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Recent Developments in the Application of Flow Cytometry to Advance our Understanding of *Mycobacterium tuberculosis* Physiology and Pathogenesis

Trisha Parbhoo,  Samantha L. Sampson,^{*}  Jacoba M. Mouton 

NRF-DST Centre of Excellence for Biomedical Tuberculosis Research, South African Medical Research Council Centre for Tuberculosis Research, Division of Molecular Biology and Human Genetics, Faculty of Medicine and Health Sciences, Stellenbosch University, Cape Town, South Africa

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*Correspondence to: Samantha L. Sampson, DST/NRF Centre of Excellence for Biomedical Tuberculosis Research/South African Medical Research Council Centre for Tuberculosis Research, Division of Molecular Biology and Human Genetics, Faculty of Medicine and Health Sciences, Stellenbosch University, Cape Town, South Africa. Email: ssampson@sun.ac.za

• Abstract

The ability of the bacterial pathogen *Mycobacterium tuberculosis* to adapt and survive within human cells to disseminate to other individuals and cause active disease is poorly understood. Research supports that as *M. tuberculosis* adapts to stressors encountered in the host, it exhibits variable physiological and metabolic states that are time and niche-dependent. Challenges associated with effective treatment and eradication of tuberculosis (TB) are in part attributed to our lack of understanding of these different mycobacterial phenotypes. This is mainly due to a lack of suitable tools to effectively identify/detect heterogeneous bacterial populations, which may include small, difficult-to-culture subpopulations. Importantly, flow cytometry allows rapid and affordable multiparametric measurements of physical and chemical characteristics of single cells, without the need to preculture cells. Here, we summarize current knowledge of flow cytometry applications that have advanced our understanding of the physiology of *M. tuberculosis* during TB disease. Specifically, we review how host-associated stressors influence bacterial characteristics such as metabolic activity, membrane potential, redox status and the mycobacterial cell wall. Further, we highlight that flow cytometry offers unprecedented opportunities for insight into bacterial population heterogeneity, which is increasingly appreciated as an important determinant of disease outcome. © 2020 The Authors. *Cytometry Part A* published by Wiley Periodicals, Inc. on behalf of International Society for Advancement of Cytometry.

• Key terms

Mycobacterium tuberculosis; physiology; pathology; flow cytometry; heterogeneity

INTRODUCTION

Several aspects of the disease tuberculosis (TB) and its causative agent, *Mycobacterium tuberculosis*, are still poorly understood, and the epidemic remains severe. There were an estimated 10 million new TB cases in 2018, and the disease is the top cause of death due to an infectious agent (1). Drug-resistant strains seriously threaten TB control programs worldwide (1), with approximately 0.5 million new cases of drug-resistant TB in 2018 (1). These figures underline the need for new and improved TB drugs. To realize this goal, it is essential to advance our understanding of *M. tuberculosis* physiology throughout the course of TB disease. It is evident that infection with *M. tuberculosis* can result in a spectrum of disease outcomes (Fig. 1). Furthermore, there is a growing appreciation that mycobacteria exhibit different physiological and metabolic states that are time- or niche-dependent, or occur as a result of inherent population heterogeneity (2, 3). Advancing our understanding of these different bacterial states is the focus of much of fundamental TB research, as

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this could provide the key to identifying the vulnerabilities that could point toward new drug targets.

Flow cytometry presents a powerful tool with enormous potential to provide insights into the physiology and metabolism of *M. tuberculosis* populations. In a flow cytometer, cells in suspension move in single file through a light source, and scattered light is measured by detectors to provide insight into various cell properties. By combining this platform with fluorescently labeled proteins, antibodies, metabolic probes and biosensors, properties including cell size, numbers, structures and metabolic states can be measured. A major benefit of flow cytometry is the speed with which it can detect and measure multiple parameters

associated with single cells, with most instruments able to process several thousand events per second. Consequently, this tool has been extensively applied in the life sciences, largely for analysis of mammalian cells (4, 5).

Flow cytometry has also been utilized in bacteriological studies (6, 7). Flow cytometry, combined with fluorescent dyes, monoclonal antibodies, and reporter strains has been successfully applied to determine bacterial numbers and viability in environmental (8, 9), industrial (10) and clinical (11, 12) samples. More recently, flow cytometry has been exploited in the basic research context to provide insights into bacterial growth, physiology and metabolism (13, 14). Flow cytometry offers a

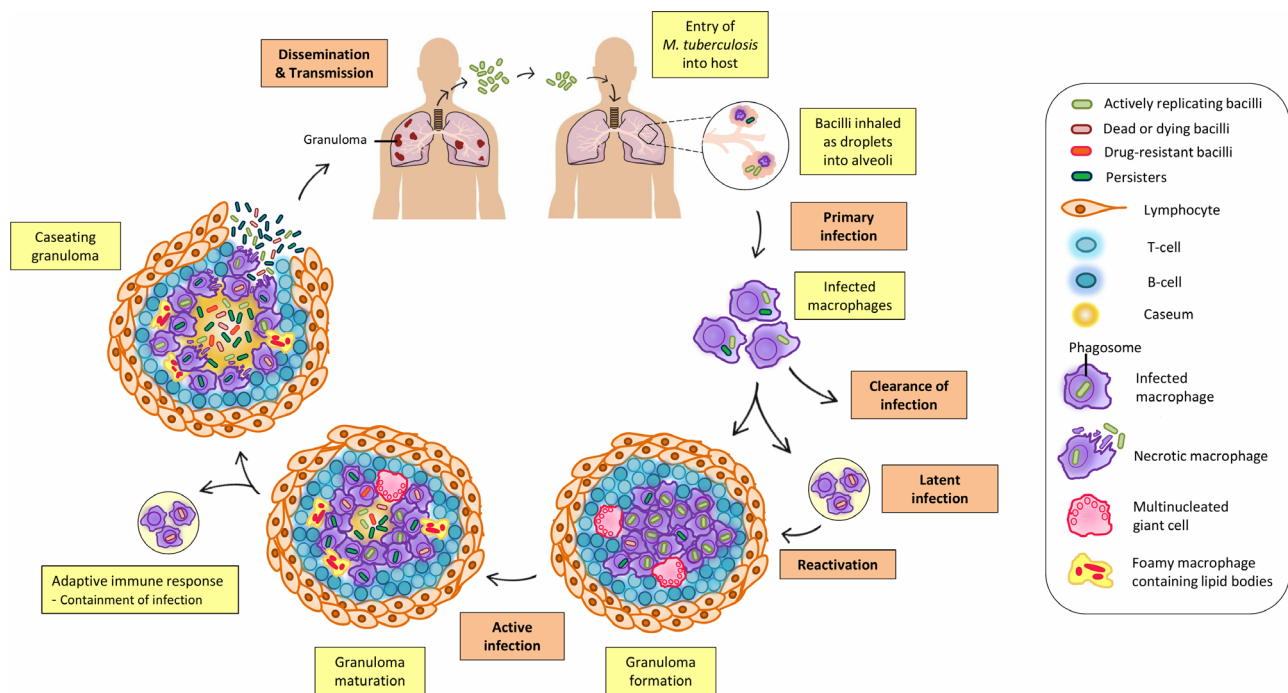


Fig 1. Infection with *M. tuberculosis* can result in a spectrum of disease outcomes. Primary infection involves inhalation of *M. tuberculosis* into the lung. Following phagocytosis of the bacilli by alveolar macrophages, infection may proceed with or without a visible lung lesion. In 90-95% of healthy individuals, infection can remain latent for decades, with potential to reactivate. During active infection, infected macrophages elicit an inflammatory response, recruiting innate immune cells to contain the infection in a granuloma. Upon induction of adaptive immunity, macrophages differentiate into specialized cells, such as multinucleated giant cells and foamy macrophages, and are surrounded by B and T cells. Infection may either be cleared by the host-immune response or upon exposure to treatment. Alternatively, the infection may persist and remain in a stable, quiescent state, attributed to physiological adaptations to intracellular stressors. The majority of bacilli are actively replicating and susceptible to the immune response and antibiotic treatment. *M. tuberculosis* could result in resistance in response to poor adherence to drug treatment or infection with a resistant strain. Other factors contributing to bacterial heterogeneity include variations in growth rate, division symmetry, metabolic rate, protein distribution and gene expression. However, increased necrotic breakdown of immune cells results in the formation of the caseum. As infection progresses this may lead to cavitation of granulomas, which could collapse into the lung, releasing the bacilli into the airway. [Color figure can be viewed at wileyonlinelibrary.com]

rapid, growth-independent means to characterize high numbers of single cells at a population-wide level, affording new insights into bacterial population heterogeneity and responses to different environmental (e.g., bacterial biofilms) and host-associated stress conditions (*M. tuberculosis* infected in vivo models such as zebra fish) (13, 15).

Certain technical limitations need to be considered for flow cytometry applications in bacteriological studies. The most obvious of these is the relatively small size of bacteria; this can make it difficult to distinguish bacteria from other cellular debris, sometimes necessitating the use of additional markers to reliably identify the bacteria of interest. Bacterial autofluorescence may also interfere with signal detection (16), and may in fact vary under different conditions (17); this property should be taken into consideration when interpreting results. Interestingly, autofluorescence has been exploited as a desirable characteristic in some settings as a means to identify bacteria in environmental and clinical samples when coupled with flow cytometry (16–18). Where reagents are adapted for use in bacteria, it is important to recognize that species- and sometimes even condition-specific optimization may be required, as has been reported with viability dyes, for example (19). Another problematic property of bacteria is the tendency of some species to clump; this is a particular concern for mycobacterial species, and measures such as sonication and filtering are advised to disperse clumps prior to flow cytometry analysis (20). A further consideration for pathogenic bacteria (such as *M. tuberculosis*) is the need for appropriate containment and decontamination measures to avoid contamination of the instrument and exposure of the user (21).

In this review, we discuss recent developments in the application of flow cytometry to *M. tuberculosis* research. We focus on how flow cytometry has been applied to

fundamental questions surrounding *M. tuberculosis* biology, such as how different host niches and stages of infection can affect the bacterial physiological state and vice versa (Fig. 1). Specifically, we consider how flow cytometry has been exploited to study how host-associated stressors influence bacterial characteristics such as metabolic activity, membrane potential, redox status and the mycobacterial cell wall (Fig. 2). Finally, we reflect briefly on how tools from other bacterial pathogens and technological platforms could be adapted for flow-cytometry-based investigation of *M. tuberculosis*.

HOW DOES THE HOST ENVIRONMENT AFFECT THE PHYSIOLOGICAL STATE OF MYCOBACTERIA?

Where Does *M. tuberculosis* Reside upon Infection of the Host?

The earliest encounter between the host and pathogen in TB infection occurs between *M. tuberculosis* and innate immune cells in the lungs, primarily macrophages, dendritic cells, monocytes, and neutrophils (43). These immune cells readily phagocytose *M. tuberculosis* and are the earliest defenders against the pathogen (Fig. 1). However, information regarding exactly which cells are initially infected and those that mediate dissemination from the alveoli to the lung interstitium is still relatively unclear. A recent study exploited a murine infection model and flow cytometry to demonstrate that early infection with *M. tuberculosis* expressing mCherry occurs almost exclusively in airway-resident alveolar macrophages (44). These infected macrophages disseminate to the lung interstitium through mechanisms requiring the pathogenicity-associated ESX-1 secretion system (44), emphasizing the dynamic interplay between host cells and *M. tuberculosis*.

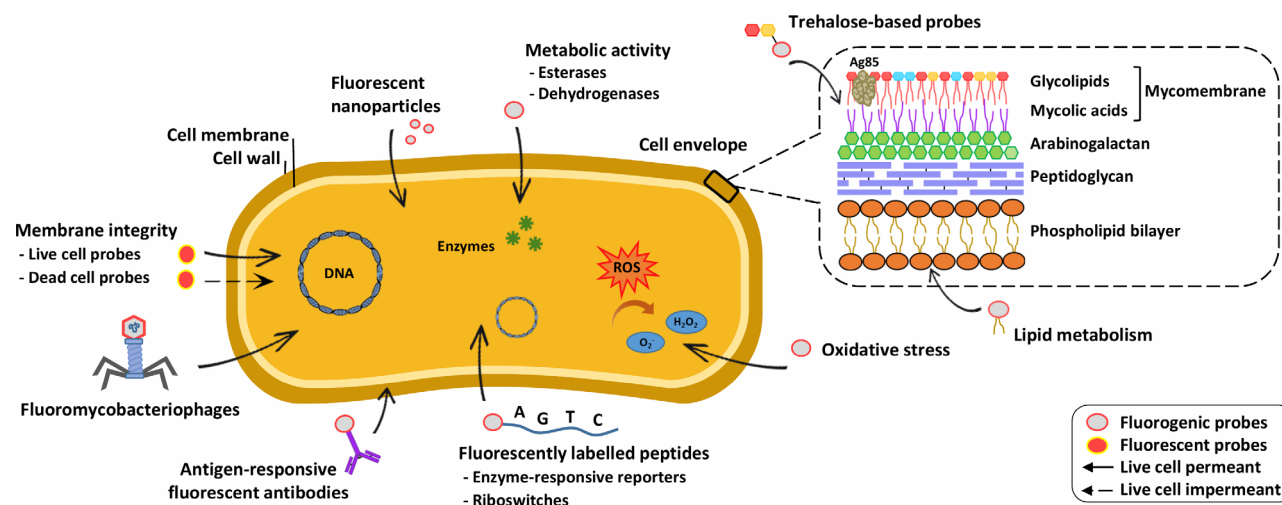


Fig 2. Fluorescent probe binding mechanisms. A combination of fluorescent probes can be applied to target distinct cellular components in *M. tuberculosis*, offering valuable applications for assessing the bacterial physiological state. The complex cellular architecture of the *M. tuberculosis* cell wall is known to affect uptake and permeability of fluorescent dyes. Recent development of probes targeting *M. tuberculosis*-specific cellular components has shown promise for improved labeling and detection. The spectral properties of a selection of fluorescent dyes used in mycobacteria are listed in Table 1. Abbreviations: Ag85; antigen 85. [Color figure can be viewed at wileyonlinelibrary.com]

Table 1 Characteristics of various fluorescent probes utilized in mycobacterial flow cytometry

CELLULAR TARGET SITES	FLUORESCENT DYES	EXCITATION (NM)	EMISSION (NM)	CELL PERMEANT	ORGANISM	REFERENCES
Metabolic activity	Fluorescein diacetate (FDA) ^a	473	514	Yes	<i>M. bovis</i> BCG vaccines	(22–24)
	Calcein-acetoxymethyl ester (calcein-AM)	495	515	Yes	<i>M. tuberculosis</i>	(25)
	Calcein violet AM ^a	400	452	Yes	<i>M. tuberculosis</i>	(26)
	Alexa fluor 488 ^a	494	517	Yes	<i>M. tuberculosis</i>	(27, 28)
	5-Carboxyfluorescein diacetate acetoxymethyl ester (CFDA) ^a	492	517	Yes	<i>M. smegmatis</i>	(29)
	5-Carboxyfluorescein-rifampicin (5-FAM-RIF)	488	519	Yes	<i>M. tuberculosis</i>	(30)
	5-Cyano-2,3-ditoyl tetrazolium chloride (CTC) ^a	450	630	Yes	<i>M. bovis</i> BCG	(29)
	BODIPY-palmitate	485	525	Yes	<i>M. tuberculosis</i>	(31, 32)
		493	503			
		485	498	Yes	<i>M. tuberculosis</i>	(33, 34)
Membrane integrity	SYTO 9 ^a	488	617	No	<i>M. tuberculosis</i>	(24, 33, 34)
	Propidium iodide (PI) ^a					
ROS	SYTOX green ^a	504	523	No	<i>M. tuberculosis</i>	(26)
	Hydroxyphenyl fluorescein (HPPF)	490	515	Yes	<i>M. tuberculosis</i>	(35–37)
	Chloromethyl-2',7'-dichlorodihydrofluoresceindiacetate (CM-H ₂ DCFDA)	492	527	Yes	<i>M. tuberculosis</i>	(38, 39)
	Superoxide	508	525	Yes	<i>M. smegmatis</i>	(29, 40, 41)
		545	565		<i>M. bovis</i> BCG	
		644	665			
		480	576	Yes	<i>M. tuberculosis</i>	(42)

^aProbes not mentioned in the text.

Macrophages eliminate *M. tuberculosis* via multiple strategies that include phagolysosomal fusion, secretion of cytokines, cell apoptosis and autophagy (45, 46). High-end technology such as imaging flow cytometry has demonstrated immensely beneficial at probing host-pathogen interactions to quantify and analyze phagocytosis, phagosome acidification and intracellular bacterial replication (47, 48). Recognition of *M. tuberculosis* by macrophages leads to phagocytosis and sequestration of the bacterium in phagosomes, which typically eradicate pathogens via fusion with lysosomes and consequent acidification of the pathogen-containing phagolysosome. However, *M. tuberculosis* utilizes a variety of strategies to survive and replicate in the phagosome by inhibiting phagosomal maturation and phagolysosomal generation to establish chronic infection. Physiological adaptation of *M. tuberculosis* to the phagosomal compartment in macrophages is an essential component of its pathogenesis, transmission, and continued survival (49, 50). The ability of mycobacteria to persist for decades during asymptomatic infection raises questions regarding their replicative and metabolic state. Several studies support that these bacteria could be in a nonreplicative, metabolically inactive state (51), while others support that these mycobacteria are nonreplicating, and metabolically active (52, 53), but contained by a dynamic host immune system. Often studies of these specific subpopulations are hampered by low bacterial numbers and difficulty to culture in broth or on agar, flow cytometry provides the ideal tool to investigate these populations without the need for preculturing or large cultures.

Is Mycobacterial Physiology Influenced by Host Niche?

The primary host niche for *M. tuberculosis* is the macrophage. Recent studies have demonstrated how *M. tuberculosis* adapts to life inside the macrophage where it encounters a nitrosative, oxidative (54, 55), hypoxic (30), and nutrient poor (56) environment. Specifically, the nucleoid-associated protein NapM, can be induced by diverse stress signals and is a broad stress-inducible protein in *M. tuberculosis* (57). It was recently shown NapM protects *M. tuberculosis* from stress-mediated killing, since it was found to enhance *M. tuberculosis* survival in macrophages by inhibiting mycobacterial DNA synthesis (57). Flow cytometry was used to measure DNA content per cell using DAPI staining, which demonstrated that NapM affects DNA replication in vivo (57). A conserved *M. tuberculosis* transmembrane protein, Rv0426c (P96272), previously shown to be essential for survival of *M. tuberculosis* in macrophages (58) and upregulated under nutrient starvation (56), was found to function as a virulence factor that suppresses apoptosis to allow mycobacterial replication within macrophages (59). Flow cytometry of FITC-conjugated Annexin V and Propidium Iodide stained macrophages that were infected with *M. smegmatis*, expressing Rv0426c, demonstrated attenuated apoptosis compared to macrophages infected with the vector only control (59), suggesting that these recombinant mycobacteria modulated their host environment to promote increased survival within macrophages.

For *M. tuberculosis* to maintain infection in macrophages and granulomas (described below), it requires import and metabolism of fatty acids. However, the mechanisms required for these processes are poorly understood. Fluorescence activated cells sorting (FACS)-based screening of mCherry-expressing *M. tuberculosis* mutants metabolically labeled with the fatty acid substrate BODIPY-palmitate (Table 1), identified several *M. tuberculosis* genes (*rv2799*, *rv0966c*, *rv0655*, and *rv0200*) required for BODIPY incorporation during macrophage infection. Furthermore, the role of the fatty acid transporter, Mce1, and Rv3723/LucA was established during this process (31). Another study recently showed that *M. tuberculosis*-infected macrophages exhibit increased accumulation of triglyceride-rich lipid droplets compared to uninfected macrophages (32). Specifically, imaging flow cytometry allowed colocalization of lipid droplets stained with BODIPY 493/503 with mCherry expressing-*M. tuberculosis*. This lipid accumulation subsequently leads to the development of foamy macrophages, which are indicative of impaired host response associated with decreased phagocytosis and respiratory burst. Several studies have suggested that lipid metabolism facilitates persistence (60, 61), which could point toward the use of these assays to improve our understanding of mycobacterial metabolic adaptation during infection.

Following the initial encounter of *M. tuberculosis* with airway macrophages, the infected macrophages may facilitate the mycobacterial dissemination through migration to distal sites in the lung and elsewhere (Fig. 1). These infected macrophages recruit uninfected macrophages and other lymphocytes to ultimately form a granuloma. The TB granuloma is an organized structure typical of infection with *M. tuberculosis* (62). Granulomas consist of several immune cells, which include macrophages, epithelioid cells (differentiated macrophages), multinucleated giant cells, and T lymphocytes (63, 64). Morphologically these cells are typically organized around a central necrotic core (caseum), surrounded by macrophages that are encircled by T cells and B cells, and may have a peripheral fibrotic border (63). Granulomas exhibit large heterogeneity and apart from the classic caseous granuloma, granulomas can be non-necrotizing, neutrophil-rich, mineralized, fibrotic, or cavitory (64). They have historically been considered to be a means for sequestering *M. tuberculosis*, but counter to this, they may also provide the mycobacterium with a niche in which it can replicate and survive (65) (Fig. 1). Further, granulomas exhibit selective and variable drug permeability, which may limit the efficacy of drugs targeted at persistent bacteria located in granulomas (66). Importantly, mycobacteria residing in granulomas are exposed to a heterogeneous cellular environment, varying oxygen levels and inflammatory milieu, environments known to drive *M. tuberculosis* into a persistent state (63, 67) (Fig. 1). The metabolic and replicative state of *M. tuberculosis* that reside inside granulomas are poorly understood; however, it is thought that these bacteria are in a dormant state (66, 68). One of the earliest studies to probe the mycobacterial granuloma using flow cytometry, focused on *Mycobacterium marinum*, a close genomic relative of *M. tuberculosis* and cause of

TB in hosts such as fish and frogs (69). This work used differential fluorescence induction to identify promoters induced in granulomas, but not in macrophages (70). Specifically, mycobacterial promoters transcriptionally fused to green fluorescent protein (GFP) enabled identification of granuloma-specific genes expressed at different stages of infection, including selected genes activated during persistence (70).

Besides stress responses mediated by specific molecular mechanisms, another, more generalized, response of *M. tuberculosis* to exposure to stressors such as those encountered in the macrophage and granuloma is to adopt a slow or non-replicating state, characteristic of “persister” bacteria (71). Bacterial persisters are a subset of drug tolerant bacteria, and may either spontaneously arise or be triggered in response to a specific stress condition, such as nutrient starvation or antibiotic treatment (71). Our working definition is consistent with that of so-called Class II persisters (72) that are slow or nongrowing drug-tolerant cells in an otherwise susceptible clonal population, in which tolerance is reversible upon removal of antibiotics (71). These bacterial populations are likely to contribute to latent TB (a clinical, asymptomatic state) and the requirement for very lengthy TB treatment regimens. Our understanding of persister biology is hampered by a lack of suitable tools to identify, isolate, and characterize these small populations of non- or slowly replicating mycobacteria. However, several culture-independent flow cytometry-based methods have recently been developed to detect viable, but non- or slowly replicating persisters not only in infected macrophages (73), but also in vitro and in sputum samples (74). One such approach, fluorescence dilution (FD), takes advantage of two fluorescent reporters, GFP, a constitutive reporter for tracking viable bacteria, and TurboFP635, an inducible reporter for measuring bacterial replication (73). This was successfully exploited in conjunction with flow cytometry in the *Salmonella* field (75, 76), and more recently for *M. tuberculosis* (73), displaying that the bacterial growth rate inside murine macrophages exhibits large heterogeneity, with emergence of intracellular viable, but non- or slowly replicating persisters.

Understanding and targeting bacterial metabolic responses that underlie entry into a viable, but non- or slowly replicating persister state during drug treatment could provide ways to prevent drug tolerance, and thereby shorten TB treatment regimens (42, 77). For example, a CRISPRi-based genetic repression strategy in combination with flow cytometry of DAPI-stained bacteria improved our understanding of the role DNA gyrase depletion has on *M. tuberculosis* physiology. Specifically, suppression of DNA gyrase as a result of treatment with second-line anti-TB drugs, fluoroquinolones, impaired intra- and extracellular *M. tuberculosis* growth in human monocyte-derived macrophages, which induced persister formation via the activation of the RexA/LexA-mediated SOS response. Subsequently, chemical inhibition of RecA in gyrase-depleted bacteria was able to reverse the persister phenotype and improve antibiotic treatment outcome (77). Since DNA gyrase plays a functional role during transcription (78), a GFP-tagged rRNA reporter was exploited to assess the

transcription rate of rRNA using flow cytometry (79). This allowed detection of rare phenotypes present at low numbers, which is critical for studying heterogeneity within *M. tuberculosis* growth and gene expression (79). In other work probing heterogeneity in *M. tuberculosis* response to drug treatment, the dual-reporter mycobacteriophage (ϕ^2 DRM) was exploited (74). This reporter comprises of constitutively expressed mVenus as a viability marker and promoter-tDTomato fusions (where the promoters are from genes that were highly expressed in response to isoniazid [INH] treatment) as a marker for persisters (74). This system was applied to detect phenotypic heterogeneity within sputum samples during the course of treatment, which is likely to influence treatment outcome (74). Surprisingly, a subpopulation of persisters were detected in sputum prior to antibiotic treatment, and were highly enriched following INH treatment (74). Expanding the number of promoter fusion constructs can assist in understanding the expression of virulence-associated genes, thereby improving understanding of disease progression and treatment response.

Do Host Cell Phenotypes Modulate Infection Outcome?

Host cell phenotypes have been shown to modulate *M. tuberculosis* infection outcome (3, 80). Using fluorescently labeled *M. tuberculosis* strains, which respond and fluoresce upon exposure to specific host stressors in combination with flow cytometry, alveolar macrophages were shown to demonstrate higher bacterial burdens compared to interstitial macrophages, suggesting that alveolar macrophages are more permissive of bacterial growth than interstitial macrophages (3). This has been supported by more recent work, which used FACS in combination with dual RNA sequencing to provide highly detailed insights into interactions between different bacterial growth phenotypes and host cells (81). Interestingly, these results suggested that increased *M. tuberculosis* growth in alveolar macrophages occurs as a result of improved access to iron and fatty acids, whereas *M. tuberculosis* growth in interstitial macrophages is restricted through iron sequestration and increased nitric oxide levels (81).

In a similar vein, the impact of heterogeneity in macrophage polarization on control of *M. tuberculosis* infection has recently been highlighted by RNA sequencing of human monocyte derived macrophages containing viable or dead mycobacteria (80). An *M. tuberculosis* reporter strain constitutively expressing long-lived mCherry was used as a measure of all bacteria, and the inducible expression of GFP, served as a proxy for cell viability. This dual reporter was exploited in combination with FACS analyses to separate macrophages containing viable bacilli from macrophages containing dead bacilli for RNA sequencing. These results demonstrated that the expression of genes involved in granulocyte-macrophage colony-stimulating factor (GM-CSF) signaling discriminates between the two macrophage subpopulations. The addition of GM-CSF resulted in inhibited mycobacterial growth, whereas blocking of GM-CSF permitted mycobacterial growth, demonstrating a critical mechanism that can be manipulated by

M. tuberculosis to drive a more favorable host environment (80).

How Do Host Stressors Affect the Metabolism of Mycobacteria?

The underlying metabolic state of bacteria is a crucial determinant of bacterial survival; shifts in central metabolic pathways have been observed in response to persistence (82), the availability of nutrients (83, 84), metabolite sensing (60, 85, 86), and antibiotic pressure (42, 87, 88). Metabolic shifts may manifest as altered levels of reactive oxygen species (ROS) (29). Different ROS-sensing probes specifically detect different species of ROS, which subsequently may provide insight into how metabolic adaptation could affect oxidative stress (Fig. 2). For example, hydroxyphenyl fluorescein (HPF) has been shown to exclusively stain hydrogen peroxide (89), while the chloromethyl derivative of chloromethyl-2',7'-dichlorodihydro fluoresceindiacetate (DCFDA or also known as H₂DCFDA) may be used for general oxidative stress measurements (Table 1). Both HPF (35–37) and CM-H₂DCFDA (38, 39) have recently been exploited *in vitro* in combination with flow cytometry to investigate *M. tuberculosis* tolerance to ROS species in response to infection and antibiotic treatment and provide useful tools for analyses of metabolic activity.

A recent study explored the role of cysteine and other small thiols, which act as cytoplasmic redox buffers, in combination with first-line anti-*M. tuberculosis* drugs in inhibiting the formation of persister and drug resistant *M. tuberculosis* in murine macrophages (42). Since mycothiol is a cysteine reservoir it was hypothesized that cysteine accumulation may be a contributing factor to INH resistance. Surprisingly, the combination of reducing compounds, such as N-acetylcysteine (42) or vitamin C (90), with INH resulted in enhanced production of ROS and prevented formation of both drug tolerant and resistant populations, as assessed by flow cytometry following staining with the superoxide indicator, dihydroethidium (Table 1) (42). This could provide ways to prevent bacteria from entering a persister state, which could have important implications for effective treatment of TB. Comparatively, a genetically-encoded biosensor (Mrx1-roGFP2) was designed by coupling redox-sensitive GFP (roGFP2) to mycothiol-dependent oxidoreductase (Mrx1), providing an extremely sensitive ratiometric sensor for flow cytometric usage that is able to detect nanomolar changes in mycothiol redox potential (91). Mrx1-roGFP2 was exploited in combination with flow cytometry to assess perturbations in mycothiol homeostasis attributed to macrophage-induced oxidative stress and antibiotic exposure (91). Additionally, the role of protein kinase G (PknG), responsible for regulating bacterial metabolism and persistence during stress, was established using Mrx1-roGFP2 to determine the impact deletion of *pknG* presents for mycothiol-linked redox heterogeneity and *M. tuberculosis* clearance during infection (92). This highlights the potential of thiols to enhance drug activity by generating an oxidative burst, thereby coercing *M. tuberculosis* persisters into a constant metabolically active state (42, 91, 92). The use of the fluorogenic probe CellROX followed by

flow cytometry recently demonstrated the heterogeneous generation of superoxide in response to antibiotic exposure and nutrient depletion in *M. smegmatis* and BCG *in vitro* (Table 1). CellROX allows the detection and quantification of ROS in live cells; it is cell permeant and nonfluorescent or weakly fluorescent in the reduced state and upon oxidation emits a strong fluorescence. Additionally, persisters were shown to accumulate superoxide (29). CellROX has previously been applied in combination with flow cytometry to determine the effect of the redox buffers ergothione and mycothiol on redox and bioenergetic homeostasis (40), and assess ROS generation in response to treatment with the electron transport chain inhibitors, bedaquiline and clofazimine (41). An inducible enzyme-responsive expression system for flow cytometric usage was developed whereby organic hydroperoxide reductase regulator (OhrR) senses and responds to intracellular peroxide stress through oxidation of its cysteine residues (93). Multiparametric staining in combination with probes that target specific types of ROS could be exploited to investigate the effect of stress (environmental, antibiotic treatment, etc.) on redox homeostasis and how this is maintained in *M. tuberculosis*.

DOES BACTERIAL ADAPTATION TO HOST STRESSORS RESULT IN A REMODELED CELL SURFACE?

The structural and functional adaptation of the mycobacterial cell envelope in response to the host environment contributes to its survival and pathogenicity (94). Mycobacteria divide and deposit new cell wall material asymmetrically (27, 95), with variable distribution of cell surface proteins, secretory, enzymatic, and regulatory complexes (27, 95). Host-encountered stressors likely contribute to varying growth rates and size of daughter cells (96, 97), and this heterogeneity may result in variable responses to host-encountered stressors. Importantly, flow cytometry provides a suitable tool to assess these heterogeneous features.

Heterogeneity in mycobacterial growth has been shown to be associated with drug tolerance (25, 26, 73), which in turn can drive treatment failure. In a FACS-based transposon screen, calcein-acetoxymethyl ester (calcein-AM) was exploited to detect genes involved in asymmetric growth *in vitro*. Calcein-AM is a nonfluorescent molecule that enters the cell through passive diffusion to produce a fluorescent molecule, calcein, in response to esterase activity (Table 1, Fig. 2). This approach identified *lamA*, encoding a member of the mycobacterial division complex that controls the pattern of cell growth by inhibiting growth at nascent poles, creating asymmetry in polar growth (25). Variability in calcein fluorescence distribution corresponded with heterogeneity in drug susceptibility, whereby cells that lack *lamA* showed more uniform and rapid killing in response to rifampicin and drugs that target the cell wall (25). The polar and septal localization of the mycolic acid synthesis enzyme, InhA, may further explain the variable activity of INH (98). Alternatively, thickening of the mycobacterial cell wall during persistence creates a permeability barrier and may contribute to the restricted

entry of drugs. Polysaccharide-specific staining with Calcofluor white revealed increased abundance of polysaccharide in the thickened cell wall of persisters compared to actively growing mid-log phase bacilli using microscopy. Additionally, flow cytometry analyses demonstrated low permeability of rifampicin-conjugated-5-carboxyfluorescein (5-FAM-RIF), an amine-reactive probe, providing support for a mechanism of persistence (Table 1) (30).

Mycolyltransferases play a crucial role in the architecture and assembly of the mycobacterial cell envelope, contributing to nutrient acquisition, cell envelope integrity, and protection from the host environment (99, 100). Abundantly secreted mycolyltransferases, consisting of the antigen 85 (Ag85) complex (Ag85A, Ag85B, and Ag85C), integrate long-chained, branched mycolic acids either covalently or noncovalently into arabinogalactan or trehalose glycolipids, respectively (Fig. 2). Since these highly immunogenic components are essential for mycobacterial viability and virulence, they are possible targets for antigen detection (99, 100). The synthesis of fluorogenic trehalose analogues exploit metabolic conversion by the Ag85 complex for incorporation into the mycomembrane as trehalose mycolates in metabolically active, live bacilli, without perturbing the arrangement of mycolic acids. Importantly, these specific cell wall glycolipids are highly antigenic and serve as essential components for cell envelope function, virulence and persistence in pathogenic mycobacteria, providing high specificity of *M. tuberculosis* detection using flow cytometry (101–103). Antigen-specific antibodies in combination with different fluorophores have been used to investigate the variation and distribution of antigens and surface proteins on the cell envelope of *M. tuberculosis* (104–107).

Dependent on probe properties, trehalose glycolipids can be detected by direct attachment to fluorophores or by click chemistry, which involves delivery of the fluorophore (to trehalose glycolipids) in a secondary labeling step. The advantage of click chemistry is that mycomembrane composition and trehalose processing can be simultaneously labeled dependent on the transference of the clickable lipid chain onto mycolyl acceptors, as comprehensively described (108). Briefly, the native mycolate is replaced by the trehalose analogue containing a terminal chemical reporter (e.g., azide, alkyne, or fluorescent tags), followed by fluorophore labeling of the tagged mycolyl acceptor using click chemistry. This allows higher metabolic incorporation efficiency and the benefit of delivering secondary tags to the trehalose analogue during the second labeling step. Additionally, mycolylated components of the mycomembrane are labeled, providing a comprehensive analysis of cell wall metabolism and glycolipid distribution during infection (108, 109). Alternatively, a FRET-based trehalose analogue facilitated understanding of mycolyltransferase activity without labeling of the mycomembrane components; whereby fluorescence was mediated via Ag85-processing of trehalose modified with a fluorophore-quencher (103). In these studies, flow cytometry allowed visualization of fluorescence (time-, binding affinity-, and growth phase-dependence of labeling), which can

improve our understanding of the biosynthetic pathways involved in mycomembrane remodeling (102, 103, 108). Several modifications of trehalose analogues have been reported, influencing labeling efficiency and spectral properties and thereby extending their potential applications (83, 101, 102, 108–111).

Enhanced understanding of the mycomembrane composition and biosynthesis could prove advantageous for the development of mycobacteria-specific probes that closely resemble native mycolates that are likely to improve metabolic incorporation within a cellular environment (108). Trehalose conjugated to the solvatochromic fluorophore 4-N,N-dimethylamino-1,8-naphthalimide, referred to as DMN-Tre, has enabled rapid detection of *M. tuberculosis* in sputum samples using flow cytometry (101). DMN-Tre labeling is dependent on mycomembrane biosynthesis, whereby the probe specifically targets trehalose through metabolic conversion into the mycomembrane, providing a measure of metabolically active, viable mycobacteria. Additionally, bacilli compromised by antibiotic treatment were distinguished by their diminished DMN-Tre labeling intensity. Contrastingly, antibiotic-compromised cells could not be differentiated using conventional auramine staining and microscopy, emphasizing the use of DMN-Tre in combination with flow cytometry as a promising therapeutic tool (101). Additionally, probe incorporation via alternative biosynthetic pathways may enhance our understanding of the extent of mycobacterial cell wall remodeling during stress (Fig. 2). For example, cell wall peptidoglycan synthesis, which is critical for providing cell wall support during elongation can be characterized using flow cytometry by labeling *M. tuberculosis* with D-amino acid probes (112). Another study utilizes a specific dual-targeting, enzyme-responsive fluorogenic probe in response to *M. tuberculosis* β -lactamase activity and synthesis of cell wall arabinans, displaying rapid labeling of live bacilli in sputum samples using flow cytometry (113).

Many *M. tuberculosis* cell-surface proteins exhibit adhesin activity, such as mannose-capped lipoarabinomannan (ManLAM) (114) and malate synthase (115), which respectively represent the key glycolipid and essential enzyme in the glyoxylate shunt. Since ManLAM can be recognized by multiple receptors on macrophages (116), the design of aptamers in combination with flow cytometry has been used to determine how interaction of various components of the cell wall with host cell receptors may lead to production of different signaling pathways to promote immune regulation (114, 115, 117). In combination with flow cytometry, the design of an aptamer binding to ManLAM potentially reorganizes the cell wall as it disrupts interaction with mannose receptors and induced production of iNOS and inflammatory cytokines associated with M1 polarization, thereby reducing progression of infection (114). Aptamers are short oligonucleotide or peptide molecules that bind with high-specificity to target molecules. Aptamer selection using Systematic Evolution of Ligands by Exponential enrichment (SELEX) technology offers the advantage that they can be more easily produced than antibodies and has the ability to modulate the level and

timing of gene expression (118, 119). Additionally, aptamers can be used to control synthetic riboswitches and have been applied for tight regulation of mycobacterial gene expression; when coupled with flow cytometry, this can be used to assess gene expression and track bacteria in *in vitro* culture or in the context of macrophage infection (73, 120).

CONCLUSION

Challenges associated with delivering effective TB treatment are intrinsically linked to its adaptation to the intracellular lifestyle of *M. tuberculosis*. However, our understanding of how *M. tuberculosis* is able to survive and persist within eukaryotic cells and organs is limited. Recent applications of tools such as flow cytometry has significantly improved our understanding of the physiology and pathology of *M. tuberculosis* in response to host stressors and antibiotic treatment and offers immense potential to resolve unanswered questions that could previously not be addressed due to a lack of suitable tools.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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