DrrS, a small non-coding *Mycobacterium tuberculosis* RNA, regulates the whole genome expression shifts consistent with adaptations for survival within host macrophages

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Abstract

Small non-coding RNAs play a significant role in regulation of bacterial transcription and translation. Their expression in response to external factors is important for the adaptation of bacteria to changing environmental conditions. We investigated the expression of DrrS, a small noncoding RNA of *Mycobacterium tuberculosis*, in the mouse model *in vivo*, in the *ex vivo* model based upon infected macrophages, and in bacterial cultures, and demonstrated its significant contribution to host-pathogen interactions. Activation of the host immune system triggers NO-inducible up-regulation of DrrS in macrophage-engulfed mycobacteria. Constitutive overexpression of DrrS in cultured mycobacteria launches a broad spectrum of shifts in the bacterial transcriptome profile very similar to those reported for *M. tuberculosis* adaptation to hostile intra-macrophage environment, and providing defense against oxidative and NO stresses. In addition, we observed dramatic up-regulation of genes for the PE/PPE proteins and proteins of the ESX-1 and ESX-5 secretion systems. Taken together, our results suggest a direct involvement on this small RNA in the interplay between mycobacteria and the host immune system during infectious process.

Author summary

Pathogenic mycobacteria, including *Mycobacterium tuberculosis*, are able to survive within host macrophages. In attempt to eliminate intracellular mycobacteria, innate and acquired immune responses of the host activate a number of effector reactions to achieve effective intracellular mycobacterial killing. Mycobacteria, in turn, evolved a plethora of molecular mechanism providing successful escape from host immunity, involving several metabolic pathways allowing transition to dormancy – the state of slow-to-no replicative activity and an increased resistance to external stresses. These mechanisms remain poorly characterized. Small non-coding bacterial RNAs are

expressed in response to external factors and play an important role in adaptation of bacteria to changing environmental conditions and escape from host immune responses. We investigated DrrS, a small non-coding RNA of *Mycobacterium tuberculosis*, in the mouse TB model *in vivo*, in infected macrophages *ex vivo* and in bacterial culture, and demonstrated that DrrS up-regulation strictly follows activation of the host immune defense. We established the strain of *M. tuberculosis* overexpressing DrrS in culture and found that DrrS contributes to mycobacterial resistance to reactive intermediates and activation of dormancy-associated genes, thus participating in bacterial metabolic adaptations and interactions with the host immune system.

Introduction

Tuberculosis (TB), a chronic disease caused by *Mycobacterium tuberculosis*, takes nearly 2 million lives annually [1]. *M. tuberculosis* persistence in the infected host involve several stages and may have different manifestations: initial infection followed by acute, semi-acute or chronic diseases; latent infection characterized by the presence of viable bacteria with slow-to-no level of replication and the lack of clinical manifestations; and transition from the latent state to reactivation processes (reviewed in [2, 3]). The spectrum of the disease manifestations depends upon a dynamic balance between protective host responses and defensive strategies of *M. tuberculosis*. Identification of molecular mechanisms of *M. tuberculosis* adaptation to the host immune defense during its persistence within macrophages and other phagocytes is an important scientific and medical problem.

Long co-evolution of *M. tuberculosis* and its human host allowed the pathogen to develop strategies that can effectively combat host defense systems. Regulatory proteins, non-coding RNAs and their targets constitute complex adaptive metabolic networks that allow the pathogen to resist host response at different stages of infection. Bacterial small RNAs participate in regulation of

63 transcription and translation by affecting the level of gene expression and mRNA stability. Mostly,
64 small RNAs are expressed in response to the external factors, helping bacteria to adaptively react to
65 the changing environmental conditions and regulate the key stages of pathogenesis (see recent
66 reviews [4-6]).

67 Application of the high throughput sequencing and computer algorithm approaches allowed
68 identification of dozens of small RNAs in mycobacterial species [7, 8]. Several *in vitro* studies have
69 elucidated the functioning of small RNAs in *M. tuberculosis* [9-12]. However, dissecting the role of
70 a particular small RNA in mycobacterial physiology appeared to be difficult, especially in *in vivo*
71 settings.

72 One of such RNAs, DrrS (DosR-associated sRNA, ncRv11733, MTS1338), is highly
73 expressed during the stationary phase of growth [13], and the dormancy state [14]. This small RNA
74 is present only in genomes of highly pathogenic mycobacteria and is highly conservative. *In vitro*
75 experiments demonstrated that its transcription is controlled by the transcriptional regulator DosR
76 and is activated under hypoxic and NO-induced stress conditions [11], suggesting that DrrS may
77 play a role during the stable phase of infection, when host responses confronts mycobacterial
78 multiplication more or less successfully. Indeed, we and others demonstrated a striking increase in
79 the DrrS transcription in animal models of chronic infection [9, 15]. Thus, it seems likely that DrrS
80 triggers adaptive biochemical cascades for intracellular bacterial persistence.

81 Here, we characterize dynamic changes in the DrrS expression in mycobacteria obtained
82 from the lungs of genetically susceptible and resistant TB-infected mice and provide a direct
83 evidence that the level of expression is regulated by the IFN-γ-dependent NO production. Using
84 high-throughput technologies, we describe the changes in the genome transcription profile that
85 accompany an increased DrrS transcription by mycobacteria. Overexpression of DrrS has led to the

transcriptional shifts similar to those previously observed during mycobacterial persistence in macrophages: an enhanced expression of several transcription factors, an up-regulated expression of the systems involved in superoxide- and NO-induced stress defense, a metabolic switch to anaerobic respiration, as well as down-regulation of the cell division- and amino acid synthesis-linked systems. Remarkably, we observed a dramatic up-regulation in the expression of genes involved in the synthesis of PE/PPE proteins, whose functioning, remaining elusive, is very likely involved in the interplay with the host immune system. Overall, we have identified the DrrS modulon that tunes the network of interacting regulons, which, in turn, activates molecular mechanisms necessary for the *M. tuberculosis* inter-macrophage survival.

Results and Discussion

DrrS expression in TB-infected mice

Earlier it was demonstrated that several *M. tuberculosis* non-coding RNAs, including DrrS, are highly transcribed *in vivo* [15, 16]. We investigated the transcription profile of DrrS in mycobacteria extracted from the mouse lungs in dynamics, from the initial to the terminal phases of infection. Aerosol infection with low doses of *M. tuberculosis* leads to a chronic and temporary effectively controlled infection in genetically resistant B6 mice, whilst in susceptible I/St mice fatal pulmonary pathology develops relatively rapidly. Differences in mycobacterial lung CFU counts between I/St and B6 mice reach about 1.5 logs during the first 2 months post challenge and remain stable until I/St mice succumb to infection [17]. We profiled the DrrS expression along the infectious course at 2 weeks (CFU numbers are similar in both strains, adaptive immune response has not developed yet), 6 - 10 weeks (well-established adaptive immune response, significant inter-strain differences in CFU numbers, bacterial transcriptome reflects adaptation), and 12 months (only B6 mice survive, the late infection phase) post challenge (Fig 1A). For each time point, total RNA

was isolated from the lungs, and the level of DrrS expression was determined using quantitative real-time PCR (Fig 1B). The highest level of expression was observed at week 10 post-challenge. In B6 mice, it remained high throughout the experiment, although slowly decreased at the very late phase of infection. At week 10 of infection, when I/St mice start to lose control of the disease progression, the level of DrrS expression in their lung mycobacterial population was significantly higher ($P < 0.01$) than that in more resistant B6 mice (Fig 1B). This may reflect an attempt of mycobacteria residing in the I/St lungs to rapidly turn down metabolism, facing severe functional failure in the surrounding tissue, providing aggressive, highly hypoxic and necrotic conditions to a large proportion of mycobacterial population [18]. Overall, at the stage of flourishing infection, the DrrS expression level in the lung-residing bacteria was more then 1000-fold higher compared to its expression level during stationary phase of growth *in vitro* [15].

The expression of DrrS is regulated by iNOS

Our *in vivo* experiments demonstrated that the level of DrrS expression peaks at the stage of fully developed adaptive immune response against mycobacteria. At this stage, B6 mice display significantly higher levels of IFN-γ production compare to their I/St counterparts [18, 19]. Since IFN-γ is the key cytokine activating macrophages for intracellular mycobacterial killing [20], we compared DrrS expression levels in infected peritoneal B6 macrophages, either activated by the external IFN-γ or not. At 2, 4, and 24 hours of macrophage infection, total RNA was isolated and the level of DrrS expression was assessed in dynamics (Fig 2A). In IFN-γ-activated macrophages, DrrS expression was significantly ($P < 0.001$, unpaired *t*-test) higher than in control macrophages at every time point, and the difference reached more than 10-fold at 24 hours post infection. Thus, pre-activation of macrophages with IFN-γ induced up-regulation of the DrrS expression in engulfed mycobacteria. Given that the efficacy of mycobacterial killing by peritoneal macrophages

significantly increases in the presence of IFN-γ [21], this result suggests that the level of DrrS expression correlates with the level of pressure emanating from infected macrophages.

Since the active nitrogen oxidative derivatives serve as the major trigger of DrrS transcription activation in vitro [11], we decided to test whether this is true for the infected macrophage system. As far as nitrogen oxidative derivatives production in macrophages depends upon inducible NO-synthase (iNOS2), we compared mycobacteria-infected IFN-γ-activated and control macrophages cultured in the presence or absence of L-NIL [N6-(1-iminoethyl)-L-lysine hydrochloride] – a selective inhibitor of iNOS2. After 24-h incubation, total RNA was isolated and analyzed (Fig 2B). Inhibition of NO production in IFN-γ-activated macrophages completely abrogated elevation in the DrrS expression, reducing it to the control levels. L-NIL itself did not affect DrrS expression in pure *M. tuberculosis* cultures. Thus, in macrophages, nitrogen oxidative derivatives are an important trigger of DrrS expression, and this regulatory pathway seems to be highly specific, since the expression of MTS0997, another small RNA abundantly present in dormant mycobacteria [14], was not dependent upon the presence of L-NIL (Fig 2C).

In the *in vitro* system, DrrS expression was shown to be induced by the transcription regulatory protein DosR [11], thus we assessed the dynamics of DosR transcription in our co-culture system (Fig 2D). Remarkably, the level of DosR transcription in mycobacteria engulfed by activated and control macrophages remained extremely low at the early stages of infection, indicating that the difference in an early up-regulation of DrrS in mycobacteria residing in activated and control macrophages was DosR-independent. In IFN-γ-activated macrophages, an elevation of the DosR transcription was observed much later, whereas in control macrophages it remained at a very low level even at 24 hours post infection (Fig 2D). Thus, regulation of DrrS transcription by *M. tuberculosis* in culture and within the host cells is different.

Transcriptome changes induced by the DrrS overexpression are consistent with mycobacterial adaptation to stress conditions

The involvement of DrrS in mycobacterial physiology suggested by the results obtained in infected mice and IFN-γ-activated infected macrophages prompted us to create an experimental system allowing the assessment of shifts in *M. tuberculosis* transcription profile resulting from elevation of the DrrS expression level. To this end, we established a new *M. tuberculosis* strain overexpressing DrrS (hereafter – OVER) and compared transcriptomes of this and of the control strain (hereafter – pMV, transfected with the "empty" pMV261 vector) at the phase of exponential growth in liquid culture using RNA-seq approaches. The DrrS expression level in the OVER strain was more than 10-fold higher compared to the pMV strain, as confirmed by qRT-PCR (S1 Fig).

Mapping the processed reads against the reference *M. tuberculosis* genome (AL123456.3, http://www.ncbi.nlm.nih.gov/), provided the following numbers of mapped reads: 22.6 x 10^6 for the OVER strain (98% of all reads) and 11.8 x 10^6 for the pMV strain (98% of all reads). The percentage of the protein-encoding part of the genome deduced from all reads mapped comprised 70% for pMV (8.2 x 10^6 reads) and 57% for OVER (12.8 x 10^6 reads). Statistical results were visualized as transcription profiles using the Artemis genome browser [22].

Using the software package edgeR [23], we identified genes the expression of which differed between the two strains. Overall, 235 genes were found to be differently expressed under the DrrS overexpression condition (S1 Table), with 88 genes demonstrating a decreased and 147 an increased expression. Further ascribing of genes to functional categories was performed using the Mycobrowser database. The most prominent differentially expressed genes, with the expression change module exceeding four, are displayed in Fig 3.

177 **Oxidative and NO-stress defense.** The most striking (>200-fold) difference between the expression
178 levels in the OVER and pMV strains was observed for the *glbN* gene encoding truncated
179 hemoglobin N. This protein protects *M. tuberculosis* from the oxidative host response in
180 macrophages by metabolizing NO into harmless nitrate [24, 25], preventing not only inhibition of
181 respiration, but also NO-mediated modification of thiol and metal centers of several enzymes and
182 regulatory proteins. The *glbN* gene is co-transcribed with the *lprI* gene (up-regulated 10-fold in
183 OVER strain) encoding the LprI lipoprotein which acts as a lysozyme inhibitor [26]. Binding of
184 LprI expressed on bacterial surface with lysozyme molecules inhibits activity of the latter and
185 supports the integrity of bacterial cells, allowing macrophage invasion. Invasion is facilitated by the
186 presence of glycosylated HbN, which protects bacilli from the macrophage-generated reactive
187 nitrogen derivatives. These two surface proteins work in concert and constitute a well-evolved
188 arsenal to disarm two important host defense mechanisms – NO and lysozyme production.

189 DrrS overexpression resulted in a significant elevation in the expression of four genes
190 involved in protection against oxidative stress. Firstly, the expression of genes for key components
191 of the peroxynitrite reductase/peroxidase antioxidant system, AhpC and AhpD, was ~10-fold higher
192 in the OVER compared to the pMV strain. AhpC is the NADH-dependent peroxidase whose thiol
193 groups in the active center are oxidized to form disulfide bridges whilst catalyzing H_2O_2 and organic
194 peroxides reduction to the water and alcohols, respectively [27]. In macrophages, AhpC catalyzes
195 the breakdown of the $ONOO^-$ group by forming an antioxidant complex bearing peroxidase and
196 peroxinitrite reductase activities, in conjunction with the dihydrolipoamide dehydrogenase (Lpd),
197 dihydrolipoamide succinyltransferase (SucB) and thioredoxin-like (AhpD) proteins [28]. Up-
198 regulation of the *ahpC* and *ahpD* transcription was previously reported for *M. tuberculosis*
199 experiencing the NO-mediated oxidative stress in macrophages [29]. Secondly, we observed up-

regulation of the genes for the peroxidase BpoA (~9-fold) and the ferric uptake regulation protein FurA (4-fold). These genes are also involved in the oxidative stress response [30].

It was previously reported that in persisting *M. tuberculosis* the nuoA-N subunits of the NADH-dehydrogenase complex are repressed [31]. Our results also demonstrate a 4-fold down-regulation of transcription of the genes for all subunits in the OVER strain.

In line with the respiratory chain inhibition, overexpression of DrrS has led to a pronounced up-regulation of genes associated with nitrogen metabolism. Under hypoxic conditions, nitrates protect *M. tuberculosis* by replacing oxygen as a final electron acceptor in the respiratory chain [32, 33]. While assessing the expression of genes encoding four subunits of the NarGHJI nitrate reductase, we observed the highest (4-fold) elevation in the expression of gene for NarG and 2-3-fold increases in the expression of genes for other three. However, there is evidence that this nitrate reductase is constitutively expressed at sufficiently high levels upon nitrate assimilation under both anaerobic and aerobic conditions [34]. Another way of nitrogen assimilation by *M. tuberculosis* is capturing organic amine-containing compounds of the host through a variety of transporters. The gene for one of these transporters, the amino acid permease RocE [35], was 6-fold up-regulated in the OVER strain. For further reduction of nitrites to ammonium, mycobacteria use the NADH-dependent nitrite reductase NirBD [33], whose expression was dramatically (~40-fold) elevated in the OVER strain. In addition, the expression of genes for NarU, NarK1 and NarK3, nitrite extrusion transporters of unrecovered nitrites from bacterial cells, was also significantly increased (6, 15 and 11-fold, respectively).

Amino acid biosynthesis. During *M. tuberculosis* dormancy, protein transcription and synthesis is profoundly inhibited due to a reduced expression of genes involved in biosynthesis of amino acids

and cofactors [36]. In line with this general tendency, we observed a ~4-fold reduction in the expression of genes involved in synthesis of several amino acids (tryptophan, arginine, cysteine, leucine, and alanine) and vitamin B6 in the OVER strain (Fig 3).

Regulators. Overexpression of DrrS significantly changed transcription of regulatory genes increasing 10 and decreasing 7 of them (Fig 3). Among up-regulated, was the *Rv0079* (8-fold) considered as a part of the DosR regulon [30]. In *E. coli* and *M. bovis* corresponding protein significantly inhibits cell growth, apparently by the interaction with the 30S ribosome subunit, thus inhibiting translation – the phenotype typical for transition to dormancy [37]. Another regulator of transcription, whose expression was increased (8-fold), is the *Rv0081* gene – one of the two key transcription factors mediating early response to hypoxia [38]. As an important "metabolic hub" working in concert with other transcription regulators, *Rv0081* is associated with the processes of lipid metabolism, protein degradation and cholesterol biosynthesis. In addition, our data confirmed up-regulation of four of seven predicted and verified Rv0081 targets: *Rv2329c, Rv2699c, Rv1057c* and *Rv3619c* [39]. Another up-regulated (~5-fold) transcription factor-encoding gene, *Rv2034*, is known to be involved in the induction of transcription of the two major stress response genes, *dosR* and *phoP* [40].

Evaluation of the expression of two transcription factors from the WhiB family provided a 22-fold increase in the *whiB3* expression, but a 4-fold decrease for *whiB2*. Up-regulation of the transcriptional regulator WhiB3 in mycobacteria has been demonstrated during responses to a number of stressful conditions, including low pH levels and drug pressure [41]. WhiB3 interacts with the sigma factor RpoV, possibly playing a role in the disease progression and development of tissue pathology [42]. In addition, WhiB3 is redox-dependent and undergoes activation in mycobacteria residing in macrophages [43]. Under conditions of acidic stress, WhiB3 controls the

245 expression of multiple genes, including genes for complex lipids syntheses (*pks2*, *pks3-pks4*),
246 transcription factors (*whiB7*, *Rv0827c*, *Rv3183*), amino acid biosynthesis (*metH*, *metK*, *sahH*, and
247 *leuB*), ESX-1 secretion system (*Rv3614c*, *Rv3616c*) and nitrite transport (*narK1*). Remarkably,
248 unlike up-regulated WhiB3 involved in the stress/pathology metabolism, down-regulated WhiB2
249 controls the expression of several genes involved in cell division processes [44]. WhiB2 down-
250 regulation balanced by the WhiB3 up-regulation was observed in dividing intracellular bacteria [45,
251 46].

252 Another up-regulated (~5-fold) transcription factor, Rv2034, is involved in the transcription
253 induction of two major stress response genes, *dosR* and *phoP* [40]. Indeed, in our system *dosR*
254 transcription was increased about 3-fold in the OVER strain.

255 **PE/PPE system.** Among the genes encoding proteins belonging to the PE/PPE superfamily, the
256 transcription levels of 26 genes significantly (at least 4-fold) differed between the OVER and pMV
257 strains. Remarkably, an elevated transcription in the OVER strain was observed for 24 genes,
258 whereas only two genes (the tandem *Rv3477-3478* encoding the PE31 and PPE60 proteins) were
259 down-regulated.

260 PE and PPE proteins influence profoundly the survival of mycobacteria within host
261 macrophages by increasing bacterial resistance to oxidative, acidic and sodium dodecyl sulfate
262 stresses [47]. There is also an evidence that a group of these proteins expressed on bacterial surface
263 possesses antigenic properties, induces host immune responses and modifies immunologic and
264 inflammatory processes in many different ways [48-50]. Thus, the PPE25 protein (8-fold
265 transcriptional increase in the OVER strain) is believed to be involved in inhibition of phagosome
266 maturation [51]. PPE18 (8-fold up-regulation) was shown to bind TLR2 and trigger secretion of the

anti-inflammatory cytokine IL-10 in macrophages via activation of p38 MAPK [52], and PPE2 (4-fold up-regulation) inhibits the reactive forms of nitrogen by inhibiting transcription of the gene for iNOS [53].

At the genome level, some genes for PE/PPE proteins are clustered within the mycobacterial ESX secretion system loci. In the experimental system based upon infection of human macrophages with virulent *Mycobacterial marinum* it was shown that a few PE/PPE proteins are transported via the ESX-5 secretion system. Moreover, loss-of-function mutations in the *ESX-5* locus resulted in the inhibition of secretion of different cytokines dependent upon signaling of several TLR via Myd88 adaptive molecule in macrophages [54, 55]. In the OVER strain, many genes for the PE/PPE components of ESX-5 (*PPE25, PE18, PPE26, PPE27, PE19*) were up-regulated compared to the pMV strain, as were the *esxN* and *esxM* genes encoding putative ESAT-6-like proteins. Similar expression shifts were found for the *PE13*, *PPE18*, *esxK* and *esxL* genes for the components of another ESX secretion system, ESX-1. Recently, it was reported that the esxL protein down-regulates the expression of CIITA/MHC-II by inducing hyper-methylation in histone H3 lysine9, leading to inhibition of antigen presentation of mycobacterial antigens to the $CD4^+$ T lymphocytes [56].

Taken together, the results obtained in the present study demonstrate tight connections between the expression of small non-coding DrrS RNA and capacity of *M. tuberculosis* to respond against stressful conditions *in vitro* and *in vivo*. Remarkably, overexpression of DrrS leads to the transcriptional *in vitro* pattern similar to that reported for mycobacteria residing within host macrophages. This particularly concerns mycobacterial responses to oxidative stresses. As the DrrS expression displays a wide range of influence onto anti-stress transcriptional responses, we suggest that this regulator works as a novel “DrrS modulon” in the mycobacterial genome.

Conclusion

In mycobacteria, small RNAs have been discovered much later then in many other bacterial species [7], and their functions mostly remain unknown. However, the growing body of evidence obtained *in vitro* and *in vivo* indicates that the DrrS small RNA may play a role in TB dormancy/latency [13]. In the present work, we demonstrate that up-regulation of DrrS *in vivo* is NO-inducible and parallels iNOS activation in infected macrophages. *In vitro*, overexpression of DrrS in *M. tuberculosis* triggers remarkable shifts in bacterial transcriptome, consistent with the activation of NO-induced and other oxidative stress defense mechanisms, thus mimicking persistence within host macrophages (Fig 4). Thus, elevated amounts of DrrS initiate changes in mycobacterial transcriptional profile that are required to increase the resistance to stressful environmental conditions. We suggest that a dramatic up-regulation of the genes for PE/PPE proteins and ESX-1 and ESX-5 secretion systems observed under DrrS overexpression conditions reflects the importance of this small RNA for a broad range of stress adaptations *in vivo* and *in vitro*.

Materials and methods

Ethics Statement

Mice were maintained under conventional, non-SPF conditions at the Animal Facilities of the Central Institute for Tuberculosis (CIT, Moscow, Russia) in accordance with the guidelines from the Russian Ministry of Health # 755, and under the NIH Office of Laboratory Animal Welfare (OLAW) Assurance #A5502-11. All experimental procedures were approved by the Bioethics Committee of the Central Research Institute of Tuberculosis (IACUC), protocols # 2, 3, 7, 8, 11 approved on March 6, 2016.

Bacterial strains, media and growth conditions

For *in vitro* experiments, *M. tuberculosis* H37Rv, pMV and OVER *M. tuberculosis* strains were initially grown from frozen stocks for 10 days in Sauton medium containing (per liter): 0.5 g KH_2PO_4, 1.4 g $MgSO_4 \times 7H_2O$, 4 g L-asparagine, 60 ml glycerol, 0.05 g ferric ammonium citrate, 2 g sodium citrate, 0.1 ml 1% $ZnSO_4$, pH 7.0 (adjusted with 1M NaOH), and supplemented with ADC growth supplement [57], 0.05% Tween 80 and 50 μg/ml kanamycin (Sigma-Aldrich, St Louis, MO, USA) at 37°C with agitation (200 rpm). The starter cultures were inoculated into fresh medium (the same composition) and incubated for another 7-10 days until its optical density at 600 nm (OD_{600}) reached 1.2 (early log-phase).

For cloning procedures, *Escherichia coli* DH5α was grown in Luria Bertani (LB) broth and LB-agar. When required, antibiotics were added at the following concentrations: kanamycin (Sigma-Aldrich, St Louis, MO, USA), 50 μg/ml (*M. tuberculosis*); ampicillin (Invitrogen, Carlsbad, CA, USA), 100 μg/ml (*E. coli*).

M. tuberculosis OVER and pMV (control) strains establishment

The DrrS gene containing vector was constructed on the basis of the pMV261 [58] as described in Ref. [14]. The plasmid was transferred into mycobacteria by electroporation. DrrS overexpression was confirmed by qPCR. The control strain was produced using an empty pMV261 vector.

RNA extraction from cultured mycobacteria

Bacterial cultures were grown up to the early log phase, rapidly cooled on ice, centrifuged, and total RNA was isolated by phenol-chloroform extraction after cell disruption with BeadBeater (BioSpec Products, Bartlesville, OK, USA) as previously described [59]. After isolation, RNA was treated with Turbo DNase (Life Technologies, Carlsbad, CA, USA) to remove traces of genomic

DNA, and purified with the RNeasy mini kit (Qiagen, Venlo, Netherlands). Amounts and purity of RNA were determined spectrophotometrically; integrity of RNA was assessed in 1% agarose gel.

Libraries for RNA-seq and RNA-seq data analyses

RNA samples were depleted of 16S and 23S rRNA using Ribo-Zero rRNA Removal Kit for Gram-Positive Bacteria (Epicentre, Madison, WI, USA). Sequencing libraries were generated using the resulting ribosomal transcript-depleted RNA and the TruSeq Stranded mRNA Library Prep Kit (Illumina, San Diego, CA, USA) according to the manufacturers' protocol. Sequencing was performed using the Illumina HiSeq 2500 as the double-ended 100 nt-long reads. Experiments were performed in triplicates.

After quality control evaluation and trimming of bad qualitative reads the reads were mapped on the reference *M. tuberculosis* genome (AL123456.3, http://www.ncbi.nlm.nih.gov/) by Bowtie2 [60]. The alignment was performed with the "local" option, which allows leaving 5' and 3' ends uncharted. Calculation of the mapped reads for all genes was performed using functions of the HTSeq-count package built into the author's script. The resulting statistics were visualized as transcription profiles using the Artemis genome browser [22].

Differentially expressed genes were identified by the software package edgeR [23]. The genes were considered to be differentially expressed, if the p-value was less than 0.05, the expected measure of false deviations (FDR) was not higher than 0.1, and the expression change module (FC, Fold change) was not less than 4. Further distribution of genes according functional categories was performed using the Mycobrowser database (https://mycobrowser.epfl.ch/).

Quantitative reverse transcription-PCR (qRT-PCR)

One microgram of total RNA was used for cDNA synthesis with random hexanucleotides and SuperScript III reverse transcriptase (Life Technologies, Carlsbad, CA, USA). Quantitative PCR

was performed using qPCRmix-HS SYBR (Evrogen, Russia) and the Light Cycler 480 real-time PCR system (Roche, Switzerland); cycling conditions were as follows: 95C for20 s, 61C for 20 s, 72C for 30 s, repeat 40 times; primers are listed in S2 Table. In the end of amplification, a dissociation curve was plotted to confirm specificity of the product. All real-time experiments were repeated in triplicate. The results were normalized against the 16S rRNA gene.

Infections in vivo and ex vivo

Mice. C57BL/6Ycit (B6) and I/StSnEgYCit strain (I/St) mice were kept under standard conditions in the Animal Facilities of the Central Research Institute of Tuberculosis. The mice had *ad libitum* access to food and water. Female mice aged 2.5–3.0 months were used in experiments.

Mycobacteria. For infection of mice and macrophage cultures, *M. tuberculosis* H37Rv (substrain Pasteur) from the collection of CIT were used. Mycobacteria were prepared to infect mice and macrophages as described previously [61]. Briefly, to obtain log-phase bacteria for challenge, 50 µl from a thawed aliquot was added to 30 ml of Dubos broth (BD Biosience, Sparks, MD, USA) supplemented with 0.5% Fatty Acid-Poor BSA (Calbiochem-Behring Corp., La Jolla, CA, USA) and oleic acid and incubated for 2 weeks at 37°C. The resulting suspension was washed two times at 3000 g, 20 min, 4°C with Ca^{2+}- and Mg^{2+}-free PBS containing 0,2 mM EDTA and 0,025% Tween 80. Cultures were filtered through a 45 µm-pore-size filter (Millipore, USA) to remove clumps. To estimate the CFU content in the filtrate, 20 µl from each 5-fold serial dilution was plated onto Dubos agar (BD), and the total number of micro-colonies in the spot was calculated under an inverted microscope (200^x magnification) after being cultured for 3 days at 37°C. The bulk of the filtered culture was stored at 4°C, and it was found that no change in the CFU content occurred during this storage period.

379 **Infection of mice**. To infect mice, mycobacteria were resuspended in supplemented PBS. Mice
380 were infected via respiratory tract with ~100 viable CFU/mouse using an Inhalation Exposure
381 System (Glas-Col, Terre Haute, IN), as described in [18, 62]. Briefly, animals were exposed for 40
382 min to aerosol produced by nebulizing 8 ml of a bacterial suspension in PBS solution with 0,05%
383 Twin-80 at the concentration of 1.5 x 10^6 bacilli/ml. The size of challenging dose was confirmed in
384 preliminary experiments by plating serial 2-fold dilutions of 2-ml homogenates of the whole lungs
385 obtained from B6 and I/St females at 2 h post-exposure onto Dubos agar and counting colonies after
386 3-wk incubation at 37^0C. To assess CFU counts, lungs from individual mice were homogenized in
387 2.0 ml of sterile saline, and 10-fold serial dilutions were plated on Dubos agar and incubated at 37°C
388 for 20-22 days.
389 **Infection of macrophages, iNOS activation, RNA extraction.** To obtain peritoneal macrophages,
390 B6 mice were injected intra-peritoneally with 3% peptone (Sigma-Aldrich) in saline. Five days later,
391 peritoneal exudate cells (PEC) were eluted from the peritoneal cavities with Ca^{2+}- and Mg^{2+}-free
392 PBS supplemented with 2% FCS and 10 U/ml heparin, washed twice with PBS, and resuspended in
393 RPMI 1640 containing 5% FCS, 10 mM HEPES and 2 mM L-glutamine. The content of nonspecific
394 esterase-positive cells in PEC exceeded 85 %. PEC were plated onto 90 mm Petri dishes (Costar,
395 Corning Inc., Corning, NY, USA) at 10 x 10^6 cells/dish in 10 ml of RPMI-1640 containing 5% FCS,
396 10 mM HEPES and 2 mM L-glutamine to obtain macrophage monolayers. The cells were allowed
397 to adhere for 2 h at 37^oC, 5% CO_2 before mycobacteria were added in 10 ml of supplemented
398 RPMI-1640 at MOI = 30, 20, 15 and 5 for further culturing for periods indicated in Fig 2.
399 Macrophage-free mycobacterial cultures served as controls.

To activate macrophages, monolayers were treated with murine rIFN-γ (100 U/ml, Sigma) for 14 h before adding mycobacteria. To block iNOS, 100 μM L-NIL (Sigma) was added 1 h before rIFN-γ administration.

To extract RNA, dishes with cell monolayers were gently shaken, culture medium was completely aspirated and macrophages were lysed with 5 ml/dish of Trisol (Invitrogen, Carlsbad, CA,USA) as recommended by the manufacturer. Mycobacteria alone in control cultures were suspended by pipetting and centrifuged at 3000 g, 20 min, 4°C. Pellets were suspended in 1 ml of Trisol.

Statistics

Statistical analysis was performed using ANOVA test and unpaired t-test by GraphPad Prism6.0 software (GraphPad Software, San Diego, CA, USA). *P*-value below 0.05 was assumed to be statistically significant.

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577

Figure captions

Fig 1. *M. tuberculosis* infection in resistant B6 and susceptible I/St mice.

(A). Lung CFU counts along the disease progression (P<0.01 at 6 and 10 weeks post challenge, ANOVA). (B). DrrS expression levels of at different time points. (**P < 0.01 and ***P < 0.001, unpaired t-test). At indicated time points, samples of total RNA were analyzed by quantitative real-time PCR, and the DrrS expression levels in the lung tissue were normalized to those of 16S rRNA. Error bars represent the SD for three biological replicates.

Fig 2. DrrS transcription is NO-dependent and correlates with activation of infected macrophages.

(A). The DrrS transcription dynamics in infected peritoneal macrophages of B6 mice. (B). The level of DrrS transcription at 24 hrs post infection: control (mφ +MTb), IFN-γ-activated (mφ +MTb + INF-γ), IFN-γ-activated and L-NIL treated (mφ +MTb + INF-γ + NIL). The levels of DrrS transcription in pure *M. tuberculosis* cultures (MTb) and L-NIL-treated cultures (MTb + NIL) serve as controls for the assessment of possible L-NIL influence onto cultured mycobacteria. (C). The level of MTS0997 transcription at 24 hrs post infection: control (mφ +MTb), IFN-γ-activated (mφ +MTb + INF-γ), IFN-γ-activated and L-NIL treated (mφ +MTb + INF-γ + NIL). (D). The dynamics of DosR transcription in infected peritoneal macrophages.

Fig 3. Differential gene expression under the DrrS overexpression conditions.

(A). The numbers of differentially expressed genes by functional categories (Mycobrowser) are given in circles. (B). The heat map of genes changing their transcription more then 4-fold under DrrS overexpression. Gene expression ratios were determined using the edgeR package, log2

601 transformed, normalized to the average level across the replicates and displayed according to the
602 color code.
603
604 **Fig 4. Schematic representation of metabolic/transcriptomic cascades triggered by the elevated**
605 **transcriptional level of DrrS in the OVER mycobacterial strain.**
606 Up-regulated genes/processes are displayed in red, down-regulated – in green.
607

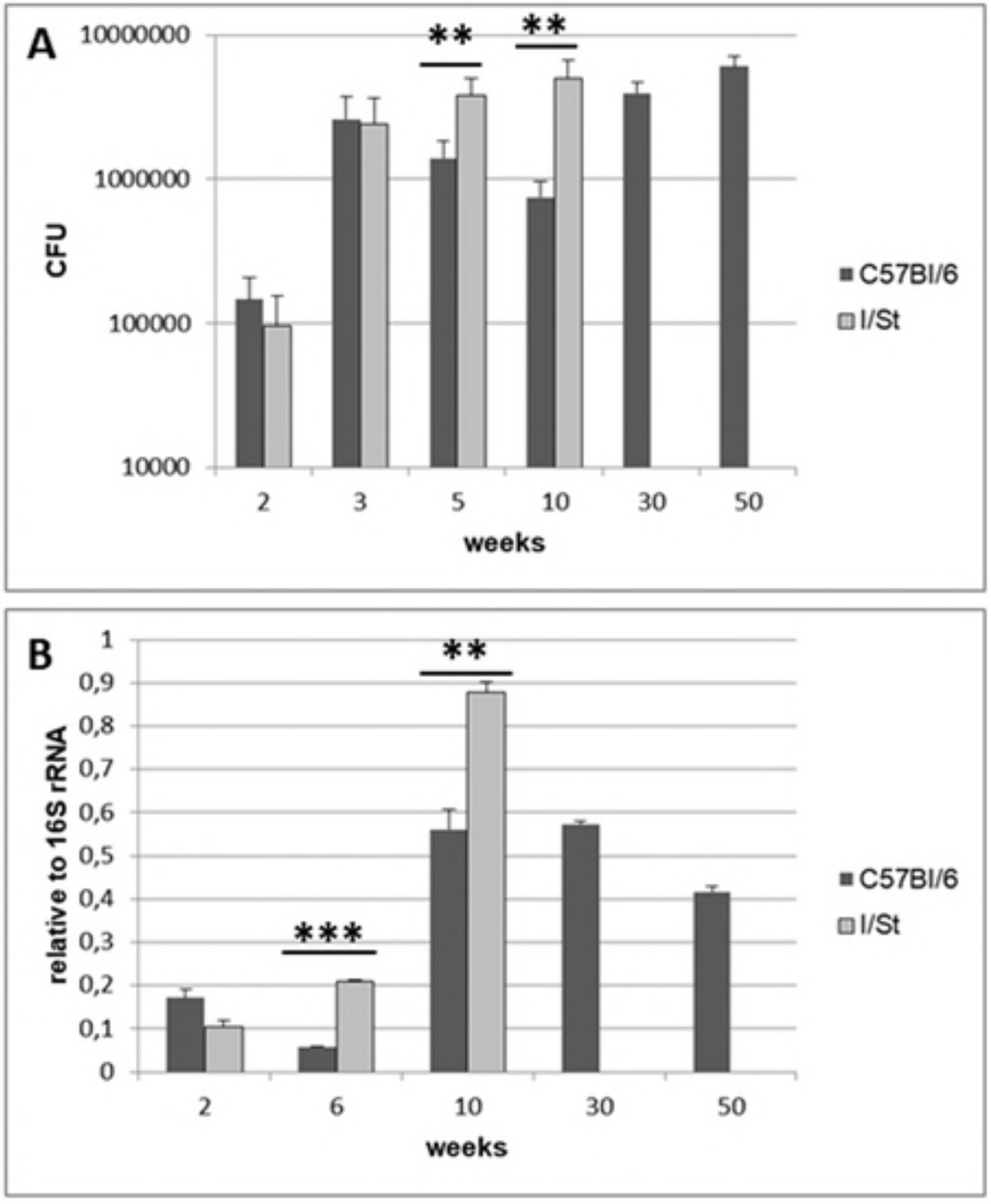
Supporting information

S1 Table. Differential expression of genes in OVER strain vs pMV strain.

S2 Table. Primers used for qPCR mRNA transcript analysis

S1 Fig. DrrS transcription level in M. tuberculosis OVER strain comparable to H37Rv (wt) and pMV strains.

Transcription was measured by qRT-PCR in phase of exponential growth in liquid culture.


A
10000000
1000000
100000
10000
CFU
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2
3
5
10
30
50
weeks
C57Bl/6
I/St
B
1
0,9
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
relative to 16S rRNA
**
***
2
6
10
30
50
weeks
C57Bl/6
I/St

Figure 1

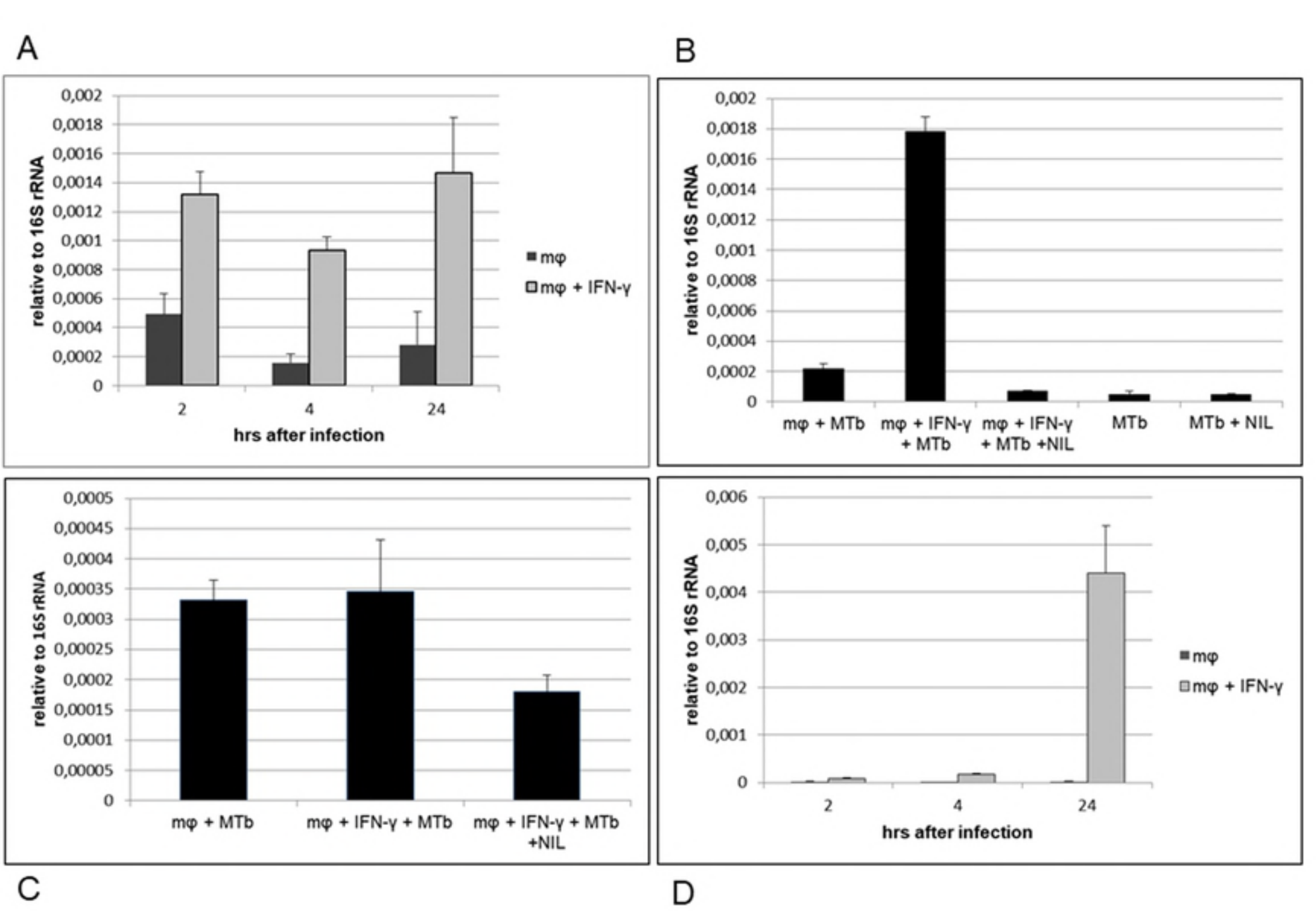
A
0,002
0,0018
0,0016
0,0014
0,0012
0,001
0,0008
0,0006
0,0004
0,0002
0
relative to 16S rRNA
2
4
24
hrs after infection
mφ
mφ + IFN-γ
B
0,002
0,0018
0,0016
0,0014
0,0012
0,001
0,0008
0,0006
0,0004
0,0002
0
relative to 16S rRNA
mφ + MTb
mφ + IFN-γ + MTb
mφ + IFN-γ + MTb +NIL
MTb
MTb + NIL
C
0,0005
0,00045
0,0004
0,00035
0,0003
0,00025
0,0002
0,00015
0,0001
0,00005
0
relative to 16S rRNA
mφ + MTb
mφ + IFN-γ + MTb
mφ + IFN-γ + MTb +NIL
D
0,006
0,005
0,004
0,003
0,002
0,001
0
relative to 16S rRNA
2
4
24
hrs after infection
mφ
mφ + IFN-γ

Figure 2

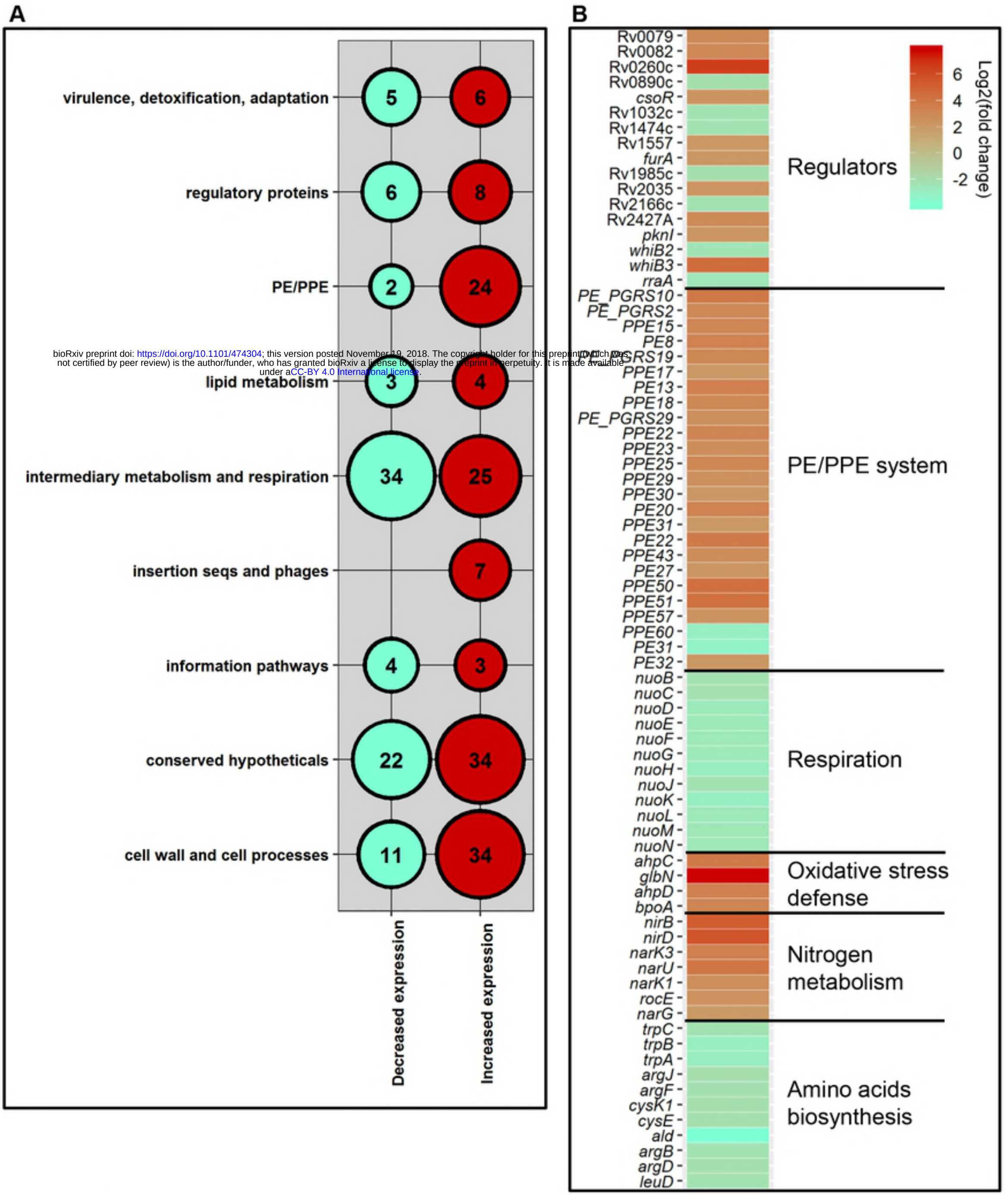

Figure 3

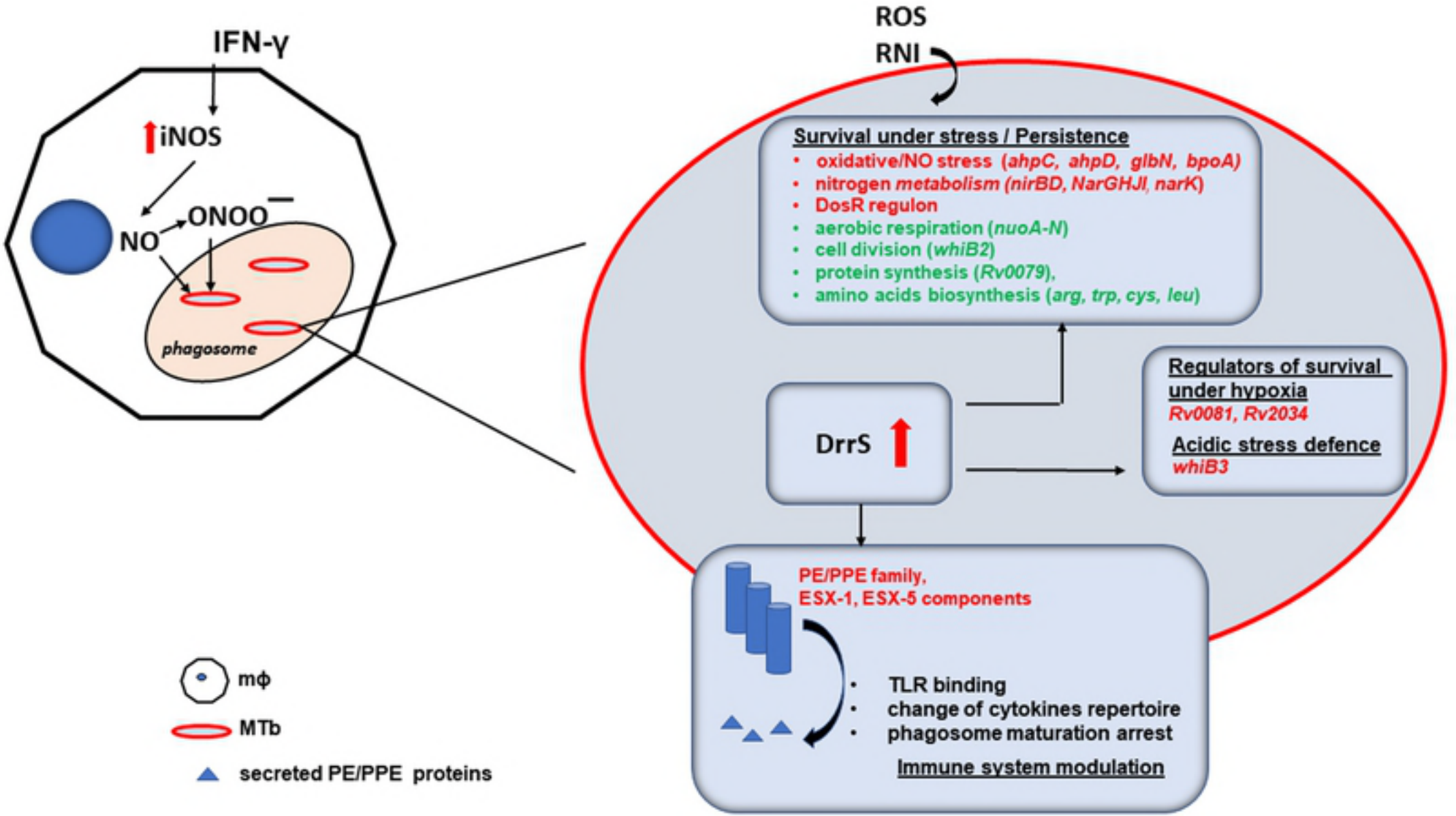
IFN-γ
iNOS
NO
ONOO⁻
phagosome
ROS
RNI
Survival under stress / Persistence
• oxidative/NO stress (ahpC, ahpD, glbN, bpoA)
• nitrogen metabolism (nirBD, NarGHJI, narK)
• DosR regulon
• aerobic respiration (nuoA-N)
• cell division (whiB2)
• protein synthesis (Rv0079),
• amino acids biosynthesis (arg, trp, cys, leu)
DrrS
Regulators of survival under hypoxia
Rv0081, Rv2034
Acidic stress defence
whiB3
PE/PPE family,
ESX-1, ESX-5 components
• TLR binding
• change of cytokines repertoire
• phagosome maturation arrest
Immune system modulation
mϕ
MTb
secreted PE/PPE proteins

Figure 4