d. Details of the research work duly signed by the applicant, for which the Sun Pharma Research Award is claimed, including references and illustrations (not to exceed 6000 words).

I: Targeting heterogeneity in redox physiology of *Mycobacterium tuberculosis* to improve therapy outcome

A major obstacle in the clinical treatment of tuberculosis (TB) is the long therapy time (6-9 months) required to clear the infection. A plausible explanation for the protracted drug regimen is the development of heterogeneity- a process whereby a genetically identical population of *Mycobacterium tuberculosis* (*Mtb*: the pathogen that causes TB) diversifies to produce drug-tolerant subpopulations. This form of drug insensitivity is called as phenotypic drug tolerance, which represents the greatest hurdle to effective chemotherapy. Heterogeneity in bacterial population is likely to contribute to phenotypic drug tolerance in the sputum of active TB patients. Molecular basis of heterogeneity is largely derived from studies performed in liquid culture medium, which indicates the role of differences in cell sizes and growth rates (actively multiplying vs slow growth) in promoting drug tolerance [1, 2]. However, *in vitro* approaches have not yielded any effective strategies to target heterogeneity and drug tolerance in *Mtb*, indicating that the significance of bacterial heterogeneity and drug tolerance needs to be examined in the context of host environment during infection. Filling this knowledge gap will contribute to new avenues through which current anti-TB drugs could be used in combination with compounds that block the capacity of *Mtb* to promote heterogeneity *in vivo*.

We identified that host environment promotes variations in the redox physiology of *Mtb* population to tolerate anti-TB drugs during infection. We used a range of cutting-edge technologies such as redox biosensor, replication clock, flow sorting, intra-phagosomal RNA-sequencing, mass spectrometry, and animal models, to mechanistically dissect host and bacterial factors responsible for redox heterogeneity and multi-drug tolerance in *Mtb* population during infection. Our findings, for the first time, provide empirical evidences showing how host and bacterial mechanisms cross talk to induce bacterial heterogeneity and drug tolerance during infection. Based on our findings a new model of drug tolerance emerged. According to this model, limited phagosomal acidification inside naïve macrophages facilitates the emergence of a redox-altered drug-tolerant subpopulation of *Mtb*. This drug tolerant subpopulation is actively multiplying, metabolically active, and showed higher expression of genes responsible for rerouting of cysteine amino acid into pathways such as Fe-S cluster biogenesis and hydrogen sulfide gas generation. We provide genetic evidences that pathways coordinating cysteine flux are important contributors of acidic pH dependent redox heterogeneity and drug tolerance in *Mtb* (Fig. 1). More-importantly, redox heterogeneity and phagosomal pH also contributes to drug tolerance when *Mtb* infects macrophages co-infected with another human pathogen-HIV-1. Our findings have the potential to understand why high rates of TB therapy failure are clinically documented in humans co-infected with HIV-TB as compared to patient infected with TB alone [3].

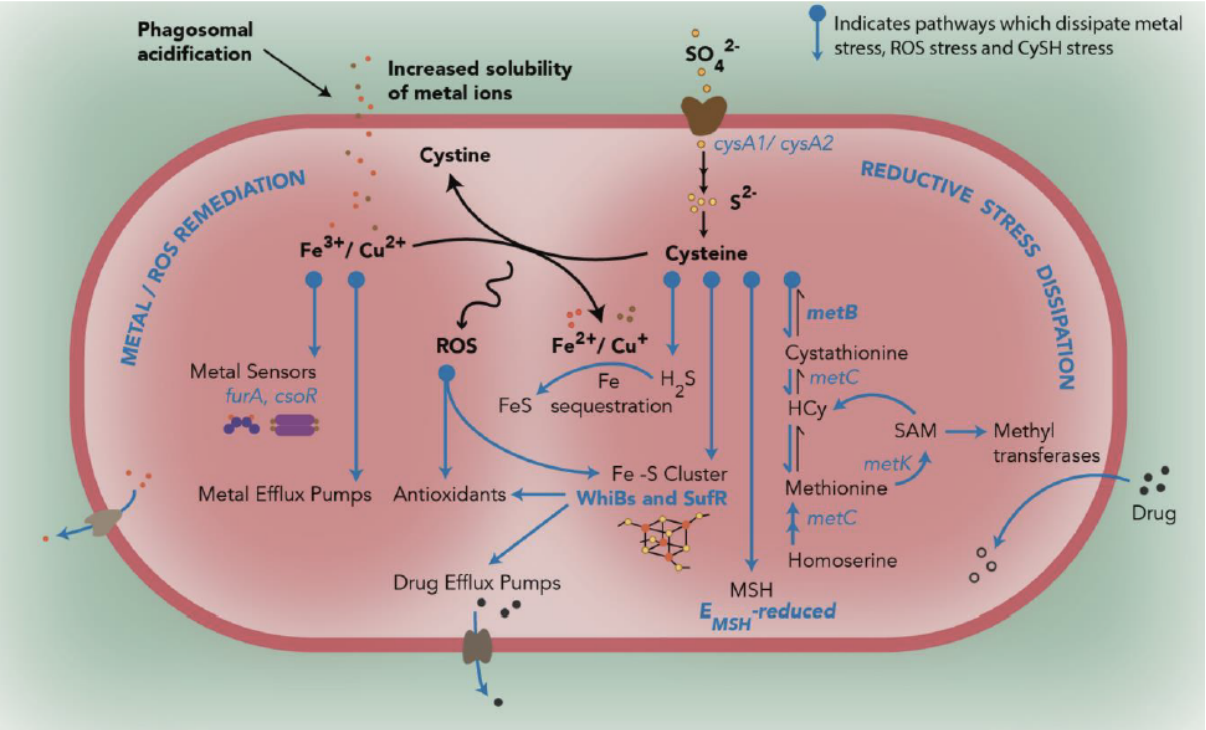

Fig. 1 Model depicting various mechanisms underlying redox-mediated drug tolerance in replicating *Mtb*. *Phagosomal acidification inside resting macrophages serves as a cue to induce redox heterogeneity and drug tolerance in Mtb. Gene expression data indicate that low pH induces accumulation of cysteine (CySH) causing reductive stress. Elevation of CySH is known to generate reactive oxygen species (ROS) through metal (Fe/Cu) catalysed oxidation to cystine (CyS2). Acidic pH also increases solubility of metals such as Fe and Cu, which drives generation of ROS via Fenton chemistry. Also, various anti-TB drugs induce oxidative stress inside Mtb during infection. To manage these stresses, Mtb efficiently channelizes the flux of CySH in reverse transsulfuration pathway (H2S generation), Fe-S cluster biogenesis (SufR/WhiBs), and MSH production, resulting a reductive shift in the EMSH of Mtb. All of these mechanisms are known to protect bacteria from drugs and oxidative stress by metal sequestration and activation of antioxidants. Increased expression of metal and drug efflux pumps further remediate metal/antibiotic triggered redox stress in Mtb. Induction of SAM-dependent methyl-transferase in EMSH-reduced bacteria can directly inactivate drugs by N-methylation. Impaired ability of metB, sufR, and whiB3 mutants in tolerating antibiotics suggests management of CySH flux as an important bacterial strategy to protect from drugs. Pharmacological inhibition of phagosomal acidity by CQ restores redox heterogeneity to subvert drug tolerance and post-therapeutic relapse.*

How can we relate our basic findings to develop new therapeutic strategies against human TB? Our study raises fascinating new possibilities for managing phenotypic antibiotic resistance. For example, inhibitors of host signal(s) that are sensed by *Mtb* to generate redox diversity could be exploited to restore phenotypic homogeneity and potentiate the killing activity of existing frontline antibiotics. On this basis, we reasoned that pharmacological inhibition of phagosomal acidification could preclude mobilization of redox-mediated drug-tolerant phenotype *in vivo*. We tested our hypothesis by using the antimalarial drug chloroquine (CQ), which is well known to increase vesicular pH [4]. We discovered that CQ in combination with front-line anti-TB drugs (isoniazid or rifampicin) eradicated drug-tolerant *Mtb*, ameliorated lung pathology, and reduced post-chemotherapeutic relapses in infected animals (mice and

guinea pigs) (Fig. 2). The pharmacokinetic study shows no adverse interaction of CQ with the first line anti-TB drugs in mice. Since CQ is clinically used, has a longer half-life, is cost-effective, and highly tolerable with fewer side effects [5], it can be conveniently repurposed to formulate new combinations with the current anti-TB regimen to reduce therapy duration.

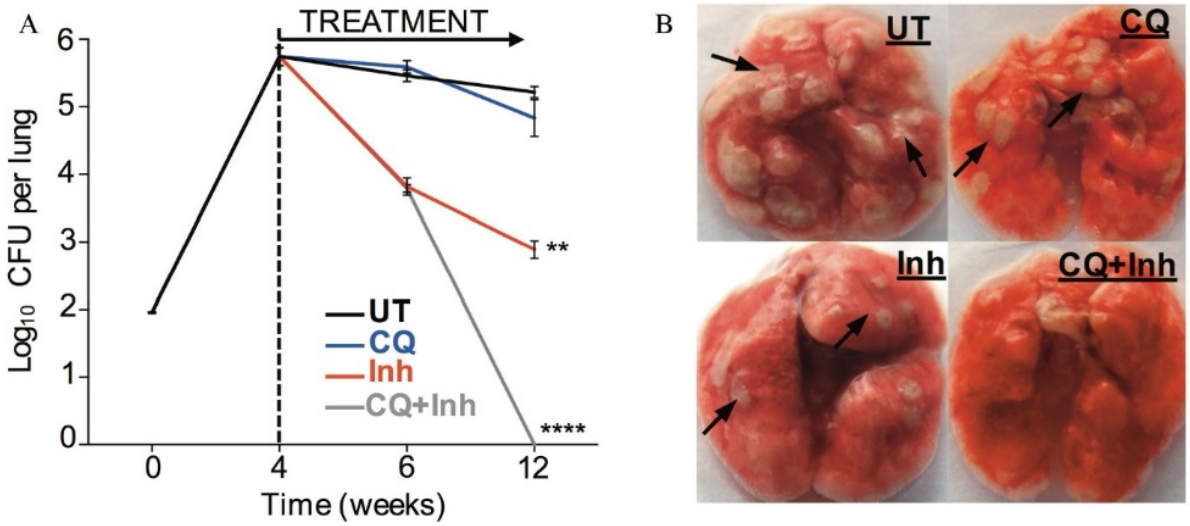

Fig. 2. Chloroquine-Isoniazid combination sterilizes Mtb in animal lungs*. (A) Survival kinetics of Mtb in the lungs of mice upon treatment with chloroquine (CQ) alone, Isoniazid alone (Inh) and CQ plus Inh combination. (B) Gross lung pathology shows healthy lungs without TB lesions in case of CQ + Inh combination.*

In conclusion, our study is a clear example of how fundamental research can generate translational opportunities to target one of the global human pathogen, which kills ~ 1.7 million people annually. Finally, redox-mediated multi-drug tolerance may be relevant to other chronic pathogens. For example, heightened antioxidant capacity is linked to the acquisition of phenotypic antibiotic resistance in the human pathogens *Pseudomonas aeruginosa* [6]. Thus our findings may have broad relevance to several human pathogens where a sterilizing cure is therapeutically challenging.

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II: Making Common antibiotics powerful against Tuberculosis:

Despite the use of effective combination chemotherapy, Tuberculosis (TB) remains a major cause of human death worldwide. One of the major factors that contributes to therapy failure include the emergence of multi-, extensively-, and totally-drug resistant strains (MDR, XDR, and TDR) of *Mycobacterium tuberculosis* (*Mtb*). New drugs are urgently needed to target drug-resistant TB. In this context, β-lactams are one of the most widely used classes of antibacterial against a large repertoire of human pathogens. Approximately 50% of clinically relevant antibacterials prescribed by clinicians belong to β-lactams. However, *Mtb*, owing to the expression of a highly active β-lactamase (BlaC) and a poorly permeable outer membrane, remains completely tolerant to β-lactams. Recently, combinations of β-lactams and β-lactamase inhibitors have been shown to effectively kill MDR and XDR strain of *Mtb* in human clinical trials. Based on this, we hypothesized that there is a need to better understand the mechanism of how β-lactams and β-lactamase inhibitor combinations function and the possible mode of resistance. Both of these understandings are critical to establish confidence on this therapeutic option and to open up new avenues of research to reverse the transmission of drug-resistance. We revealed the contribution of intramycobacterial redox physiology in influencing tolerance of *Mycobacterium tuberculosis* (*Mtb*) against a new and potent anti-mycobacterial drug combination i.e. β-lactam (Amoxycillin) and β-lactamase inhibitor (clavulanate) during infection. This combination is commonly referred as Augmentin (AG). Using exhaustive network biology, biochemical, redox biosensor, bacterial genetics, macrophages infection and animal experiments, we generated a new insight on the mode of AG action and tolerance mechanisms in *Mtb*. We found that *Mtb* responds to AG by modulating the expression of genes specifically associated with β -lactam resistance (*e.g.,* β-lactamase and peptidoglycan biosynthesis) as well those, which maintain cellular homeostasis (*e.g.,* oxidative stress response, central carbon metabolism, and respiration). Further, our data indicate a more central role for the major *Mtb* cytoplasmic antioxidant buffer, mycothiol (MSH), in mediating response to AG. Any change in mycothiol redox potential (E_{MSH}) was found to alter *Mtb's* response to AG *in vitro* and during infection. Lastly, what is striking in our study is the capacity to identify one gene *whiB4*, which encodes a redox responsive Fe-S cluster transcription factor, as the major actor of the process. In aggregate, the functional linkage between β-lactams, redox balance, and WhiB4 can be exploited to potentiate AG action against drug-resistant *Mtb* infections (Fig. 3).

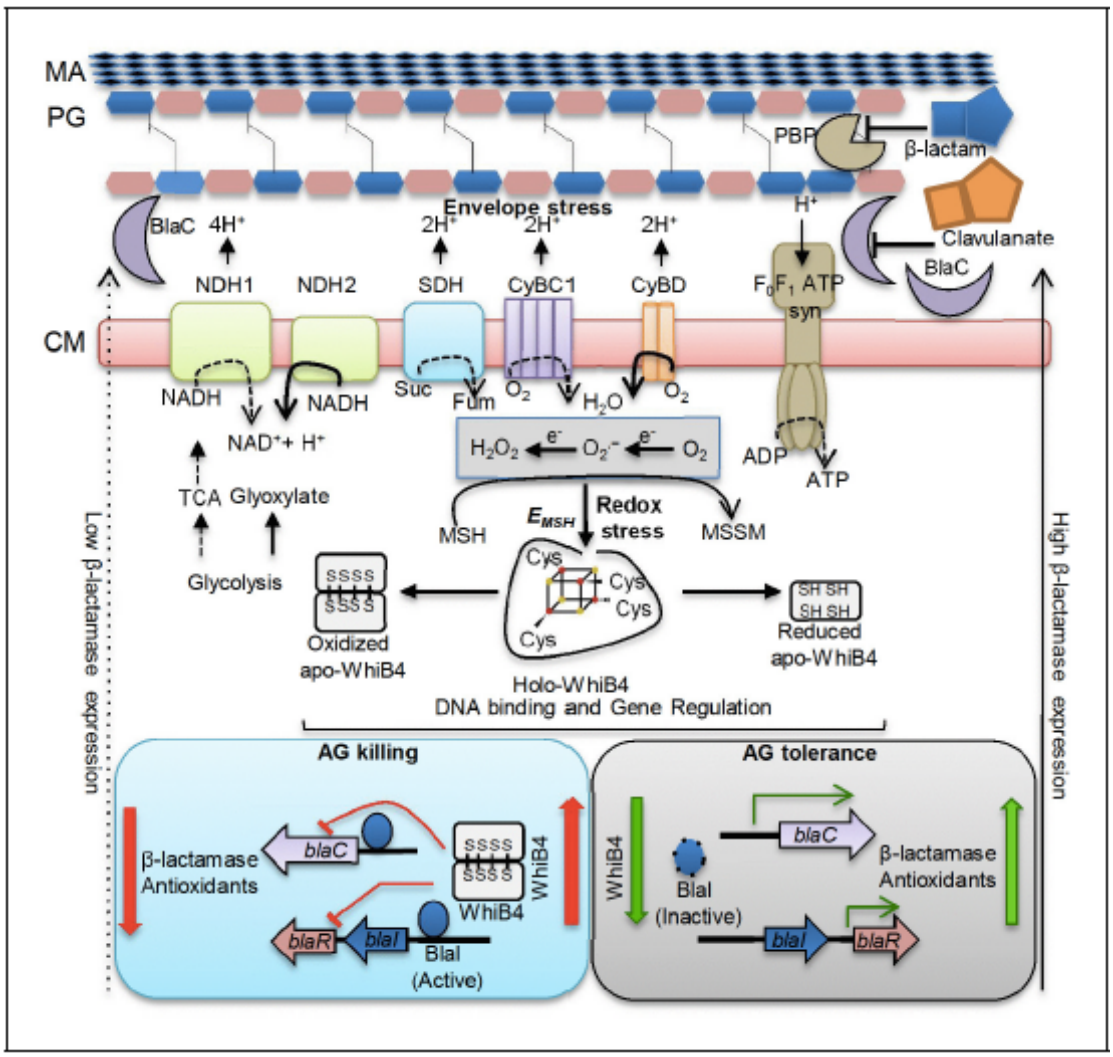

Fig. 3 Model showing redox basis of AG tolerance in *Mtb*. *Cell wall damage caused by AG can perturb the membrane integrity thereby affecting respiratory chain, redox balance, and ATP generation. All of this results in metabolic instability and AG-induced killing. To tolerate AG, Mtb redirects respiration from the energetically*

efficient route (e.g. NDH1, CyBC1) to the energetically poor one (e.g. NDH2, CyBD), and carbon metabolism from the TCA cycle to glyoxylate, glycolysis and gluconeogenesis. Rerouting of electron flux through CyBD can trigger generation of ROS by univalent reduction of O2 via metal-, flavin-, and quinone-containing respiratory enzymes. The intramycobacterial redox buffer, MSH, detoxifies ROS to protect Mtb from AG. The oxidative shift in EMSH of Mtb in response to AG serves as a cue to calibrate the expression of b-lactamase, PG enzymes, carbon metabolism, antioxidants, and alternate respiration via WhiB4. Under native conditions, O2- induced loss of WhiB4 Fe-S cluster generates oxidized apo-WhiB4, which binds and represses the expression of blaR and blaC. Reduction of oxidized apo-WhiB4 disulfides reversed this effect. Down-regulation of whiB4 in response to AG derepresses blaR and stimulates expression of blaC directly and/or indirectly via BlaR-mediated cleavage of the blaC repressor (i.e. BlaI) to induce AG tolerance. Accumulation of oxidized apo-WhiB4 upon overexpression led to hyper-repression of BlaC activity and oxidative shift in EMSH to potentiate mycobactericidal activity of AG. Since genes associated with alternate-respiration (e.g. CyBD) and energy metabolism (e.g. ATP synthase) are also regulated by BlaI, our results suggest cross-talk between WhiB4 and BlaI pathways resulting in AG tolerance of Mtb. Altogether, WhiB4 couples the changes in the redox physiology of Mtb triggered by AG to the

expression of genes involved in antibiotic tolerance and redox homeostasis. MA: Mycolic acid, CM: Cytoplasmic membrane, NDH1: NADH-dehydrogenase I (nuo operon), NDH2: NADH dehydrogenase 2 (ndh), CyBD: Cytochrome BD oxidase, CyBC1: Cytochrome BC1-aa3 oxidase, F0F1 ATP syn: ATP Synthase, PBP: Penicillin-binding proteins and SDH: Succinate Dehydrogenase. Bold or dashed arrows indicate increased or decreased electron flow through respiratory complexes, respectively, based on gene expression data.

Our work represents the first report describing a redox-based mechanism underlying tolerance to a novel β-lactam- β -lactamase inhibitor combination against a human pathogen. The use of AG is actively considered to treat drug-resistant tuberculosis infection (under human clinical trials). Further, this work should be viewed in the light of recent controversies regarding the role of antibiotic-induced oxidative stress in antibiotic action and tolerance. Several studies in recent years have tested, validated, and supported the linkage between ROS and antibiotics (**Kohanski et al., Cell, 2007, Foti et al., Science, 2012, Brynildsen et al., Nature Biotechnology, 2013**). However, some studies questioned the role of ROS-mediated killing of bacterial cells by antibiotics (**Liu and Imaly, *Science*, 399, 1210-1213, 2013 and Keren et al., *Science*, 399, 1213-1216, 2013**). Our findings provide a missing link of how secondary consequences of antibiotic exposure (redox stress and metabolism) is functionally associated with its primary drug targets by a redox-sensitive transcription factor, WhiB4, in *Mtb*. Considering that drug-resistance in *Mtb* is a global problem with a very little insight, our results showing that WhiB4 mediated changes in redox potential of *Mtb* can potentiate killing of clinical drug-resistant forms of *Mtb* by AG are unique.

Our results emphasize the importance of redox physiology in targeting drug-resistance in *Mtb* and can be extended to all human pathogens. Consequently, if priority is maintained, this study will serve as an invaluable resource for defining future research directions towards understanding antibiotics action, and development of high-throughput cellular screens at least for mycobacterial infections. Here the importance of *Mtb*, and its drug resistant forms, as a globally re-emerging infection cannot be overstated. Lastly, our innovative strategy to understand antibiotic actions in a natural context of infection will remain a priority, as the need for developing new drugs is as urgent as ever. Therefore, we also anticipate that this work will benefit from frequent citations by the TB, bacterial, and pharmaceutical research communities.

III: HIV- TB Coinfection: importance of redox and mitochondrial bioenergetics

Globally, individuals co-infected with the AIDS virus, HIV-1, and with *Mycobacterium tuberculosis* (*Mtb*- causative agent of tuberculosis [TB]), pose major obstacles in the clinical management of both the diseases. At the heart of this issues is the apparent synergy between the two human pathogens [1]. On one hand, mechanisms induced by HIV-1 responsible for reactivation of TB and progression to active TB diseases in AIDS patients (*e.g.,* CD4 decay) are well characterized [2]. On the other hand, despite that clinical data clearly identify TB as a risk factor for HIV-1 reactivation and associated mortality [3], the mechanisms by which *Mtb* exacerbates HIV-1 replication and infection remain poorly characterized. Addressing this issue should help generating new knowledge explaining the mechanism of synergy, which can be exploited for therapeutic benefits.

Lung macrophages are the primary host for *Mtb* [4], whereas HIV-1 mainly resides in CD4+ T cells[2]. Recent data seem to suggest an important contribution of macrophages in HIV-1 pathogenesis and HIV-TB co-infection [5]. How *Mtb* infected macrophages modulates HIV-1 infected lymphocytes and monocytes remain elusive. In the present study, we identified that

Mtb infected macrophages secrete exosomes to realign redox metabolism and bioenergetic parameters of lymphocytes and monocytes latently infected with HIV-1. These physiological events precede *Mtb* induced HIV-1 reactivation and replication. We used a range of cutting-edge technologies such as redox biosensor, Seahorse XF Flux analyzer, Nanostring nCounter Profiling, and LC-MS/MS, to mechanistically dissect redox and respiratory events associated with *Mtb* induced HIV-1 reactivation. Our findings, for the first time, provide empirical evidences showing how *Mtb* and HIV infected cells crosstalk via exosomes to promote viral reactivation. Based on our findings, we propose a new model of HIV-TB synergy. According to this model, *Mtb*-specific exosomes are selectively enriched for proteins (*e.g.,* HIF-1α, galectins, and HSP90) to alter transcriptome, redox physiology, inflammation, and oxygen consumption capacity of cells latently infected with HIV-1. The subtle variations in the redox potential and non-mitochondrial oxygen consumption rate of HIV-1 infected cells mediated by *Mtb*-specific exosomes reactivate HIV-1 without triggering any detrimental influence on the host physiology.

How can we relate our basic findings to develop new therapeutic strategies to reduce the risk of TB induced exacerbation of HIV morbidity and mortality? Our study raises fascinating new possibilities. For example, we present data showing that pharmacological pre-treatment with the antioxidant (N-acetyl cysteine) or the inhibitors of Galectins (lactose) and HSP90 (17-(N-allylamino)-17-demethoxygeldanamycin [17-AAG]) attenuated *Mtb*-exosomes mediated reactivation of HIV-1. HSP90 inhibitor, SNX-5422, shows a good safety profile in patients with solid tumors [6]. One can envisage using these inhibitors along with HAART to repress HIV-1 reactivation and replication in HIV-TB co-infected patients. Furthermore, reactivation of latent virus coupled with HAART has been put forward as a possible "Kick-and-Kill" approach to eliminate latent reservoir. However, most of the screening efforts identified latency-reversing agents that are cytotoxic. Since the *Mtb*-specific exosomes mediate HIV-1 reactivation without causing overwhelming oxidative stress and cytotoxicity, we anticipate that co-treatment of *Mtb* exosomes with HAART can target HIV-1 reservoir without triggering global cytotoxicity. Exosomes derived from *Mtb* infected macrophages were already reported to potentiate the antimycobacterial activity of anti-TB drugs *in vivo* [7], suggesting that a combination of *Mtb* exosomes with HAART and/or anti-TB drugs can be exploited to reduce the burden of HIV-TB co-infection.

In sum, our findings uncovered new paradigms for understanding the redox and bioenergetics basis of HIV-TB co-infection, which will enable the design of effective therapeutic strategies.

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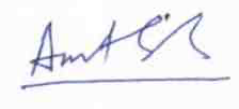

(Amit Singh- Signature)