Cytometry

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 🅾

● 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

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
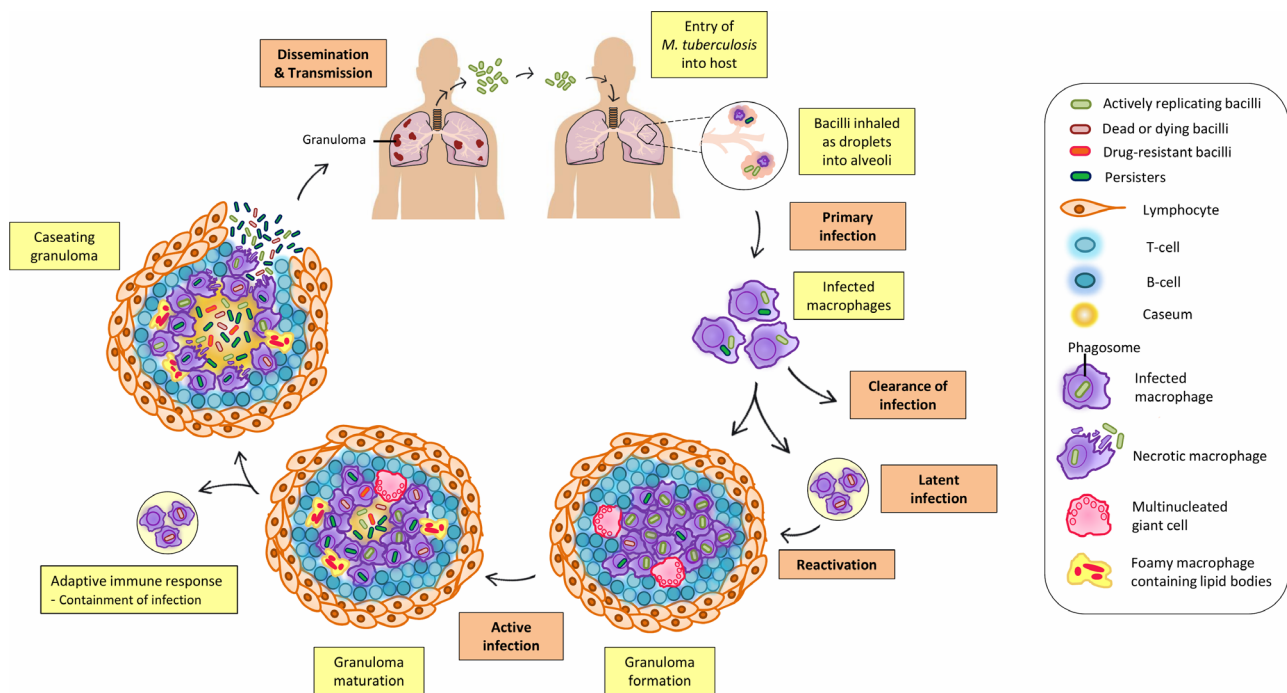

Fig 1. Infection with *M. tuberculosis* can result in a spectrum of disease outcomes. Primary infection involves inhalation of *M. tuberculosis* into the lung. Following phagocytosis of the bacilli by alveolar macrophages, infection may proceed with or without a visible lung lesion. In 90-95% of healthy 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*.
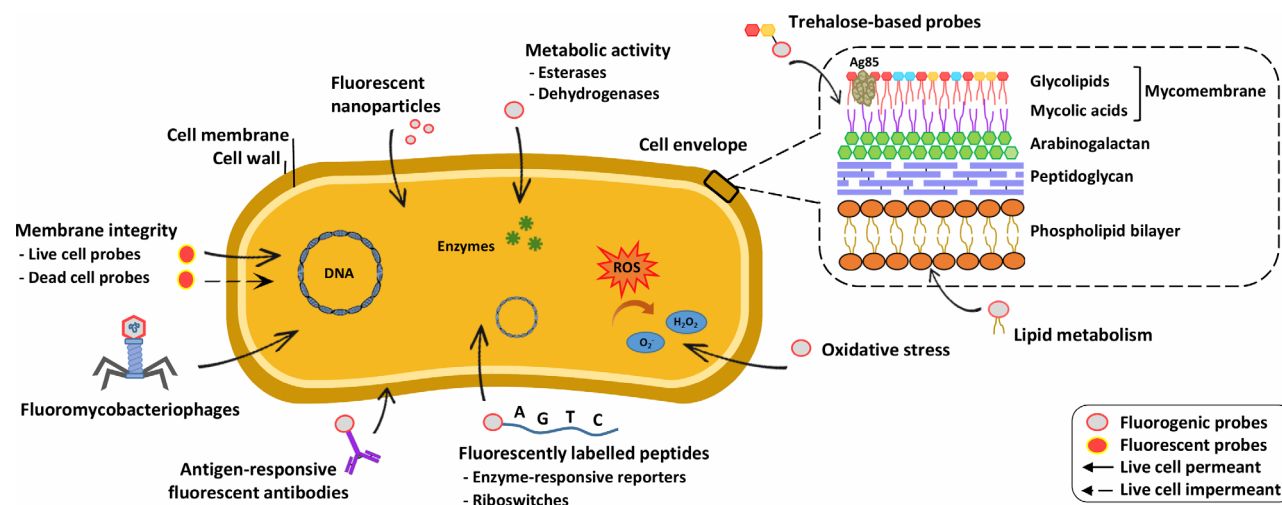
Fig 2. Fluorescent probe binding mechanisms. A combination of fluorescent probes can be applied to target distinct cellular components in *M. tuberculosis*, offering valuable applications for assessing the bacterial physiological state. The complex cellular architecture of the *M. tuberculosis* cell 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
		Fluorescein diacetate (FDA)[a]	473	514	Yes	M. bovis BCG vaccines M. tuberculosis	(22–24)
Metabolic activity	Esterases	Calcein-acetoxymethyl ester (calcein-AM)	495	515	Yes	M. smegmatis M. tuberculosis	(25)
		Calcein violet AM[a]	400	452	Yes	M. tuberculosis	(26)
		Alexa fluor 488[a]	494	517	Yes	M. smegmatis	(27, 28)
		5-Carboxyfluorescein diacetate acetoxymethyl ester (CFDA)[a]	492	517	Yes	M. bovis BCG	(29)
		5-Carboxyfluorescein-rifampicin (5-FAM-RIF)	488	519	Yes	M. tuberculosis	(30)
	Dehydrogenases	5-Cyano-2,3-ditolyl tetrazolium chloride (CTC)[a]	450	630	Yes	M. bovis BCG	(29)
	Lipid metabolism	BODIPY-palmitate	485	525	Yes	M. tuberculosis	(31, 32)
			493	503			
Membrane integrity	Nucleic acid	SYTO 9[a]	485	498	Yes	M. tuberculosis	(33, 34)
		Propidium iodide (PI)[a]	488	617	No	M. tuberculosis	(24, 33, 34)
		SYTOX green[a]	504	523	No	M. tuberculosis	(26)
ROS	Hydrogen peroxide	Hydroxyphenyl fluorescein (HPF)	490	515	Yes	M. tuberculosis	(35–37)
	Overall oxidative stress	Chloromethyl-2',7'-dichlorodihydrofluoresceindiacetate (CM-H2DCFDA)	492	527	Yes	M. tuberculosis	(38, 39)
	Superoxide	CellROX Green	508	525	Yes	M. smegmatis	(29, 40, 41)
		CellROX Orange	545	565		M. bovis BCG	
		CellROX Deep Red	644	665			
		Dihydroethidium	480	576	Yes	M. tuberculosis	(42)

[a]Probes not mentioned in the text.

Improved Understanding of Mycobacterial Physiology

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 cavitary (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^2DRM) 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 H_2DCFDA) may be used for general oxidative stress measurements (Table 1). Both HPF (35–37) and CM-H_2DCFDA (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 ergothioneine and mycothiol on redox and bioenergetic homeostasis (40), and assess ROS generation in response to treatment with the electron transport chain inhibitors, bedaquiline and clofazamine (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.

Acknowledgments

Conflict of Interest
The authors declare that they have no conflicts of interest.

Literature Cited

1. World Health Organization. World Health Statistics 2019: Monitoring Health for the SDGs, Sustainable Development Goals. Geneva: World Health Organization, 2019.

2. Russell DG, Huang L, VanderVen BC. Immunometabolism at the interface between macrophages and pathogens. Nat Rev Immunol 2019;19:291–304.

3. Huang L, Nazarova EV, Tan S, Liu Y, Russell DG. Growth of *Mycobacterium tuberculosis in vivo* segregates with host macrophage metabolism and ontogeny. J Exp Med 2018;215:1135–1152.

4. Adan A, Alizada G, Kiraz Y, Baran Y, Nalbant A. Flow cytometry: Basic principles and applications. Crit Rev Biotechnol 2017;37:163–176.

5. McKinnon KM. Flow cytometry: An overview. Curr Protoc Immunol 2018;120: 5.1.1–5.1.11.

6. Müller S, Davey H. Recent advances in the analysis of individual microbial cells. Cytometry Part A 2009;75A:83–86.

7. Robinson JP. Overview of flow cytometry and microbiology. Curr Protoc Cytom 2018;84:e37.

8. Emerson JB, Adams RI, Román CMB, Brooks B, Coil DA, Dahlhausen K, Ganz HH, Hartmann EM, Hsu A, Justice NB, et al. Schrödinger's microbes: Tools for distinguishing the living from the dead in microbial ecosystems. Microbiome 2017;5:86.

9. Frossard A, Hammes F, Gessner MO. Flow cytometric assessment of bacterial abundance in soils, sediments and sludge. Front Microbiol 2016;7:903–911.

10. Perera IA, Abinandan S, Subashchandrabose SR, Venkateswarlu K, Naidu R, Megharaj M. Advances in the technologies for studying consortia of bacteria and cyanobacteria/microalgae in wastewaters. Crit Rev Biotechnol 2019;39:709–731.

11. Mejuto P, Luengo M, Díaz-Gigante J. Automated flow cytometry: An alternative to urine culture in a routine clinical microbiology laboratory? Int J Microbiol 2017; 2017:1–8.

12. Rubio E, Zboromyrska Y, Bosch J, Fernandez-Pittol MJ, Fidalgo BI, Fasanella A, Mons A, Román A, Casals-Pascual C, Vila J. Evaluation of flow cytometry for the detection of bacteria in biological fluids. PLoS ONE 2019;14:e0220307.

13. Ambriz-Aviña AV, Contreras-Garduño JA, Pedraza-Reyes M. Applications of flow cytometry to characterize bacterial physiological responses. Biomed Res Int 2014; 2014:e461941.

14. Tracy BP, Gaida SM, Papoutsakis ET. Flow cytometry for bacteria: Enabling metabolic engineering, synthetic biology and the elucidation of complex phenotypes. Curr Opin Biotechnol 2010;21:85–99.

15. Dhar N, McKinney J, Manina G. Phenotypic heterogeneity in *Mycobacterium tuberculosis*. Microbiol Spectr 2016;4:671–697.

16. Ammor MS. Recent advances in the use of intrinsic fluorescence for bacterial identification and characterization. J Fluoresc 2007;17:455–459.

17. Renggli S, Keck W, Jenal U, Ritz D. Role of autofluorescence in flow cytometric analysis of *Escherichia coli* treated with bactericidal antibiotics. J Bacteriol 2013; 195:4067–4073.

18. Dartnell LR, Roberts TA, Moore G, Ward JM, Muller J-P. Fluorescence characterization of clinically-important bacteria. PLoS ONE 2013;8:e75270.

19. Buysschaert B, Byloos B, Leys N, Van Houdt R, Boon N. Reevaluating multicolor flow cytometry to assess microbial viability. Appl Microbiol Biotechnol 2016;100: 9037–9051.

20. Cheng N, Porter MA, Frick LW, Nguyen Y, Hayden JD, Young EF, Braunstein MS, Hull-Ryde EA, Janzen WP. Filtration improves the performance of a high-throughput screen for anti-mycobacterial compounds. PLoS ONE 2014;9:e96348.

21. Pina-Vaz C, Costa-de-Oliveira S, Rodrigues AG. Safe susceptibility testing of *Mycobacterium tuberculosis* by flow cytometry with the fluorescent nucleic acid stain SYTO 16. J Med Microbiol 2005;54:77–81.

22. Gweon E, Choi C, Kim J, Kim B, Kang H, Park T, Ban S, Bae M, Park S, Jeong J. Development of a new approach to determine the potency of Bacille Calmette–Guérin vaccines using flow cytometry. Osong Public Health Res Perspect 2017;8: 389–396.

23. Reis RS, Neves I, Lourenço SLS, Fonseca LS, Lourenço MCS. Comparison of flow cytometric and alamar blue tests with the proportional method for testing susceptibility of *Mycobacterium tuberculosis* to rifampin and isoniazid. J Clin Microbiol 2004;42:2247–2248.

24. Kurthkoti K, Amin H, Marakalala MJ, Ghanny S, Subbian S, Sakatos A, Livny J, Fortune SM, Berney M, Rodriguez GM. The capacity of *Mycobacterium tuberculosis* to survive iron starvation might enable it to persist in iron-deprived microenvironments of human granulomas. MBio 2017;8:1092–1109.

25. Rego EH, Audette RE, Rubin EJ. Deletion of a mycobacterial divisome factor collapses single-cell phenotypic heterogeneity. Nature 2017;546:153–157.

26. Hendon-Dunn CL, Doris KS, Thomas SR, Allnutt JC, Marriott AAN, Hatch KA, Watson RJ, Bottley G, Marsh PD, Taylor SC, et al. A flow cytometry method for rapidly assessing *M. tuberculosis* responses to antibiotics with different modes of action. Antimicrob Agents Chemother 2016;60(7):3869–3883.

27. Hayashi JM, Luo C-Y, Mayfield JA, Hsu T, Fukuda T, Walfield AL, Giffen SR, Leszyk JD, Baer CE, Bennion OT, et al. Spatially distinct and metabolically active membrane domain in mycobacteria. Proc Natl Acad Sci 2016;113:5400–5405.

28. Zhu J-H, Wang B-W, Pan M, Zeng Y-N, Rego H, Javid B. Rifampicin can induce antibiotic tolerance in mycobacteria via paradoxical changes in rpoB transcription. Nat Commun 2018;9:4218.

29. McBee ME, Chionh YH, Sharaf ML, Ho P, Cai MWL, Dedon PC. Production of superoxide in bacteria is stress- and cell state-dependent: A gating-optimized flow cytometry method that minimizes ROS measurement artifacts with fluorescent dyes. Front Microbiol 2017;8:459.

30. Jakkala K, Ajitkumar P. Hypoxic non-replicating persistent *Mycobacterium tuberculosis* develops thickened outer layer that helps in restricting rifampicin entry. Front Microbiol 2019;10:2339.

31. Nazarova EV, Montague CR, Huang L, La TTT, Russell DG, VanderVen BC. The genetic requirements of fatty acid import by *Mycobacterium tuberculosis* within macrophages. Elife 2019;8:e43621.

32. Guerrini V, Prideaux B, Blanc L, Bruiners N, Arrigucci R, Singh S, Ho-Liang HP, Salamon H, Chen P-Y, Lakehal K, et al. Storage lipid studies in tuberculosis reveal that foam cell biogenesis is disease-specific. PLoS Pathog 2018;14: e1007223.

33. Burdz TVN, Wolfe J, Kabani A. Evaluation of sputum decontamination methods for *Mycobacterium tuberculosis* using viable colony counts and flow cytometry. Diagn Microbiol Infect Dis 2003;47:503–509.

34. Soejima T, Iida K, Qin T, Taniai H, Yoshida S. Discrimination of live, anti-tuberculosis agent-injured, and dead *Mycobacterium tuberculosis* using flow cytometry. FEMS Microbiol Lett 2009;294:74–81.

35. Dwyer DJ, Belenky PA, Yang JH, MacDonald IC, Martell JD, Takahashi N, Chan CTY, Lobritz MA, Braff D, Schwarz EG, et al. Antibiotics induce redox-related physiological alterations as part of their lethality. Proc Natl Acad Sci U S A 2014;111:E2100–E2109.

36. Sebastian J, Swaminath S, Nair RR, Jakkala K, Pradhan A, Ajitkumar P. De novo emergence of genetically resistant mutants of *Mycobacterium tuberculosis* from the persistence phase cells formed against antituberculosis drugs *in vitro*. Antimicrob Agents Chemother 2017;61:e01343–e01316.

37. Nair RR, Sharan D, Sebastian J, Swaminath S, Ajitkumar P. Heterogeneity of ROS levels in antibiotic-exposed mycobacterial subpopulations confers differential susceptibility. Microbiology 2019;165:668–682.

38. Aguilar-López BA, Correa F, Moreno-Altamirano MMB, Espitia C, Hernández-Longoria R, Oliva-Ramírez J, Padierna-Olivos J, Sánchez-García FJ. LprG and PE_PGRS33 Mycobacterium tuberculosis virulence factors induce differential mitochondrial dynamics in macrophages. Scand J Immunol 2019;89:e12728.

39. Mishra S, Shukla P, Bhaskar A, Anand K, Baloni P, Jha RK, Mohan A, Rajmani RS, Nagaraja V, Chandra N, et al. Efficacy of β-lactam/β-lactamase inhibitor combination is linked to WhiB4-mediated changes in redox physiology of Mycobacterium tuberculosis. Elife 2017;6:e25624.

40. Saini V, Cumming BM, Guidry L, Lamprecht D, Adamson JH, Reddy VP, Chinta KC, Mazorodzo J, Glasgow JN, Richard-Greenblatt M, et al. Ergothioneine maintains redox and bioenergetic homeostasis essential for drug susceptibility and virulence of Mycobacterium tuberculosis. Cell Rep 2016;14:572–585.

41. Lamprecht DA, Finin PM, Rahman MA, Cumming BM, Russell SL, Jonnala SR, Adamson JH, Steyn AJC. Turning the respiratory flexibility of Mycobacterium tuberculosis against itself. Nat Commun 2016;7:12393.

42. Vilchèze C, Hartman T, Weinrick B, Jain P, Weisbrod TR, Leung LW, Freundlich JS, Jacobs WR. Enhanced respiration prevents drug tolerance and drug resistance in Mycobacterium tuberculosis. Proc Natl Acad Sci U S A 2017;114: 4495–4500.

43. Sia JK, Rengarajan J. Immunology of Mycobacterium tuberculosis infections. Microbiol Spectr 2019;7:1056–1086.

44. Cohen SB, Gern BH, Delahaye JL, Adams KN, Plumlee CR, Winkler JK, Sherman DR, Gerner MY, Urdahl KB. Alveolar macrophages provide an early Mycobacterium tuberculosis niche and initiate dissemination. Cell Host Microbe 2018;24:439–446.e4.

45. Weiss G, Schaible UE. Macrophage defense mechanisms against intracellular bacteria. Immunol Rev 2015;264:182–203.

46. BoseDasgupta S, Pieters J. Macrophage-microbe interaction: Lessons learned from the pathogen Mycobacterium tuberculosis. Semin Immunopathol 2018;40:577–591.

47. Johansson J, Karlsson A, Bylund J, Welin A. Phagocyte interactions with Mycobacterium tuberculosis—Simultaneous analysis of phagocytosis, phagosome maturation and intracellular replication by imaging flow cytometry. J Immunol Methods 2015; 427:73–84.

48. Haridas V, Ranjbar S, Vorobjev IA, Goldfeld AE, Barteneva NS. Imaging flow cytometry analysis of intracellular pathogens. Methods San Diego Calif 2017;112: 91–104.

49. Russell DG. Mycobacterium tuberculosis: Here today, and here tomorrow. Nat Rev Mol Cell Biol 2001;2:569–577.

50. Wayne LG, Sohaskey CD. Nonreplicating persistence of Mycobacterium tuberculosis. Annu Rev Microbiol 2001;55:139–163.

51. Gengenbacher M, Rao SPS, Pethe K, Dick T. Nutrient-starved, non-replicating Mycobacterium tuberculosis requires respiration, ATP synthase and isocitrate lyase for maintenance of ATP homeostasis and viability. Microbiology 2010;156: 81–87.

52. Zimmermann M, Kuehne A, Boshoff HI, Barry CE, Zamboni N, Sauer U. Dynamic exometabolome analysis reveals active metabolic pathways in non-replicating mycobacteria. Environ Microbiol 2015;17:4802–4815.

53. Nikitushkin VD, Trenkamp S, Demina GR, Shleeva MO, Kaprelyants AS. Metabolic profiling of dormant Mycolicibacterium smegmatis cells' reactivation reveals a gradual assembly of metabolic processes. Metabolomics 2020;16:24.

54. Mehta M, Singh A. Mycobacterium tuberculosis WhiB3 maintains redox homeostasis and survival in response to reactive oxygen and nitrogen species. Free Radic Biol Med 2019;131:50–58.

55. Srivastava S, Battu MB, Khan MZ, Nandicoori VK, Mukhopadhyay S. Mycobacterium tuberculosis PPE2 protein interacts with p67phox and inhibits reactive oxygen species production. J Immunol 2019;203:1218–1229.

56. Betts JC, Lukey PT, Robb LC, McAdam RA, Duncan K. Evaluation of a nutrient starvation model of Mycobacterium tuberculosis persistence by gene and protein expression profiling: Nutrient starvation of M. tuberculosis. Mol Microbiol 2002;43: 717–731.

57. Liu Y, Xie Z, Zhou X, Li W, Zhang H, He Z-G. NapM enhances the survival of Mycobacterium tuberculosis under stress and in macrophages. Commun Biol 2019; 2:65.

58. Rengarajan J, Bloom BR, Rubin EJ. From the cover: Genome-wide requirements for Mycobacterium tuberculosis adaptation and survival in macrophages. Proc Natl Acad Sci 2005;102:8327–8332.

59. Ruan C, Li J, Niu J, Li P, Huang Y, Li X, Duan W, Yan S, Zhen J, Xie J. Mycobacterium tuberculosis Rv0426c promotes recombinant mycobacteria intracellular survival via manipulating host inflammatory cytokines and suppressing cell apoptosis. Infect Genet Evol 2020;77:104070.

60. Pandey AK, Sassetti CM. Mycobacterial persistence requires the utilization of host cholesterol. Proc Natl Acad Sci 2008;105:4376–4380.

61. Peyron P, Vaubourgeix J, Poquet Y, Levillain F, Botanch C, Bardou F, Daffé M, Emile J-F, Marchou B, Cardona P-J, et al. Foamy macrophages from tuberculous patients' granulomas constitute a nutrient-rich reservoir for M. tuberculosis persistence. PLoS Pathog 2008;4:e1000204.

62. Ehlers S, Schaible UE. The granuloma in tuberculosis: Dynamics of a host-pathogen collusion. Front Immunol 2012;3:411.

63. Ramakrishnan L. Revisiting the role of the granuloma in tuberculosis. Nat Rev Immunol 2012;12:352–366.

64. Flynn JL, Chan J, Lin PL. Macrophages and control of granulomatous inflammation in tuberculosis. Mucosal Immunol 2011;4:271–278.

65. Mayito J, Andia I, Belay M, Jolliffe DA, Kateete DP, Reece ST, Martineau AR. Anatomic and cellular niches for Mycobacterium tuberculosis in latent tuberculosis infection. J Infect Dis 2019;219:685–694.

66. Prideaux B, Via LE, Zimmerman MD, Eum S, Sarathy J, O'Brien P, Chen C, Kaya F, Weiner DM, Chen P-Y, et al. The association between sterilizing activity and drug distribution into tuberculosis lesions. Nat Med 2015;21:1223–1227.

67. Russell DG, Cardona P-J, Kim M-J, Allain S, Altare F. Foamy macrophages and the progression of the human tuberculosis granuloma. Nat Immunol 2009;10: 943–948.

68. Sarathy JP, Via LE, Weiner D, Blanc L, Boshoff H, Eugenin EA, Barry CE, Dartois VA. Extreme drug tolerance of Mycobacterium tuberculosis in caseum. Antimicrob Agents Chemother 2018;62:2266–2283.

69. Cosma CL, Swaim LE, Volkman H, Ramakrishnan L, Davis JM. Zebrafish and frog models of Mycobacterium marinum infection. Curr Protoc Microbiol 2006;3: 10B.2.1–10B.2.33.

70. Ramakrishnan L. Granuloma-specific expression of mycobacterium virulence proteins from the glycine-rich PE-PGRS family. Science 2000;288:1436–1439.

71. Balaban NQ, Helaine S, Lewis K, Ackermann M, Aldridge B, Andersson DI, Brynildsen MP, Bumann D, Camilli A, Collins JJ, et al. Definitions and guidelines for research on antibiotic persistence. Nat Rev Microbiol 2019;17:441–448.

72. Gold B, Nathan C. Targeting phenotypically tolerant Mycobacterium tuberculosis. Microbiol Spectr 2017;5:317–360.

73. Mouton JM, Helaine S, Holden DW, Sampson SL. Elucidating population-wide mycobacterial replication dynamics at the single-cell level. Microbiol 2016;162(6): 966–978.

74. Jain P, Weinrick BC, Kalivoda EJ, Yang H, Munsamy V, Vilcheze C, Weisbrod TR, Larsen MH, O'Donnell MR, Pym A, et al. Dual-reporter mycobacteriophages (Φ2DRMs) reveal preexisting Mycobacterium tuberculosis persistent cells in human sputum. MBio 2016;7:e01023–e01016.

75. Helaine S, Thompson JA, Watson KG, Liu M, Boyle C, Holden DW. Dynamics of intracellular bacterial replication at the single cell level. Proc Natl Acad Sci 2010; 107:3746–3751.

76. Helaine S, Cheverton AM, Watson KG, Faure LM, Matthews SA, Holden DW. Internalization of Salmonella by macrophages induces formation of nonreplicating persisters. Science 2014;343:204–208.

77. Choudhary E, Sharma R, Kumar Y, Agarwal N. Conditional silencing by CRISPRi reveals the role of DNA gyrase in formation of drug-tolerant persister population in Mycobacterium tuberculosis. Front Cell Infect Microbiol 2019;9:70.

78. Ashley RE, Blower TR, Berger JM, Osheroff N. Recognition of DNA supercoil geometry by Mycobacterium tuberculosis gyrase. Biochemistry 2017;56:5440–5448.

79. Manina G, Dhar N, McKinney JD. Stress and host immunity amplify Mycobacterium tuberculosis phenotypic heterogeneity and induce nongrowing metabolically active forms. Cell Host Microbe 2015;17:32–46.

80. Bryson BD, Rosebrock TR, Tafesse FG, Itoh CY, Nibasumba A, Babunovic GH, Corleis B, Martin C, Keegan C, Andrade P, et al. Heterogeneous GM-CSF signaling in macrophages is associated with control of Mycobacterium tuberculosis. Nat Commun 2019;10:2329.

81. Pisu D, Huang L, Grenier JK, Russell DG. Dual RNA-Seq of Mtb-infected macrophages in vivo reveals ontologically distinct host-pathogen interactions. Cell Rep 2020;30:335–350.e4.

82. Cumming BM, Addicott KW, Adamson JH, Steyn AJ. Mycobacterium tuberculosis induces decelerated bioenergetic metabolism in human macrophages. Elife 2018;7: e39169.

83. Dutta AK, Choudhary E, Wang X, Záhorszka M, Forbak M, Lohner P, Jessen HJ, Agarwal N, Korduláková J, Jessen-Trefzer C. Trehalose conjugation enhances toxicity of photosensitizers against mycobacteria. ACS Cent Sci 2019;5:644–650.

84. Lovewell RR, Sassetti CM, VanderVen BC. Chewing the fat: Lipid metabolism and homeostasis during M. tuberculosis infection. Curr Opin Microbiol 2016;29: 30–36.

85. Namugenyi SB, Aagesen AM, Elliott SR, Tischler AD. Mycobacterium tuberculosis PhoY proteins promote persister formation by mediating Pst/SenX3-RegX3 phosphate sensing. MBio 2017;8:e00494–e00417.

86. Sharma S, Ryndak MB, Aggarwal AN, Yadav R, Sethi S, Masih S, Laal S, Verma I. Transcriptome analysis of mycobacteria in sputum samples of pulmonary tuberculosis patients. PLoS ONE 2017;12:e0173508.

87. Maglica Ž, Özdemir E, McKinney JD. Single-cell tracking reveals antibiotic-induced changes in mycobacterial energy metabolism. MBio 2015;6:e02236–e02214.

88. Bald D, Villellas C, Lu P, Koul A. Targeting energy metabolism in Mycobacterium tuberculosis, a new paradigm in antimycobacterial drug discovery. MBio 2017; 8:272–289.

89. Setsukinai K, Urano Y, Kakinuma K, Majima HJ, Nagano T. Development of novel fluorescence probes that can reliably detect reactive oxygen species and distinguish specific species. J Biol Chem 2003;278:3170–3175.

90. Vilchèze C, Hartman T, Weinrick B, Jacobs WR. Mycobacterium tuberculosis is extraordinarily sensitive to killing by a vitamin C-induced Fenton reaction. Nat Commun 2013;4:1881.

91. Bhaskar A, Chawla M, Mehta M, Parikh P, Chandra P, Bhave D, Kumar D, Carroll KS, Singh A. Reengineering redox sensitive GFP to measure mycothiol redox potential of Mycobacterium tuberculosis during infection. PLoS Pathog 2014; 10:e1003902.

92. Khan MZ, Bhaskar A, Upadhyay S, Kumari P, Rajmani RS, Jain P, Singh A, Kumar D, Bhavesh NS, Nandicoori VK. Protein kinase G confers survival advantage to Mycobacterium tuberculosis during latency-like conditions. J Biol Chem 2017;292:16093–16108.

Improved Understanding of Mycobacterial Physiology

93. Garnica OA, Das K, Dhandayuthapani S. OhrR of *Mycobacterium smegmatis* senses and responds to intracellular organic hydroperoxide stress. Sci Rep 2017; 7:1–12.

94. Hett EC, Rubin EJ. Bacterial growth and cell division: A mycobacterial perspective. Microbiol Mol Biol Rev 2008;72:126–156.

95. Meniche X, Otten R, Siegrist MS, Baer CE, Murphy KC, Bertozzi CR, Sassetti CM. Subpolar addition of new cell wall is directed by DivIVA in mycobacteria. Proc Natl Acad Sci U S A 2014;111:E3243–E3251.

96. Aldridge BB, Fernandez-Suarez M, Heller D, Ambravaneswaran V, Irimia D, Toner M, Fortune SM. Asymmetry and aging of mycobacterial cells lead to variable growth and antibiotic susceptibility. Science 2012;335:100–104.

97. Santi I, Dhar N, Bousbaine D, Wakamoto Y, McKinney JD. Single-cell dynamics of the chromosome replication and cell division cycles in mycobacteria. Nat Commun 2013;4:2470.

98. Carel C, Nukdee K, Cantaloube S, Bonne M, Diagne CT, Laval F, Daffé M, Zerbib D. *Mycobacterium tuberculosis* proteins involved in mycolic acid synthesis and transport localize dynamically to the old growing pole and septum. PLoS ONE 2014;9:e97148.

99. Kremer L, Maughan WN, Wilson RA, Dover LG, Besra GS. The *M. tuberculosis* antigen 85 complex and mycolyltransferase activity. Lett Appl Microbiol 2002;34: 233–237.

100. Dulberger CL, Rubin EJ, Boutte CC. The mycobacterial cell envelope—A moving target. Nat Rev Microbiol 2020;18:47–59.

101. Kamariza M, Shieh P, Ealand CS, Peters JS, Chu B, Rodriguez-Rivera FP, Sait MRB, Treuren WV, Martinson N, Kalscheuer R, et al. Rapid detection of *Mycobacterium tuberculosis* in sputum with a solvatochromic trehalose probe. Sci Transl Med 2018;10:eaam6310.

102. Rodriguez-Rivera FP, Zhou X, Theriot JA, Bertozzi CR. Visualization of mycobacterial membrane dynamics in live cells. J Am Chem Soc 2017;139:3488–3495.

103. Hodges HL, Brown RA, Crooks JA, Weibel DB, Kiessling LL. Imaging mycobacterial growth and division with a fluorogenic probe. Proc Natl Acad Sci 2018;115: 5271–5276.

104. Danilchanka O, Sun J, Pavlenok M, Maueröder C, Speer A, Siroy A, Marrero J, Trujillo C, Mayhew DL, Doornbos KS, et al. An outer membrane channel protein of *Mycobacterium tuberculosis* with exotoxin activity. Proc Natl Acad Sci 2014;111: 6750–6755.

105. Mitra A, Speer A, Lin K, Ehrt S, Niederweis M. PPE surface proteins are required for heme utilization by *Mycobacterium tuberculosis*. MBio 2017;8:e01720– e01716.

106. Schierloh P, Klepp L, Vazquez C, Rocha RV, Blanco FC, Balboa L, López B, Ritacco V, Bigi F, Sasiain M d C. Differential expression of immunogenic proteins on virulent *Mycobacterium tuberculosis* clinical isolates. Biomed Res Int 2014.2014, 1–13. https://doi.org/10.1155/2014/741309.

107. Sampson SL, Lukey P, Warren RM, van Helden PD, Richardson M, Everett MJ. Expression, characterization and subcellular localization of the *Mycobacterium tuberculosis* PPE gene Rv1917c. Tuberculosis 2001;81:305–317.

108. Fiolek TJ, Banahene N, Kavunja HW, Holmes NJ, Rylski AK, Pohane AA, Siegrist MS, Swarts BM. Engineering the mycomembrane of live mycobacteria with an expanded set of trehalose monomycolate analogues. Chembiochem 2019;20: 1282–1291.

109. Foley HN, Stewart JA, Kavunja HW, Rundell SR, Swarts BM. Bioorthogonal chemical reporters for selective in situ probing of mycomembrane components in mycobacteria. Angew Chem Int Ed Engl 2016;55:2053–2057.

110. Rundell SR, Wagar ZL, Meints LM, Olson CD, O'Neill MK, Piligian BF, Poston AW, Hood RJ, Woodruff PJ, Swarts BM. Deoxyfluoro-D-trehalose (FDTre) analogues as potential PET probes for imaging mycobacterial infection: Rapid synthesis and purification, conformational analysis, and uptake by mycobacteria. Org Biomol Chem 2016;14:8598–8609.

111. Swarts BM, Holsclaw CM, Jewett JC, Alber M, Fox DM, Siegrist MS, Leary JA, Kalscheuer R, Bertozzi CR. Probing the mycobacterial trehalome with bioorthogonal chemistry. J Am Chem Soc 2012;134:16123–16126.

112. García-Heredia A, Pohane AA, Melzer ES, Carr CR, Fiolek TJ, Rundell SR, Lim HC, Wagner JC, Morita YS, Swarts BM, et al. Peptidoglycan precursor synthesis along the sidewall of pole-growing mycobacteria. Elife 2018;7:e37243.

113. Cheng Y, Xie J, Lee K-H, Gaur RL, Song A, Dai T, Ren H, Wu J, Sun Z, Banaei N, et al. Rapid and specific labeling of single live *Mycobacterium tuberculosis* with a dual-targeting fluorogenic probe. Sci Transl Med 2018;10(454):eaar4470.

114. Pan Q, Yan J, Liu Q, Yuan C, Zhang X-L. A single-stranded DNA aptamer against mannose-capped lipoarabinomannan enhances anti-tuberculosis activity of macrophages through downregulation of lipid-sensing nuclear receptor peroxisome proliferator-activated receptor γ expression: Aptamer enhances macrophages against *Mtb*. Microbiol Immunol 2017;61:92–102.

115. Dhiman A, Kumar C, Mishra SK, Sikri K, Datta I, Sharma P, Singh TP, Haldar S, Sharma N, Bansal A, et al. Theranostic application of a novel G-quadruplex-forming DNA aptamer targeting malate synthase of *Mycobacterium tuberculosis*. Mol Ther Nucleic Acids 2019;18:661–672.

116. Zhou K-L, Li X, Zhang X-L, Pan Q. Mycobacterial mannose-capped lipoarabinomannan: A modulator bridging innate and adaptive immunity. Emerg Microbes Infect 2019;8:1168–1177.

117. Tang X-L, Wu S-M, Xie Y, Song N, Guan Q, Yuan C, Zhou X, Zhang X-L. Generation and application of ssDNA aptamers against glycolipid antigen ManLAM of *Mycobacterium tuberculosis* for TB diagnosis. J Infect 2016;72:573–586.

118. Ansari N, Ghazvini K, Ramezani M, Shahdordizadeh M, Yazdian-Robati R, Abnous K, Taghdisi SM. Selection of DNA aptamers against *Mycobacterium tuberculosis* Ag85A, and its application in a graphene oxide-based fluorometric assay. Microchim Acta 2017;185:21.

119. Kalra P, Mishra SK, Kaur S, Kumar A, Prasad HK, Sharma TK, Tyagi JS. G-quadruplex-forming DNA aptamers inhibit the DNA-binding function of HupB and *Mycobacterium tuberculosis* entry into host cells. Mol Ther Nucleic Acids 2018;13:99–109.

120. Seeliger JC, Topp S, Sogi KM, Previti ML, Gallivan JP, Bertozzi CR. A riboswitch-based inducible gene expression system for mycobacteria. PLoS ONE 2012;7: e29266.