at pH 6.5. Clofazimine (CFM, 0.05–3.2 µg/ml) and tetrahydrolipstatin (THL, 80 µM) were added to cultures at day 0 along with vit C. Efflux pump inhibitors (EPI), verapamil (VER) and piperine (PIP) each 25–125 µg/ml were added at 24 h post-vit C treatment in combination with INH (4 µg/ml) or RIF (1 µg/ml). When added in combination, INH, RIF, EMB, PZA, Ver/ Pip were added at 4 × MIC₉₀ (concentrations of 0.12 µg/ml, 1 µg/ml, 16 µg/ml, 50 µg/ml, 125 µg/ml, respectively) at 24 h post addition of vit C. CFUs were analyzed 4 days post-vit C treatment and colonies were counted after 5 weeks incubation at 37 °C. Bacterial survival rates (% survival) were determined by normalizing bacterial Cfus enumerated in drug-treated cultures (vit C-treated/ control) versus that in time-matched no drug cultures.

2.9. THP-1 cell infection and drug susceptibility of intracellular bacteria

THP-1 cells (5 × 10⁵ per well of 24 well plate) were differentiated with PMA (30 nM for 16–18 h) and infected with *Mtb* (H37Rv or 1886/13 *katG* mutant) at MOI 1:10 (cell:*Mtb*) in 24-well tissue culture plates. At 4 h post-infection, the cells were washed twice with PBS and replenished with complete media. Infected cells were either left untreated or treated with vit C (2 mM final concentration). At 24 h of vit C exposure, cells were exposed to INH, RIF, EMB, PZA, Ver or Pip added at concentrations of 4 × MIC₉₀ (0.12 µg/ml, 1 µg/ml, 16 µg/ml, 50 µg/ml, 125 µg/ml), respectively at 24 h post addition of vit C individually or in combination. The plates were further incubated for 3 days (total of 4 days post-infection) and intracellular bacterial survival was determined by Cfus enumeration after lysing THP-1 cells with 0.025% SDS and plating the lysates on MB 7H11 Agar. Bacterial survival rates (% survival) in THP-1 cells after 72 h of drug treatment were determined by normalizing bacterial Cfus enumerated in drug-treated infections (vit C-treated/ control) versus that in time-matched no drug infections.

2.10. Redox potential measurements/measuring EMSH of mycobacteria during infection

PMA-differentiated THP-1 cells were infected with *Mtb* H37Rv expressing Mrx1-roGFP2 as described above. Infected cells were either left untreated or treated with 0.1 mM vit C. INH treatment (4 µg/ml) was given 24 h post infection. At indicated time points, cells were treated with 10 mM N-ethylmaleimide (NEM) for 5 min, fixed with 4% PFA for 15 min and washed thrice with PBS. Scraped cells were analyzed by Flow Cytometry (BD FACS Verse, BD Biosciences). Violet (V500) and blue (FITC) lasers were used to obtain the values of emission (510/10 nm) after excitation at 408 nm and 488 nm, respectively. Data was analyzed using the FACSsuite software. The obtained 408/488 nm ratios were normalized with the ratios obtained by treatment of infected cells with 10 mM DTT (0% oxidation, ratio set to 0.1) and with 1 mM CHP (100% oxidation, ratio set to 1). Normalized ratios were then converted to redox potential (*E*_{MSH}, mV) by putting the values in the calibration curve obtained by titrating Mrx1-roGFP2 expressing bacilli with different ratios of oxidized DTT (trans-4,5-dihydroxy-1,2-dithiane) to

reduced DTT [27]. Bacilli were divided into 3 subpopulations based on average mycothiol redox potential (*E*_{MSH}): Oxidized (– 240 mV), Basal (– 270 mV) and Reduced (– 300 mV).

2.11. In-vitro DNA damage and protection assay

The in-vitro DNA damage and protection assay was performed as described [28]. Reaction mixtures (20 µl) containing 50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 200 ng supercoiled pUC19 DNA and GroEL1 (concentration range 2–8 µM) were incubated at room temperature for 30 min followed by the addition of Fenton reagent (10 mM vit C plus DTA and 50 µM Ferric ammonium citrate). The reaction mixtures were incubated for 10 min at room temperature, stopped with 5 mM EDTA and were electrophoresed on a 1% agarose gel in 0.5% TBE buffer and stained with ethidium bromide.

2.12. Statistical analysis. The data was statistically analyzed from 3 to 4 independent replicates wherever applicable using unpaired two tailed t-test.

3. Results

3.1. *Mtb* mounts a cohesive and robust transcriptional response to vit C. Previous studies to decipher bacterial responses to vit C were restricted to single time points and could not capture the breadth of the response [16,17]. Here we used Weighted Gene Co-expression Network Analysis (WGCNA) of 21 datasets to examine temporal co-expression over a period of 24 h and obtain a systems-level understanding of the coordinated response of bacteria to vit C (Fig. 1a and Fig. S1). The approach goes beyond a simple listing of differentially expressed genes and enables one to deconstruct gene expression datasets into co-expressed functional clusters/ networks for understanding the regulatory roles of key genes and the dynamics of gene-gene connections in biological pathways [21]. A massive co-expression response was detected which involved ~ 67% of the *Mtb* genome and mapped to turquoise, brown and blue modules having 2312, 199 and 200 genes, respectively (Fig. 1b, c and Table S1). The turquoise module was highly connected; 733 hub genes with a high connectivity score (*k*_{ME} ≥ 0.85, indicating a high degree of correlation with other genes in the network) were identified and disclosed a large-scale cohesive bacterial response to vit C. A functional assignment of top connected genes revealed a major realignment of pathways involved in growth processes (*dnaE1*, *gyrA*, *rho*, *infC*, *rpl* and *rps*), redox homeostasis (*katG*, *mshB*), DNA repair (*ligAB*, *mutY*, *mutT3*, *nei*, *rsfA*, *dinP*, *radA*), cell wall lipid metabolism (*accD5*, *mmaA3*, *mmaA4*, *lpp*, *lpr*, *lipX*, *lipY*), respiration and energy pathways (*icl*, *cydBD*, *nirB*, *atp*), dormancy switch (*devS*, *Rv1738*), ion transporters (*ctpJ*, *ctpV*, *narK2*) and drug efflux (*Rv1258c*; Fig. 1d). The key predictions from gene expression data that indicated a dormancy inducing activity of vit C were functionally investigated.

3.2. Vit C induced dormancy progresses to a viable but non-culturable (VBNC) condition. Transcriptome analysis revealed a major realignment of growth related genes in response to vit C; these included genes coding for ribosome biogenesis (*rps*, *rpl* genes), partitioning (*parAB*), cell division (*gid* and *ftsZ*) as well as DNA and RNA synthesis (*dnaAB*, *gyrAB*, *ssb*, *rho*, *rpoAB*) which were coordinately down-regulated (Table S2). This indicated a slowing down of bacterial growth and is consistent with growth stasis observed in vit C-treated bacteria in contrast to control cultures that grew ~ 15-fold over a 4-day period. A decrease in Cfus on day 6 was followed by a failure to recover colonies on MB solid agar from day 8 onwards (Fig. 2a). Vit C induced growth stasis maybe attributed to the dual effects of acidification (vit C lowered the pH of Dubos culture media to moderately acidic pH of 5.5) and hypoxia generation [16], both of which are reported to cause bacterial growth arrest [19,29]. The induction of protective responses to pH (*whiB3* and *Rv3761c* [30,31]) and hypoxia (*devRS*, *Rv0081* [32,33]) and of stress-induced sigma factors (*sigB*, *sigE*, *sigF*, *sigH*, and

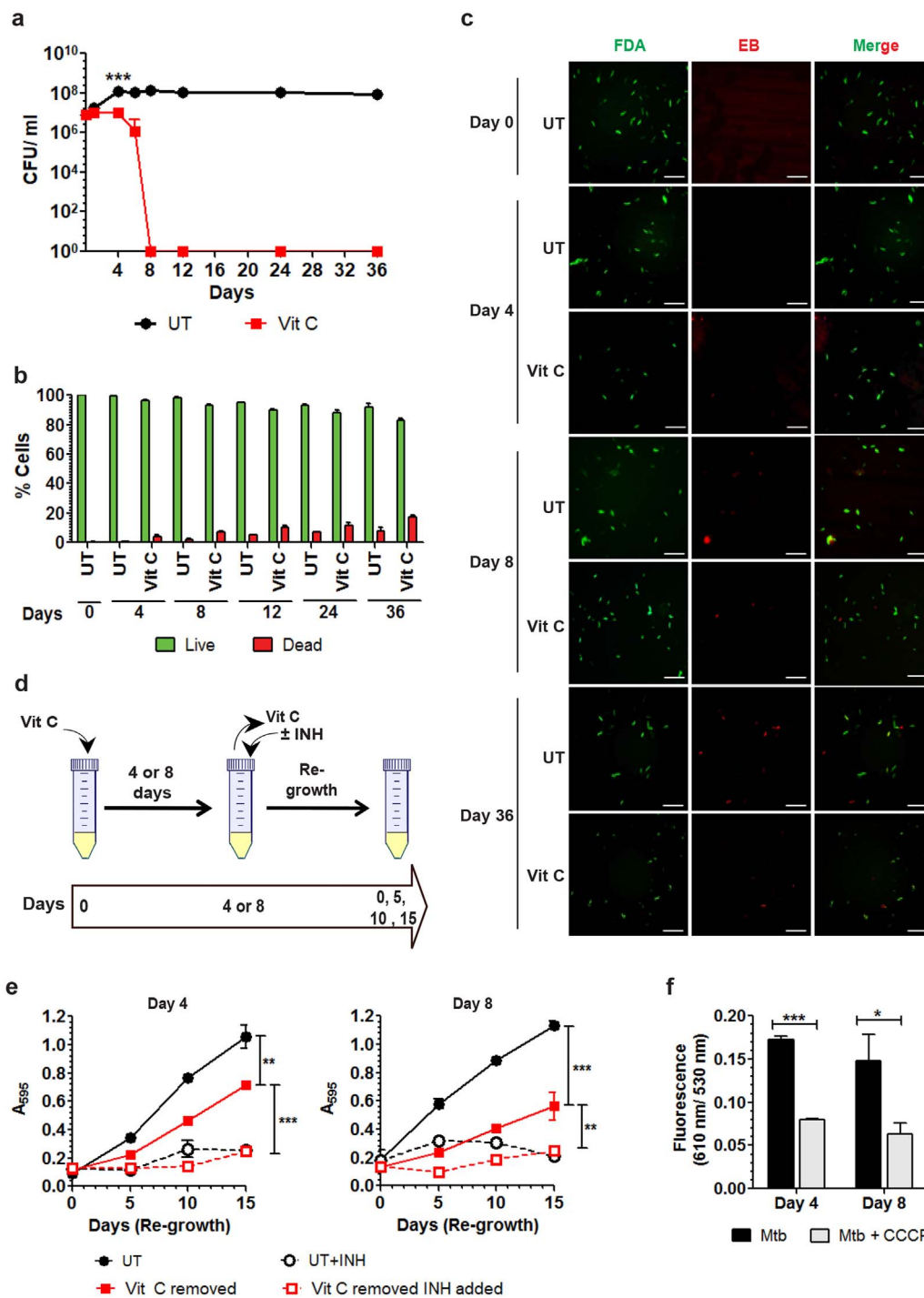


Fig. 2. Vit C treatment leads to growth arrest and entry into VBNC state in Mtb. a) Growth analysis of vit C-treated Mtb culture by enumeration of CfU. b) and c) Live-dead staining of Mtb with FDA (live cells; green) and EB (dead cells; red). At least 250 bacteria were counted per time point to determine the percentage of live and dead bacteria. Images from representative time points are shown in (c). d) Schema for analyzing re-growth of vit C-treated Mtb cells. e) Re-growth of Mtb cultures treated with 10 mM vit C for 4 or 8 days. Washed bacteria were resuspended in fresh Dubos media and re-growth was monitored (A₅₉₅) post-removal of vit C. INH (4 μg/ml) was added to day 4 or day 8 h cultures during re-growth. f) Membrane potential of day 4 and day 8 vit C-treated Mtb cultures measured using DiOC₂ probe on day 10. [UT, untreated culture; vit C, vit C-treated culture; Mean ± SD from 3 to 4 replicates is plotted for all panels; pvalue, *** < 0.001, ** < 0.01, * < 0.05. Scale bar, 5 μm].

sigJ [34]) further pointed towards a concerted bacterial effort for resisting vit C effects and adapting to a state of growth stasis (Table S2).

It is reported that adaptation of bacterial species to adverse conditions, such as exposure to antibiotics and host-derived stresses, can occur through bacterial entry into a VBNC state ensuring long-term survival (defined here as viable but not-culturable bacteria on solid media) [35,36]. However from the perspective of disease control, the inherent failure of VBNC bacteria to grow on solid media causes an underestimation/ non-detection of these bacteria by the CfU assay. Importantly, growth is restored under suitable conditions, such as incubating specimens in liquid cultures [5,7,35,37]. Considering the inability to recover CfU on solid media from vit C-treated cultures (Fig. 2a), and to distinguish this condition from the reported cidal effect

of vit C [17], we tested for bacterial entry into a VBNC state using live-dead staining technique. Live bacteria stained with fluorescein diacetate (FDA) were detected in vit C-treated cultures; ~ 83% of bacteria were determined to be live on day 36 (Fig. 2b and c), which indicated that treated bacteria were viable and failed to grow only on solid culture media. Importantly, the block in cell division was reversible; bacterial growth and colony forming ability were restored in VBNC cells when suitable conditions were provided (Fig. 2d). This growth recovery resembled the reactivation of in-vitro persisters upon removal of drugs [6]. Vit C-treated bacteria re-grew slowly; ~ 7-fold as opposed to ~ 14-fold increase in turbidity in case of control cultures revived on day 4 (pvalue < 0.01) and ~2-fold increase in vit C cultures revived on day 8 (pvalue < 0.001; Fig. 2e), likely due to a lag in re-adaptation to

conditions favorable for growth. The re-grown bacteria regained the ability to form colonies on solid agar (data not shown) and displayed membrane potential (measured by DiOC₂), a characteristic of viable cells, which could be disrupted by the addition of protonophore CCCP (Fig. 2f). More importantly, revived control and vit C-treated cultures were equally sensitive to isoniazid (INH), confirming their INH susceptible status after they emerged from dormant (on day 4, *p*value < 0.001) or VBNC state (on day 8, *p*value < 0.05). Thus vit C-adapted bacteria fulfilled four cardinal characteristics of VBNC cells namely, non-culturability on solid medium, viability, re-growth in liquid culture medium under suitable conditions and recovery of drug susceptibility.

3.3. Vit C-mediated reductive stress is dissipated by accumulation of TAG storage lipids. The decreased expression of aerobic respiratory pathway genes *nuoA-N* (encoding Ndh-1) and *atpA-H* (encoding ATP synthase, Fig. 1d and Table S2) indicated bacterial adaptation to the hypoxic and acidic environment generated by vit C [16]. Such a down regulation has been documented earlier during adaptation to various stresses [38–40]. A decrease in aerobic respiration under these conditions is also associated with decreased regeneration of NAD⁺ and an accumulation of reduced cofactors [31,41]. Hence we probed the mechanism by which vit C-treated bacteria could mitigate reductive stress and maintain redox balance for survival. Notably, the anabolic pathways of triacylglycerol (TAG) and sulfolipid (SL-1) synthesis (Fig. S2a) that consume reducing equivalents, and WhiB3, the redox-dependent regulator of TAG/SL-1, were induced (Table S2). WhiB3-regulated lipid synthesis is reported as a mechanism to dispose of reductants accumulated during hypoxia and/or acidic pH mediated stress [42]. Further, 11 out of 15 known TAG biosynthetic genes (*tgs*) including DevR-regulated *tgs1*, were strongly induced in response to vit C (Fig. 3a), which resembled their induction during in-vitro dormancy [43]. Consistent with the enhanced expression of *tgs* genes, we detected greater accumulation of TAG lipid droplets in vit C-treated bacteria (*p*value < 0.05; Fig. 3b and c). Thus TAG accumulation over 36 days established an important phenotypic correlate of dormancy in vit C-adapted cultures. In addition, a decrease in Auramine-O stained bacteria indicated a loss of acid-fastness in a larger proportion of vit C-treated bacteria (*p*value < 0.05; Fig. 3b and c), a feature reported for dormant and hypoxia-adapted mycobacteria [19,44,45]. Loss of acid-fastness is considered to be an outcome of shorter chain mycolic acids [46], and can be attributed to the lowered expression of the FAS-II system in vit C-treated cells (Fig. S2a). Vit C-treated Mtb also underwent a rapid reduction in length from 2 μm to ~ 1.2 μm (~ 60% reduction) on day 1 and to ~ 1 μm by day 36 (*p*value < 0.001, Fig. S2b), which was consistent with the size reduction reported in in-vitro dormancy models [45].

The twin activities of TAG accumulation and its utilization through β-oxidation pathway have been linked to Mtb virulence, persistence and re-growth under suitable conditions. It is reported that fatty acids, released from acylglycerols by lipase-mediated hydrolysis, serve as an energy source [43,47]. Thirteen of 24 lipase (*lip*) genes including LipY, a major TAG lipase necessary for survival during nutrient starvation [48], were induced by vit C (Fig. 3a), suggesting the possibility of enhanced TAG hydrolysis. Indeed, TAG-degrading lipase activity was increased in vit C-treated bacteria (*p*value < 0.001; Fig. 3d and e). As TAGs could be synthesized by any one of 11 *tgs* gene products up-regulated by vit C (Fig. 3a), we used a pathway-independent lipase inhibitor tetrahydrolipstatin (THL) to verify the relevance of TAGs in bacterial survival strategy. TAG-degrading lipase activity was decreased by treatment with THL (*p*value < 0.001, Fig. 3e) and was accompanied by a survival defect (*p*value < 0.05, Fig. 3f), pointing to the importance of fatty acids released from accumulated TAGs for β-oxidation. This can be a favored pathway for generating energy under hypoxia as aerobic respiration is ruled out. The up-regulation of genes belonging to the β-oxidation pathway was consistent with these observations (Fig. S2c). In contrast to THL + vit C-treated cultures, THL-treated aerobic cultures

exhibited increased growth (*p*value < 0.05) likely due to greater metabolic flux of glucose through the TCA cycle as reported [49]. Lastly, vit C + THL treated cultures were compromised in re-growth on removal of vit C (*p*value < 0.001), in contrast to THL-treated control cultures (Fig. 3g). Taking together the increase in TAG stained cells over 36 days, lipase enzyme activity and survival data, we establish that up-regulated TAG synthases balance the activity of lipases in vit C-treated bacteria. Thus TAG accumulation appears to be an active mechanism for dissipating reductive stress and the survival of vit C-treated bacteria depends on TAG utilization.

Vit C has antioxidant as well as prooxidant properties and both are relevant to redox stress mechanism. Efforts to maintain redox homeostasis in vit C-treated bacteria were also suggested by the increased expression of genes (Fig. S2d) that encode the cytoplasmic redox buffer systems, mycothiol and ergothioneine [50,51]. Vit C-induced reductive stress was confirmed by measuring the redox potential of mycothiol (E_{MSH}), the major redox buffer of Mtb, in infected THP-1 cells. The E_{MSH} of intraphagosomal Mtb upon exposure to INH/Vit C/Vit C + INH was measured using a mycothiol-specific biosensor, Mrx1-roGFP2 [27] (Fig. 3h). INH is reported to induce a significant oxidized shift in the E_{MSH} of Mtb during infection of THP-1 cells, whereas a reductive shift in intra-mycobacterial E_{MSH} promotes drug tolerance [27]. As expected, INH induced a significant oxidative shift inside bacilli (E_{MSH} = -240 mV, *p*value < 0.001; Fig. 3i), whereas vit C exposure resulted in a significant increase in the E_{MSH} -reduced subpopulation (-300 mV; *p*value < 0.001; Fig. 3i). More importantly, THP-1 cells infected with Mtb expressing the biosensor and co-treated with vit C + INH combination significantly reduced the fraction of E_{MSH} -oxidized subpopulation as compared to INH alone (*p*value < 0.05, Fig. 3i), indicating that vit C has a protective role in mitigating INH-derived oxidative stress.

3.4. Mtb exerts a robust protective response to vit C-mediated oxidative stress. The anti-oxidant and pro-oxidant activities of vit C include (1) O₂ scavenging function which generates H₂O₂, (2) a reductant activity which generates Fe²⁺ in-vitro (ferric ammonium citrate is present in Dubos medium), which together with H₂O₂ could potentially mediate Fenton reaction, and (3) a potent ROS neutralizing activity (Eqs. (1)–(4), Text S1). Taking together our observations that vit C exposure leads to dormancy and further entry into VBNC state, we next sought to understand the mechanism underlying the protective bacterial response to vit C-generated oxidative stress.

First, although vit C generated H₂O₂ while scavenging O₂, H₂O₂ concentration decreased significantly in culture supernatants after 8 h to a nontoxic concentration of ~ 65 μM (*p*value < 0.001; Fig. 4a). Mtb is reported to be highly resistant to H₂O₂-derived stresses and the maximal concentrations achieved in culture supernatants (~ 160 μM) were substantially lower than the reported cidal concentrations of > 10 mM [52].

Second, transcriptome profiling revealed a robust genetic response to mitigate oxidative and cell envelope stress through sigma factors σ^H and σ^E, which converge in the activation of σ^B (Table S3 and S4). The repair or degradation of affected biomolecules is an important protective response to oxidative stress. Consistent with this function, genes involved in the synthesis, repair/protection of iron-sulfur clusters (*cysD*, *cysK2*, *cysM* and *cysN*), DNA repair [nucleotide excision repair (*uvrA-D*), base excision repair (*alkA*), recombination repair (*recBCD*, *ruvAB* and *ligD*), reversal of guanine oxidation (*fpg*, *nei*, *mutY*) [53], and proteins (chaperones *clpB*, *groEL2*, *hsp*, *hspX* and *htpX*) were highly induced (Table S3). Iron homeostasis mechanisms were also activated in vit C-treated bacteria, including regulators IdeR and FurA. *mbt1*, *mbt2* clusters and iron transporters (*irtAB*) were up-regulated, while *bfr* genes coding for iron storage proteins were down-regulated, reflecting a cellular need to acquire iron [54]. Genes encoding CtpJ and CtpD transporters that may protect from redox stress [55] were up-regulated, and their Fe²⁺ efflux activity can be hypothesized to protect bacteria from Fenton reaction-mediated effects. The enzyme activity of aconitase

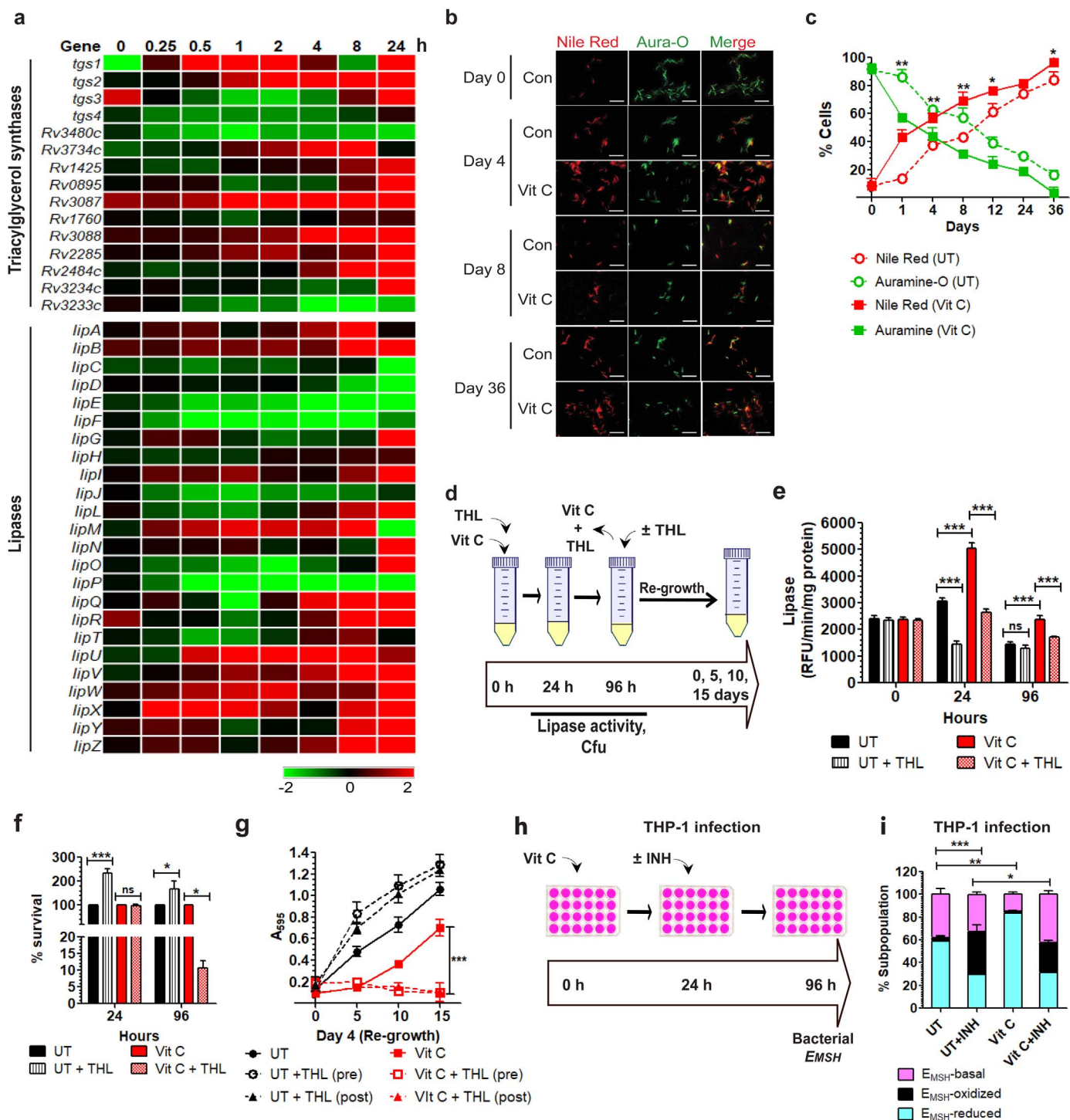


Fig. 3. Accumulation and utilization of TAGs. **a**) Temporal expression of Mtb *tgs* and *lip* genes upon vit C treatment. **b**) Accumulation of intra-bacterial lipid droplets detected by Nile Red staining. Scale bar, 5 μ m. **c**) Percentages of bacterial cells (from a minimum of 250 cells) which were either Auramine-O (green) or Nile Red (red) positive are plotted. Scale bar, 5 μ m. **d**) Schema for lipase activity and survival assays in in-vitro vit C-treated cultures. **e**) Lipase activity measured in Mtb cell-free extracts in vit C- treated cultures and time matched untreated controls. **f**) Effect of lipase inhibitor tetrahydropstatin (THL) on the survival of vit C-treated and UT cultures at 24 h and 96 h post-treatment with vit C and THL. The percentage survival is calculated from Cfu/ml with respect to no THL control (for UT and vit C sets), 24 h and 96 h post-vit C treatment. **g**) Re-growth of THL-pretreated cultures ('pre') monitored by increase in A_{595} . One subset of UT and vit C-treated cultures was treated with THL at the time of revival ('post'). **h**) Schema for reductive shift measurement using Mtb H37Rv expressing Mrx-1 roGFP2 infected in THP-1 cells post vit C and INH treatment. **i**) Vit C-induced reductive shift in redox potential E_{MSH} of intracellular Mtb is shown using Mtb H37Rv expressing Mrx-1 roGFP2. Treatment with vit C at 4 h of infection was followed by INH treatment at 24 h post-infection induced an oxidative shift in the E_{MSH} of the bacteria. All measurements were made at 4 days-post infection. [UT, untreated culture; vit C, vit C-treated culture; Mean \pm SD from 3 to 4 replicates is plotted for all panels; p value*** < 0.001, ** < 0.01, * < 0.05, ns, not significant].

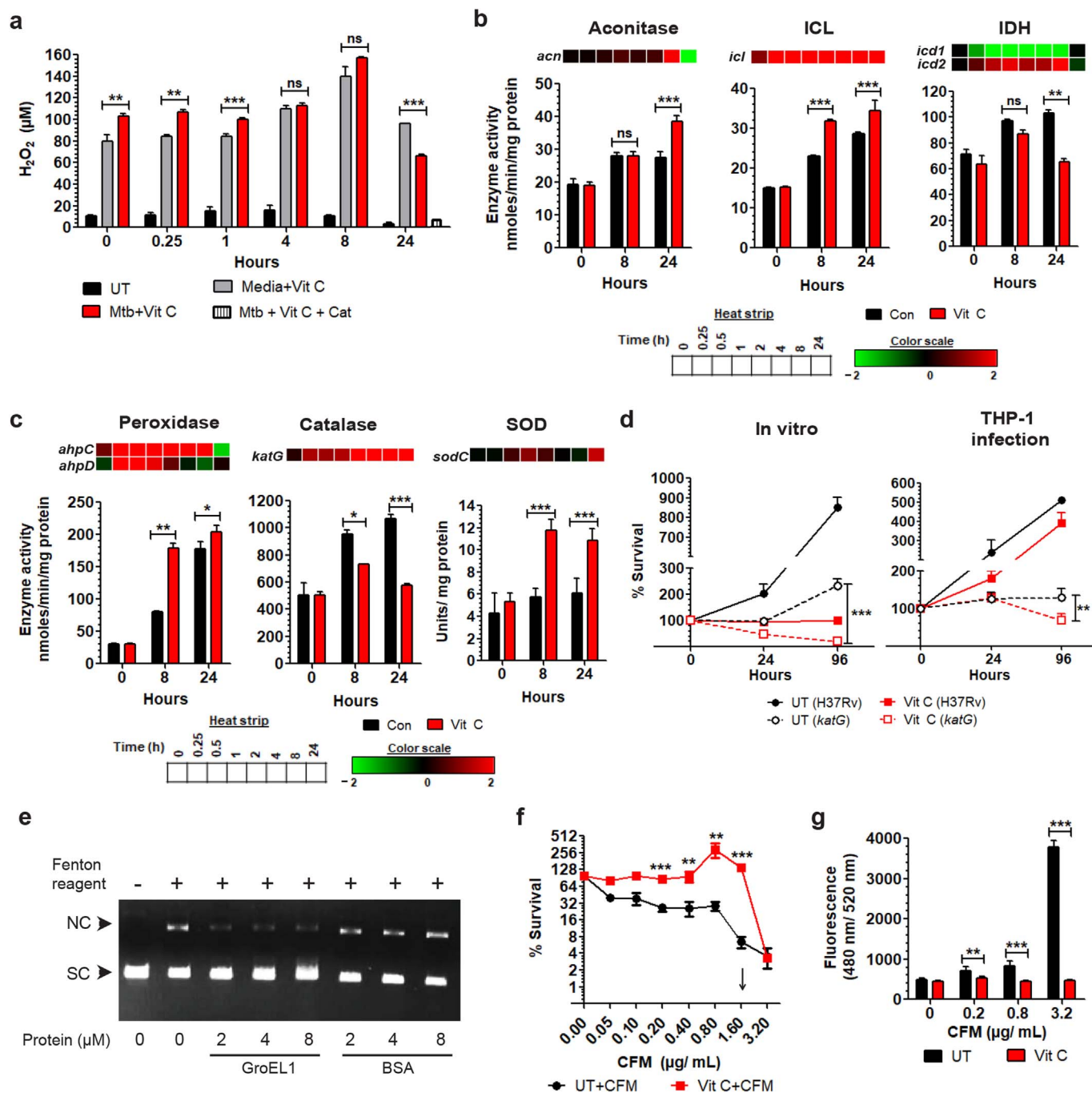


Fig. 4. Oxidative stress management in vit C-treated Mtb. a) Quantitation of H₂O₂ in culture filtrates. Catalase addition (Cat, 10 U, 5 min at RT) results in decomposition of H₂O₂ (only representative 24 h data is shown). b) Activities of metabolic enzymes in cell-free extracts of Mtb treated with vit C and time matched UT controls. c) Activities of ROS scavenging enzymes in cell-free extracts of Mtb treated with vit C and time matched UT controls. Color strips in panel (b) and (c) represents the range of gene expression values. d) Survival of Mtb *katG* mutant in the presence and absence of vit C in-vitro and in THP-1 cell infection model. The percentage survival is calculated from Cfu/ml with respect to Cfu/ml at 0 h time point. e) DNA damage assay. Protection of pUC19 plasmid DNA damage by GroEL1 in the presence of vit C and 50 µM ferric ammonium citrate (Fenton-like reagent). BSA, negative control. A representative of 3 separate experiments is depicted. NC, nicked circular DNA; SC, supercoiled DNA. f) Effect of clofazimine (CFM) + vit C co-treatment on Mtb survival. The percentage survival is calculated with reference to Cfu/ml of respective CFM minus controls. MIC₉₀ of CFM is marked with a black arrow. g) ROS detection using H₂DCFDA in CFM-treated Mtb. [UT, untreated culture; Vit C, vit C-treated culture. Mean ± SD from 3 to 4 replicates is plotted for all panels; pvalue*** < 0.001, ** < 0.01, * < 0.05, ns, not significant].

(Acn), a TCA cycle enzyme which is highly sensitive to oxidation of its solvent-exposed iron-sulfur cluster [56] and was used as a surrogate for integrity of its iron-sulfur cluster, was increased in vit C-treated bacteria (pvalue < 0.01), and demonstrates minimal damage to iron-sulfur cluster containing enzymes due to oxidative stress (Fig. 4b).

Third, genes coding for ROS scavenging enzymes KatG, superoxide dismutases (SODs), and peroxidase-peroxynitrite reductase (AhpC) complex, major components of oxidative stress mitigation in Mtb, were

induced (Fig. 4c). This was accompanied by significant increases in the activity of ROS scavenging enzymes, peroxidase and superoxide dismutase, and may aid bacterial survival (pvalue < 0.05; Fig. 4c). The quenching of H₂O₂ by exogenously added catalase in vit C-treated bacteria (Fig. 4a) suggested a role for KatG in quenching of H₂O₂. Hence, a *katG* loss-of-function mutant was used to assess the functional utility of Mtb KatG against vit C-mediated redox stress. This mutant is growth attenuated without vit C treatment under in-vitro as well as

intracellular conditions and we took this defect as a baseline measure to assess KatG protective effects. The survival data from both in-vitro and intracellular setups support a protective role for KatG in vit C-treated bacteria (p values < 0.001 and < 0.01; Fig. 4d).

Fourth, there are reports that the glyoxylate shunt, comprising of Icl and malate synthase, is preferentially utilized by dormant and antibiotic-treated bacteria [44,57] for replenishing TCA cycle intermediates and for gluconeogenesis and other biosynthetic processes. Therefore, Icl competes with the TCA cycle enzyme isocitrate dehydrogenase (Idh) for isocitrate [58]. Icl mRNA transcripts and activity were also reported to be increased by antibiotic-mediated oxidative stress [59]. Vit C-treated cultures showed elevated *icl* and decreased *idh* transcript levels that was accompanied by increased Icl enzyme activity and diminished Idh enzyme activity, indicating preferential routing of carbon through Icl (i.e. glyoxylate cycle) and not Idh (i.e. TCA cycle, p value < 0.01; Fig. 4b). This metabolic realignment resembled the rerouting of glucose carbon observed in in-vitro persistence and stress models [44,57] and in antibiotic-treated bacteria [59]. The preferred use of this pathway in vit C-adapted cultures may also enhance antioxidant defense mechanism consistent with the observed mitigation of oxidative stress upon decreasing carbon flux through the TCA cycle [59].

Fifth, *groEL1* encoding GroEL1 that is reported to shield DNA from oxidative damage [60] was up-regulated, suggested the possibility of GroEL1-mediated protection of DNA in vit C-treated bacteria (Table S3). Minimal damage to supercoiled DNA in-vitro was observed in the presence GroEL1 + vit C (Fig. 4e).

Lastly, diminished bacterial susceptibility to clofazimine (CFM) confirmed the antioxidant function of vit C through ROS neutralization mechanism. CFM is an anti-leprosy drug repurposed for the treatment of multi-drug resistant TB [61] and its cidal effect is attributed to its ROS generation capacity mediated through the respiratory enzyme Ndh-2 [62]. Enhanced bacterial survival was observed in cultures co-treated with vit C + CFM added at MIC₉₀ concentration (p value < 0.001, Fig. 4f). This finding indicated that quenching of ROS by cellular detoxifying pathways (enzymes and redox buffers) and by vit C (Eqn 4, Text S1) underlies the observed resistance to CFM-mediated killing. ROS quenching was confirmed by minimal intracellular ROS accumulation in vit C + CFM-treated cultures (p value < 0.01, Fig. 4g). The drop in bacterial viability observed at higher CFM concentration (3.2 µg/ml) is attributed to a ROS-independent killing mechanism (Fig. 4f). Taken together, these observations establish that the anti-oxidant property of vit C mitigates oxidative stress and bacteria employ a range of defense strategies to cope with vit C-mediated redox stresses.

3.5. Vit C induces tolerance to first line TB drugs that is reversed by efflux pump inhibitors. An important survival mechanism in stress-adapted bacteria is the acquisition of phenotypic drug tolerance [9]. Because vit C-adapted bacteria exhibit cardinal features of dormancy (foregoing sections), we investigated whether vit C exposure led to a state of generalized tolerance to TB drugs having diverse mechanisms of action. Rifampicin (RIF), INH (isoniazid); streptomycin (STR) or ethambutol (EMB) was added individually or in combination to vit C-treated cultures and their survival rates were assessed after 72 h (Fig. 5a and Fig. S3a). Vit C-treated Mtb exhibited a significantly higher survival rate in the presence of antibiotics; ~ 15 to 50% of the bacteria were tolerant to drugs added at concentrations as high as 32 × to 128 × of their MIC₉₀. In contrast, untreated bacteria were drug susceptible; their survival rate was < 10% at their MIC₉₀ and < 1% at 2 × MIC (p value < 0.05; Fig. 5b, c and Fig. S3a). These findings established the drug tolerant property of vit C-treated Mtb.

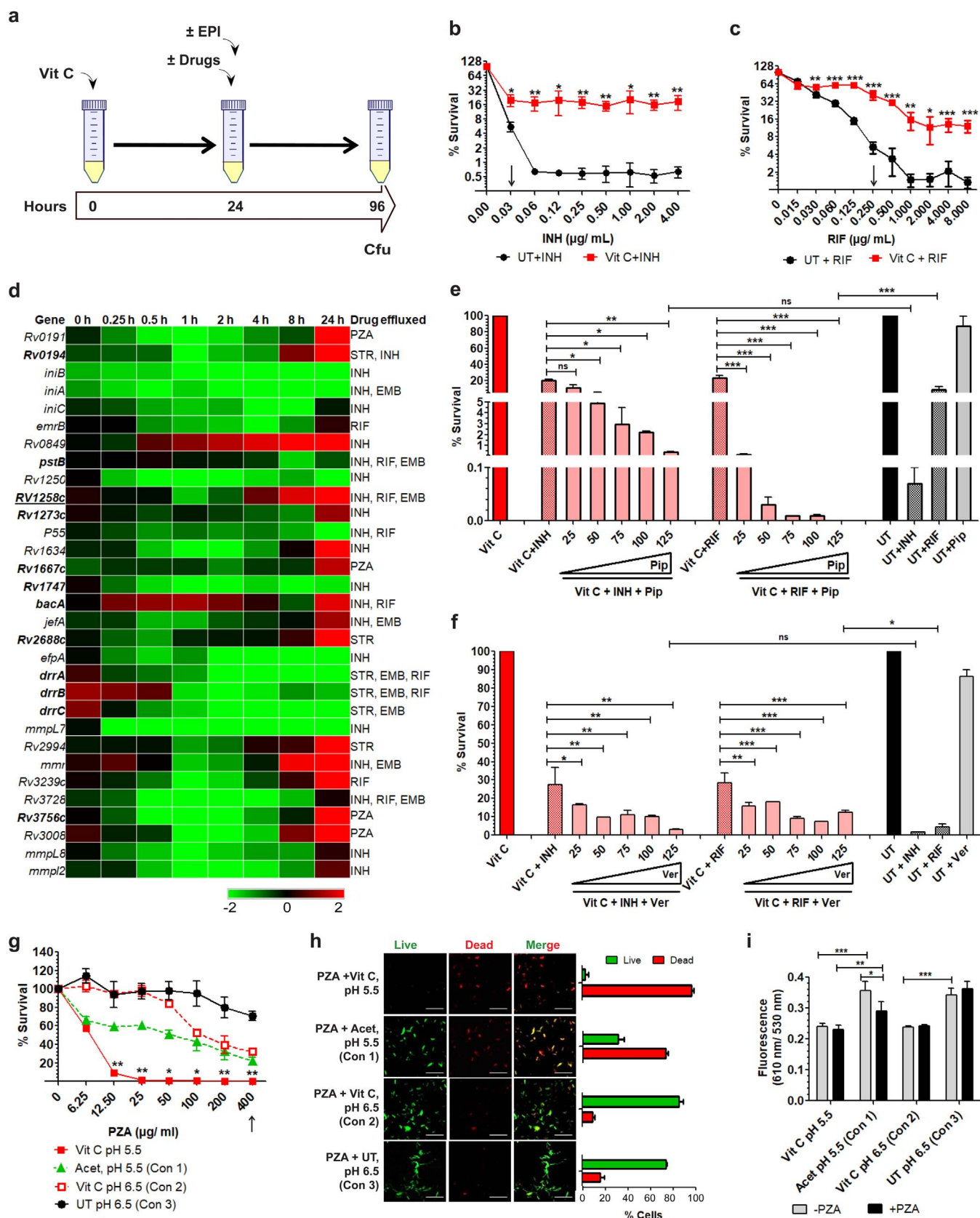
We infer a possible role for drug targets in tolerance to RIF, INH and STR, which target growth related functions. As vitamin C exposure causes growth stasis (in-vitro) or slows it down (intracellular bacteria), the observed downregulation of target genes, *rpoB* (RIF), *inhA* (INH) and *rpsL* (STR) (Fig. S3b) may possibly contribute to tolerance. It can be argued that the development of drug tolerance under these conditions

was counter-intuitive, since a lower level of drug target can be expected to lower drug MIC and not raise it. An explanation for raised MIC could be a change in bacterial redox homeostasis, a major confounding factor in drug susceptibility. RIF generates oxidative stress by inducing hydroxyl radical formation in Mtb to exert its killing action [63]. Likewise, ROS arising during INH activation can be involved in its anti-Mtb activity [64,65]. The upregulation of *msh* and *egt* genes along with other antioxidant genes (*ahpC*, *katG*, *sod*) is likely to boost redox status and mitigate oxidative stress. The activation of these oxidative stress compensatory mechanisms together with chemical neutralizing activity of vit C may underlie the observed tolerance of Mtb to RIF and INH in this study. Another possibility is that these antibiotics are not deleterious to drug tolerant bacteria owing to lowered growth related activity [66].

Efflux pumps are proposed to confer drug tolerance in Mtb [67,68]. Interestingly, several efflux pump coding genes, including *Rv1258c*, that are implicated in tolerance to RIF, INH and STR, were up-regulated by vit C (Fig. 5d). We next investigated the modulatory activity of efflux pump inhibitors (EPIs), Verapamil (Ver) and Piperine (Pip), in vit C-adapted Mtb. EPIs are proposed to act by raising intracellular drug concentrations [67]. Ver or Pip were added individually to vit C-adapted or control cultures that were simultaneously exposed to RIF or INH and bacterial survival rates were assessed after 72 h (Fig. 5a). These EPIs mediated a reversal of vit C-induced tolerance to RIF and INH. More importantly, the cidal activity of antibiotic + EPI combination against vit C-treated bacteria was restored to levels comparable to that in control cultures (Fig. 5e and f). Based on these findings we conclude that (1) vit C-adapted bacteria exhibit tolerance to major TB drugs irrespective of their mode of action, and (2) the activation of efflux pumps prominently contributes to the tolerance mechanism. Notably, the induction of tolerance to first line TB drugs by vit C is contrary to the proposed killing of Mtb by vit C [17].

3.6. Vit C potentiates the cidal action of pyrazinamide. Pyrazinamide (PZA) is an indispensable first line TB drug having a unique role in TB treatment due to its cidal activity against non-metabolizing/ slowly metabolizing persister organisms that are refractory to other drugs [69]. It has long been thought to be selectively active at acidic pH [69,70]. To assess if vit C modulates PZA activity owing to its inherent acidifying property, the drug was added to vit C-adapted cultures and the bacterial survival rate was quantitated after 72 h (Fig. 5a). Consistent with the selective activity of PZA at acidic pH, vit C significantly enhanced the potency of PZA. The MIC of PZA (50 µg/ml) [71,72] decreased 4-fold in vit C-treated cultures (Fig. 5g) and the survival rate of bacteria decreased from < 10% at 12.5 µg/ml of PZA to < 1% at higher drug concentrations. In comparison, control cultures were not effectively killed by PZA (Con 3, DTA medium having a pH of 6.5; Con 2, vit C-containing medium adjusted to pH 6.5; Con 1, medium acidified to pH 5.5 with acetic acid (p values < 0.05; Fig. 5g). Live-dead staining confirmed the sterilizing activity of PZA + vit C combination and ruled out bacterial adaptation to VBNC state (Fig. 5h).

The expression of *pncA*, encoding PZase enzyme that deaminates PZA to active pyrazinoic acid (POA, [69]) was induced and suggested the possibility of more efficient conversion of PZA to POA in treated cultures. PZA is reported to inhibit multiple targets/processes, such as disruption of membrane energetics, trans-translation, acidification of the cytosol and inhibition of pantothenate and coenzyme A synthesis [69]. The known targets of POA, namely *rpsA* (trans-translation) and *panD* (pantothenate synthesis), were down-regulated (Fig. S3b), suggesting a possible basis for lowering of drug MIC. The bactericidal activity of PZA is reported to be highest when bacterial energy status is diminished, as in persister populations [6,69]. Consistent with their growth stasis and dormant condition, Vit C-treated bacteria (pH 5.5) exhibited significantly lower membrane potential (p -value < 0.001, Fig. 5i) and vit C + PZA co-treatment did not cause a further lowering of membrane potential (Fig. 5i). Notably, vit C-treated cultures buffered to pH 6.5 (Con 2) were less susceptible to PZA in spite of a lowering of



(caption on next page)

Fig. 5. Tolerance to TB drugs reversed by efflux pump inhibitors and enhanced susceptibility to PZA establish a dichotomous response of Mtb to vit C. a) Schema for drug tolerance assay. Bacteria exposed to vit C for 24 h were treated with varying concentrations of (b) INH and (c) RIF. The percentage survival of Mtb was calculated from the enumerated Cfu with respect to no drug control for UT and vit C sets on day 4 post-vit C treatment. MIC₉₀ for INH and RIF are marked with black arrows. d) Expression profile of genes reported to code for drug efflux pumps for INH, RIF, STR, EMB and PZA in response to vit C. *Rv1258c* is marked in bold and underlined and ABC transporters are indicated in bold. e) Reversal of vit C-mediated antibiotic tolerance in the presence of EPIs piperine (Pip) and (F) verapamil (Ver) co-treated with INH (4 µg/ml) or RIF (1 µg/ml) at 24 h post vit C treatment. Cfu recovered on day 4 post-vit C treatment were used to calculate the percentage of bacteria surviving the treatment respective to their no drug controls. g) Activity of PZA against Mtb pre-adapted with vit C for 24 h. PZA activity against vit C-treated Mtb was compared to that in media buffered with acetic acid to pH 5.5 (Con 1), vit C-treated cultures adjusted to pH 6.5 (Con 2) and untreated control at pH 6.5 (Con 3). Cells were plating for Cfu enumeration on day 4 post-vit C treatment. Maximum killing of 80% by PZA in Con 1 at pH 5.5 is depicted by a black arrow. MIC₉₀ could not be obtained for the same. *p* values are calculated between vit C-treated Mtb (pH 5.5) and Con 1. h) Live-dead staining of PZA-treated Mtb (50 µg/ml) at day 4 post-vit C treatment. Scale bar, 5 µm. i) Membrane potential of 4 days Mtb culture measured using DiOC₂. [UT, untreated culture; Vit C, vit C-treated culture; Mean ± SD from 3 replicates is plotted for all panels; *p* value *** < 0.001, ** < 0.01, * < 0.05, ns, not significant].

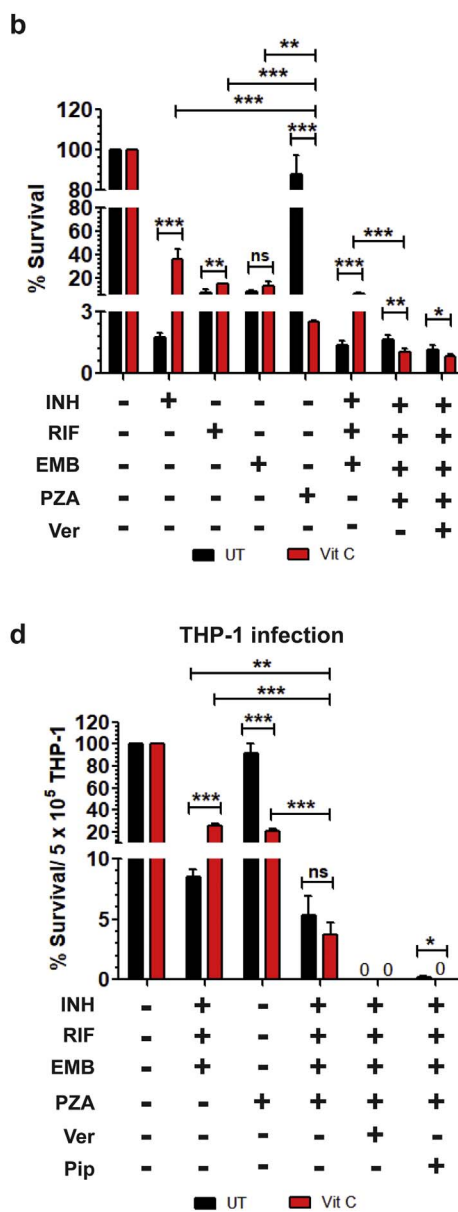
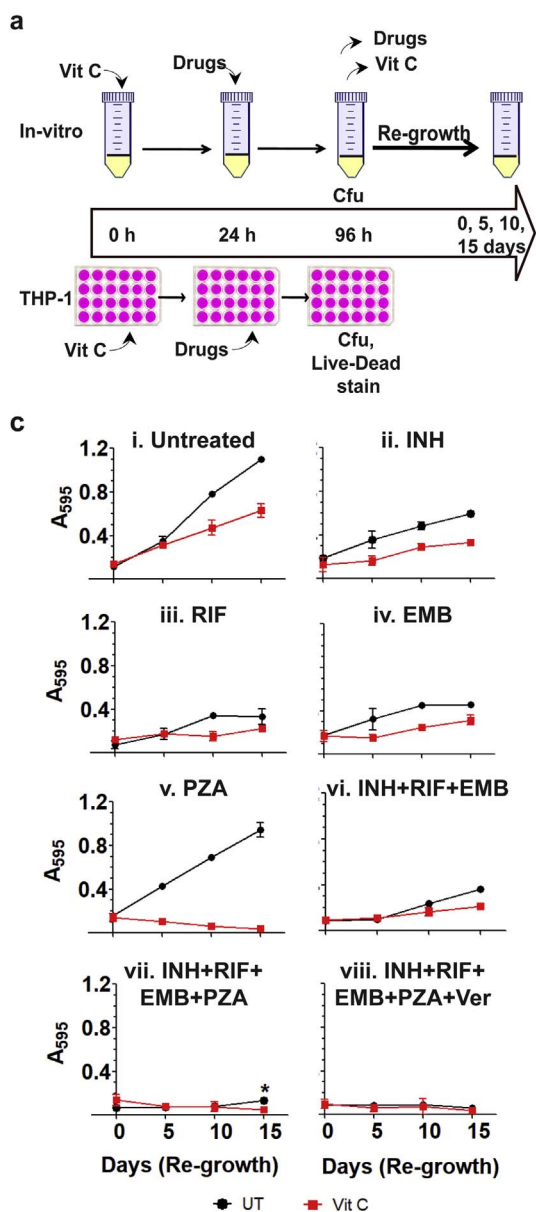


Fig. 6. Vit C enhances the clearance of heterogeneous Mtb populations by TB drugs. a) Schema for survival and re-growth assays for assessing drugs in-vitro and in THP-1 cells. b) Mtb cultures pre-treated with vit C for 24 h, were either treated singly or co-treated for 4 days with 4 × MIC of drugs, namely, INH (0.12 µg/ml), RIF (1 µg/ml), EMB (16 µg/ml), PZA (50 µg/ml), or combinations thereof, plus Ver or Pip (50 µg/ml each) as indicated. Percentage survival refers to Cfu/ml of drug-treated bacteria with reference to their respective UT and vit C no-drug controls. c) Assessment of cidal activity by re-growth of drug-treated vit C and drug-treated UT cultures for 15 days. d) Mtb-infected THP-1 cells were treated with 2 mM vit C for 24 h and then co-treated with 4 × MIC of drugs, INH (0.12 µg/ml), RIF (1 µg/ml), EMB (16 µg/ml), PZA (50 µg/ml) plus Ver or Pip (50 µg/ml each) as indicated. The enumeration of Cfu was performed on day 4 post-vit C treatment. [UT, untreated culture; Vit C, vit C-treated culture; Mean ± SD from 4 replicates is plotted for all panels; *p* value*** < 0.001, ** < 0.01, * < 0.05, ns, not significant].

membrane potential (*p*value < 0.001; Fig. 5i). These observations collectively indicate that the dormancy-inducing and membrane potential-lowering properties of vit C collectively contribute to the enhanced cidal activity of PZA. The synergy between vit C and PZA assumes special prominence in view the unique sterilizing activity of PZA among all TB drugs.

3.7. Vitamin C enhances clearance of bacterial subpopulations by a combination of TB drugs. A noteworthy finding was the dichotomous

role of vit C in modulating antibiotic activity. On one hand, vit C led to INH and RIF tolerance at concentrations that exceeded their MICs by 32 × to 128 ×, and in contrast it potentiated the cidal activity of PZA at 4 × lower concentration than its MIC (Fig. 5b, c and g). The standard treatment of drug susceptible TB includes these drugs in combination with EMB, therefore we extended our study to determine vit C effects on standard treatment in in-vitro cultures and intracellular Mtb. Control and 24 h vit C-exposed cultures were treated with drugs (with/without EPI) for 72 h and bacterial survival quantitated by the enumeration of

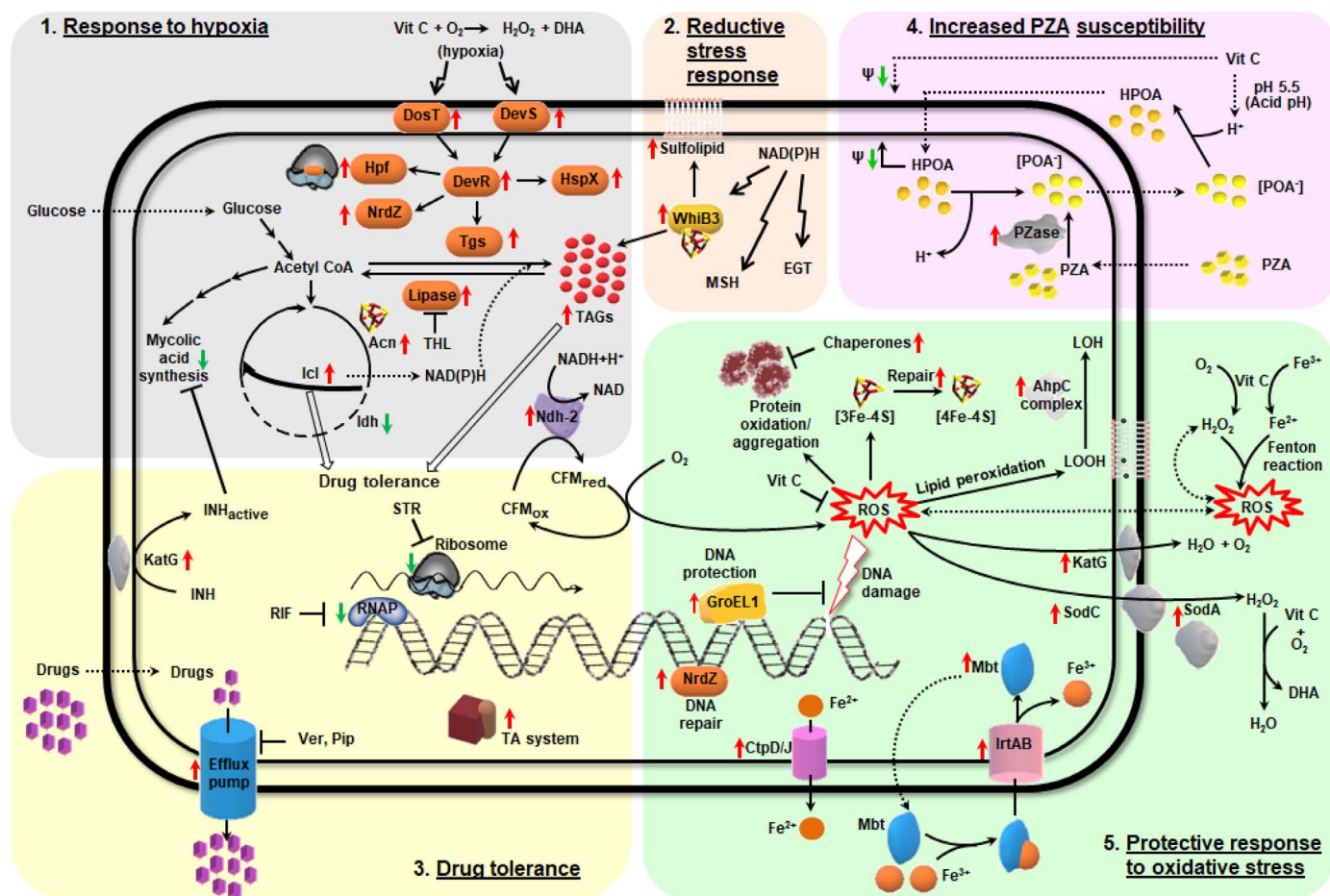


Fig. 7. Salient features of vit C-induced dormancy. (1) **Response to hypoxic stress.** Scavenging O_2 by vit C induces the DevR regulon through DevS and DosT, including chaperone HspX, DNA repair protein NrdZ, hibernation promoting factors (Hpf; Rv1738 and Rv0079) and triacylglycerol synthase (Tgs1). Tgs1 (and other Tgs) activity induces the accumulation of TAGs, which is utilized as a fuel during vit C-induced dormancy and re-growth through lipases and eventually generates acetyl-CoA that enters the TCA cycle. The enhanced expression and activity of Fe-S cluster containing enzyme aconitase (Acn) and isocitrate lyase (Icl) with concomitantly lowered expression and activity of isocitrate dehydrogenase (Idh) indicate the preferable usage of the glyoxylate pathway for metabolism. (2) **Response to reductive stress.** The reducing equivalents accumulated from a slowdown of growth processes are a stimulus for the redox regulator WhiB3 that activates lipid anabolic processes which serves as a reductive sink. The expression of mycothiol (MSH) and ergothioneine (EGT) redox buffers-synthesizing and reducing enzymes is also up-regulated. (3) **Drug tolerance.** Bacterial tolerance to INH, RIF and STR is mediated by the accumulation of TAGs, enhanced activity of efflux pumps and Icl, reduced availability of targets to drugs, and possibly through toxin-antitoxin (TA) systems. (4) **Increased susceptibility to PZA.** Vit C induces the expression of *pncA* (PZase) and acidifies the medium to pH 5.5. The enhanced conversion of PZA to active POA, and subsequent formation of HPOA reduces the membrane potential. Increased susceptibility is attributed to membrane potential-lowering and dormancy-inducing properties of vit C. (5) **Protective response of Mtb to oxidative stress.** Vit C-mediated Fenton reaction leads to the formation of ROS that affect the vital cellular components including DNA, protein, iron-sulfur (4Fe-4S) clusters and lipids. The enzymatic degradation of ROS by catalase-peroxidase (KatG), superoxide dismutase and alkyl hydroperoxidase (AhpC complex), Fe^{2+} efflux as well as chemical quenching of ROS by vit C effectively neutralizes oxidative stress. Further protection by DNA binding and repair proteins GroEL1 and NrdZ, respectively, protein refolding by chaperones and repair of iron-sulfur clusters provides molecular basis of cell adaptation to oxidative stress.

Cfu (Fig. 6a). The observed tolerance of vit C-treated bacteria to INH + RIF + EMB combination (~ 6%) was reversed to < 1% survival by adding PZA (p value < 0.001; Fig. 6b). Bacterial survival was compromised further by inclusion of Pip or Ver (p value < 0.05; Fig. 6b) and the cidal activity of PZA-containing combinations was confirmed in the re-growth assay (Fig. 6a and c, panels vii, viii). Infection models more closely mirror the intracellular milieu and in the THP-1 model, vit C-exposed Mtb exhibit modest growth (not stasis) (Fig. S4). Intracellular bacteria treated with vit C also exhibited higher levels of tolerance to INH + RIF + EMB (~ 25%) compared to control intracellular bacteria (~ 8%, p value < 0.001; Fig. 6d). PZA reversed the vit C-induced tolerance; a combination of 4 drugs with vit C caused ~8-fold enhanced killing relative to 3 drugs (~3% survival; p value < 0.001). Notably, intracellular bacterial killing by 4 drugs co-treated with vit C is greater than that by PZA + vit C (~3% survival vs. ~ 20% survival, p value < 0.001), or by INH + RIF + EMB (~ 8% survival; p value < 0.01; Fig. 6d). Vital staining of the intracellular recovered pool of bacteria confirmed the cidal action of this

combination (data not shown). Our findings conclusively establish that the inclusion of PZA in drug combinations significantly improves killing of vit C-induced dormant bacteria and also efficiently negates vit C-induced tolerance to INH and RIF in both axenic culture and intracellular models. More importantly, the 4 drug combination with either vit C or Ver/ Pip was equally effective in clearing bacteria (Fig. 6b), demonstrating the value of combining TB drugs with vit C, a nutritional supplement.

4. Discussion

The multitude of bacterial adaptation responses to vit C gleaned from this study are summarized in Fig. 7. Large-scale remodeling of the bacterial transcriptome by vit C is explainable by its hypoxia, acidic pH, redox and metabolic stress conferring properties, each of which would elicit a specific bacterial response. Indeed, the integrated response to vit C is consistent with bacterial responses to resist these individual conditions [16,31,32,42,73–76]. The protective response to vit C has twin consequences for Mtb: (1) slowdown or arrest of growth and growth-

related functions followed by progression to a non-culturable state, and (2) activation of dormancy survival functions. The latter is accomplished by collective mechanisms, namely dissipation of reductive stress through TAG accumulation, TAG utilization for survival, protective response to ROS and activation of antibiotic tolerance mechanisms. Various in-vitro models designed to mimic the host environment have revealed that Mtb utilizes multiple pathways for the development of dormancy and drug tolerance [18]. Taken together with the extensive regulatory network of Mtb [77], our data point to the convergence of multiple pathways to achieve dormancy in vit C-adapted bacteria. Importantly, the adapted bacteria possess the cardinal features described for other models of dormancy. An additional feature of the vit C model is progression to a VBNC state. Taking together our observations and reports of VBNC bacilli in 100-day cultures and sputum [1,4–6], we conclude that entry into a VBNC state is achieved quite rapidly in vit C-treated bacteria.

TAG is highly relevant as a storage fuel during prolonged bacterial survival, dormancy and re-growth [43,44,47]. Here we show that vit C strongly induces the accumulation of TAGs. In addition to DevR which mediates Tgs1-dependent accumulation of TAGs [44], the WhiB3 regulator was strongly induced. WhiB3 facilitates metabolic shift to lipid anabolism to maintain redox balance and resist acid stress [31,42], suggesting its relevance in reductive stress dissipative mechanism in vit C models. More importantly, THL-mediated inhibition of lipase led to a severe survival defect which points to the functional relevance of TAG hydrolysis and β -oxidation during both vit C-induced dormancy and re-growth. A defect in TAG accumulation was linked to an inability to arrest bacterial growth and an increased sensitivity to antibiotics [76]. Taking this together with the drastically reduced survival of THL-treated bacteria, we establish these pathways as novel targets to interrupt dormancy and promote bacterial clearance.

It was previously reported that vit C antibacterial activity in-vitro is due to an activation of Fenton reaction [17]. Here we provide several lines of experimental evidence to establish that the bacterial response to vit C-induced ROS is protective, several of which were confirmed in intracellular bacteria. Other features also support the development of dormancy in vit C-adapted bacteria; these include growth stasis, TAG accumulation and utilization, and antibiotic tolerance. Other possible contributory factors for the differing observations made in the present study and that of Vilcheze and colleagues [17] are differences in experimental systems (culture medium, vit C treatment protocol). Notably, the development of VBNC status prevents bacterial growth on solid medium, a criterion that was used to infer the cidal activity of vitamin C [17]. In this context, our findings sound a general cautionary note against scoring bacterial growth on solid medium to infer the cidal activity of drugs and drug candidates.

Antibiotic tolerance is considered to be an adaptive response to growth restricting conditions, and consequential growth slowdown may also progress to a VBNC state [9,78]. In the vit C models, antibiotic tolerance was associated with either growth stasis in-vitro or slow growth in the intracellular scenario, suggesting that bacterial replication and antibiotic tolerance are not necessarily linked. Alternatively, the short-term nature of the infection model may preclude the development of intracellular VBNC bacteria. Antibiotic tolerance in slowly growing intracellular bacteria in the vit C infection model is consistent with an earlier report in replicating intracellular bacteria [79]. Efflux pump mechanism was a significant contributor to antibiotic tolerance in the vit C models, in line with their proposed role in the antibiotic tolerance of stress-adapted bacteria [78]. The toxin-antitoxin (TA) systems have been proposed as another mechanism that contributes to drug tolerance is [80–82]. Several TA systems were up-regulated in response to vit C (Table S5) and their role in vit C-induced antibiotic tolerance awaits further studies.

A highlight of this study is the modulatory effects of vit C on first line TB drugs. Importantly, in combination treatment, 4 first line TB drugs with vit C clear replicating and dormant subpopulations of

bacteria (both in in-vitro and infection models) by synergizing with PZA and negating any tolerance to INH and RIF. Antibiotic tolerance is believed to be the primary cause underlying the long duration of TB treatment, as drug sensitive bacilli persist through the initial phase of treatment by entry into dormancy [3]. By enriching for a larger subpopulation of dormant bacteria than standard cultures, along with its membrane potential-lowering activity, vit C significantly enhanced the efficacy of PZA in combination treatment.

The long duration of TB treatment is also attributed in part to the existence of bacterial subpopulations in diverse niches that are differentially susceptible to TB drugs [3,18]. Our findings have led to the evolution of vit C-based axenic and intracellular infection models that offer unique advantages over existing set ups: the coexistence of non-replicating/slowly growing/VBNC bacteria and replicating organisms provide opportunities to screen for compounds/combinations with vit C that are effective against these diverse subpopulations, a scenario that prevails in vivo. We showed recently by expression profiling of THP-1 cells that vit C boosts host immune defense functions [83]. Taken together with the present findings, vit C can be seen to modulate two key determinants of TB drug efficacy, namely host factors and antibiotic tolerance in bacteria.

5. Conclusions

In conclusion, the present study presents convincing evidence that vit C induces dormancy in Mtb that progresses to a VBNC state. The mechanisms underlying dormancy development are achieved through a well-coordinated and multifaceted bacterial response which results in growth stasis, reductive stress dissipation through TAG deposition, oxidative stress attenuation through chemical and enzymatic mechanisms, and the activation of antibiotic tolerance mechanisms. By the use of all 4 first line TB drugs to derive conclusions more relevant to the human disease condition, we show that the dormancy-inducing activity of vit C is actually beneficial for combination treatment both in-vitro as well as in intracellular Mtb. Although vit C is not itself antibacterial, its unique properties potentiate the activity of pyrazinamide (a cidal drug that targets dormant subpopulation) and negate tolerance to rifampicin and isoniazid in combination treatment. These findings demonstrate the potential of vit C adjunctive therapy for treatment-shortening combination chemotherapy using existing TB drugs.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.redox.2017.12.020>

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