Exposure to nitric oxide drives transition to resuscitation-promoting factor-dependency in mycobacteria

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Abbreviations

BCG – Bacillus Calmette–Guérin

CC – control compound

CFU – colony forming unit

CSN – culture supernatant

DAF-FM diacetate - 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate

DCB – differentially culturable bacilli

MPN – most probable number

Mtb – *Mycobacterium tuberculosis*

NO – nitric oxide

ND – nitric oxide donor

PMA - phorbol myristate acetate

PI – propidium iodide

Rpf – Resuscitation-promoting factor

TFOE - Transcription Factor Over Expression

TB - tuberculosis

Author's contributions

Conception and design of the study: GVM, VM, SMG, BGG, SJW, JG. Data acquisition: SMG, BGG, SJW, MC, JH, OR, NM, GVM, OT. Data analysis and interpretation: all authors. Generation of essential materials and reagents: SLK, JB, NM, OR, VM. Manuscript drafting or critical revision: all authors. Final approval of the submitted version: all authors.

Running title

Nitric oxide induces Rpf-dependency

ABSTRACT

Rationale: Mycobacterium tuberculosis (Mtb) has a devastating impact on global health. Mtb is the causative agent of human pulmonary tuberculosis (TB), which claims 1.4 million lives per annum. During infection, differentially culturable bacilli (DCB) that fail to produce colonies on solid media but grow in liquid media supplemented with resuscitation-promoting factor (Rpf) proteins are formed. These Rpf-dependent mycobacteria are a major barrier to developing improved TB treatments and diagnostic tools.

Objectives: To evaluate whether exposure to nitric oxide (NO), a prominent host defense molecule, drives the generation of Rpf-dependent DCB.

Methods: A new NO donor (ND) for sustained NO release was synthesized. Growth assays, flow cytometry analysis, and global transcriptional profiling, were used to characterize ND-treated Mtb and *Mycobacterium bovis* BCG *in vitro*. Mycobacterial DCB phenotypes were also investigated after infection of THP-1 human cells, which were activated with retinoic acid and vitamin D_3.

Measurements and Main Results: DCB were generated after exposure to ND and during infection of activated THP-1 cells. Resuscitation of these DCB was severely impaired by a specific Rpf inhibitor (3-nitro-4-thiocyanato-phenyl)-phenyl-methanone. Global transcriptomic analyses revealed the down-regulation of three (*rpfA*, *rpfB* and *rpfE*) of the five genes coding for Rpf proteins.

Conclusion: A new model for investigation of the transition of Mtb into an Rpf - dependent differentially culturable state is presented. The system reveals mechanistic insights into the generation of Rpf-dependent Mtb during TB disease and demonstrates the role of NO in this process.

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Key words: Tuberculosis, *Mycobacterium tuberculosis*, differentially culturable bacilli, Resuscitation-promoting factor, nitric oxide

INTRODUCTION

Mycobacterium tuberculosis (Mtb) is the causative agent of tuberculosis (TB), which resulted in the death of 1.4 million people in 2019 [1]. Standard TB treatment lasts at least 6 months and significant resources are directed towards developing shorter and more effective treatments [2]. One barrier to achieving this goal is the generation of tubercule bacilli populations during infection that are not detectable by standard tools [3]. These bacilli do not produce colonies on agar and only grow in liquid medium, often require supplementation with culture supernatant (CSN) [3, 4] and therefore called differentially culturable bacilli (DCB) [4]. In published literature they have been also defined as non-culturable cells [5], viable but non-culturable bacilli [6] and differentially detectable cells [7]. DCB have higher tolerance to some anti-TB drugs [3,

8], and drug treatment may increase the proportion of DCB in patients [3, 7, 9, 10]. These cells are believed to be in a special dormant state that requires resuscitation in liquid media or CSN, however, DCC are currently poorly characterised.

CSN contains resuscitation-promoting factor (Rpf) proteins, which are peptidoglycan remodeling enzymes [11] that are able to resuscitate DCB from the sputum of TB patients [3, 12] and animal lungs [13]. While resuscitation of these Rpf-dependent Mtb has been linked to TB relapse in mice [14], the importance of this process in human TB remains to be established.

The molecular mechanism of DCB generation is currently unknown, although the *in vivo* environment has been proposed as a key factor [13]. Nitric oxide (NO) is a well-characterized component of the innate immune system that is deployed to control intracellular pathogens [15]. While its precise role in controlling human TB is unclear, the inducible NO synthase (iNOS) of macrophages is critical for controlling TB in mice [16] and humans [reviewed in 17]. NO may limit Mtb intracellular growth due to its direct bactericidal effects or by activation of inflammatory cytokines [17] and interference with granulocytic inflammation [18]. Compounds that release NO (NO donors) produce a range of outcomes from bacteriostatic to bactericidal when added to Mtb cultures and dependent in part on the stability and sustained release of NO from the donor [17].

Here, it is shown that sustained exposure to a novel NO donor (ND) rapidly generated DCB of Mtb and *Mycobacterium bovis* (BCG). Resuscitation of these DCB in liquid medium, measured by Most Probable Number (MPN) assay was abolished by a specific Rpf inhibitor 3-nitro-4-thiocyanato-phenyl)-phenyl-methanone [13, 19], suggesting that NO exposure drives Rpf-dependency. Transcriptomic analysis revealed that ND dramatically down-regulated expression of three of the five genes

coding for Rpf proteins (*rpfA*, *rpfB* and *rpfE*). Importantly, NO activation of THP-1 macrophages with retinoic acid and vitamin D_3 promoted NO production and formation of DCB. Taken together, these observations suggest that NO plays a role in generation of DCB during TB infection.

METHODS

Organisms and media

Liquid cultures were grown at 37°C with shaking (100 rpm) for Mtb and static for *M. bovis* BCG Glaxo in Middlebrook 7H9 broth, supplemented with 0.2% (v/v) glycerol, 10% (v/v) albumin-dextrose-catalase (ADC), and 0.05% (w/v) Tween 80 (hereafter 7H9 medium). Culture supernatants (CSN) were prepared from exponentially growing cultures (OD_{580nm} of 0.6-0.8) and sterilized by double filtration (0.22 μm filters). For MPN assays, 50% sterile CSN was mixed with 7H9 medium. The Rpf inhibitor (3-nitro-4-thiocyanato-phenyl)-phenyl-methanone [19] was added to a final concentration of 35 μM. The *dosR* mutant and complemented strain have been previously described [20]. *M. bovis* BCG stocks for macrophage infection were prepared from exponentially growing cultures (OD_{580nm} ~0.4-0.5). THP-1 human monocyte cell line (ATCC® TIB-202™) was grown at 37°C with 5% CO_2 in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% (v/v) heat inactivated fetal bovine serum.

Assessment of viable counts

MPN counts were quantified as previously described [3, 8]. Briefly, sealed 48-well plates with serially diluted mycobacteria in 7H9 medium (MPN_7H9) or 7H9 medium supplemented with 50% (v/v) culture supernatant (MPN_CSN) were incubated at 37°C for 8 weeks. The MPN calculator program was used for determination of MPN counts [21]. For CFU counts, serially diluted samples were spotted onto Middlebrook 7H10

agar plates supplemented with 10% (v/v) ADC and 0.5% glycerol and incubated at 37°C for up to 8 weeks. Resuscitation Index (RI) was defined as RI=Log_{10} MPN - Log_{10} CFU. Limits of detection for CFU and MPN counts were 24 CFU/ml and 4.6 cells/ml, respectively.

Synthesis of NO donor (ND) and control compound (CC) and quantification of NO release

NO donor 3-cyano-5-nitropyridin-2-yl diethyldithiocarbamate (ND) and Control compound 3-cyano-4,6-dimethyl-5-nitropyridin-2-yl piperidine-1-carbodithioate (CC) were synthesized as described in the Online Supplement. The concentration of NO generated by ND and CC were quantified using Griess reagents as previously described [22]. ND or CC were incubated in 200 mM sodium phosphate buffer (pH 6.0) at a final concentration of 200 μM for 24 hours and absorbance at 540 nm was measured at different time points. Known concentrations of sodium nitrite were used to construct a calibration curve.

Treatment of Mtb with ND and CC

Mtb was grown in 7H9 medium to OD_{580nm} of 0.8-1.0. Cells were diluted with fresh 7H9 medium to an OD_{580nm} of 0.1 in a total volume of 5 ml. ND or CC were dissolved in DMSO and added to cultures to a final concentration of 100 μM. Cultures were incubated at 37°C without shaking for 24 hours. In other experiments, mycobacteria were treated with 200 μM spermine-NONOate (equivalent to up to 400 μM NO; Sigma), or 5 mM $NaNO_2$.

Flow cytometry staining and analysis

For detection of NO in ND-treated *M. bovis* BCG, DAF-FM diacetate (4-amino-5-methylamino-2´,7´-difluorofluorescein; ThermoFisher) at concentration of 5 μM was used [23]. Stained bacteria were analysed in CytoFLEX flow cytometer using filters

for excitation 495 nm and emission 515 nm and CytExpert software (Beckman Coulter).

LIVE/DEAD BacLight (ThermoFisher) stained *M. bovis* BCG were studied in FACSAria flow cytometer using BD FACSAria™ software (BD Bioscience). The following parameters were applied: SYTO 9 excitation/emission at 480/500 nm; PI excitation/emission at 490/635 nm; 10,000 events were recorded from each sample. Single parameter fluorescence overlay plots and dual parameter dot plots were generated as previously described [24]. The gating strategy is summarized in the Online Supplement. Heat-killed *M. bovis* BCG were included as a control.

Transcriptomic analyses

Three biological replicates of RNA from Mtb were extracted 4 hours after treatment with either ND or CC using the GTC/Trizol method [25]. RNA (2 µg) was labeled with Cy3 fluorophore and hybridized to a Mtb microarray (ArrayExpress accession number ABUGS-41) as previously described [26]. Fully annotated microarray data are available in ArrayExpress (E-MTAB-10776).

For quantitative RT-PCR, RNA was reverse transcribed to cDNA using SuperScript II reverse transcriptase kit (ThermoFisher Scientific) with mycobacterial genome-directed primers [27]. The qPCRs were run in the Rotor-gene 600 instrument (Corbett Research) using the 2×SYBR green master mix (ThermoFisher Scientific) and primers (Table S1). Levels of expression were normalized to 16S rRNA.

Macrophage infection

Human THP-1 monocytes were seeded at 2×10^5 cells/ml in 24 well plates (Greiner) followed by activation either with retinoic acid (1 µM) and vitamin D_3/cholecalciferol (1 µM) or 10 ng/ml PMA (phorbol myristate acetate) for 72 hours. The macrophages were infected with *M. bovis* BCG at an MOI of 0.5 and incubated at 37°C with 5% CO_2 for 3

hours, followed by treatment with 200 μg/ml amikacin for 1 hour. For CFU and MPN counts, the infected macrophages were washed three times with PBS and lysed with 0.2% (v/v) Triton X-100.

Detection of Rpf peptides using mass-spectrometry

Mtb was grown in Sauton medium to OD_{580} 0.8. Filtered CSN was enriched on DEAE Sepharose and digested with trypsin. LC-MS/MS was carried out using an RSLCnano HPLC system (Dionex, UK) and an LTQ-Orbitrap-los mass spectrometer (Thermo Scientific). The raw data were processed using Proteome Discoverer (version 1.4.0.288, Thermo Scientific), Mascot (version 2.2.04, Matrix Science Ltd).

Statistical analyses

Unpaired t-test (Excel) or one-way ANOVA (Prism 9) were used to analyze growth and flow cytometry datasets. Differentially expressed genes in ND- compared to CC-treated cultures, 4 hours after exposure, were identified using a modified t-test (GeneSpring 14.5; Agilent Technologies) with Benjamini and Hochberg multiple testing correction, and defined as those with $p<1\times10^{-5}$ and minimum fold change of 2.0.

RESULTS

Design, synthesis and testing of a novel NO donor

Exposure of mycobacteria to reactive nitrogen species lowers CFU counts [17, 28,29], which could indicate the presence of DCB. Mtb incubation with acidified nitrite in 7H9 medium at concentrations above 5 mM reduced Mtb CFU counts, but resuscitation in liquid medium was not achieved (Fig. S1A). Treatment of Mtb with spermine NONOate

(half-life of 39 min at 37°C, pH 7.4; [30]) did not significantly decrease CFU counts (Fig. S1A). We wondered if NO release might be too rapid for alteration of Mtb CFU counts and that sustained exposure to NO might be required for the generation of DCB. Therefore a novel NO donor (ND), 3-cyano-5-nitropyridin-2-yl diethyldithiocarbamate (Figure 1A) and a structurally related control compound (CC), 3-cyano-4,6-dimethyl-5-nitropyridin-2-yl piperidine-1-carbodithioate (Figure 1B) were designed and synthesized. The nitro group of CC was protected from hydroxyl or enzymatic attack by two methyl groups to impair release of NO in culture media. NO was progressively released from ND at 20°C, pH 6.0 with the highest concentration detected after 8 hours. NO was not produced from CC (Figure 2A).

DAF-FM diacetate can be used to detect the presence of intracellular NO [31]. DAF-FM diacetate positive *M. bovis* BCG were significantly higher ($p<0.0001$) in ND-treated bacteria compared to CC-treated and untreated bacteria (Figure 2B). Heat-killed *M. bovis* BCG did not stain with DAF-FM diacetate after exposure to ND. This suggested that NO released from ND entered mycobacteria and was therefore suitable for testing the effects of NO exposure on growth phenotypes using CC as a control.

Exposure to ND generated Rpf-dependent DCB

M. bovis BCG treated with ND was stained with SYTO 9 and propidium iodide (PI) to determine cell viability (Figure S2A). Three distinct populations were detected: unstained (PI-, SYTO 9-), dead (PI+, SYTO 9+), and live (PI-, SYTO 9+) (Figure S2A). After 24 hours there was no significant difference ($P>0.05$, t-test) in the percentage of live or dead bacteria for ND-treated or CC-treated *M. bovis* BCG (Figure 2C), which suggested that NO was not directly bactericidal. More than 50% of cells in both samples were PI-, SYTO 9+, and likely remained viable.

While CC treatment for 24 hours had no effect on CFU counts (Figure S3), exposure to ND resulted in a 1000-fold reduction in CFU (Figure 3A). Furthermore 1.2×10^5 bacteria were recovered in 7H9 medium 24hour post-treatment with ND, and supplementation with CSN increased this to 1.7×10^6 (Figure 3A). This suggested that the majority of the ND-treated cells were differentially culturable, in line with observations with Mtb from clinical TB samples [3, 4, 8]. Similar results were observed with *M. bovis* BCG; however, the number of resuscitated bacteria was generally lower and more variable (Figure S4).

The addition of a Rpf inhibitor [19] abolished the resuscitation of ND-treated Mtb in 7H9 medium, and significantly ($p<0.05$) impaired resuscitation in CSN-supplemented medium (Figure 3B). In accordance with previous results [13, 19], the Rpf- inhibitor had no effect on actively growing Mtb (Figure S5). Proteomics analysis confirmed that RpfA and RpfB were present in the Mtb CSN (Figure 3C). Together, these data suggest that Rpf proteins play an important role in resuscitation of ND-generated DCB.

Generation of Rpf-dependent Mtb by ND does not require DosR

DosR is a response regulator that has been previously shown to respond to NO [33, 34]. Treatment of the Mtb *dosR* deletion mutant, a complemented strain, and the parent Mtb with ND for 24 hours resulted in similar numbers of DCB (Figure S6). All strains were equally resuscitated in CSN-supplemented medium (Figure S6), indicating that transition to the Rpf-dependent differentially culturable state after ND exposure and subsequent resuscitation by Rpf proteins did not require DosR.

ND-treatment induces SigH- and SigF-transcriptional alteration and lower *rpf* gene expression

Transcript profiling was used to investigate the transition of Mtb to differential culturability. Compared to treatment with CC, 640 (407 induced, 233 repressed) genes were differentially expressed in response to ND 4 hours after exposure (Figure 4A, Table S2). Analysis of up-regulated genes (using Transcription Factor Over Expression (TFOE) tool [35]) indicated this transcriptional response was coordinated by the alternative sigma factors SigH (TFOE output significance of enrichment of the regulon 2.3×10^{-10}) and SigF (pvalue 1.2×10^{-7}), both of which genes were also up-regulated in response to ND (Figure 4B). SigH is activated by oxidation of its anti-sigma factor RshA, which can be triggered by NO, and by phosphorylation of RshA by the serine-threonine protein kinase, PknB [36]. SigF is associated with cell envelope integrity and is controlled by anti-sigma factor agonists, RsfA and RspB, which sequester the anti-sigma factor RsbW and also by the anti-sigma factor Rv1364c. In *M. smegmatis* the SigF regulon was induced by respiratory inhibitory conditions, such as might occur in the presence of NO, in a RsfB-dependent manner [37]. The up-regulation of *sigH* (3.82 fold) and *sigF* (5.79 fold) was accompanied by down-regulation of the primary sigma factor, *sigA* (4.56-fold), and 35 genes from the *rpl*, *rpm* and *rps* families, coding for ribosomal proteins, in accordance with impaired growth in the presence of ND (Table S2).

In contrast to other models of NO exposure [33, 34, 38], the DosR regulon was not induced after ND treatment (Figure 4B). Moreover*, cmr*, coding for a repressor of *dosR* expression [39], was up-regulated. Thus, the transcriptional profiling is consistent with the conclusion from our DosR deletion experiments that transition to the Rpf protein-dependent differentially culturable state after ND exposure does not require DosR .

Mtb is regarded as an obligate aerobe using aerobic respiration to support growth and NO is an inhibitor of respiration [34]. Under stress conditions Mtb can deploy two alternative terminal electron acceptors, nitrate reductase (Nar) and fumarate reductase (Frd). In accord with the lack of induction of the DosR-regulon, *narK1* was down-regulated in response to ND and expression of the *narG*, *narH*, *narI*, and *narX* genes, coding for nitrate reductase, was not significantly changed. In contrast, the *frdABCD* genes, coding for fumarate reductase, were up-regulated, suggesting utilization of fumarate reduction to maintain redox balance and membrane potential [40]. Interestingly, the serine-threonine protein kinase G (encoded by *pknG*), which contains a rubredoxin domain with two thioredoxin motifs and has been implicated in the regulation of intracellular NADH concentrations [41], was up-regulated. PknG controls central metabolism by sensing glutamate and aspartate availabilities and regulating activities of α-ketoglutarate dehydrogenase, glutamate dehydrogenase, and glutamine synthase [42]. Glutamate has been previously shown as a major carbon source for mycobacteria exposed to NO *in vitro* and during growth in macrophages [43]. In addition to these changes in operation of the Krebs cycle, the *pta-ackA* operon, coding for phosphotransacetylase and acetate kinase, was induced, consistent with impaired aerobic respiration and increased reliance on substrate-level phosphorylation.

Inhibition of bacterial growth by NO is linked to redox chemistry [44]. As a radical, NO itself reacts with transition metals and other molecules with unpaired electrons, such as superoxide. The latter reaction produces peroxynitrite, which can oxidize cysteine thiol to sulfenic acid. Hence, NO perturbs redox balance by damaging iron-sulfur clusters and protein thiol groups [38, 45]. Accordingly, exposure to ND induced expression of anti-oxidant genes, such as the SigH/AosR-activated non-canonical

cysteine biosynthetic genes (*mec-cysO-cysM*), consistent with increased synthesis of cysteine-based anti-oxidants (e.g. up-regulation of ergothioneine biosynthesis genes; *rv3700c and 3704c*). In addition, other thiol-related anti-oxidant activities were up-regulated, including sulfate-containing compound ABC transporter (*cysA1*), sulfate adenyltransferase (*cysDN*), methionine sulfoxide reductases (*msrA* and *msrB*), a rhodanese domain protein (*rv1674c*), a thioredoxin reductase and thioredoxin proteins (*trxB2-C*, *thiX*, *trxB1*). Damage to iron-sulfur proteins resulting in disrupted iron homeostasis was suggested by up-regulation of mycobactin biosynthetic genes (*mbtE*, *mbtG* and *mbtH*) and the Esx-3 genes required for siderophore-mediated iron uptake (*rv0282-rv0292*). These anti-oxidant responses are consistent with repair of NO-mediated oxidative damage.

The up-regulation of *murI,* coding for glutamate racemase [46]*, cwlM* [47], and *pbpA* [48] suggested that remodeling of peptidoglycan was involved in the transition to Rpf-dependency. Indeed, expression of *cwlM* was previously shown to be up-regulated by treatment with spermine NONOate [49]. Consistent with the observation that ND-treatment resulted in Rpf-dependent mycobacteria, three *rpf* genes were down-regulated (*rpfA, rpfB* and *rpfE*; 9.5-, 3.9- and 33.2-fold, respectively). This was confirmed by qRT-PCR (Figure 4C).

Differentially culturable mycobacteria were produced in activated THP-1 cells

Mycobacterial pathogens can survive and replicate in macrophages where they are exposed to NO [50]. The human THP-1 monocytic cell line has been shown to produce NO after exposure to retinoic acid and THP-1 cells treated with retinoic acid and vitamin D_3 (RAVD) were proposed as a model for investigation of Mtb persistence [51].

We treated THP-1 cells with either PMA, the typical stimulant for differentiation of these cells into macrophages, or RAVD, and stained them with DAF-FM diacetate to

detect NO (Figure 5A). The number of DAF-FM positive macrophages was significantly higher in samples treated with RAVD compared to untreated and PMA-treated cells ($p<0.05$, Figure 5A).

Next, either PMA- or RAVD-derived macrophages were infected with *M. bovis* BCG. CFU and MPN counts of *M. bovis* (BCG) were determined during a 7-day infection (Figures 5B, C). In both cases, there was a significant reduction of CFU after 2 days of infection; however MPN and MPN_CSN values remained unchanged for RAVD-treated THP-1 cells. Resuscitation indices calculated for 7H9 medium and CSN-supplemented medium were >1, suggesting that at early stages of infection DCB are produced in RAVD-treated macrophages. Results obtained from PMA-derived macrophages were more variable and overall did not reveal reliable generation of DCB. Thus, there was a positive relationship between macrophage NO concentration and the generation of DCB.

DISCUSSION

During infection pathogenic mycobacteria produce heterogenous populations that differ in their metabolic activity, growth characteristics and tolerance to antimicrobials [8, 13, 52]. Amongst these populations, differentially culturable mycobacteria are of particular interest, because they are difficult to detect and eradicate, but frequently represent the dominant Mtb population in clinical TB samples [3-4, 8-12, 53-55]. In the laboratory, DCB are induced *in vitro* by prolonged incubation in stationary phase [56], gradual acidification [57], alteration of sodium-potassium ratio [26], treatment with antimicrobials [14, 58, 59] or exposure to vitamin C [60]. Here we describe a new model system based on a novel NO donor (ND) that simulates the *in vivo* environment

by generating a heterogenous population of Mtb thereby permitting investigation of the physiologically distinct states of Mtb produced during the pathogenesis of TB.

In our ND-induced system transition to the differentially culturable state did not require activation of the DosR regulon, but involved two alternative sigma factors, SigH and SigF and their regulons, previously implicated in adaptation to redox stress and respiration-inhibitory conditions [36, 37, 61]. Overall, the transcriptomic signature of ND-treated DCB was associated with inhibition of cell division, repair of NO-induced damage, metabolic reprogramming to restore redox balance and membrane potential (Figure 6).

While there is an ongoing debate concerning the importance of NO in controlling Mtb infection in humans, NO is detected in TB patients and our data suggest that it contributes to the generation of DCB by causing the down-regulation of *rpfA*, *rpfB* and *rpfE* expression. Rpf are cell wall cleaving enzymes and their expression under conditions when the peptidoglycan producing machinery is likely to be damaged can be fatal for mycobacteria resulting in uncontrollable cell lysis and death. In addition, NO also has the potential to directly damage Rpf proteins, which require a disulfide bond for correct folding and activity [62]. Hence the simplest explanation for the observed Rpf-dependency of ND-treated mycobacteria is that DCC cells cannot initiate Rpf production when grown in standard media and require supplementation with recombinant Rpf or Rpf-containing CSN to restart growth and peptidoglycan biosynthesis.

Expression of Rpf coding genes is tightly controlled by multiple regulators [63], Fig. S7, including Lsr2 [64] and MtrA [65]. The precise mechanism underpinning the response to ND is unknown.

Exposure to various levels of NO drives Mtb heterogeneity in the infected host (Figure 6). These heterogenous populations have different growth requirements and respond differently to drugs, may induce differential immune responses and create a unique microbial community (8,13). Our observation that NO down-regulates the expression of *rpfA*, *rpfB* and *rpfE* and generates Rpf-dependent DCB *in vitro* and intracellularly in macrophages provides the foundation for further investigations to understand the molecular mechanisms that underpin these adaptations and elucidate the precise role of DCB in TB pathogenesis and treatment outcomes.

Conflict of Interest Statement: Authors declare no conflict of interests.

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FIGURE LEGENDS

Figure 1. Chemical structures of nitric oxide donor (ND) and a control compound (CC) used in this study. *(A)* The NO donor, 3-cyano-5-nitropyridin-2-yl diethyldithiocarbamate 3-cyano-4 (ND). *(B)* The control compound, 6-dimethyl-5-nitropyridin-2-yl piperidine-1-carbodithioate (CC).

Figure 2. *(A)* Quantifying NO release from ND and CC. Both compounds were incubated in sodium phosphate buffer for 24 hours and the amount of NO released was measured using Griess reagent. *(B)* Monitoring NO release from ND or CC after treatment of *M. bovis* BCG for 24 hours using DAF-FM diacetate and flow cytometry. The percentage of DAF-FM diacetate stained shown as mean ± SEM (N=3). *(C)* Assessing the viability of *M. bovis* BCG following treatment with ND and CC. Bacteria were dually stained with PI and SYTO 9 at 24 hours post-treatment with either ND or CC and analyzed by flow cytometry. Percentages of live (SYTO 9+), dead (SYTO 9+ PI+), or unstained bacteria are shown as mean ± SEM (N=3).

Figure 3. Generation of Rpf-dependent Mtb *in vitro*. *(A)* Mtb were treated with 100 μM NO donor (ND) for 24 hours at 37°C without shaking. CFU, MPN and MPN_CSN counts were determined before treatment and after 24 hours. *(B)* The effect of Rpf-inhibitors on the resuscitation of ND-treated mycobacteria. Rpf-inhibitor (3-nitro-4-thiocyanato-phenyl)-phenyl-methanone) 35 μM was added to both 7H9 medium (7H9 plus Rpf-inhibitor) and 7H9 supplemented with CSN (CSN plus Rpf-inhibitor). Results are the mean ± SEM (n=3). Resuscitation Index=Log_{10} MPN - Log_{10} CFU. *(C)* Detection of RpfA and RpfB peptides in Mtb CSN by mass-spectrometry.

Figure 4. Exposure of Mtb to ND for 4 hours results in activation of distinct regulatory mechanisms. *(A)* Volcano plot showing 407 genes significantly induced (red) and 233 genes repressed (blue) by ND compared to CC at 4 hours. Significantly differentially

expressed genes were identified using a moderated t-test ($p<1\times10^{-5}$ with Benjamini and Hochberg multiple testing correction) and fold change >2.0. *(B)* Box and whisker plots showing the distribution of expression ratios (log2) of the SigH regulon [66], aerobic respiration genes functional classification I.B.6.a [67] and DosR regulon [66] after 4 hours exposure to ND or CC. *(C)* Expression of selected genes was confirmed by qPCR from Mtb exposed to ND relative to CC at 4 hours normalised to 16S rRNA transcript abundance. Data shown as the $\log_2$ (relative fold change) ± SEM (N=3).

Figure 5. THP-1 macrophages generate Rpf-dependent *M. bovis* BCG. *(A)* NO levels in PMA-activated and RAVD-activated THP-1 cells detected using DAF-FM diacetate and flow cytometry. The percentages of DAF-FM diacetate-stained cells are shown as the mean+/-SEM. * $p<0.05$. RAVD-treated *(B, C)* or PMA-treated *(D, E)* THP-1 cells infected with *M. bovis* BCG at MOI 0.5. CFU, MPN and MPN_CSN counts were determined at 0, 2, 4 and 7 days post-infection. **CFU counts were significantly lower than MPN counts ($p<0.01$); ***CFU counts were significantly lower than MPN_CSN counts ($p<0.001$). The RI (RI= Log_{10}(MPN/ml) - Log_{10}(CFU/ml)) were calculated for both PMA-activated *(C)* and RAVD-activated THP-1 cells *(E)*. *(C)* *RI_7H9 and RI_CSN at 2 days were significantly higher those at the start of infection ($p<0.05$).

Figure 6. NO-mediated generation of Rpf-dependent differentially culturable Mtb. NO exposure *in vitro* induces global transcriptional changes coordinated by SigH and SigF which result in growth shut-down, activation of alternative metabolic pathways, detoxification of NO damage and down-regulation of *rpfA, rpfB* and *rpfE* expression. This leads to generation of a heterogenous population of Mtb, including a Rpf-dependent sub-population. This population heterogeneity mimics *in vivo* observations where NO is produced by the inducible NO synthase (iNOS) and drives formation of

Rpf-dependent and differentially culturable Mtb that can be detected in infected tissue and sputum samples.

FIGURES

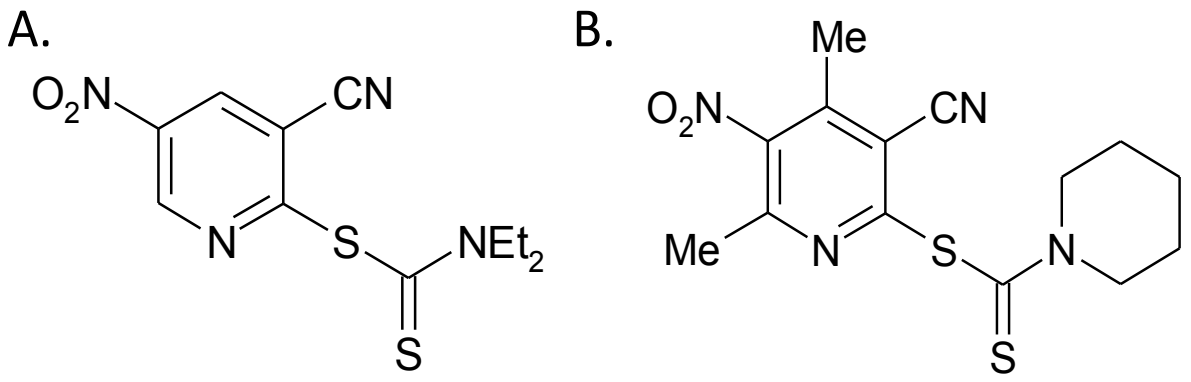

FIGURE 1.

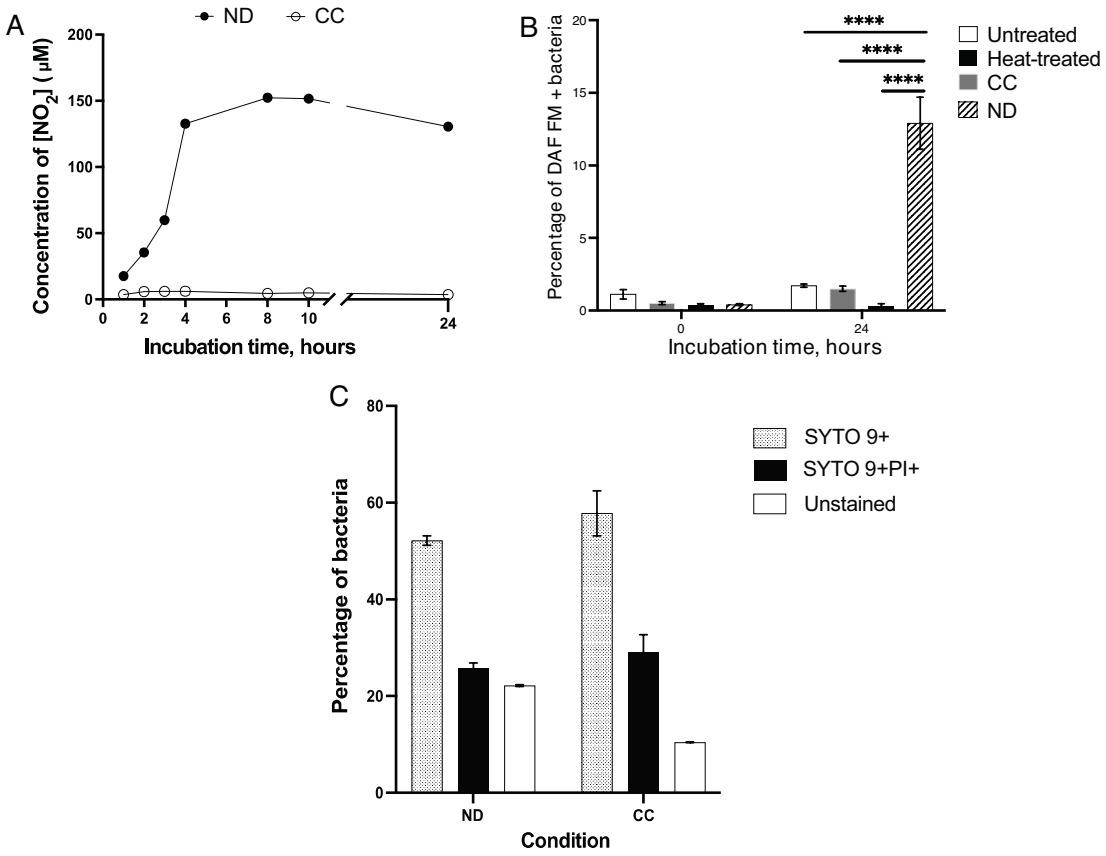
A
ND
CC
Concentration of [NO2] (μM)
200
150
100
50
0
0
2
4
6
8
10
24
Incubation time, hours
B
Percentage of DAF FM + bacteria
20
15
10
5
0
****
****
****
Untreated
Heat-treated
CC
ND
0
24
Incubation time, hours
C
Percentage of bacteria
80
60
40
20
0
SYTO 9+
SYTO 9+PI+
Unstained
ND
CC
Condition

FIGURE 2.

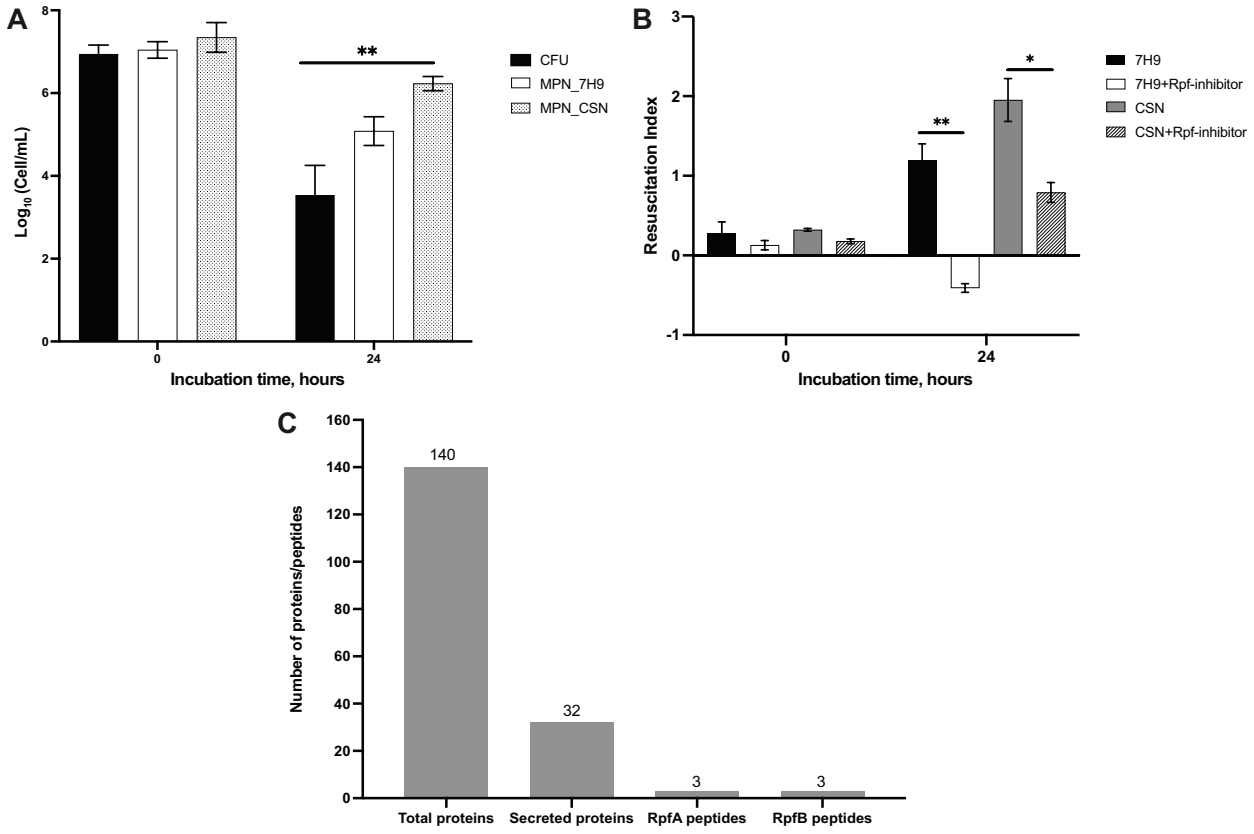
A
Log10 (Cell/mL)
Incubation time, hours
CFU
MPN_7H9
MPN_CSN
**
B
Resuscitation Index
Incubation time, hours
7H9
7H9+Rpf-inhibitor
CSN
CSN+Rpf-inhibitor
**
*
C
Number of proteins/peptides
140
32
3
3
Total proteins
Secreted proteins
RpfA peptides
RpfB peptides

FIGURE 3.

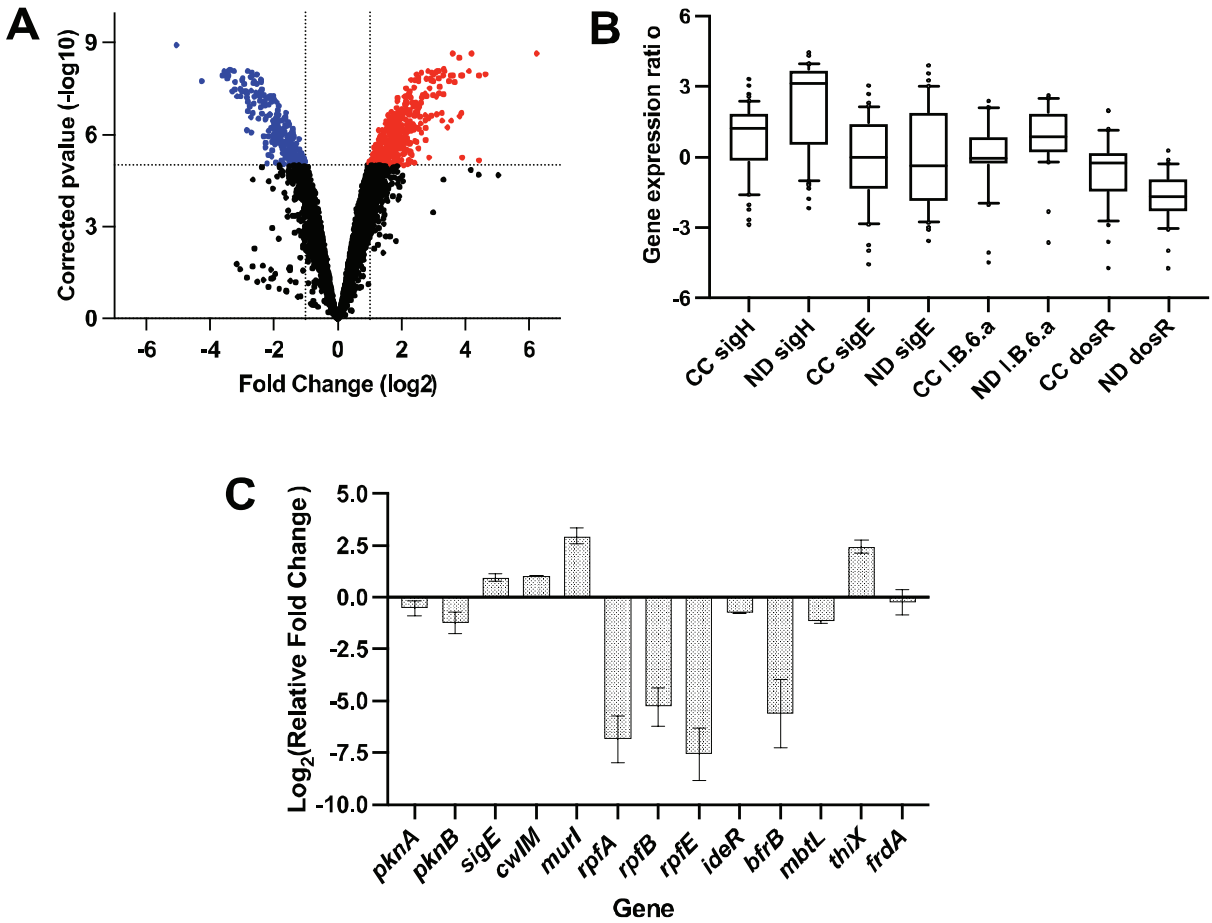
A
Corrected pvalue (-log10)
9
6
3
0
-6
-4
-2
0
2
4
6
Fold Change (log2)
B
Gene expression rati o
6
3
0
-3
-6
CC sigH
ND sigH
CC sigE
ND sigE
CC I.B.6.a
ND I.B.6.a
CC dosR
ND dosR
C
Log2(Relative Fold Change)
5.0
2.5
0.0
-2.5
-5.0
-7.5
-10.0
pknA
pknB
sigE
cwlM
murI
rpfA
rpfB
rpfE
ideR
bfrB
mbtL
thiX
frdA
Gene

FIGURE 4.

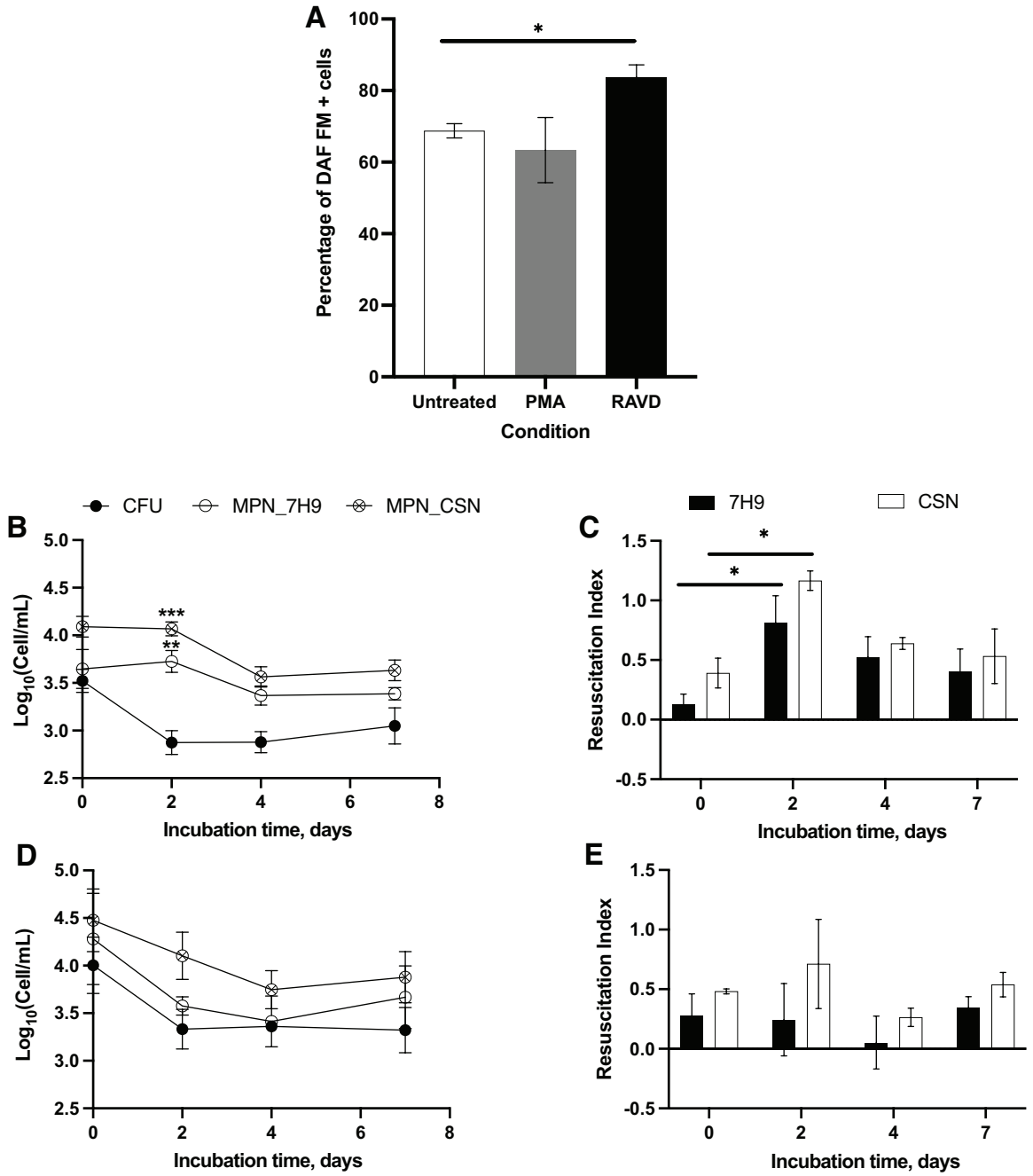
A
Percentage of DAF FM + cells
Untreated
PMA
RAVD
Condition
B
CFU
MPN_7H9
MPN_CSN
Log10(Cell/mL)
Incubation time, days
C
7H9
CSN
Resuscitation Index
Incubation time, days
D
Log10(Cell/mL)
Incubation time, days
E
Resuscitation Index
Incubation time, days

FIGURE 5.

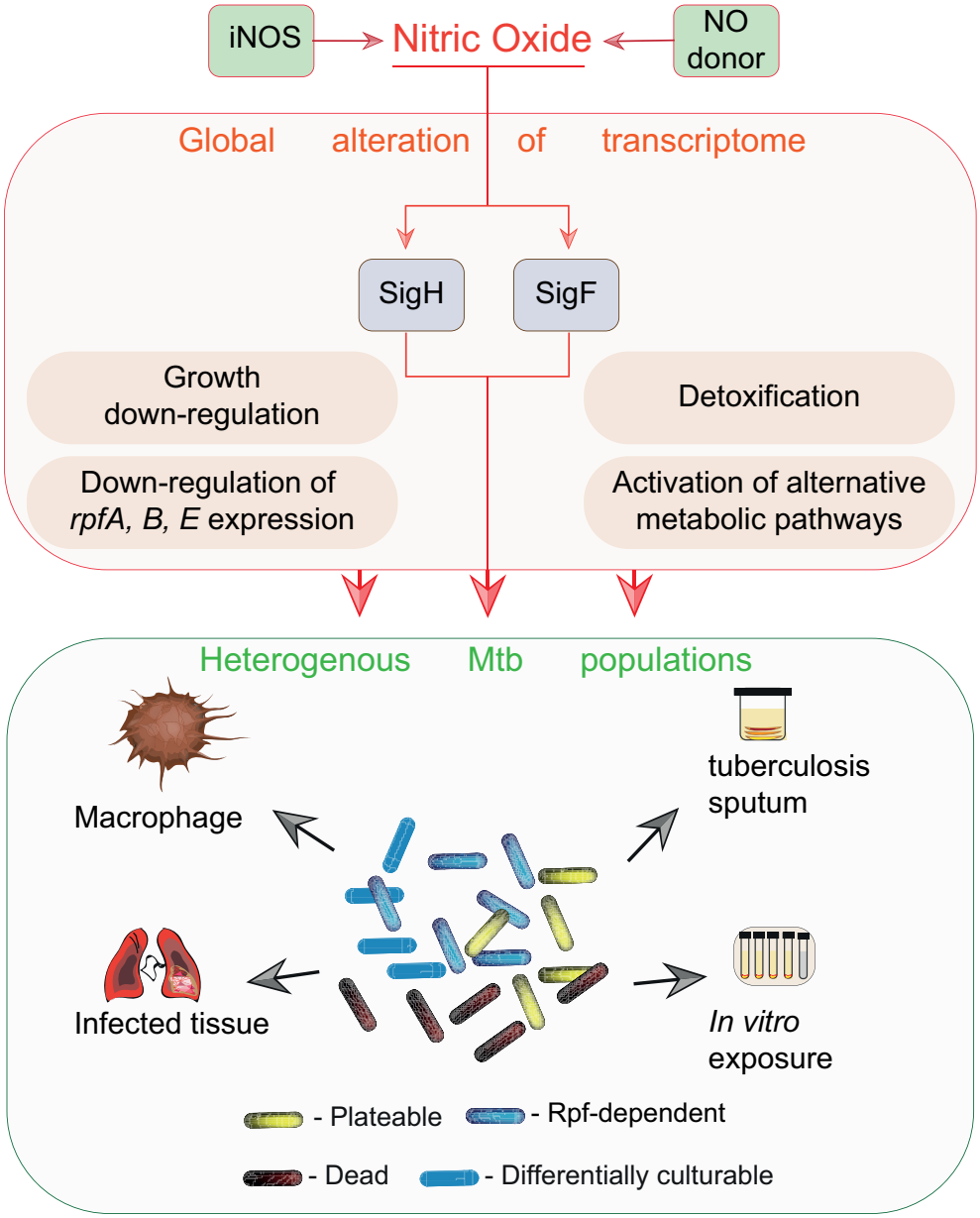
iNOS
Nitric Oxide
NO donor
Global alteration of transcriptome
SigH
SigF
Growth down-regulation
Detoxification
Down-regulation of rpfA, B, E expression
Activation of alternative metabolic pathways
Heterogenous Mtb populations
Macrophage
tuberculosis sputum
Infected tissue
In vitro exposure
- Plateable
- Rpf-dependent
- Dead
- Differentially culturable

FIGURE 6.